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Anti-HIV activity of amphotericin B-cholesteryl sulfate colloidal dispersion in vitro

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Abstract

We examined whether the anti-HIV-1 activity of the polyene antibiotic Amphotericin B (AMB) is retained following incorporation into sterically stabilized 'Stealth' liposomes (L-AMB) with prolonged circulation in vivo, or cholesteryl sulfate colloidal dispersions (CD-AMB). The effects of the different preparations on acute infection of H9 cells with HIV-1_{IIIB}, spreading of the virus from chronically infected H9/HTLV-IIIB cells to SupT1 cells, and HIV-1-induced syncytium formation were evaluated. Infection was monitored by p24 levels in culture supernatants. L-AMB did not affect HIV-1 infection. When present only during initial infection, AMB (3–20 μg/ml) reduced p24 levels by 70–80% after 7 and 10 days post-infection, while CD-AMB inhibited p24 production by ~30–40% at day 7 and 50–60% at day 10. The inhibitory effect of CD-AMB and AMB was enhanced by continuous treatment of acutely infected cells. The reduction of p24 production during continuous treatment was not due to cytotoxicity. During spreading of infection from infected to uninfected cells, AMB almost completely inhibited virus production while CD-AMB reduced both p24 production and the cytopathic effect in a dose-dependent manner. HIV-1 induced syncytium formation was slightly inhibited by AMB but not by CD-AMB. Because CD-AMB is considerably less cytotoxic than AMB, its ability to inhibit HIV infection in vivo needs to be evaluated further.

Keywords: Amphotericin B; Amphotericin B colloidal dispersion; Human immunodeficiency virus (HIV); Liposomes; Sterically stabilized liposomes; Antivirals

Abbreviations: AMB, Amphotericin B; L-AMB, sterically stabilized 'Stealth' liposomes containing AMB; CD-AMB, cholesteryl sulfate colloidal dispersion of Amphotericin B; AME, Amphotericin B methyl ester; SAB, sulfated Amphotericin B; MS8209, *N*-methylglucamine salt of 1-deoxy-1-amino-4,6-*O*-benzylidene-D-fructosyl-Amphotericin B; MCG, *N*-(*N′*-(2-(4′-methylmorpholinio)ethyl)*N″*-cyclohexylguanyl) Amphotericin B methyl ester; DAPEG, *N*-(*N′*-(3-dimethylaminopropyl)*N″*-ethylguanyl) Amphotericin B; HSPG, hydrogenated soy phosphatidylglycerol; HSPC, hydrogenated soy phosphatidylcholine; CHOL, cholesterol; PEG-DSPE, poly(ethylene glycol)-distearoylphosphatidylethanolamine; UDA, *Urtica dioica* lectin.

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1. Introduction

Amphotericin B (AMB) is a polyene macrolide antibiotic that has a high affinity for membrane sterols. It preferentially binds to ergosterol in fungal membranes, rather than to cholesterol found in mammalian membranes, altering the permeability and function of membranes of fungal cells (Norman et al., 1976; Bolard, 1986). AMB has a broad spectrum of activity against pathogenic fungi and it has been the drug of choice for the treatment of systemic fungal infections (Medoff et al., 1983; Gallis et al., 1990). However, administration of the most common preparation of AMB, a sodium deoxycholate colloidal suspension (Fungizone), is associated with severe, dose-limiting, acute and chronic toxicity, particularly nephrotoxicity (Walsh and Pizzo, 1988). In 1981, New et al. (1981) reported the decreased toxicity of liposomal AMB in an animal model of leishmaniasis. Since then several lipid formulations of AMB have been developed to increase its antifungal specificity and decrease its toxicity (Lopez-Berestein et al., 1983; Szoka et al., 1987; Wiebe and DeGregorio, 1988; Wasan and Lopez-Berestein, 1995; Hiemenz and Walsh, 1996; Kohno et al., 1997; van Etten et al., 1998). Three lipid formulations of AMB have been evaluated clinically and received regulatory approval for the treatment of systemic mycosis: AMB lipid complex (Abelcet, The Liposome Company, Princeton, NJ), AMB colloidal dispersion (Amphocil, Sequus Pharmaceuticals, Menlo Park, CA) and AmBisome (Nexstar, San Dimas, CA) (Hiemenz and Walsh, 1996).

AMB and its methyl ester (AME) are also active against a variety of lipid-enveloped viruses including vesicular stomatitis, herpes simplex and vaccinia viruses (Stevens et al., 1975; Jordan et al., 1978; Jordan and Seet, 1978). AMB, AME and sulfated AMB (SAB), can inhibit infection of lymphocytes or $CD4^+$ cell lines by human immunodeficiency virus type 1 (HIV-1) (Schaffner et al., 1986; Hansen et al., 1990; Otake et al., 1991; Hansen et al., 1992; Selvam et al., 1993). AMB and AME bind to membrane sterols and were therefore thought to interact both with the cholesterol-rich envelope of HIV and the target cell membrane (Schaffner et al., 1986; Aloia et al., 1993). The effect of SAB was attributed to inhibition of virus binding to target cells and a direct inactivation of the virus. SAB also inhibits HIV-1 induced syncytium formation and it has been suggested that this compound acts similarly to dextran sulfate (Otake et al., 1991). The anti-HIV-1 activity was also reported for MCG (*N*-(*N′*-(2-(4′-methylmorpholinio)ethyl)*N″*-cyclohexylguanyl-AMB methyl ester) and DAPEG (*N*-(*N′* - (3 - dimethylaminopropyl)*N″* - ethylguanyl-AMB) (Hansen et al., 1990) and for a non-ester AMB derivative MS8209 (*N*-methylglucamine salt of 1-deoxy-1-amino-4,6-*O*-benzylidene-D-fructosyl-AMB) (Cefai et al., 1991; Pleskoff et al., 1995, Pleskoff et al., 1996; Magierowska-Jung et al., 1996). None of these compounds, however, is currently being used for in vivo treatment of fungal diseases. In spite of the facts that (i) lipid formulations of AMB have been undergoing extensive clinical investigations and (ii) patients infected with HIV who are particularly susceptible to fungal infections, are already treated with liposomal AMB (Coker et al., 1993; Davidson et al., 1994; Torre-Cisneros et al., 1994), the in vitro effect of clinically employed lipid formulations of AMB on HIV-1 infection has not been investigated. Only one study has compared the anti-HIV effect of free AMB and AMB incorporated in dimyristoylphospahtidylcholine: dimyristoyl phosphatidylglycerol (7:3) liposomes, and concluded that liposomal AMB is less toxic but as effective against HIV-1 replication as free AMB (Pontani et al., 1989).

Here, we examined whether the activity of AMB against HIV-1 is retained following incorporation into sterically stabilized 'Stealth' liposomes (L-AMB) with prolonged circulation in vivo, or association with cholesteryl sulfate colloidal dispersion (CD-AMB). Some of our results have been presented earlier in preliminary form (Konopka et al., 1998).

2. Materials and methods

2.1. Reagents

Stealth AMB Liposomes (AMB:HSPG:HSPC:CHOL:PEG-DSPE, 5.1:9.5:48.2:34.6:2.6, mole%)

(L-AMB), Amphotericin B Colloidal Dispersion (CD-AMB) (Guo et al., 1991), control Liposomes (HSPG:HSPC:CHOL:PEG-DSPE, 9.5:48.2:34.6:2.6, mole%) and sCD4 were obtained from Sequus Pharmaceuticals, Inc., Menlo Park, CA. A sodium deoxycholate colloidal suspension of Amphotericin B (Fungizone® Intravenous) (AMB; MW. 924.09) was obtained from Bristol-Myers Squibb, Princeton, NJ. *Urtica dioica* lectin (UDA) was a gift from Dr W.J. Peumans, Katholieke Universiteit Leuven, Heverlee, Belgium.

2.2. Cells and virus

SupT1 and H9 cells were obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH, Bethesda, MD) from Drs J. Hoxie and R.C. Gallo, respectively. Chronically infected H9/HTLV-IIIB cells were kindly provided by Drs T. El-Beik and J. McGrath (San Francisco General Hospital). All cell lines were maintained at 37°C, under 5% CO_2 in RPMI 1640 medium (Irvine Scientific) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (RPMI/10). The cells were passaged 1:6 every 3–4 days. The supernatant of H9/HTLV-IIIB cells was used as a source of the HIV-1 strain, HIV-1_{IIIB}. The p24 concentration of the stock was 150 ng/ml as determined by ELISA.

2.3. Infection and treatment

H9 cells (1×10^7 cells/ml) were exposed to HIV-1_{IIIB}, at 4 ng p24/10^6 cells, for 2 h at 37°C. Cells were then washed three times to remove unbound virus, resuspended at 0.5×10^6/ml in RPMI/10 and cultured in 48-well plates (1 ml per well). The effect of L-AMB, CD-AMB, AMB or control liposomes was investigated using two protocols: (i) the test compounds were only present during initial infection or (ii) these compounds were present both during initial infection and for the duration of the experiment. For treatment during initial infection, the test compounds were added to cells immediately prior to the addition of the virus. The additions were only present during the 2 h infection period. For continuous treatment, cells were infected as described above and fresh dilutions of the test compounds were added with each medium replacement. Every 3–4 days 0.75 ml of supernatant was removed and replaced with fresh medium with or without treatments.

Infection was monitored by the viral p24 level in harvested culture supernatants using ELISA plates obtained from the AIDS Vaccine Program (NCI-Frederick Cancer Research and Development Center; Frederick, MD). The results are expressed as the mean ± standard deviation (S.D.) of duplicate determinations from four wells following infection in duplicate tubes for each condition. The results are compared to control cells infected in the absence of additions.

2.4. Spreading of HIV infection

The coculture experiments were performed as described by Berger et al. (1989). SupT1 cells (2×10^5) were mixed with 2×10^2 H9/HTLV-IIIB cells in a volume of 1 ml RPMI/10 in the absence or presence of appropriate additions. Every 2 or 3 days the cytopathic effect was evaluated by scoring syncytium formation and samples of 0.5 ml were removed for p24 determination. The incubation was continued by transferring 0.1 ml of each cell suspension to new wells containing 0.9 ml of RPMI/10 ± appropriate additions. The results are expressed as the mean ± S.D. of duplicate determinations from two wells for each condition. The results are compared to control cocultures in the absence of additions.

2.5. Assay for CD4-dependent HIV-induced cell fusion

The assay was performed as described by Lifson (1993) and evaluated by inverted phase contrast microscopy at 25× magnification. Uninfected SupT1 cells were used as indicator cells and H9/HTLV-IIIB cells, expressing HIV glycoproteins, served as effector cells. After 16–20 h of cocultivation, syncytium formation had proceeded to its full extent and cell fusion was typi-

cally extensive enough to warrant a +4 score (numerous large syncytia with a low number of viable cells). A +3 score represented numerous large syncytia with a higher number of viable cells. A +2 score was given when less numerous syncytia were observed. An *N*-acetylglucosamine-specific plant lectin UDA, and sCD4, known to block HIV-1-induced syncytium formation, served as positive controls for inhibition of cell fusion.

2.6. Cell viability assay

Cell morphology was evaluated by inverted phase contrast microscopy. The number of viable cells used for experiments was determined by Trypan Blue exclusion. Cell viability was quantified by a modified Alamar Blue assay (Fields and Lancaster, 1993). Briefly, 1.0 ml of 10% (v/v) Alamar Blue dye in RPMI/10 was added to each well. After incubation for ~4 h at 37°C, 200 μl of the supernatant was collected from each well and transferred to 96-well plates. The absorbance at 570 and 600 nm was measured with a microplate reader (Molecular Devices, Menlo Park, CA). Cell viability (as a percentage of control cells) was calculated according to the formula, $(A_{570} - A_{600})$ of treated cells $\times 100/(A_{570} - A_{600})$ of control cells. Previous studies have indicated a good correlation between the Alamar Blue assay and Trypan Blue staining (Konopka et al., 1996).

3. Results

3.1. Effect of L-AMB, CD-AMB and AMB on infection of H9 cells with HIV-1IIIB

We first examined whether L-AMB, CD-AMB and AMB, at the dose range between 1 and 20 μg/ml, would inhibit infection if present only at the time of exposure of H9 cells to HIV-1_{IIIB}. No p24 was detected after 3 days post-infection. Virus production into the culture medium increased from 0.4 ± 0.1 at day 7 to 6.3 ± 0.9 at day 10 post-infection. The results are shown as percent of control at day 7 and 10 (Fig. 1). The presence of L-AMB during initial infection did not affect p24 production in H9 cells (Fig. 1A), while AMB (3–20 μg/ml) reduced p24 levels by 70–80% (Fig. 1C). Empty liposome controls, at the same concentration of lipid as that of 5 or 20 μg/ml L-AMB, were included in the experiments; the p24 values in these wells were similar to that in the untreated controls (data not shown). Under the same experimental conditions CD-AMB (3–20 μg/ml), inhibited p24 production by approximately 30–40% at day 7 and 50–60% at day 10 (Fig. 1B). Cholesteryl sulfate is insoluble in water and stable colloidal dispersions of this lipid are not available. When subject to the high temperature solvent injection technique, cholesteryl sulfate does form liposome-like particles but they aggregate and precipitate within hours after the preparation. Therefore, cholesteryl sulfate controls were not included in our experiments.

3.2. Continuous treatment of acutely infected H9 cells with CD-AMB and AMB

Having determined that CD-AMB had a modest inhibitory effect on p24 production when present only during the 2 h period of HIV-1 binding to and entry into the cells (Fig. 1B), we examined whether this effect could be enhanced by the continuous treatment of acutely infected cells. H9 cells were infected with HIV-1_{IIIB} in the presence of CD-AMB or AMB. The cells were washed to remove unbound virus and cultured with CD-AMB or AMB (1–20 μg/ml). The tested concentrations were kept constant for 10 days. The results are shown as percent of control at day 7 and 10 (Fig. 2A,B). Under these conditions free AMB (1–20 μg/ml) reduced p24 production by over 90% after 7 days, and completely inhibited p24 production on day 10 (Fig. 2B). The p24 levels were also significantly reduced in all of the CD-AMB treated wells. The inhibition of virus replication was dose-dependent. On day 7, CD-AMB at 1, 3 and 5 μg/ml inhibited p24 production by ~50%, 70 and 80%, respectively, and by over 90% at 10 and 20 μg/ml. On day 10, over 90% inhibition of p24 production was observed at 3 to 20 μg CD-AMB/ml, and 70% inhibition with 1 μg CD-AMB/ml.

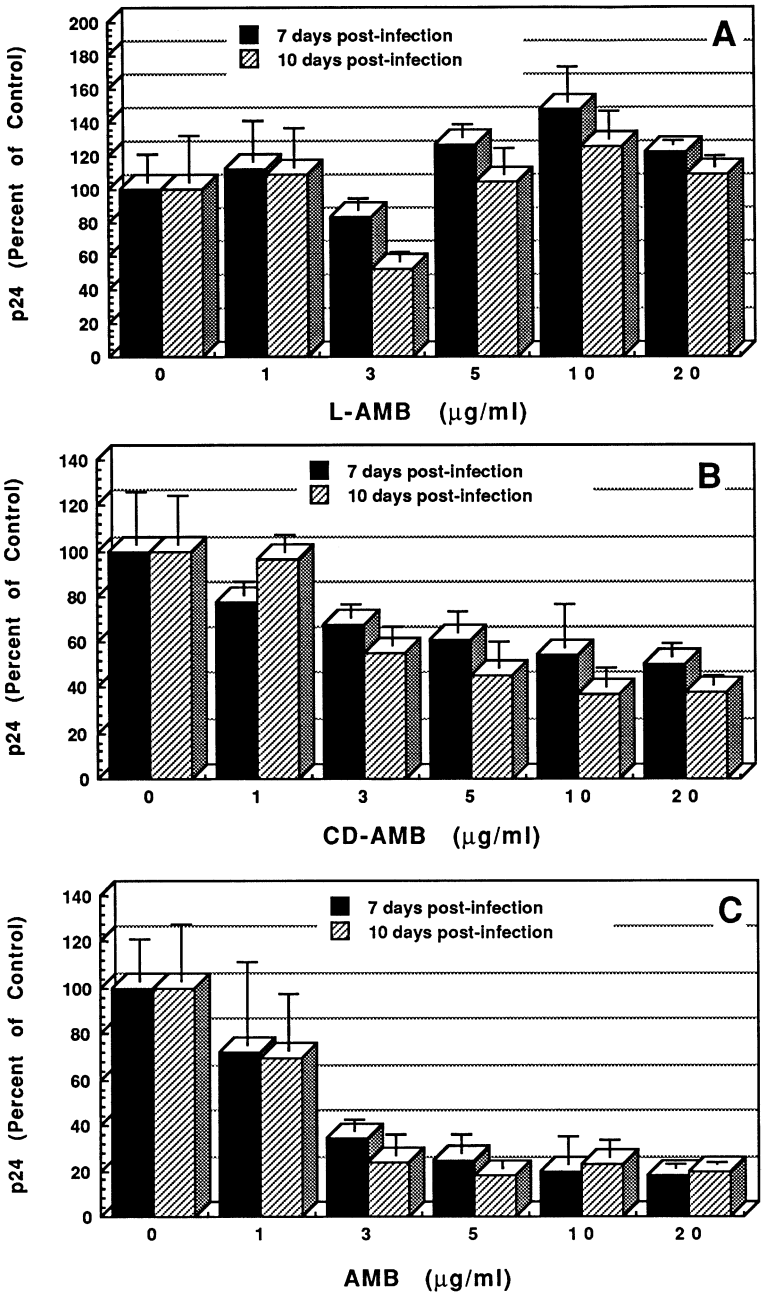

Fig. 1. Dose-dependence of viral p24 production by H9 cells infected with HIV-1_{IIIB} in the absence or presence of L-AMB, CD-AMB or AMB. The cells were exposed to the virus at 4 ng p24 per 10^6 cells as described in Section 2. The production of p24 antigen within the previous 96 or 72 h period was determined in culture supernatants on days 7 and 10 post-infection, and was expressed as percent of control (100%: 0.4 ± 0.1 and 6.3 ± 0.9 ng p24/ml on day 7 and 10, respectively. (A) L-AMB; (B) CD-AM; (C) AMB. Data represent the mean $\pm$ S.D.

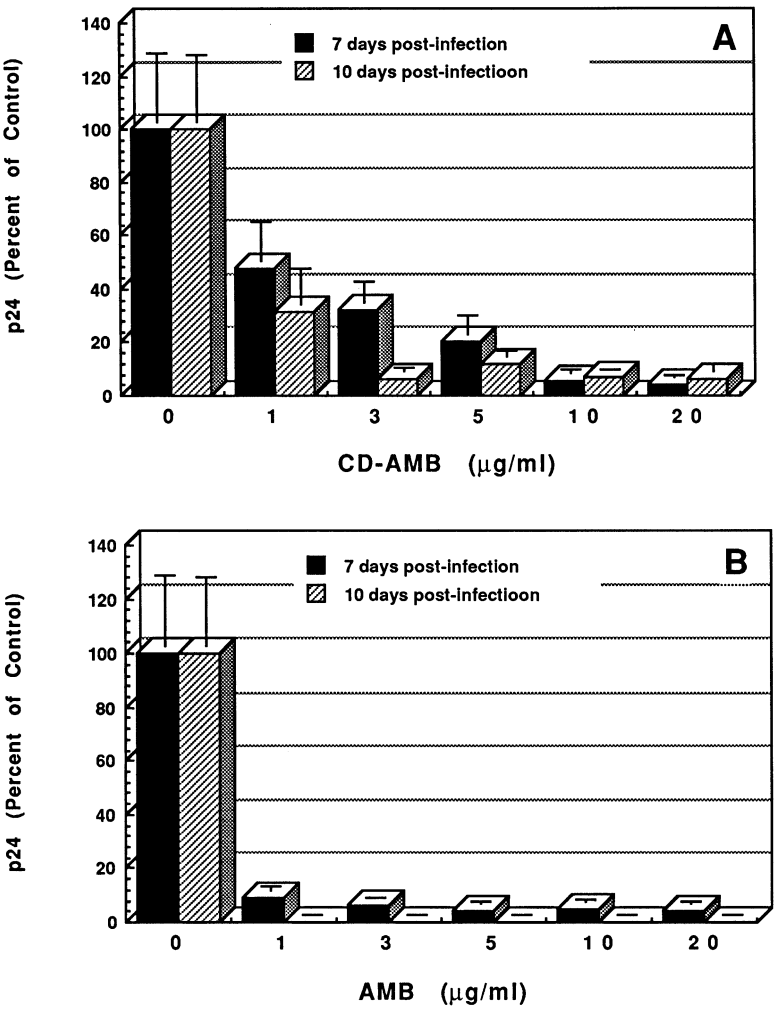

Fig. 2. Continuous treatment of acutely infected H9 cells with CD-AMB or AMB. H9 cells were infected as described in Section 2 in the presence of the test compounds and then treated continuously for 10 days. The production of p24 antigen within the previous 96 or 72 h period was determined in culture supernatants on days 7 and 10 post-infection, and was expressed as percent of control (100%: 1.1 ± 0.3 and 3.4 ± 0.85 ng p24/ml on day 7 and 10, respectively). (A) CD-AMB; (B) AMB. For AMB all values were zero on day 10. Data represent the mean $\pm$ S.D.

3.3. Effect of continuous treatment with CD-AMB and AMB on viability of H9 cells

Determination of the anti-HIV effect of the test compounds was based on monitoring the p24 level in the culture supernatant. The significant reduction of p24 production observed throughout the continuous treatment could be a result of the toxicity of the test compounds. To ascertain the cytotoxicity of the compounds H9 cells were incubated continuously with CD-AMB and AMB, and cell viability was quantified by the Alamar Blue assay on day 10 (Fig. 3). A 10-day exposure to CD-AMB (1–20 μg/ml), did not reduce cell viability. AMB decreased cell viability by ~ 30–40% at concentrations of 10 and 20 μg/ml. Thus, the reduction of p24 production observed during the continuous treatment (Fig. 2) cannot be attributed to a cytotoxic effect.

3.4. Effect of continuous treatment with L-AMB, CD-AMB and AMB on spreading of infection between H9/HTLV-IIIB and SupT cells

We next investigated whether CD-AMB and AMB were also inhibitory against transmission of HIV-1 from infected to uninfected cells. In HIV-infected individuals only a small fraction of peripheral blood lymphocytes are infected. It has been proposed that the coculture of infected cells with uninfected cells at a ratio of 1:1000 may approximate in vivo conditions (Vasudevachari et al., 1992). We used a coculture system in which HIV-infected cells (H9/HTLV-IIIB) were mixed with 1000-fold excess of uninfected cells (SupT1); HIV spread was monitored both by the cytopathic effect and by the appearance of p24 in the culture supernatant. SupT1 cells were chosen for the uninfected population because $>98\%$ of the cells expressed CD4, the cells are permissive for replication and are highly sensitive to the cytopathic effects of the virus. H9/HTLV-IIIB is an uncloned human T-cell line derived from H9 cells that survived acute infection with HTLV-IIIB (Popovic et al., 1984). A high percentage of these chronically infected cells constitutively express HIV proteins, including the envelope glycoprotein, and release infectious virus.

The absolute p24 levels (ng/ml) of the untreated mcocultures were 0.3 ± 0.6, 4.63 ± 0.6, and 51.9 ± 25.6, after 2, 4 and 7 days, respectively, and the cytopathic effect was characterized by a $3+$ score (numerous large syncytia) throughout the experiment. The determined p24 levels reflected only virus replication in de novo infected SupT1 cells; no p24 was detected in wells containing only H9/HTLV-IIIB cells (2×10^2/ml) and split 1:5 every 2 or 3 days (data not shown). The presence of L-AMB affected neither p24 production nor the cytopathic effect (data not shown). On day 2 and 4, AMB almost completely inhibited both p24 production (Table 1) and the cytopathic effect (data not shown). On day 2, CD-AMB at 1, 3 and 5 μg/ml inhibited p24 production by ~ 50, 85 and 90%, respectively, while at 10 and 20 μg CD-AMB/ml p24 was not detected. On day 4, $\sim 50\%$ inhibition of p24 production was observed at 3 to 20 μg CD-AMB/ml, and $\sim 30\%$ inhibition with 1 μg CD-AMB/ml (Table 1). The reduction in p24 levels corresponded to the decrease in the cytopathic effect (data not shown). On day 7, AMB significantly inhibited p24 production, while CD-AMB failed to reduce the p24 level when compared to untreated controls (Table 1).

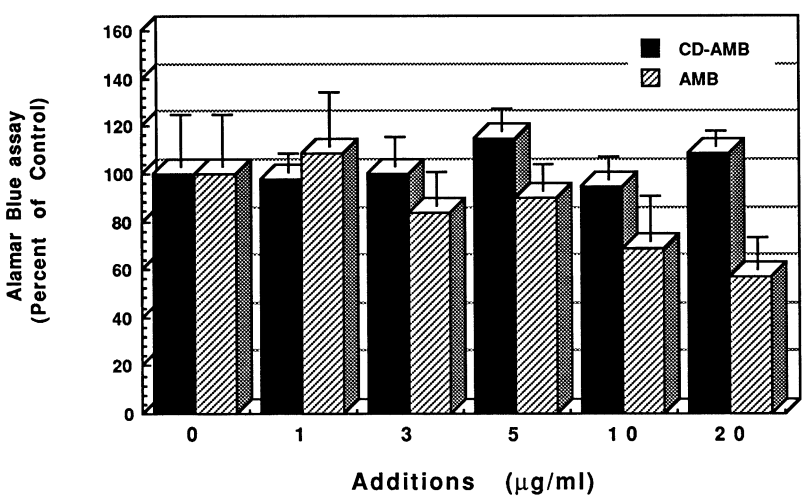

Fig. 3. Effect of CD-AMB and AMB on the viability of uninfected H9 cells. The cells in 48-well plates were exposed to the test compounds for 10 days as described in Section 2. Cell viability was measured by the Alamar Blue assay on day 10 (incubation for 4 h at 37°C) and was expressed as percent of the control. Data represent the mean ± S.D. from duplicate wells or quadruplicate wells (controls).

Table 1
Effect of CD-AMB and AMB on spreading of infection between H9/HTLV-IIIB and SupT cells

Treatment	2 Days		4 Days		7 Days	
	p24 (ng/ml)[a]	%[b]	p24 (ng/ml)	%	p24 (ng/ml)	%
Control	0.30 ± 0.06	100	4.63 ± 0.57	100	51.9 ± 25.6	100
1 μg/ml CD-AMB	0.16 ± 0.07	53.3	3.13 ± 0.55	67.6	54.4 ± 10.8	104.8
3 μg/ml CD-AMB	0.05 ± 0.03	16.7	2.42 ± 0.11	52.5	47.4 ± 4.1	91.3
5 μg/ml CD-AMB	0.03 ± 0.01	10.0	2.56 ± 0.40	55.3	54.5 ± 19.0	105.0
10 μg/ml CD-AMB	0		2.09 ± 0.56	45.1	51.6 ± 2.4	99.4
20 μg/ml CD-AMB	0		1.95 ± 0.26	42.1	48.8 ± 8.2	94.0
1 μg/ml AMB	0		0.53 ± 0.11	11.4	2.0 ± 1.5	3.9
3 μg/ml AMB	0		0		0.09 ± 0.06	0.2
5 μg/ml AMB	0		0		0.09 ± 0.01	0.2
10 μg/ml AMB	0		0		0.10 ± 0.06	0.2
20 μg/ml AMB	0		0		0.06 ± 0.03	0.1

[a] The production of p24 antigen within the previous 48 or 72 h period was determined in cell culture supernatants on days 2, 4, and 7 post-infection. Data represent mean values ± S.D.
[b] Calculated as % of control infection (without additions).

3.5. Effect of CD-AMB and AMB on HIV-1-induced syncytium formation

We investigated the effect of CD-AMB and free AMB on syncytium formation between chronically infected H9/HTLV-IIIB cells and uninfected $CD4^+$ SupT1 cells. Interestingly, no inhibitory effect was found for CD-AMB and only a slight inhibitory activity was observed in the presence of free AMB at 10 and 20 μg/ml (Table 2). Thus, the reduction in p24 production observed during spreading of infection in the presence of CD-AMB or AMB, cannot be attributed to the inhibition of fusion between infected and uninfected cells. Complete inhibition of fusion was observed in control wells treated with sCD4 and UDA, both at 10 μg/ml. In the presence of sCD4, which inhibits the binding of gp120 to CD4, the cells were uniformly spread and no clusters of cells were observed. The plant lectin UDA does not interfere with HIV-1 binding but blocks the fusion between the virus or virus-expressing cell and the target cell membrane (Balzarini et al., 1992). In wells treated with UDA, cells formed clusters and clumps but no syncytium formation could be observed.

4. Discussion

In this study, we compared the anti-HIV-1 effect of liposome-incorporated AMB (L-AMB) and cholesteryl sulfate colloidal dispersion AMB (CD-AMB) with that of the deoxycholate/AMB micelles (AMB). Our results show that AMB, at non-toxic concentrations, was highly effective in inhibiting the production of virions both in acute infection of H9 cells and during spreading of infection from HIV-infected cells to uninfected cells. The incorporation of AMB into sterically stabilized 'Stealth' liposomes with prolonged cir-

Table 2
Effect of CD-AMB and AMB on syncytium formation between H9/HTLV-IIIB and SupT1 cells

Compounds	Concentration (μg/ml)[a]					
	0	1	3	5	10	20
CD-AMB	4+	4+	4+	4+	4+	3+/4+
AMB	4+	4+	4+	4+	3+	2+/4+
sCD4					–	
UDA					–	

[a] Syncytia were scored between 16 and 20 h after the addition of chronically infected cells, H9/HTLV-IIIB to uninfected Sup-T1 cells. CD-AMB and AMB remained in the assay during the co-incubation period.

culation in vivo, completely abolished its anti-HIV-1 activity. It is likely that the bulky poly (ethylene glycol) moiety of these liposomes prevented the transfer of AMB from the liposome membrane to the viral or cell membranes. In contrast, AMB incorporated in dimyristoylphospahtidylcholine:dimyristoylphosphatidylglycerol (7:3) liposomes was as effective against HIV-1 replication as AMB (Pontani et al., 1989).

Amphotericin B is a macrolide polyene antibiotic with an asymmetrical distribution of hydrophobic and hydrophilic groups. The structure of AMB confers a very low solubility of the drug both in water as well as in many organic solvents (Bolard, 1986). This low solubility has made it difficult to study the pharmacology of AMB. The formulation of AMB currently used in the clinic is a micellar complex of AMB with deoxycholate (Fungizone). After intravenous administration, the deoxycholate/AMB micelles dissociate rapidly (Edmonds et al., 1989) and AMB binds to lipoproteins and to accessible sterol-containing cell membranes (Brajtburg et al., 1984). The widely accepted model of the anticellular effect of AMB involves the formation of 1:1 AMB/sterol aggregates which subsequently associate into a transmembrane barrel pore. However, numerous studies of AMB channel ion selectivity, sterol requirements, and mode of delivery have demonstrated multiple AMB channel structures and modes of action (Hartsel et al., 1993).

CD-AMB is a uniform disc-shaped complex of AMB and sodium cholesteryl sulfate, in a molar ratio of 1:1. Interaction of AMB with cholesteryl sulfate may form rigid and tightly packed lipid layers that are unable to bend sufficiently to form closed vesicular structures (Guo et al., 1991; Guo and Working, 1993). CD-AMB is thermodynamically stable and remains intact during passage through a Sephacryl A S-1000 column (Pharmacia). More than 98% of AMB and 95% of cholesteryl sulfate mass co-eluted as a single peak (Guo et al., 1991). Analysis of eleven batches of CD-AMB demonstrated that 101.6 $\pm$ 2.5% of AMB was recovered from the column in the complex form (Guo, unpublished data). Therefore, free AMB is not present in CD-AMB.

Incubation of deoxycholate/AMB and CD-AMB with human plasma results in a time-dependent dissociation of AMB. After 2 h at 37°C, 91 and 20% of AMB is released from the micellar formulation and CD-AMB, respectively, and bound to plasma lipoproteins (Guo et al., 1991; Guo, unpublished data). Although these studies were performed in the absence of cells, it can be postulated that a slow release of AMB from CD-AMB would reduce the transfer of AMB from the lipid to serum components and to cell membranes. Under our experimental conditions, when deoxycholate/AMB or CD-AMB were incubated with cells and the virus in the presence of 10% FBS, AMB dissociated from these formulations binds to viral and cell membranes, and to serum components. Toxicity of the drug depends on the rate and the form (monomer vs. aggregates) of AMB transfer into membranes. The anti-HIV effect of AMB and CD-AMB observed during continuous treatment was not related to cytotoxicity, e.g. formation of channels in cell membranes and cell death. Thus, it appears that AMB released from deoxycholate/AMB or CD-AMB under these conditions, does not accumulate in cell membranes but rather binds to viral membranes and to serum lipoproteins. Under in vivo conditions the tissue distribution of AMB is modulated by the rate of clearance of the AMB formulations from the blood.

Although CD-AMB forms a tight complex that does not readily dissociate after intravenous injection, this complex retains the antifungal spectrum and potency of AMB. CD-AMB delivers drug to fungi even though it reduces its delivery to many tissues in the host (Guo et al., 1991). The fungicidal concentrations of CD-AMB appear similar to those of AMB with some differences in specificity against some species of fungi (Hanson and Stevens, 1992). Studies comparing toxicity of CD-AMB with AMB in animals and humans have shown that this lipid formulation is significantly less toxic than equivalent doses of deoxycholate/AMB (Guo et al., 1991; Guo and Working, 1993; Hiemenz and Walsh, 1996). CD-AMB is also effective in treating patients with systemic mycosis and is less nephrotoxic than AMB (White et al., 1997; Anaissie et al., 1998).

AMB and its water soluble derivatives inhibit HIV-1 infection, but the precise mechanism of this effect is not known. These agents have to be present during the initial infection to exhibit an inhibitory effect (Hansen et al., 1990; Pleskoff et al., 1995, Pleskoff et al., 1996; Magierowska-Jung et al., 1996). They do not block virus binding to host cells. The results of time-of-addition experiments indicate that they interfere with an early event in viral replication. The target seems viral rather than cellular, since the pretreatment of cells with MS8209 has no effect on their infection by HIV-1, while the pretreatment of viral stocks reduces infectivity (Cefai et al., 1991; Pleskoff et al., 1995). It has been suggested that MS8209 blocks HIV-1 entry at a step following attachment to the CD4 receptor, possibly membrane fusion, by interacting with the cholesterol-rich envelope of HIV (Pleskoff et al., 1995). The anti-HIV activity of MS8029 seems to depend also upon its interaction with viral envelope proteins, in particular the V3 domain of gp120 (Pleskoff et al., 1996).

We have found that CD-AMB is less effective than AMB in reducing virus production in acutely infected cells, but the antiviral effect could be significantly enhanced by maintaining the drug throughout the culture period. This observation is in agreement with results obtained with MS8209 (Magierowska-Jung et al., 1996). The enhancement of the anti-HIV effect by continuous treatment may be explained by three, not mutually exclusive, mechanisms: (i) additional inhibitory effect on other steps of viral replication, (ii) the inhibition of virus entry occurring after the adsorption step, and (iii) the inhibition of the secondary spread of infection occurring during the 10 day culture. Previous in vitro experiments indicate that MS8209 does not interfere with reverse transcription (Cefai et al., 1991). Thus, the continuous presence of CD-AMB or AMB most likely inhibited virus entry. Bicyclams, anti-HIV compounds which are specifically targeted to the virus-cell fusion process, are still effective when added 1–2 h following virus adsorption (Donzella et al., 1998).

Both CD-AMB and AMB were much less effective at inhibiting cell-cell fusion than virus-cell fusion. A slight reduction of syncytium formation was observed only at 10 and 20 μg AMB/ml. These observations are in agreement with results obtained with MS8209 (Pleskoff et al., 1995). Moreover, several studies have shown that AMB can enhance virus-induced fusion of cells normally permissive to infection (Kataoka and Koprowski, 1975; Pinter et al., 1986; Hansen et al., 1990). AMB can also facilitate fusion of cells expressing HIV-2 or SIV envelope proteins with $CD4^+$ murine cells (McKnight et al., 1994). Other compounds, e.g. heparin (Baba et al., 1990) and bicyclams (De Clercq et al., 1992; De Vreese et al., 1996) have a similar mode of action, in that they are not inhibitory to syncytium formation at concentrations that are inhibitory to HIV entry. Interestingly, it has been shown recently that the bicyclam AMD3100 interacts with CXCR4, the coreceptor used by T-cell tropic viruses to infect target cells (Schols et al., 1997; Donzella et al., 1998). It is tempting to speculate that binding of AMB to the HIV envelope, especially to the V3 domain (Pleskoff et al., 1996), may affect the interaction of the virus with the CXCR4 coreceptor. On the other hand, interaction of AMB with cell membranes may change the affinity of the virus for CXCR4 after binding to the CD4 receptor.

The question raised by our findings is why cell-cell fusion should be less affected by AMB and CD-AMB than virus-cell fusion. The viral and cellular requirements of HIV-1 entry and syncytium formation may not be totally identical (Callebaut et al., 1994; Konopka et al., 1995; Pleskoff et al., 1995) and it is possible that AMB can interact more effectively with the membrane accessory molecules that may be involved in virus-cell but not in cell-cell fusion. The bicyclam AMD3100 that interacts with the CXCR4 coreceptor, inhibits cell-cell fusion at concentrations that are about 100-fold higher than those required for inhibition of HIV entry (Schols et al., 1997; Donzella et al., 1998). Thus, even interaction with the same molecule on the cell surface does not result in an identical effect on cell-cell and virus-cell fusion.

There was no direct correlation between the effects of CD-AMB and AMB on syncytium for-

mation and the spread of infection. CD-AMB did not inhibit syncytium formation while it significantly delayed spreading of infection. In spite of its only slightly inhibitory effect on syncytium formation, AMB completely blocked virus spread. These findings most likely support the hypothesis that the spread of infection occurs mostly by newly released virions, not by cell-cell transmission. The presence of CD-AMB during spreading of infection did not prevent replication of the virus. Although p24 production was markedly reduced on day 2, it rose throughout the course of the experiment and reached the level of untreated controls on day 7. This observation may be due to the rapid kinetics of virus production in the course of the highly cytopathic infection of SupT1 cells.

It is not straightforward to translate our in vitro results into in vivo antiviral efficacy. Nevertheless, they support the possibility of designing effective and less toxic lipid formulations of AMB, which may not only treat opportunistic fungal diseases associated with HIV infection, but also act on HIV itself.

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