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# Derivatives of amphotericin inhibit infection with human immunodeficiency virus in vitro by different modes of action

John-Erik S. Hansen[1], Niels M. Witzke[2], Claus Nielsen[2], Lars R. Mathiesen[1], Lars S. Teglbjærg[1], Carsten M. Nielsen[2] and Jens O. Nielsen[1]

[1]*Department of Infectious Diseases, Hvidovre Hospital, Denmark and*
[2]*Department of Virology, State Serum Institute, Copenhagen, Denmark*

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## Summary

Three water-soluble derivatives of amphotericin B were tested for inhibition of HIV infection in vitro. The compounds amphotericin B methyl ester (AME) and *N*-(*N'*-(2-(4'-methylmorpholinio)ethyl)*N''*-cyclohexyl guanyl) amphotericin B methyl ester (MCG) inhibited HIV infection by 50% at 1 μg/ml; *N*-(*N'*-(3-dimethylaminopropyl)*N''*-ethyl guanyl) amphotericin B (DAPEG) did so at 5–11 μg/ml. While the virus-inhibitory effect of AME was due to an interaction with target lymphocytes, the effect of MCG was due to a direct anti-viral action. AME increased the potential of infected cells to fuse with uninfected cells, but MCG had no significant effect on cell fusion. All compounds had a lower cellular toxicity than amphotericin B and were not toxic at concentrations below 20 μg/ml.

HIV; Amphotericin B; Syncytium formation

## Introduction

Human immunodeficiency virus (HIV) is an enveloped retrovirus (Gallo, 1987). HIV obtains its envelope from infected cells during budding but the envelope has a high cholesterol:phospholipid ratio of approximately 1, as compared to approximately 0.6 for the cell membranes (Aloia et al., 1988; Crews et al., 1988). This indicates that HIV has a very rigid envelope, as also found in other enveloped viruses (Aloia et al., 1988; Crews et al., 1988). The fluidization of the HIV envelope obtained by reducing the cholesterol content by 30% corresponds to the fluidization obtained by heating to 56°C (Aloia et al., 1988).

*Correspondence to:* J-E.S. Hansen, Department 144, Hvidovre Hospital, 2650 Hvidovre, Denmark.

The polyene macrolide antibiotics amphotericin B (AB) and amphotericin B methyl ester (AME) have antiviral properties against a variety of lipid-enveloped viruses like vesicular stomatitis virus, herpes simplex virus and vaccinia virus (Jordan et al., 1978 a,b). AB and AME act by binding to cholesterol in cell and virus membranes and lower the permeability barrier of the lipid membrane by channel formation (Saha et al., 1986; Cybulska et al., 1986; Bolard, 1986). AME has been shown to inhibit the in vitro infection of HIV by either a direct antiviral or a cytoprotective mechanism (Schaffner et al., 1986).

The compound AL-721 has been shown to inhibit HIV infection in vitro (Sarin et al., 1985) probably by lowering the cholesterol content and thus fluidizing the HIV envelope (Crews et al., 1988). The binding of AB to cholesterol in cellular membranes has been shown to result in an enhanced fusion of virus-infected cells (Kataoka and Koprowski, 1975; Pinter et al., 1986), although this has not previously been investigated for HIV.

Modification of the lipid composition of the viral envelope thus offers new potential in antiviral chemotherapy. However, AB is not ideal because of low water-solubility and high toxicity. Its effect on HIV-induced cell fusion is unknown. Small modifications of AB result in both different physico-chemical as well as functional properties (Fisher et al., 1975). Thus AME is both water-soluble and is less toxic to cells than AB but retains the antiviral potency (Jordan and Seet, 1978). Liposomal AB is also less toxic (Pontani et al., 1989).

We have compared the effects on HIV infection and virus-induced cell fusion properties of three different water-soluble derivatives of AB: *N*-(*N'*-(2-(4'-methylmorpholinio)ethyl)*N''*-cyclohexyl guanyl) amphotericin B methyl ester (MCG), *N*-(*N'*-(3-dimethylaminopropyl)*N''*-ethyl guanyl) amphotericin B (DAPEG) and amphotericin B methyl ester (AME).

## Materials and Methods

### *Amphotericin B and derivatives*

The amphotericin B derivatives DAPEG and MCG were prepared as previously described (Witzke, 1981). AME was a gift from Dr C.P. Schaffner, Waksman Institute of Microbiology, Piscataway, New Jersey. The purity of the derivatives was checked by TLC and HPLC as described (Witzke, 1981). Fungizone (E.R. Squibb & Sons) was used as source of amphotericin B.

### *Anti-HIV assay*

A supernatant from H9 cells chronically infected with the reference HIV-1 strain HTLV-$III_B$ (Popovic et al., 1984) was filtered (0.22 $\mu$m), aliquoted, and stored at $-80°C$ until use as virus source for infectivity experiments. Before use, the $CCID_{50}$ (50% cell culture infectious dose) of the virus preparation was determined by end-point titration in MT-4 cells (Harada et al., 1985).

MT-4 cells ($1 \times 10^6$ cells) were suspended in 500 $\mu$l growth medium (RPMI 1640, 10% fetal calf serum, containing 100 IU/ml penicillin, 20 $\mu$g/ml gentamicin and 100 IU/ml streptomycin) containing an appropriate concentration of derivative and incubated with 20 $CCID_{50}$ HTLV-$III_B$ (50 $\mu$l) for 2 h at 37°C. After extensive washing, the infected cells were resuspended in 5 ml growth medium containing an appropriate concentration of derivative, and quadruplicates of 1 ml suspension (200 000 cells) were transferred to a 24-well cell culture plate. The cells were cultured for 7 days at 37°C, 5% $CO_2$. After 4 days, 500 $\mu$l cell-free supernatant was exchanged with fresh medium containing an appropriate concentration of the derivative. In some experiments, either the MT-4 cells or the virus-inoculum were preincubated with the derivative prior to inoculation of the cells. In these experiments, inoculation was performed in growth medium without derivative.

HIV antigen production was measured by a double antibody sandwich ELISA on cell-free supernatants in 96-well immunoplates as described previously (Nielsen et al., 1987). Each plate included a dilution series of a standard HIV antigen preparation, and extinction values (OD 490 nm) were expressed relative to this standard preparation (arbitrary units). All in vitro infection experiments included an untreated control.

To assess the effect on the infectivity of virus produced in the presence of derivative, H9 cells chronically infected with HTLV-$III_B$ were cultured in 10 $\mu$g/ml of each derivative along with an untreated control culture. After 1 week, viable cell counts were obtained by trypan blue exclusion and HIV antigen concentrations in the culture supernatants were determined. The $CCID_{50}$ of these supernatants was then determined by end-point titration: 30 000 MT-4 cells in each well of a 96-well cell culture plate were infected in quadruplicates by five-fold dilutions of the H9/HTLV-$III_B$-supernatants. After 1 week of culture, HIV antigen concentrations were measured (cut-off: mean of negative controls + 3 SD) and the $CCID_{50}$ determined (by the Reed-Muench method).

*Syncytium assay*

Uninfected CEM cells or HTLV-$III_B$-infected H9 (H9/HIV) cells were cultured for 1 week in 0, 10 or 20 $\mu$g derivative per ml growth medium. Then, a two-fold dilution of H9/HIV cells ($10^5$–$10^2$ cells per well) were incubated in a 96-well cell culture plate together with $2 \times 10^5$ CEM cells in a total volume of 100 $\mu$l medium per well. Syncytial activity was defined as the maximal dilution of H9/HIV cells in which syncytia were found after 18 h incubation at 37°C, and expressed relative to untreated control cultures. A compound which inhibits syncytium formation will thus result in a syncytial activity below 1, and an syncytium-enhancing compound will result in a syncytial activity above 1.

*Toxicity assay*

MT-4 cells (200 000) in 1 ml growth medium containing a dilution series of derivative were cultured in 24-well cell culture plates for 7 days. After 4 and 7

days, 50 $\mu$l cell-suspension from each well was used to obtain viable cell counts using trypan blue exclusion.

*PCR and dot-blot hybridization*

PCR was performed as described previously (Hansen et al., 1990). Briefly, samples were boiled for 10 min to release DNA, and 10 $\mu$l DNA (from approximately $10^4$ cells) was used for PCR. Forty rounds of amplification were carried out in a DNA Thermal Cycler (Perkin-Elmer Cetus). Each cycle started with denaturation for 1 min at 94°C, followed by cooling to 65°C for 1 min to allow primer annealing. The primer extension step was carried out at 72°C for 1 min. The primers used were SK29/SK30 and the probe was SK31 (Ou et al., 1988). Subsequently, 10 $\mu$l of the amplified product were spotted onto a Zeta-probe membrane, hybridized overnight with a $^{32}$P-labelled probe and exposed to Kodak XAR autoradiography films.

## Results

*Inhibition of virus infection*

Three water-soluble derivatives of amphotericin B (Fig. 1) inhibited HIV infection of the T cell line MT-4 (Fig. 2). When target cells were inoculated with the HIV isolate HTLV-III$_B$ in a dilution series of each compound, the 50% inhibitory

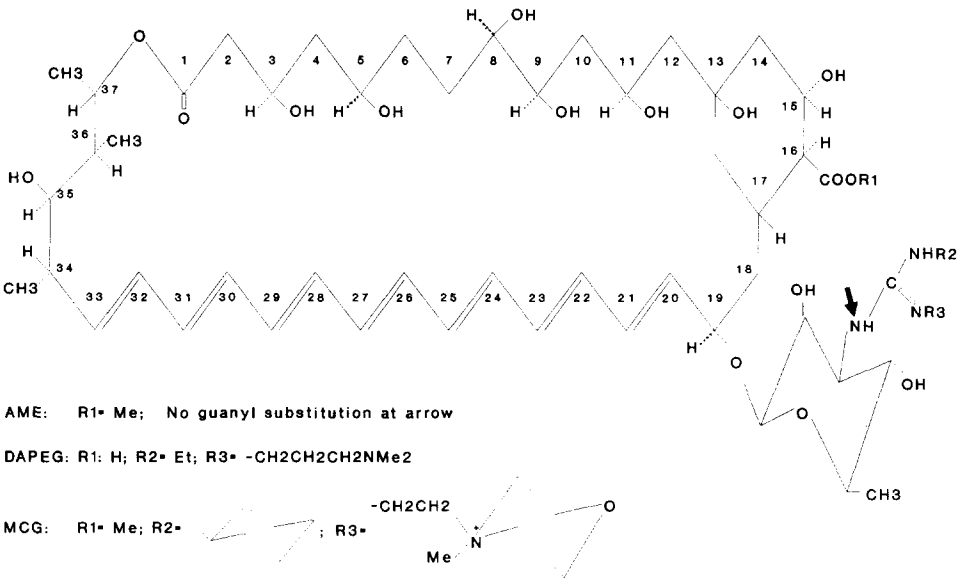


Fig. 1. Structure of three water-soluble derivatives of amphotericin B. AME: amphotericin B methyl ester; MCG: *N*-(*N'*-(2-(4'-methylmorpholinio)ethyl)*N''*-cyclohexyl guanyl) amphotericin B methyl ester; DAPEG: *N*-(*N'*-(3-dimethylaminopropyl)*N''*-ethyl guanyl) amphotericin B.

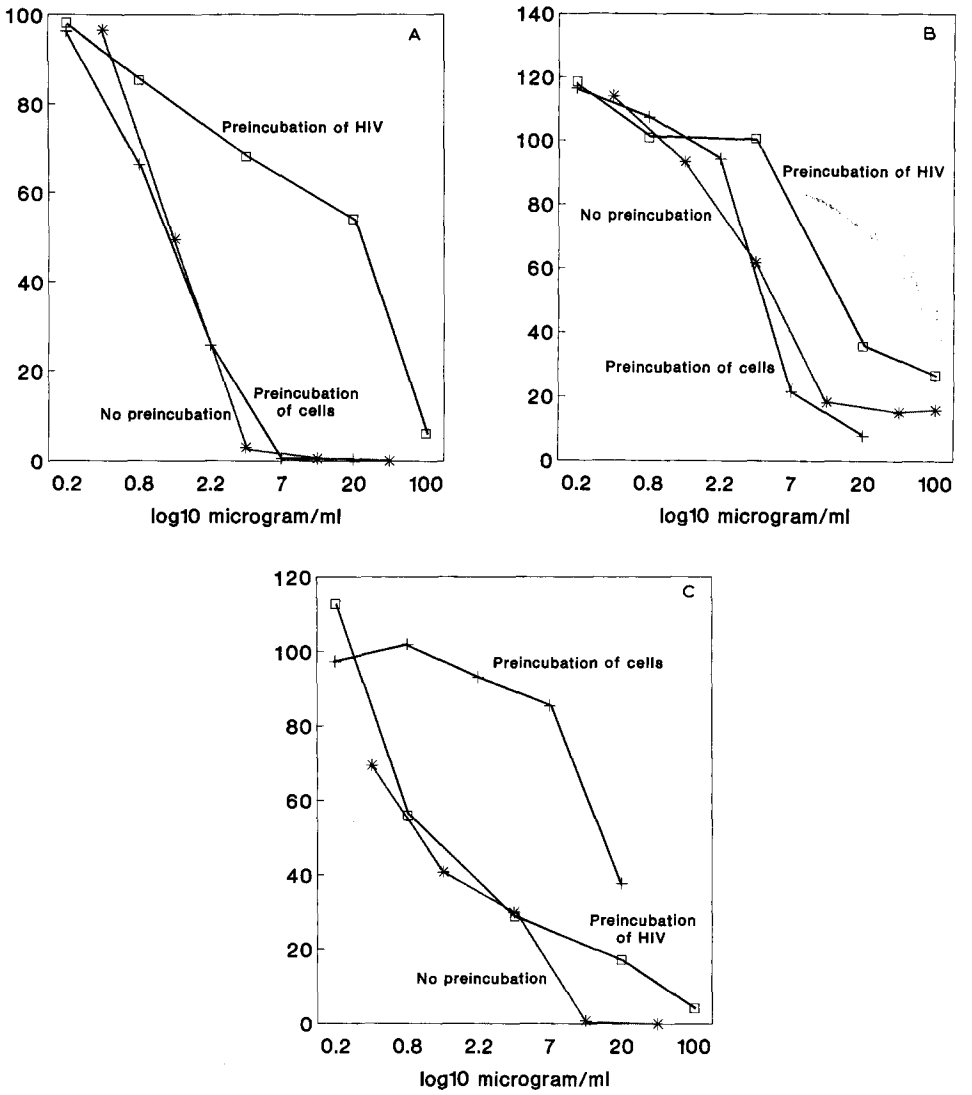


Fig. 2. Inhibitory effects of three derivatives of amphotericin (AME, DAPEG and MCG; panels A, B and C, respectively) on HIV infection in MT-4 cultures. MT-4 cells were inoculated with 20 $CCID_{50}$ $HTLV\text{-}III_B$ in the presence of varying concentrations of the derivative, and cultured for 7 days (no preincubation); or either MT-4 cells or HIV were preincubated with the derivative prior to inoculation. Results are expressed as HIV antigen level in culture medium at day 7 (in percent of the antigen level in untreated control cultures).

concentrations ($IC_{50}$), as evaluated by HIV antigen concentration in culture supernatants after 7 days of culture, were 1–6 $\mu$g/ml (Table 1). To see whether the compounds exerted their anti-HIV effect by interaction with virus or with target cells, either the viral inoculum or the target cells were preincubated with a dilution

TABLE 1
Inhibitory concentrations (IC) of three derivatives of amphotericin whether or not preincubated with either lymphocytes (overnight) or HIV (1 h) before inoculation

| Derivative | $IC_{50}$ ($\mu$g/ml) | | |
|---|---|---|---|
| | Preincubated with | | No preincubation |
| | Cells | HIV | |
| AME | 1.2 | 21.3 | 1.1 |
| DAPEG | 5.3 | 11.3 | 6.2 |
| MCG | 16.2 | 1.1 | 1.7 |

IC values are expressed as compound concentrations ($\mu$g/ml), interpolated by logaritmic regression, resulting in 50% reduction of HIV antigen concentration in culture medium after 7 days incubation.

series of the compounds prior to inoculation in compound-free medium (Fig. 2 and Table 1). This revealed an apparent difference in mode of action. Preincubation of the cells with AME showed the same dose-response and $IC_{50}$ (1.2 $\mu$g/ml) as no preincubation ($IC_{50}$ 1.1 $\mu$g/ml) while preincubation of HIV had a different dose-response and $IC_{50}$ (21.3 $\mu$g/ml). MCG showed the opposite: the dose-response and $IC_{50}$ (1.1 $\mu$g/ml) of preincubation of HIV was similar to no preincubation ($IC_{50}$ 1.7 $\mu$g/ml), but preincubation of cells was different ($IC_{50}$ 16.2 $\mu$g/ml). DAPEG behaved similarly to AME but with less pronounced differences between preincubation of cells and preincubation of HIV.

Cells were then incubated in a relatively high concentration of each compound (10 $\mu$g/ml), inoculated with 20 $CCID_{50}$ HIV and cultured for 7 days. After 4 and 7 days, HIV antigen concentration in culture supernatants was measured by ELISA, and HIV-DNA in samples of cells was analyzed by polymerase chain reaction. This showed (Table 2) that the cytoprotective effect of AME at 10 $\mu$g/ml was complete, as neither HIV antigen in culture supernatants nor cellular HIV-DNA appeared. Both DAPEG and MCG retarded the expression of HIV antigen in culture supernatants as no significant amounts were detectable after 4 days of culture, although the cells of were infected as revealed by the presence of cellular HIV-DNA. After 7 days of culture also HIV antigen expression in these cultures became apparent.

TABLE 2
MT-4 cells were preincubated with 10 $\mu$g/ml amphotericin B derivative, washed, inoculated with 20 $CCID_{50}$ HTLV-$III_B$ in growth medium and subsequently incubated for 7 days in the presence of 10 $\mu$g/ml of each compound. HIV antigen concentration in culture medium was measured at days 4 and 7 and expressed as percent of untreated control. Presence of cellular HIV-DNA at days 4 and 7 was analyzed by hybridization with an HIV-specific probe after amplification using the polymerase chain reaction

| Compound: | AME | | DAPEG | | MCG | |
|---|---|---|---|---|---|---|
| Day: | 4 | 7 | 4 | 7 | 4 | 7 |
| | HIV antigen concentration (%) | | | | | |
| | 0.2 | 3.2 | 3.2* | 92.9* | 3.1* | 70.2* |

*Cells from MCG- and DAPEG-treated cultures contained HIV-DNA. AME-treated cultures were negative for HIV-DNA.

*Reduction of HIV infectivity*

Permissively HTLV-$III_B$-infected H9 cells were cultured with 10 μg/ml of the three derivatives and the infectivity of the virus produced during this treatment was estimated by end-point titration in MT-4 cells (Table 3). All three derivatives reduced the $CCID_{50}$ of the respective culture supernatants compared to an untreated control-culture ($CCID_{50}^{-1}$ = 6250). The most effective was AME ($CCID_{50}^{-1}$ = 250) and the least effective was DAPEG ($CCID_{50}^{-1}$ = 2000). The cell counts in the derivative-treated cultures were higher than in the untreated cultures, and HIV antigen concentration in the supernatants from the derivative-treated cultures was only slightly reduced as compared to the untreated cultures.

*Enhancement of syncytial activity*

The ability of the three amphotericin derivatives to influence the formation of syncytia was assessed by preincubating chronically infected H9 cells and uninfected CEM cells with 0–20 μg/ml of each compound and comparing the syncytial activity (end point) in series of cocultures consisting of decreasing numbers of infected cells and a fixed number of uninfected cells (Fig. 3). We found that this modification of traditional syncytium assays (using fixed amounts of both cell-types and counting the number of individual syncytia) eliminated the inaccuracies of counting and evaluating the size of syncytia, which we found especially difficult when compounds enhanced syncytium formation. By this modified assay it was thus possible to evaluate both inhibition and enhancement of syncytium formation. The results showed that AME enhanced the syncytial activity when H9/HIV cells were preincubated with this derivative in high concentration (20 μg/ml). The magnitude of this enhancement was similar to the enhancement by AB (not shown). MCG did not show significant enhancement of syncytial activity compared to untreated controls. The effect of DAPEG on syncytial activity was intermediary between those of AME and MCG, i.e. enhancement (4 times control) at 20 μg/ml and borderline inhibition (0.5 times control) at 10 μg/ml.

TABLE 3
Reduction of HIV-infectivity by derivatives of amphotericin B

| | No drug | AME | MCG | DAPEG | |
|---|---|---|---|---|---|
| Number of viable cells: | 1.44 | 1.64 | 1.78 | 1.64 | $\times 10^6$/ml |
| Amount of HIV antigen: | 600 | 510 | 400 | 440 | arbitrary units |
| Infectivity | 6250 | 250 | 500 | 2000 | $CCID_{50}^{-1}$ |

H9 cells chronically infected with HTLV-$III_B$ were cultured in the presence of 10 μg/ml derivative. After 1 week, live cell and HIV antigen concentrations were measured and $CCID_{50}$ of the culture supernatants was determined in MT-4 cells by end-point titration.

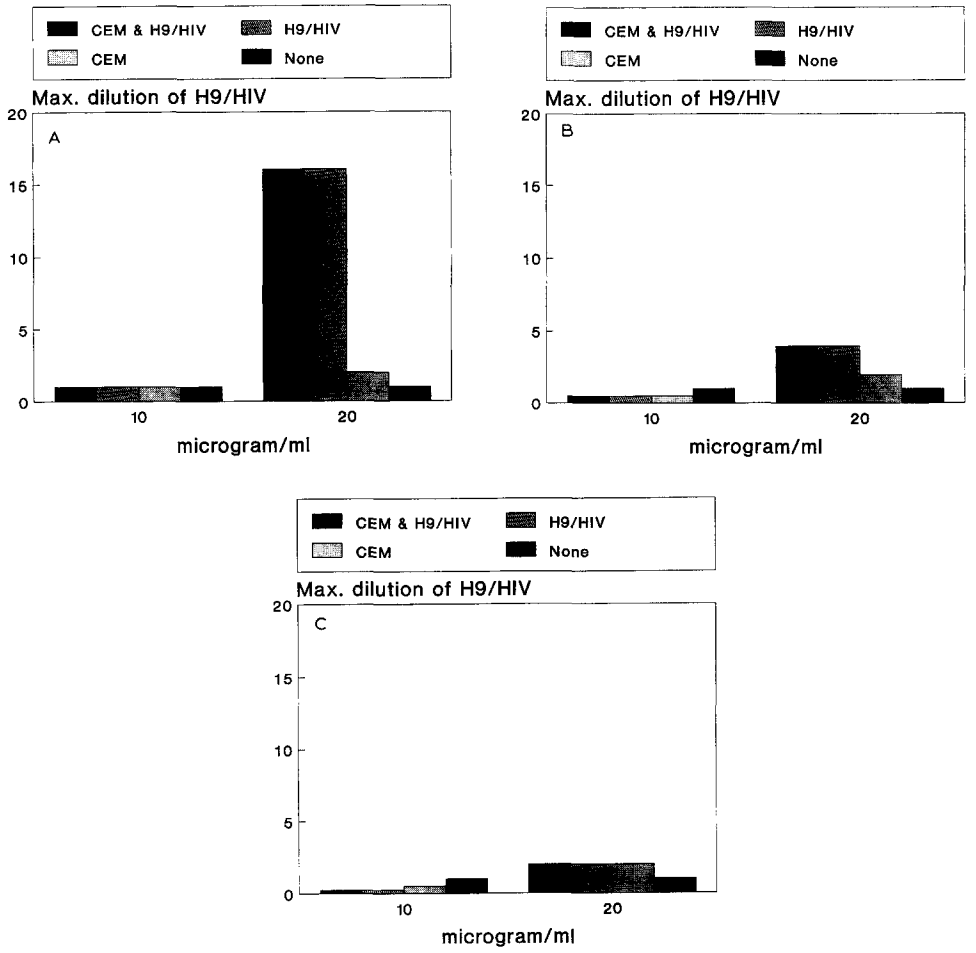


Fig. 3. Syncytial activity after preincubation of uninfected CEM cells and/or HIV infected H9 cells for 1 week with derivatives of amphotericin B (AME, DAPEG and MCG; panels A, B and C, respectively). Syncytial activity was defined as the maximal dilution of H9/HIV cells in which syncytia were found after 18 h of coculture, and expressed relative to untreated control cultures. A compound which inhibits syncytium formation will thus result in a syncytial activity below 1, and a compound which enhances syncytium formation will result in a syncytial activity above 1.

## *Toxicity*

Toxicity of the three amphotericin derivatives was assessed by culturing MT-4 cells in dilution series of compound (from 100 $\mu$g/ml). All three derivatives behaved essentially identically and showed, compared to amphotericin B, a low toxicity (Table 4). Viable cell counts showed no significant reduction in cell viability or growth potential up till 20 $\mu$g/ml. At 100 $\mu$g/ml there was some reduction in the growth rate.

TABLE 4
Toxicity of three derivatives of amphotericin B in MT-4 cells. Cells were cultured in 24-well cell culture plates in 1 ml growth medium containing varying concentrations of the derivative. Viable cell counts were determined by trypan blue exclusion. Results are expressed as number of viable cells × $10^4$ per well

| Derivative | μg/ml | Day 0 | Day 4 | Day 7 |
|---|---|---|---|---|
| AME | 0 | 46 | 86 | 358 |
| | 0.16 | 46 | 80 | ND |
| | 0.8 | 46 | 90 | ND |
| | 4 | 46 | 94 | 331 |
| | 20 | 46 | 104 | 322 |
| | 100 | 46 | 70 | 133 |
| DAPEG | 0 | 46 | 78 | 368 |
| | 0.16 | 46 | 92 | ND |
| | 0.8 | 46 | 86 | ND |
| | 4 | 46 | 84 | 339 |
| | 20 | 46 | 90 | 327 |
| | 100 | 46 | 58 | 225 |
| MCG | 0 | 46 | 60 | 363 |
| | 0.16 | 46 | 90 | ND |
| | 0.8 | 46 | 88 | ND |
| | 4 | 46 | 74 | 326 |
| | 20 | 46 | 94 | 303 |
| | 100 | 46 | 66 | 156 |

Percentage of viable cells was > 85%. ND, not determined. For comparison, cells cultured in the presence of 50 μg/ml amphotericin B (fungizone) yielded a viable cell count of 5 × $10^4$ at day 4.

## Discussion

Three derivatives of amphotericin B were all able to inhibit in vitro infection by HIV. These compounds also reduced the infectivity of virions produced in cultures containing non-toxic amounts of any derivative. Of the three derivatives, AME was the most effective both in inhibiting HIV infection and HIV infectivity. In a previous study (Schaffner et al., 1986), AME was found to inhibit HIV infection although it was not clear whether the effect of AME was preferentially caused by a direct antiviral action or by interference with the target cells. We found that AME seems to protect the target cells against infection rather than act on the HIV envelope, as AME inhibited infection when preincubated with target cells but not when preincubated with virus. This inhibition was apparent both from the absence of HIV antigen in the culture medium as well as from the absence of HIV-DNA in the target lymphocytes after 7 days of culture. How this protection is achieved is unclear. AME is known to bind to cholesterol in lipid membranes (Bolard, 1986) and this might interfere with the penetration of the virus/receptor complex in target lymphocytes.

Although almost as effective as AME, MCG did not seem to act in the same way as AME. Infection was inhibited by preincubation of the virus but less so by preincubation of the target cells. A direct antiviral effect of this differently modified amphotericin B derivative could be due to either a fluidization of the viral envelope with disturbances in orientation and presentation of viral glycoproteins (Crews et

al., 1988) or it could be caused by a lowering of the permeability barrier of HIV virions by channel-formation in the viral envelope (Cybulska et al., 1986). The preferential susceptibility of virions to the harmful effects of channel formation could be due to lack of repair mechanisms present in cells, as suggested previously (Jordan and Seet, 1978).

The 50% HIV inhibitory concentrations ($IC_{50}$) of AME and MCG were 1 $\mu$g/ml, which corresponds to the $IC_{50}$ of AME against other viruses (Jordan and Seet, 1978; Stevens et al., 1975) and to the $IC_{50}$ of liposomal AB against HIV (Pontani et al., 1989). AME in this dosage range has earlier been found to be non-toxic in humans (Hoeprich et al., 1976), and we found no cell toxicity of the derivatives at 20 $\mu$g/ml. Protein-binding of the derivatives (Witzke, 1981) was similar to that of AB (70–90% binding to human serum) and this may influence the antiviral dose-response profile in vivo.

The derivatives which protected target-lymphocytes against infection, AME and, to a lesser extent, DAPEG, enhanced fusion between infected, gp120-bearing lymphocytes and uninfected, CD4-bearing lymphocytes. This enhancement was seen after preincubation of the compound with infected but not uninfected lymphocytes. The derivative MCG, which had a direct antiviral effect but no protective effect on uninfected target cells, did not show significant enhancement of syncytium formation. Enhancement of syncytial activity has been previously described for other retroviruses (Pinter et al., 1986; Kataoka and Koprowski, 1975), but the mechanism of this differential mode of action, i.e., that a compound which interacts with uninfected cells and protects them against infection also interacts with infected cells and enhances cell fusion, is puzzling. It does indicate, however, that the molecular events leading to virus-cell penetration may not be completely identical to the mechanisms responsible for cell-cell fusion.

AME was the most effective inhibitor of infection with cell-free virus, but as the mechanism of action seemed to involve both a protective interaction with uninfected lymphocytes and a fusion-enhancing effect on already infected lymphocytes, this compound could theoretically enhance cell-cell transmission of an already established infection. The inhibitory effect of MCG seems to be due to a direct action on the virions and this derivative does not show significant enhancement of syncytial activity. Thus, this study supports the notion that interference with the lipid composition of the cell and/or viral envelope has potential as an anti-HIV strategy. Furthermore, this study shows that small modifications of the polyene macrolide structure can modify both the toxicity and the mode of action of such compounds.

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