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Anti-HIV-1 activity of sulfated amphotericin B in vitro

Toru Otake[1], Keiichi Miyano[1], Haruyo Mori[1], Motoko Morimoto[1], Noboru Ueba[1], Nobuharu Kunita[1], Hideki Nakashima[2] and Takashi Kurimura[3]

[1]*Osaka Prefectural Institute of Public Health, Osaka,* [2]*Department of Microbiology, Tokyo Medical and Dental University School of Medicine, Tokyo and* [3]*Research Institute for Microbial Diseases, Osaka University, Suita, Japan*

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Summary

To reduce the toxicity of amphotericin B methyl ester (AME), which shows some anti-HIV-1 activity, sulfated amphotericin B (SAB) was prepared from amphotericin B (AB), and its anti-HIV-1 activity was examined in vitro. SAB at concentration of 7.8 μg/ml completely suppressed the HIV-1-induced cytopathic effect in MT-4 cells, at 3.9 μg/ml inhibited the expression of HIV-1 antigen in peripheral blood mononuclear cells infected with freshly isolated HIV-1 and at 22 μg/ml completely suppressed formation of giant cells in co-cultures of MOLT-4 with MOLT-4/HIV-1 cells. Reverse transcriptase activity was inhibited by SAB, but only at higher concentrations (0.2–1 mg/ml). Furthermore, the toxicity of SAB was lower than that of AME or AB, and SAB did not affect the proliferation of MT-4 cells at concentrations up to 0.5 mg/ml. The anti-coagulant effect of SAB was 10-fold less than that of dextran sulfate (MW = 8000). The anti-HIV-1 effect of SAB is attributed to inhibition of binding of virions to target cells.

Human immunodeficiency virus type 1 (HIV-1); Sulfated amphotericin B; Amphotericin B methyl ester; AIDS

Correspondence to: Dr. T. Otake, Osaka Prefectural Institute of Public Health, 1-3-69, Nakamichi, Higashinari-ku, Osaka 537, Japan.

Introduction

Several therapeutic or prophylactic drugs have been developed for patients with acquired immunodeficiency syndrome (AIDS) or human immunodeficiency virus (HIV) carriers; azidothymidine (AZT) (Mitsuya et al., 1985), recombinant CD4 (Smith et al., 1987; Traunecker et al., 1988) or interferon (Gelmann et al., 1985; Ho et al., 1985) are known as representative drugs that inhibit HIV replication. For recent review, see Mitsuya and Broder (1990), De Clercq (1990) and, in this journal, De Clercq (1989). AZT is the only drug that has been approved as a drug for AIDS patients. It shows an excellent anti-HIV effect in vitro and prolongs life of AIDS patients (Fischl et al., 1987). However, the drug has serious side effects on hematopoietic functions (Richman et al., 1987). Sulfated polysaccharides such as dextran sulfate and heparin also exhibit anti-HIV activity (Ito et al., 1987; Nakashima et al., 1987b), and clinical studies with dextran sulfate have been conducted in the U.S.A. However, both drugs have an anti-coagulation effect, which limit their clinical usefulness.

Amphotericin B methyl ester (AME) (Mechlinski et al., 1972) is a water-soluble derivative of amphotericin B (AB) that has been used as a therapeutic drug for the treatment of candidiasis of AIDS patients and possesses an inhibitory effect on the replication of enveloped viruses such as herpesviruses and retroviruses (Chirigos et al., 1973; Stevens et al., 1975). Furthermore, its anti-HIV-1 effect has been described a few years ago (Schaffner et al., 1986). However, AB is known to be highly toxic in vivo and in vitro, which was confirmed by our experiments. However, AB is advantageous in that it can be absorbed by the oral route. We reasoned that sulfation may enhance anti-HIV activity of AB and reduce its toxicity, and, therefore, synthesized and evaluated the in vitro anti-HIV-1 activity of sulfated AB.

Materials and Methods

Preparation of test compounds

Sulfatation of AB was conducted as described by Lloyd (1962). AB was reacted with pyridine-sulfur trioxide complex under the presence of pyridine at 60 C for 6 h. The product was purified with a cation exchange column, and SAB (potassium salt) was obtained. The chemical structure of this material is unknown, but HPLC of this material showed only 1 major peak. It is a water-soluble (pH 7) brown material. Sulfur content of SAB is about 12%.

AME was prepared according to the method described by Mechlinski and Schaffner (1972), and it was used as its ascorbic acid salt.

Cells

Established cell lines derived from T lymphocyte The HLTV-I-carrying cell line MT4 and the human leukemic T-cell line MOLT-4 were used. They were

maintained at 37°C under 5% CO_2 in RPMI-1640 medium (Flow Laboratories) supplemented with 10% fetal calf serum (FCS) and 100 μg/ml of streptomycin (Meiji Seika) and 100 U/ml of penicillin G (Banyu Pharmaceutical).

Peripheral blood mononuclear (PBM) cells from healthy donors PBM cells were separated from peripheral blood of healthy donors by the Ficoll-Paque (Pharmacia) centrifugation method. Then, the cells were cultured for 3 or 4 days in medium containing 0.1% PHA (Difco Laboratories), 20% FCS and antibiotics.

Virus

The LAV-1 and HTLV-III strains of HIV-1 were obtained from culture supernatant of MOLT-4 cells which had been persistently infected with LAV-1 or HTLV-III. The KK-1 strain of HIV-1 used in the PBM cells assays (Otake et al., 1990) was isolated from a Japanese AIDS patient.

Assay of cytopathic effect of HIV-1 in MT-4 cell line

The inhibitory effect of the test compounds on cytopathic activity of HIV-1 was measured by the method of Harada et al. (1985). MT-4 cells were infected with the LAV-1 strain of HIV-1 at 0.001 $TCID_{50}$/cell for 1 h at 37°C. They were washed once and resuspended at 1×10^5 cells/ml in medium containing the test compound. Two hundred microliters of the suspension was cultured in each well of a 96-well culture plate for 5 days; the assays were run in quadruplicate. The number of viable cells was assessed by the trypan blue exclusion method.

To examine the effect of SAB on virus adsorption, the test compound was added to the medium for 1 h during the virus adsorption period. Then, the cells were washed twice and further cultured in the absence of SAB.

Anti-HIV-1 activity assay with KK-1 strain in PBM cells

PHA-stimulated PBM cells were infected with the freshly isolated KK-1 strain of HIV-1 for 3 h at 37°C. Then, cells were washed twice and resuspended at 2×10^6 cells/ml, in medium containing 200 U/ml of interleukin-2 (Shionogi Laboratories), 20% FCS and test compound. One ml of the suspension was cultured in each well of a 24-well culture plate for 6 days, and half of the suspension was collected. The number of viable cells was determined by the trypan blue exclusion method. The PBM cells were examined for HIV-1-specific antigen expression by indirect immunofluorescence. The cells were fixed with cold acetone in glass slides, and incubated with human polyclonal antibody to HIV-1 for 30 min at 37°C. Then the cells were washed with phosphate-buffered saline (PBS), incubated with FITC-labeled goat anti-human IgG for 30 min at 37°C and washed with PBS. More than 600 cells were counted under a fluorescence microscope, and the percentage of IF-positive

cells was determined. A total of 1×10^6 cells (in 0.5 ml of the medium containing the test compound) was added to the remaining half of the cells, and the procedure described above was repeated after 4 days.

Suppressive effect on giant cell formation

According to the method described by Nakashima et al. (1987a), the suppressive effect of the test compounds on HIV-1-induced giant cell formation was assayed. MOLT-4 and MOLT-4/HTLV-III were mixed at a ratio of 1:1 (total cell number of 5×10^5 cells/ml), and the mixture was cultured. After a 24-h co-culture period, the number of viable cells was counted by the trypan blue exclusion method, and the fusion index (FI) was calculated as follows:

$$FI = 1 - \frac{\text{Cell number in test well (MOLT-4 + MOLT/HTLV-III)}}{\text{Cell number in control (MOLT-4 cells)}}$$

With this definition, the FI can vary between 0 (no fusion) and 1 (total fusion, no viable cells remain). The FI values obtained for each concentration of the compound can be expressed as a fraction of the control value. Thus, percentage of fusion inhibition was calculated as:

$$\% \text{ fusion inhibition} = \left(1 - \frac{FI_1}{FI_2}\right) \times 100$$

where FI_1 is the fusion index of the test sample and FI_2 is the fusion index of the control sample.

Virus binding assay

Binding of HIV-1 particles to the cell surface was evaluated as described by Schols et al. (1989). MT-4 cells were exposed to HIV-1 (at 100 × concentrate of the culture supernatant of MOLT-4/HTLV-III) and the various concentrations of SAB. After incubation at 37 C for 1 h, the cells were washed twice to remove unbound virus. Then the cells were processed for indirect immunofluorescence using polyclonal antibody to HIV-1 and analyzed for HIV-1 particles bound to the cells by laser flow cytofluorography. The binding inhibitory activity ratio (BI) was calculated as follows:

$$BI = 1 - \frac{\%\ MF\ (VC) - \%\ MF\ (CC)}{\%\ MF\ (V) - \%\ MF\ (C)} \times 100$$

where MF (VC) = mean fluorescence for the HIV-infected cells treated with compound,
MF (CC) = mean fluorescence for the control cells (not exposed to HIV-1) treated with compound,

MF (V) = mean fluorescence for the HIV-infected cells not treated with compound and
MF (C) = mean fluorescence for the control cells (not exposed to HIV-1 and not treated with compound).

Direct effect of SAB on HIV-1

Culture medium of MOLT-4/LAV-1 cells was incubated with SAB for 2 h at room temperature. Then, the suspension was ultracentrifuged at 40 000 rpm for 30 min (Beckman rotor SW 50). The precipitate was resuspended in medium without the test compound. The infectious titers of HIV-1 were assayed both before and after treatment. The cytopathic effect of HIV-1 was measured in MT-4 cells.

Inhibitory effect on reverse transcriptase (RT) activity in vitro

The effect of the test compounds on RT activity was assayed according to the RT activity assay method described by Lee et al. (1987). Culture medium of MOLT-4/LAV-1 cell line was ultracentrifuged at 28 000 rpm for 1 h (Beckman rotor SW28). One-tenth volume of virus-solubilizing buffer was added to the precipitate. Fifty microliters of the test compound solution were added to 50 μl of the buffer, and the mixture was incubated for 30 min at room temperature.

Then 90 μl of RT reaction buffer were added to the mixture. To 95 μl of the mixture, 10 μCi of [^{3}H]dTTP (ICN Biomedicals) and 2.5 μg/5 μl template-primer [poly(rA)-$(dT)_{12-18}$] (Pharmacia) were added, and the mixture was incubated for 22 h at 37°C. The reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid. Precipitated DNA was collected on a glass filter, and acid-insoluble radioactivity was assayed by a liquid scintillation spectrometer.

Assay of anti-coagulation effect

The anti-coagulation effect of the test compounds was assayed following the Zucker's activated partial thromboplastin time (APTT) method (Zucker et al., 1969). To 0.1 ml of human plasma containing 3.8% citric acid, 0.01 ml of the test compound solution (Tris-HCl buffered solution, pH 4.0, 50 mM) was added. The mixture was incubated for 2 min at 37°C and 0.1 ml of ACTIN (activated cephaloplastin reagent) was added. Then 0.1 ml of calcium chloride solution (0.02 M) was added and the time required until coagulation (APTT) was measured.

Results

Effect of SAB on HIV-1 cytopathogenicity in MT-4 cells

When MT-4 cells were infected with HIV-1, the total number of viable cells was reduced from 1.4×10^6 cells/ml to 5×10^4 cells/ml at 5 days after infection. When SAB was added to the medium at a concentration of $\geqslant 7.8$ μg/ml, proliferation of HIV-1-infected MT-4 cells was similar to that of uninfected cells. Thus, under these conditions, the cytopathic effect of the virus was completely suppressed (Fig. 1). Only at 2000 μg/ml, the cytotoxic effect of SAB was apparent and at this concentration, cell proliferation was reduced by about 50%. As shown in Fig. 2, AME suppressed the proliferation of MT-4 cells at a concentration of 33 μg/ml, and the cytopathic effect of HIV-1 was not markedly affected at any concentration of AME.

When SAB was only present at the period of adsorption of HIV-1 to the cells, it again completely suppressed the cytopathic effect of HIV-1 at a concentration of $\geqslant 6.7$ μg/ml (Fig. 3). In fact, the results obtained under these conditions were quite similar to those obtained with SAB that had remained in contact with the cells for the full 5-day observation period.

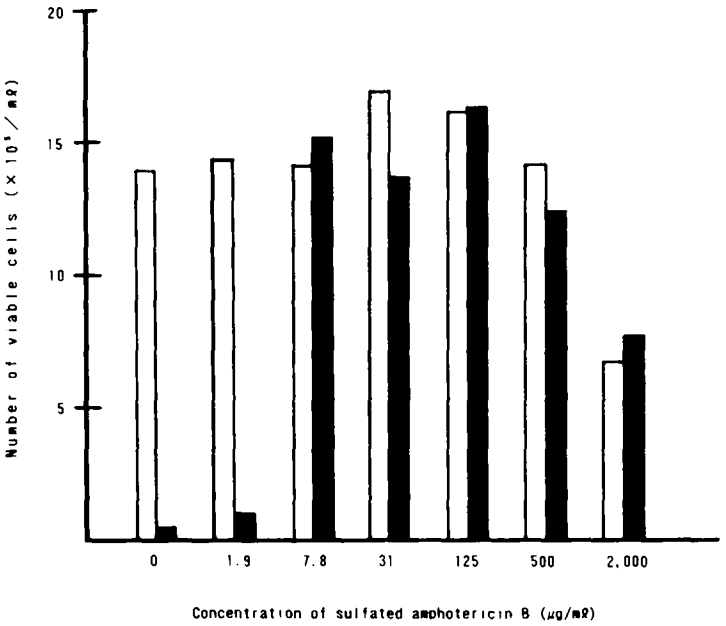

Fig. 1. Effect of SAB on the proliferation of MT-4 cells, infected or not with HIV-1. MT-4 cells were mock-infected or infected with HIV-1 and cultured for 5 days in the presence or absence of SAB. The HIV-1-infected cells are indicated by solid columns (■) and the mock-infected cells are indicated by open columns (□).

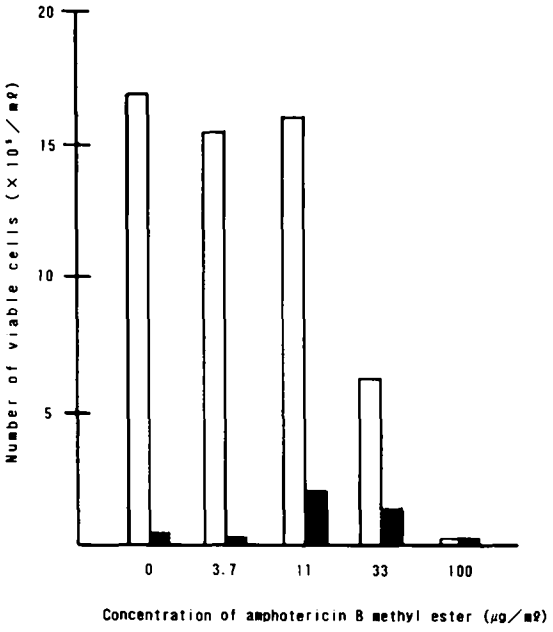

Fig. 2. Effect of AME on the proliferation of MT-4 cells, infected or not with HIV-1. MT-4 cells were mock-infected or infected with HIV-1 and cultured for 5 days in the presence or absence of AME. The HIV-1-infected cells are indicated by solid columns (■) and the mock-infected cells are indicated by open columns (□).

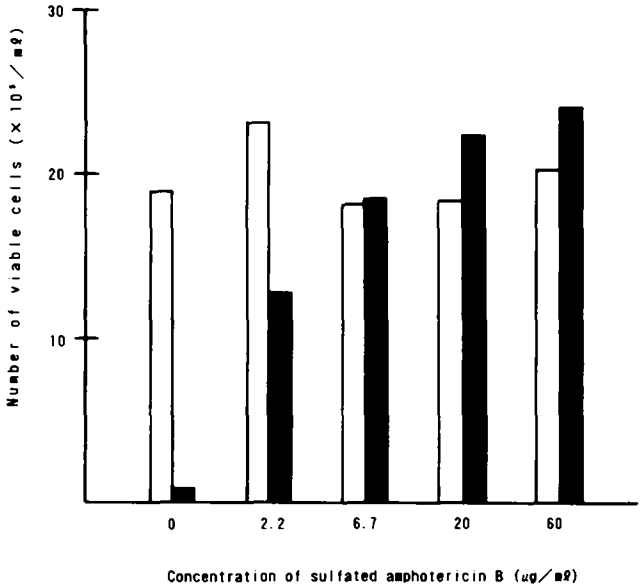

Fig. 3. Effect of SAB on the proliferation of MT-4 cells, infected or not with HIV-1: SAB was present only during the HIV adsorption period. MT-4 cells were washed and then cultured for 5 days in the absence of SAB. The HIV-1-infected cells are indicated by solid columns (■) and the mock-infected cells are indicated by open columns (□).

TABLE 1

Inhibitory effects of SAB on the expression of HIV antigens in peripheral blood mononuclear (PBM) cells infected with freshly isolated HIV (KK-1 strain)

SAB concentration (μg/ml)	Percentage of fluorescence-positive PBM cells	
	Day 6	Day 10
1000	0.68	0
250	0.68	0
62.5	0.76	3.7
15.6	1.2	17.6
3.9	1.3	23.5
0	3.1	35.0

Inhibitory effect of SAB on the replication of the freshly isolated HIV-1 strain KK-1 in PBM cells

Table 1 shows the expression of HIV antigen on PBM cells infected with the KK-1 strain of HIV-1. About 50% reduction in antigen expression was noted at a concentration of 3.9 and 15.6 μg/ml at 6 or 10 days after infection, respectively.

Effect of SAB on giant cell formation

At a concentration of $\geqslant$ 22 μg/ml SAB completely inhibited the formation of giant cells in co-cultures of MOLT-4 with MOLT-4/HTLV-III cells. The 50% inhibitory concentration for giant cell (syncytium) formation was 16 μg/ml. AME did not inhibit syncytium formation even at a concentration of 100 μg/ml.

Effect of SAB on virus adsorption

At a concentration of $\geqslant$ 4 μg/ml SAB effected a concentration-dependent inhibition of HIV-1 binding to MT-4 cells (Table 2).

TABLE 2

Inhibitory effect of SAB on HIV-1 binding to MT-4 cells

SAB concentration (μg/ml)	% Fluorescence-positive cells		BI[a]
	HIV-infected	Mock-infected	
100	1.7	0.3	97.8
20	3.9	1.4	96.1
4	14.5	1.6	79.6
0.8	41.6	1.3	36.1
0	64.0	0.8	

[a]Binding inhibitory activity ratio.

TABLE 3

Direct effect of SAB on HIV-1 infectivity

SAB concentration (μg/ml)	HIV titer measured in MT-4 cells based on viral cytopathogenicity ($TCID_{50}$/ml)
200	$10^{4.5}$
20	$10^{4.5}$
2	$10^{5.25}$
0	$10^{5.5}$

In additional experiments, the possibility was examined of whether SAB might interfere with the binding of OKT-4A/Leu3a monoclonal antibody to MT-4 cells. SAB did not inhibit the binding of OKT-4A/Leu3a to the surface receptor of MT-4 cells, when tested at 4, 20 or 100 μg/ml (data not shown).

Direct effect of SAB on HIV-1 infectivity

Table 3 shows the infectious titer of HIV-1 after exposure to varying concentrations of SAB. The infectious titer of HIV-1 decreased by one $\log_{10}$ after the virus had been exposed to SAB concentrations of 20 and 200 μg/ml.

Inhibitory effect on HIV-1 RT activity

As shown in Table 4, SAB suppressed the RT activity in a concentration-dependent fashion. RT activity was decreased by 30% at 40 μg/ml, 80% at 200 μg/ml, and 98.8 at 1000 μg/ml. AME did not inhibit HIV-1 RT activity.

Anti-coagulant activity

As shown in Table 5, the anti-coagulant activity of SAB was about 1/10 that of dextran sulfate (MW = 8000).

TABLE 4

Effect of SAB and AME on HIV-1 reverse transcriptase (RT) activity

Concentration (μg/ml)	RT activity ($\times 10^4$ cpm)	
	SAB	AME
1000	5.2	398
200	85	401
40	286	420
0	417	417

TABLE 5

Anti-coagulant activity of SAB and dextran sulfate

Test compound	Concentration required to obtain 2-fold APTT[a] (μg/ml, mean ± SD)
SAB	29.3 ± 0.58
Dextran sulfate (MW = 8000)	3.2 ± 0.32

[a]Plasma collected from 3 normal volunteers.

Discussion

In the present study we have demonstrated that SAB inhibits HIV-1 replication in vitro. The minimum concentration of SAB required to totally suppress HIV-1 replication in MT-4 cells (7.8 μg/ml) is comparable to that of dextran sulfate (Ito et al., 1987; Nakashima et al., 1987b; Ueno et al., 1987; Baba et al., 1988b). Furthermore, to examine the anti-HIV-1 activity of SAB in a system that is more closely related to natural infection, PBM cells prepared from healthy donors were infected with the KK-1 strain of HIV-1 isolated from a Japanese AIDS patient. The results of this experiment confirmed the anti-HIV-1 effect of SAB.

AME has been reported to suppress HIV-1 replication in H9 cells at a concentration of 10 μg/ml (Schaffner et al., 1986). However, no anti-HIV-1 effect of AME was noted in MT-4 cells (Fig. 2). The reason(s) for this discrepancy remain(s) to be clarified.

The cytotoxicity of SAB for MT-4 cells was about 50-fold less than that of AME. Up to a concentration of 500 μg/ml SAB did not affect the proliferation of MT-4 cells. Nor did SAB show cytotoxicity for PBM cells at a concentration of 1000 μg/ml. A preliminary acute toxicity study with SAB in BALB/c male mice indicated that SAB was not lethal to the mice if administered intraperitoneally as a single dose of 250 mg/kg (unpublished data). The 50% lethal dose of AB in CD-1 mice appears to be 88 mg/kg (Keim et al., 1973). Thus, SAB is apparently less toxic than AB.

Baba et al. (1988b) and Mitsuya et al. (1988) have shown that the anti-HIV activity of dextran sulfate is due to inhibition of the binding of HIV to $CD4^+$ cells. SAB may act in a similar fashion as dextran sulfate and other sulfated compounds. In fact, it was directly shown by flow cytofluorography that SAB inhibits HIV-1 binding to the cell surface. Also, SAB suppressed the cytopathic effect of HIV-1 when it was present only during the HIV-1 absorption period. In accord with other sulfated compounds (Baba et al., 1990b), SAB also suppressed the formation of giant cells in the MOLT-4 and MOLT-4/HTLV-III co-culture system.

Schaffner et al. (1986) postulated that anti-HIV activity of AME may be due to binding of AME to the HIV-1 envelope protein and/or host cell membrane

sterols. In our experiments, pretreatment of MT-4 cells with SAB did not affect the cytopathic effect of HIV-1 (data not shown). Nor did SAB inhibit the binding of OKT-4A/Leu3a to MT-4 cells. On the other hand, pretreatment of HIV-1 with SAB brought about a 10-fold decrease in HIV-1 infection titer.

Our results suggest that the anti-HIV-1 activity of SAB results from an inhibition of HIV-1 binding to cells, combined with a direct inactivating effect on the virus. As the inhibitory effect of SAB on reverse transcriptase activity is seen only at higher concentrations, it is not considered to play a main role in the inhibition of HIV-1 replication by SAB.

The low toxicity of SAB makes it a potential candidate drug for therapeutic use in AIDS patients. Its oral bioavailability remains to be determined. Sulfated polysaccharides, which have anti-HIV activity but small anti-coagulant effects, were reported (Baba et al., 1988a). The anti-coagulant activity of SAB is weaker than that of dextran sulfate. This proves that the anti-HIV activity of polysulfated substances can be dissociated from their anti-coagulant activity, as has been demonstrated previously for sulfated polysaccharides (Baba et al., 1988a, 1990a).

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