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Sulfated pentagalloyl glucose (Y-ART-3) inhibits HIV replication and cytopathic effects in vitro, and reduces HIV infection in hu-PBL-SCID mice

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Abstract

To evaluate the efficacy of Y-ART-3 as an antiviral drug for HIV infections, its anti-HIV activity was assessed in vitro in cell culture systems and in vivo in hu-PBL-SCID mice. The results indicated that Y-ART-3 invariably inhibited not only HIV-1, but also HIV-2 and SIV strains. Its mechanism of action is ascribed to inhibition of viral adsorption to CD4-positive cells. In an in vivo study, human Ig- and CD4-positive cells were detected at similar levels in Y-ART-3-treated hu-PBL-SCID mice that were infected with HIV, and in PBS-treated control hu-PBL SCID mice that were not infected with HIV. If HIV positivity was calculated using the number of tests in which HIV was detected (i.e. PCR, and p24 from co-cultures of spleen and peritoneal wash cells), a significant effect of Y-ART-3 at a dose of 4 mg/kg was noted. Therefore, Y-ART-3 may be considered to be a potent and effective anti-HIV compound.

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Abbreviations: HIV, human immunodeficiency virus; PBL, peripheral blood leucocytes; SCID, severe combined immunodeficiency; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RT, reverse transcriptase; $TCID_{50}$, 50% tissue culture infectious doses; CC_{50}, 50% cytotoxic concentration; EC_{50}, 50% effective concentration; SI, selectivity index; BI, binding inhibitory activity ratio; FI, fusion index.

1. Introduction

The relative complexity of the human immunodeficiency virus (HIV) replicative cycle suggests that several stages of the replication process might represent potential targets for anti-HIV therapy (De Clercq, 1991). The reverse transcriptase (RT) encoded by HIV catalyzes the conver-

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sion of viral genomic RNA into proviral DNA (Barré-Sinoussi et al., 1983; Ratner et al., 1985). Since RT is essential for virus replication and exhibits no closely related identified cellular homology, it has been the prime target for anti viral therapy against the acquired immune deficiency syndrome (AIDS) (De Clercq, 1992). This strategy is appropriate since 3'-azido-2',3'-dideoxythymidine (AZT), a nucleoside analog inhibitor of reverse transcription, was the first drug shown to be of benefit to HIV- infected individuals (Fischl et al., 1987). In addition, 2',3'-dideoxyinosine (ddI) and 2',3'-dideoxycytidine (ddC) showed promise on clinical evaluation (Yarchoan et al., 1989a; Yarchoan et al., 1989b). However, the administration of these drugs to patients is usually limited by their serious toxicity (Richman et al., 1987). Moreover, prolonged use of nucleoside analogs has led to the development of HIV mutants resistant to these drugs, suggesting that the emergence of a resistant virus will limit the drugs efficacy (Larder et al., 1989). Thus, effective prolonged treatment of HIV infection requires the discovery of other antiviral compounds.

Previously, we have reported that a sulfated derivative of a 5'-nucleotidase-inhibitory polyphenol, NF-86II-S, inhibited HIV replication in vitro (Toukairin et al., 1992). We also synthesized simple sulfated plant polyphenols, such as tannic acid sultate, rutin sulfate, ellagic acid sulfate, (-)-epicatechin sulfate and (-)-epigallocatechin gallate sulfate, and found that they are inhibitory to HIV infection in vitro (Mizuno et al., 1992; Nakashima et al., 1992b). In the present study, we evaluated the anti HIV effectiveness of sulfated pentagalloyl glucose (Y-ART-3) in vitro. Secondly, to determine whether or not Y-ART-3 administered to animals can pass into the bloodstream in a form effective against HIV, mice were given an intravenous injection of the test compound, and the anti-HIV activity in their serum was then quantitatively analyzed by a bioassay. Finally, the in vivo anti-HIV activity of Y-ART-3 was evaluated in the SCID (homozygous for severe combined immunodeficiency) mouse engrafted with human cells, which is believed to be a valuable animal model for studies on HIV infection.

2. Materials and methods

2.1. Compounds

Sulfated pentagalloyl glucose (Y-ART-3) was synthesized as described previously (Toukairin et al., 1992; Mizuno et al., 1992). Briefly, SO_3NMe_3 (109.25 g) was added to a solution of pentagalloyl glucose (20 g) in dry DMF (200 ml) and the mixture was stirred for 93 h at 55°C. After cooling to room temperature, the mixture was poured into a solution of $NaHCO_3$ (124 g) in H_2O (200 ml) and filtered. A solution of HSO_4NBu_4 (68 g) and $NaHCO_3$ (18 g) in H_2O (100 ml) was added to the filtrate. The solution was extracted with CH_2Cl_2, washed with H_2O, dried over Na_2SO_4 and evaporated in vacuo. Ion-exchange resin (Amberlight IR-120B, Na + form, 2L) was added to a solution of the obtained residue in H_20 (200 ml) and the mixture was kept at room temperature under agitation for 7 h. The mixture was filtered and the filtrate applied onto a column of HP-20 (1L). The column was eluted with H_2O. The filtrate was concentrated under reduced pressure to ca 500 ml, washed with CH_2Cl_2 and the solvent was removed to yield Y-ART-3 as a white powder (22.74 g). The molecular weight of Y-ART-3 was estimated to be 1655.01 to 1961.15 and its chemical structure is shown in Fig. 1. Dextran sulfate was purchased from Kowa Co., Ltd. (Tokyo, Japan), and 3'-azido-2',3'-dideoxythymidine (AZT) and 2',3'-dideoxyinosine (ddI) were obtained from Yamasa Shoyu Co., Ltd. (Chiba, Japan).

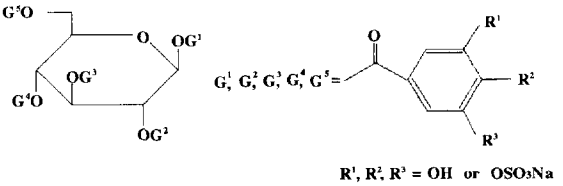

Fig. 1. Chemical structure of sulfated pentagalloyl glucose (Y-ART-3).

2.2. Cells

Human T-lymphotropic virus type I (HTLV-I)-positive human T cell line, MT-4, and HTLV-I non-infected T cell line, MOLT-4, were grown and maintained in RPMI 1640 medium supplemented with 10% (vol./vol.) heat-inactivated fetal calf serum (FCS), 100 IU/ml of penicillin and 100 μg/ml of streptomycin. Human peripheral blood lymphocytes (PBL) were obtained from healthy donors, stimulated with phytohemagglutinin (PHA) for 3 days, then cultured in RPMI 1640 medium containing 20% FCS, antibiotics and 5 ng/ml of recombinant interleukin 2 (IL-2).

2.3. Viruses

Three strains of HIV-1 (HIV-1_{IIIB}, HIV-1_{A012B} and HIV-1_{012D}), one strain of HIV-2 (HIV-2_{ROD}), and simian immunodeficiency virus (SIV_{MAC}) were used as cell-free virus to conduct antiviral assays. HIV-1_{A012D} is the AZT-resistant strain and HIV-1_{A012B} is the corresponding AZT-sensitive clinical isolate. These viruses were obtained from the culture supernatant of MOLT-4 cells or MT-4 cells, which were infected with the virus and stored in a small volume (1 ml) at -80°C until used. The titer of the virus stocks was determined as 50% tissue culture infectious doses ($TCID_{50}$) in MT-4 cells.

2.4. Anti-HIV assay

The anti-HIV activity of the test compounds was determined from the protection they provided against HIV-induced cytopathic effects. MT-4 cells were infected with HIV-1_{IIIB} using 1000 $TCID_{50}/10^5$ cells. HIV- or mock-infected MT-4 cells (1.5×10^5 cells/ml, 200 μl) were placed into 96-well microtiter plates and incubated in the presence of various concentrations of test compounds. Drugs were diluted five-fold, and nine different concentrations of each compound were examined. All experiments were performed in triplicate. After 5 days of culture at 37°C in a CO_2 incubator, cell viability was quantified by a colorimetric assay which monitors the ability of viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a blue-colored formazan product (Pauwels et al., 1988; Nakashima et al., 1992a).

The anti-HIV-1 efficacy was also determined from the inhibitory effect on virus-specific antigen expression. Briefly, HIV-1-infected MT-4 cells (1000 $TCID_{50}/10^5$ cells) were cultured with various concentrations of the test compound, then viral antigen expression was examined by indirect immunofluorescence (IF), using anti-HIV-1 antibody-positive human serum obtained from an asymptomatic HIV-1 infected patient as a probe, and monitored by laser flow cytometry (FACScan, Becton Dickinson, Mountain View, CA).

The antiviral activity against HIV-2_{ROD} and SIV_{MAC} was also determined by the MTT method in virus-infected MT-4 cells. The assay procedure for measuring the anti-HIV-1 activity of the test compounds in PBL was based on the quantitative detection of p24, which was determined with a sandwich enzyme-linked immunosorbent assay kit (Abbott GmgH Diagnostika, Wiesbaden-Delkenheim, Germany) on day 12 after infection.

2.5. Virus binding assay

The procedure used to detect binding of HIV-1 particles to the cell surface has been described previously (Schols et al., 1989). Briefly, MT-4 cells were exposed to an HIV-1 preparation (which had been concentrated 100-fold from the supernatant of MOLT 4/HIV-1_{IIIB} cultures) in the absence or presence of the test compounds. After incubation at 37°C for 60 min, the cells were washed twice in PBS to remove unbound virus particles. The cells were then processed for indirect immunofluorescence measurement. The binding inhibitory activity ratio (BI) was calculated as follows:

$$BI = [1 - (\%MF_{VS} - \%MF_{CS}) / (\%MF_V - \%MF_C)] \times 100$$

where MF = mean fluorescence; VS = HIV-infected cells treated with test compound; CS = control cells (not exposed to HIV) treated with test compound; V = HIV-infected cells without test compound; C = control cells (not exposed to HIV and not treated with test compound).

Table 1
The protocol for Y-ART-3 treatment of hu-PBL-SCID mice

Group	No. of mice (M/F)	HIV infection	Treatment
1	6/7	100 $TCID_{50}$ HIV-1_{IIIB}	PBS i.p. days 15–28 (once a day injection)
2	7/6	100 $TCID_{50}$ HIV-1_{IIIB}	1 mg/Kg Y-ART-3 i.p. days 15–28 (once a day injection)
3	7/7	100 $TCID_{50}$ HIV-1_{IIIB}	4 mg/Kg Y-ART-3 i.p. days 15–28 (once a day injection)
4	4/4	No	PBS i.p. days 15–28 (once a day injection)

2.6. Syncytium formation inhibition assay

MOLT-4 cells and MOLT-4/HIV-1_{IIIB} cells were mixed in the ratio of 1:1 (final cell concentration, 1×10^6/ml). The mixed cell suspension was then co-cultured with various concentrations of the test compound at 37°C in a CO_2 incubator. Twenty hours after the co-culture, the number of viable cells was determined by the trypan blue dye exclusion method and the fusion index (FI) was calculated as follows:

$$FI = 1 - [\textit{cell number in a test well (MOLT-4 + MOLT-4/HIV-1}_{IIIB})] / [\textit{cell number in control well (MOLT-4 cells)}].$$

The FI values obtained for each concentration of a compound can be expressed as a fraction of the control value, leading to the following definition of percent fusion inhibition:

$$\%\ \textit{Fusion Inhibition} = [1 - (FI_T/FI_C)] \times 100$$

where FI_T is the FI of the test sample and FI_c is that of the control sample (Tochikura et al., 1988).

2.7. Quasi-in-vivo assay

Mice (Crj:CD-1[ICR]; 5-week-old, average weight : 28 g) were used for quasi-in-vivo studies. The animals were given an intravenous injection of the test compound, then blood was collected by means of cardiac puncture at different times after injection and kept at 4°C for 6 h. Subsequently, serum was collected by centrifugation for 15 min at 1500 rpm and stored at −80°C until assayed. The anti-HIV activity of the serum was monitored by means of the MTT assay, as described previously. In this assay, the dilution steps of the test serum were two-fold and anti-HIV activity was expressed as the reciprocal number of the dilution which represented 50% or 90% protection from HIV-induced CPE (Witvrouw et al., 1990; Shimizu et al., 1993; Nakashima et al., 1995).

2.8. Evaluation of Y-ART-3 efficacy in reducing HIV infection of hu-PBL-SCID mice

Homozygous C.B.- 17 *scid/scid* (SCID) mice were bred and maintained in micro-isolator cages (Lab Products, Maywood, NJ) in our animal facility. The animals were given sterile food (Purina Lab Chow, Newco, San Diego, CA) ad libitum, and provided with acidified, sterile, Millipore-filtered water. Forty-eight mice were injected with $32–27 \times 10^6$ human peripheral blood leukocytes in 0.5 ml, which were obtained from two EBV seropositive donors. On screening for human Ig 12 days later, all 48 mice were found to have human Ig levels of over 50 μg/ml, and thus served as hu-PBL-SCID mice for this study. Two weeks later, HIV infection and treatment of mice was initiated according to the protocol shown in Table 1. To provide quantitative information on the prevention or retardation of HIV infection, the following assays were performed on half the mice in each group at 14 days post-HIV challenge (sacrificed 4 weeks after PBL grafting) and on the remaining mice at 21 days post-HIV challenge (sacrificed 5 weeks after PBL grafting). At the time of sacrifice, an anesthetized animal was bled, then its peritoneal cavity was flushed with 10 ml

of sterile PBS. The harvested cells were centrifuged and resuspended to 2 ml in RPMI 1640 with 10% FCS. A 500-μl aliquot of the cell suspension was added to a culture in 1.5 ml of 2×10^6 PHA and IL-2 activated human PBLs for p24 antigen detection. The remaining cell suspension was stored in small volumes (500 μl/tube) at -80°C until used for PCR analysis and cytometry. The spleen was removed and placed in a petri dish containing 2 ml of RPMI medium, and converted into a cell suspension. A 500-μl aliquot was placed in culture for p24 antigen detection and the remainder was frozen at 80°C until assayed: 500 μl for PCR analysis, another 500 μl for cytometry and a further 500 μl for use as a reserve sample.

2.8.1. p24 assaying of co-cultures of peritoneal wash and spleen cells with human PHA blasts

An aliquot of harvested peritoneal wash cells or spleen cells from each SCID mouse was placed in culture with 2×10^6 PHA and IL-2 activated hu-PBL in a total volume of 2 ml. The supernatant of each culture was fed weekly with fresh PHA and IL-2-activated hu-PBL, with replacement of the medium withdrawn for sampling. The expression of p24 antigen in culture supernates of the co-cultures of peritoneal wash and spleen cells was assayed using a Coulter p24 assay kit (Coulter Immunology, Hialeah, FL).

2.8.2. PCR assay for HIV and HLA DNA sequences from peritoneal wash and spleen cells

The polymerase chain reaction (PCR) method for amplifying DNA was used to assay spleen and peritoneal lavage cells for the presence of proviral HIV and human HLA sequences. Cells were resuspended in K buffer and DNA was isolated as described previously (Nakashima et al., 1992a). The oligonucleotide primers used for human HLA-DQ alpha gene, GH26 and GH27, were purchased from Roche Diagnostics (Branchburg, NJ). The nucleotide sequences of 115 bp fragment bordered by primers from the *gag* gene of the HIV-1 isolates ARV-2 were as follows: SK38, 5′-ATAATCCACCTATCCCAGTAGGAGAAA-T-3′ (*gag* 1551-1578); SK39, 5′TTTGGTCCTT-GTCTTATGTCCAGAATGC-3′ (*gag* 1665-1638); and SK19, 5′ATCCTGGGATTAAATAAAATA-GGTAAGAATGTATAGCCCTAC-3′ (*gag* 1595-1635). Amplification of approximately 2 μg of DNA was performed in a total reaction volume of 50 μl containing 2.5 units/100 μl of Taq DNA polymerase (Promega, Madison, WI), 25 mM Tris HCl (pH 8.0), 50 mM NaCl, 4 mM $MgCl_2$, 2 mM dNTP, and 50 pmol of each primer. Amplification was achieved by means of 45 two-temperature cycles, each consisting of 30 sec at 95°C (denaturation) and 30 sec at 60°C (annealing and extension). To interpret the presence of HIV-1, a lymphocyte cell line (AC-1), which has one copy of HIV-1 DNA per cell, was included in the PCR analysis: the AC-1 cell was used to spike negative whole blood to produce a standard curve to determine the analytical sensitivity. Amplified DNA was detected in microtiter plates (Immulon II). An oligonucleotide complementary to the internal sequence of the HIV-1 or HLA DQ alpha amplification target was immobilized on microtiter plates. Amplification was performed with biotinylated extension primers and the biotinylated amplification product was hybridized to the immobilized complementary probe. The captured amplification product was detected with an avidin-horseradish peroxidase conjugate and TMB (tetramethylbenzidine) substrate. The absorption value of each sample was read at 450 nm and regarded as positive for PCR if the average OD for duplicate wells was greater than 0.400.

2.8.3. ELISA for serum human immunoglobulin levels

Following grafting with human leukocytes, mice were bled periodically and their sera tested for the level of human Ig. Blood collected 12 days after PBL grafting was also tested for the serum content of mouse IgM. The capture antibody for the human Ig assay was goat anti-human immunoglobulin G + M + A (Cappel, West Chester, PA) at a concentration of 2 μg/ml. The standards were either a mouse IgM antibody (Binding Site, San Diego, CA) or a total human immunoglobulin standard (Binding Site, San Diego, CA). The controls were either a calibrated murine serum standard (Binding Site, San Diego, CA) or purified human IgG (Sigma, St. Louis, MO). The

plates were incubated for 2 h at room temperature with serial dilution of SCID serum in PBS, pH 7.4, or with standards in the same dilution. The bound antibody was detected using either peroxidase-conjugated goat anti-mouse IgM antibody (Tago, Burlingame, CA) or peroxidase-conjugated goat anti-human IgG + M + A antibody (Cappel, West Chester, PA), with incubation in the dark for 1 h at room temperature. After peroxidase-catalyzed color development using ortho phenylenediamine and hydrogen peroxide, absorption at 490 nm was quantitated using a Titertek Multiscan Plus MK II ELISA reader (Flow Labs, MacLean, VA).

2.8.4. Flow cytometry assay for CD4$^+$ and CD8$^+$ T cells in peritoneal wash

One aliquot of peritoneal wash cells from each animal was treated with appropriate fluorescein or phycoerythrin-conjugated antibodies, i.e. to CD4 or CD8 (Sigma and Pharmingen, respectively), then cells were gently vortexed and allowed to stand at 4°C for 30 min. After the incubation, the cells were washed in cold Hank's without BSA or azide. After the wash, the supernatants were removed and the cells were resuspended in 1 ml of 2% formaldehyde in saline and analyzed with a Becton-Dickinson FACScan. Whenever possible, a minimum of 3000 events was collected, and the data were analyzed for forward and side scatter-gated events.

3. Results

3.1. In vitro anti-HIV activity of Y-ART-3

The anti-HIV-1 activity of Y-ART-3 was assessed as the protection provided against HIV-1-induced cytopathogenicity and inhibition of virus-specific antigen expression in MT-4 cells in vitro (Fig. 2). Although HIV-1-infected MT-4 cells were not able to survive under virus infection, Y-ART-3 showed concentration-dependent protective activity against virus-induced CPE, the 50% effective concentration (EC_{50}) of Y-ART-3 was 0.58 μg/ml. The 50% cytotoxic concentration (CC_{50}) of Y-ART-3 was 197 μg/ml and the selectivity index (ratio of CC_{50} to EC_{50}) was 340. We also investigated the antiviral activity of Y-ART-3 against the clinical isolate in normal human peripheral blood lymphocytes (PBL) (Table 2). Irrespective of the criteria (such as inhibition of viral cytopathicity, antigen expression or p24 production) used to assess anti-HIV activity, Y-ART-3 invariably inhibited not only HIV-1 strains including the AZT-resistant, but also the HIV-2 and SIV strains. The cytotoxicity of Y-ART-3 was also investigated in MOLT-4 cells and PBL. The CC_{50} values in these cells were 150 μg/ml to 500 μg/ml.

3.2. Inhibition of virus binding

To elucidate the mechanism of action of Y-ART-3, we examined whether Y-ART-3 inhibited the binding of HIV-1 particles to MT-4 cells, as assessed by laser flow cytometry/immunofluorescence assay. As shown in Fig. 3A and Fig. 3C,

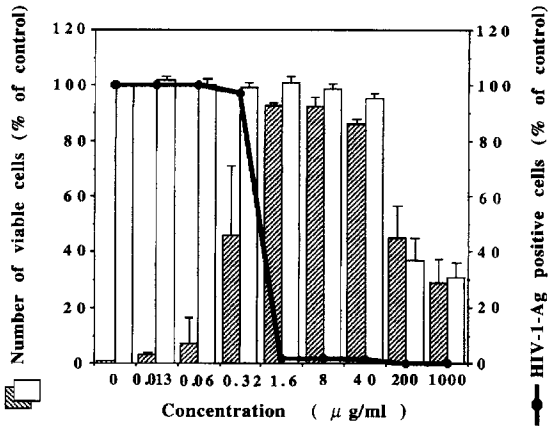

Fig. 2. Inhibitory effect of Y-ART-3 on HIV-1-induced cytopathogenicity and viral antigen expression in MT-4 cells. The viability of HIV-infected (hatched columns) and mock-infected MT-4 cells (open columns) was measured by the MTT method 5 days after infection. The number of viable cells was expressed as the percentage of mock-infected drug-free control cells. HIV-1 antigen-positive cells were detected by indirect immunofluorescence and laser flow cytometry, using a polyclonal antibody as a probe. The number of viral antigen-positive cells was expressed as a percentage of the HIV-1-infected drug-free control cells. Each experiment was performed at least three times and the results are the means of three experiments.

Table 2
Antiviral activity spectra of Y-ART-3, dextran sulfate and dideoxynucleoside analogues

Virus strain	Cell	Assay	Day of analysis	Antiviral activity (EC_{50}^{a})			
				Y-ART-3 (μg/ml)	Dextran sulphate (μg/ml)	AZT (μM)	ddI (μM)
$HIV\text{-}1_{IIIB}$	MT-4	MTT	5	0.58	0.78	0.024	17.49
	PBL[b]	p24	12	2.55	2.68	0.011	ND
$HIV\text{-}1_{A012B}$	MT-4	IF	5	0.72	0.54	0.012	12.93
$HIV\text{-}1^{c}_{A012D}$	MT-4	IF	5	0.68	0.48	1.43	12.22
$HIV\text{-}2_{ROD}$	MT-4	MTT	5	0.22	0.86	0.014	12.21
SIV_{MAC}	MT-4	MTT	5	0.58	4.41	0.011	13.06

[a]Anti-HIV-1 activity (EC_{50}), concentration required for 50% inhibition HIV-1 induced cytopathogenicity in MT-4 cells, as determined by the MTT method.
[b]EC_{50} of PBL was calculated as 50% reduction of p24 expression in the culture supernatant.
[c]AZT-resistant HIV-1 strain.

Y-ART-3 inhibited HIV-1 binding by 99% at 25 μg/ml. Dextran sulfate (25 μg/ml) also inhibited HIV-1 binding by 95% (Fig. 3B).

Fig. 3D demonstrated that Y-ART-3 caused a concentration-dependent inhibition of HIV-1 binding to MT-4 cells.

3.3. Inhibition of syncytium formation

When we investigated the inhibitory activity against multinuclear giant cell (syncytium) formation in co-cultures of persistently HIV-1-infected MOLT-4 cells (MOLT-4/$HIV\text{-}1_{IIIB}$) and uninfected MOLT-4 cells, Y-ART-3 was found to inhibit giant cell formation more efficiently than dextran sulfate; the 50% inhibitory concentrations of these compounds were 0.46 μg/ml and 1.79 μg/ml, respectively (Fig. 4).

3.4. Effect of serum on in vitro anti-HIV activity of Y-ART-3

To determine whether the anti-HIV activity of Y-ART-3 was altered in the presence of a high concentration of human or animal serum, the anti-HIV activity of Y-ART-3, dextran sulfate and ddI was investigated in the presence of various concentrations of FCS. Results showed that the anti-HIV activity of Y-ART-3 was affected by the FCS concentration used in the HIV-infected MT-4 cell culture. In the presence of 10% FCS, the EC_{50S} value of Y-ART-3 was 0.64 μg/ml. In the presence of higher concentrations of FCS, the activity of Y-ART-3 was reduced; EC_{50S} increased to 3.72 and 4.56 μg/ml in the presence of 50 and 80% FCS, respectively (Table 3). The reduction of Y-ART-3 in terms of anti-HIV activity was somewhat less than that of dextran sulfate; EC_{50S} of dextran sulfate were 0.83, 6.27 and 13.43 μg/ml in the presence of 10, 50 and 80% FCS, respectively. The anti-HIV activity of ddI was not significantly affected by different concentrations of FCS (Table 3).

3.5. Anti-HIV activity of serum from mice injected with Y-ART-3 (Quasi-in-vivo assay)

To determine the antiviral efficacy in the bloodstream, the anti-HIV activity of mouse serum obtained at appropriate times after injection was measured. Various amounts of Y-ART-3 were administered to mice intravenously and the anti-HIV activity of the serum was dose-dependent. Fig. 5A demonstrates that mouse serum diluted 1134-fold and 101-fold) obtained 30 min after injection of 100 mg/kg and 10 mg/kg of Y-ART-3, respectively, inhibited HIV-induced cytopathogenicity by 50% in MT-4 cells. These data were reproducible, and when 100 mg/kg of Y-ART-3 was administered intravenously, strong anti-HIV activity was observed and maintained for at least 120 min (Fig. 5B). In this experiment, 1:2469 diluted serum, which was obtained 1 min after injection, inhibited HIV-induced cytopathogenicity

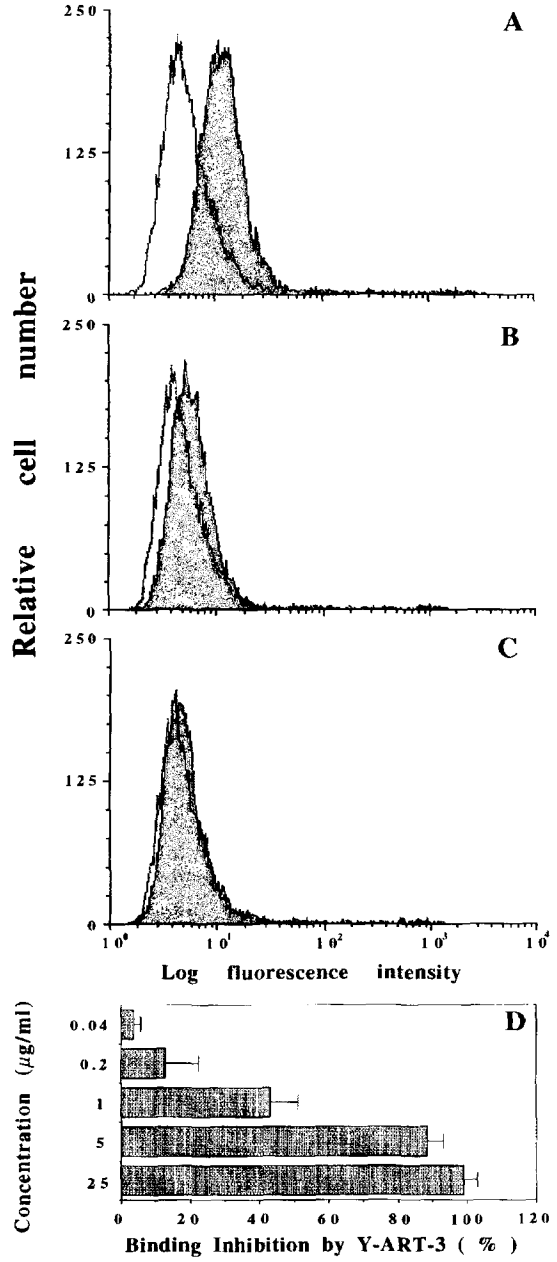

Fig. 3. Effects of Y-ART-3 and dextran sulfate on HIV-1 binding to MT-4 cells. The thick-line histograms represent cellular fluorescence resulting from nonspecific binding of anti-HIV-1 antibody to MT-4 cells (which had not been exposed to HIV-1). The thin-line histograms with shading represent the cellular fluorescence resulting from specific binding of anti-HIV-1 antibody to virus-exposed MT-4 cells in the absence of the test compound (A), or in the presence of dextran sulfate (25 μg/ml) (B) or Y-ART-3 (25 μg/ml) (C). Concentration-dependent inhibitory activity of HIV-1 binding by Y-ART-3 is shown in panel D.

by 50% MT-4 cells. This indicated that sufficient activity was revealed under the cell culture conditions if the culture medium had a serum concentration as low as 0.04%. The reciprocal numbers of dilution of serum obtained 30 min after injection for 50% or 90% inhibition of HIV-induced CPE were 1382 and 643, respectively, and those of the serum 2 h after injection were 964 and 509, respectively.

3.6. Reduction of HIV infection of hu-PBL-SCID mice

The total human Ig levels are reported for two time points. The first time point was at day 12 after human PBL grafting, at which point a level of 50 μg/ml was considered to be the initial positive evidence of grafting. The human Ig level at sacrifice (day 28 or day 35) was regarded as the final indication of positive PBL grafting. At this time point, an animal was judged to show survival of the human PBL graft if the human Ig level was at least 100 μg/ml. With these criteria, all mice in this study showed evidence of human PBL engraftment both at the time of HIV infection and at sacrifice. Fig. 6A shows the average level of human Ig at sacrifice for all mice, which was not significantly different between groups. Fig. 6B shows the average percentage of CD4-positive human cells detected among peritoneal wash cells, including a group which had not received HIV. Although a trend towards protection by the compound was evident from the reduction in the CD4 percentage due to HIV infection, the differences between groups were not significant.

To quantify HIV burdens in hu-PBL-SCID mice, the levels of p24 in supernatants harvested from 4-day co-cultures of spleen cells and peritoneal wash cells were measured quantitatively. This was followed by qualitative determination after 1 week and 2 weeks in culture. Supernatants with negative p24 results after 1 and 2 weeks were re-tested after 4 weeks of culture. Spleen or peritoneal wash cell samples were regarded as p24-positive if the results were positive at any day of the three time points tested (Table 4). The detection of human HLA DQ alpha sequences confirms the presence of human DNA, thereby validating the negative results for HIV PCR

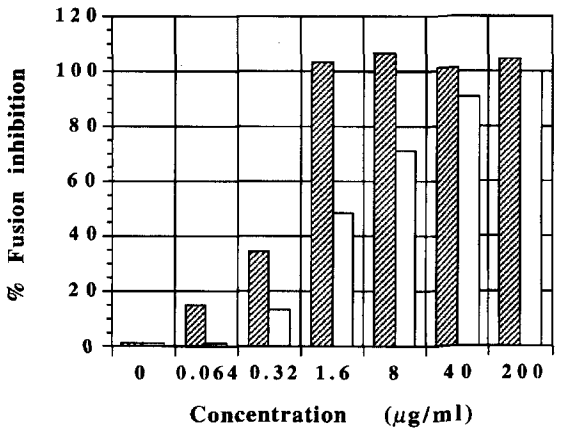

Fig. 4. Inhibitory effects of dextran sulfate (open columns) and Y-ART-3 (hatched columns) on syncytium formation in a co-culture of MOLT-4 and MOLT-4/HIV-1_{IIIB} cells. The number of viable cells was determined at 20 h after co-culture in the presence of various concentrations of the compounds, and percent fusion inhibition was calculated as described under 'Materials and methods'.

(Table 4). Mice were regarded as HIV-positive if any of four detection tests (p24 and HIV PCR of spleen cells and peritoneal wash cells) were positive. Fig. 6C illustrates the percentage of hu-PBL-SCID mice in which HIV-1 could be detected in each treatment group.

The final columns in Table 4 shows the number of positive assays of each mouse. As described previously, an animal was judged to be positive for HIV infection if either the p24 or PCR assay was positive for either spleen or peritoneal wash (i.e. four tests in total). The number entered in the positive assay column, therefore, is 0 if none of these was positive. A positive number indicates how many of the four tests were positive, and may be useful as a semi-quantitative measure of the level of HIV infection in each mouse. The average for the number of positive assays in the hu-PBL-SCID mice of each group is demonstrated in Fig. 6D. The mice were considered to be infected with HIV-1 if the virus was isolated on co-culture or if HIV-1 proviral sequences were amplified by PCR. According to these criteria, Y-ART-3 reduced the frequency of mice infected with HIV, though not with statistical significance (Fig. 6C). If, however, HIV positivity is calculated using the number of tests in which HIV was detected (i.e. PCR and p24 from co-cultures of spleen and peritoneal wash cells), a significant effect for Y-ART-3 at 4 mg/kg was noted (Fig. 6D).

4. Discussion

The present study indicates that sulfated pentagalloyl glucose (Y-ART-3) shows potent anti-HIV activity in vitro, possibly due to interference with the adsorption of virus particles to CD4-positive cells. Reverse transcription process could be excluded as a target for this compound because Y-ART-3 did not cause a marked reduction in the HIV-1 RT activity (data not shown). Moreover, we examined whether Y-ART-3 suppressed the production of HIV-1 in chronically infected cells. In this experiment, we used HIV-1 chronically infected MOLT-4/HIV-1 cells and U1 cells. U1 cells are the subclones of a monocytic cell line, U937 cells, chronically infected with HIV-1. Upon treatment with TPA (10 ng/ml), these cells produce viral antigen, as revealed through a HIV-1 p24 antigen assay (Murakami et al., 1992). Results indicated that Y-ART-3 (50 ug/ml) did not inhibit HIV-1 production in MOLT-4/HIV-1 and HIV-1 induction in the TPA-treated U1 cells (data not shown).

We also demonstrated that the anti-HIV activity of Y-ART-3 was reduced in the presence of high concentrations of FCS. This reduction in anti-HIV activity of Y-ART-3 was somewhat less than that of dextran sulfate. Clinical reports have revealed the poor absorption (bioavailability, tissue distribution) of dextran sulfate (Abrams et al., 1989; Lorentsen et al., 1989; Flexner et al., 1991). In the present study, we found that Y-ART-3 might be more stable in an effective form in the bloodstream. When Y-ART-3 was administered intravenously to mice, the compound was present in an effective anti-HIV form for at least several hours after injection. Our results indicate than mouse serum diluted 2469-fold, obtained 1 min after the injection of 100 mg/kg of Y-ART-3, is able to inhibit HIV-induced CPE by 50%. When we injected 10 mg/kg of Y-ART-3 into mice,

Table 3
Decreases in anti-HIV activity of Y-ART-3, dextran sulfate and ddI with high concentrations of FCS in vitro

Compound	EC_{50}		
	10%	50%	80%
Y-ART-3 (μg/ml)	0.64 (1)	3.72 (5.8)	4.56 (7.1)
Dextran sulfate (μg/ml)	0.83 (1)	6.27 (7.6)	13.43 (16.2)
ddI (μM)	19.13 (1)	15.41 (0.81)	18.12 (0.95)

HIV-1-infected MT-4 cells were cultured with the test compound in the presence of three different concentrations (10, 50 and 80%) of heat-inactivated FCS for 5 days. EC_{50} was calculated on the basis of the 50% inhibition concentration of HIV-1-induced cytopathogenicity. Values in parentheses denote fold differences compared with the EC_{50} determined in the presence of 10% FCS.

significant anti-HIV activity was also observed in their serum. It remains to be excluded that injection of Y-ART-3 might induce a substance, such as an anti-HIV cytokine, that is effective against HIV, although this possibility can be considered as unlikely.

In any case, a high level of anti-HIV activity was observed and maintained for a certain period in the serum of experimental animals that had been injected with Y-ART-3. Furthermore, mice injected with 10 mg/kg/day of Y-ART-3 for 2 weeks showed neither biochemical nor pathological changes. Only one of 5 experimental mice injected with Y-ART-3 (10 mg/kg/day for 2 weeks) showed mild hepatocyte swelling and vacuole degeneration of renal tubule epithelial cells, while all 5 mice that had been continuously given 100 mg/kg/day showed more pronounced toxic effects. Y-ART-3 caused no significant increase in the clotting time or prothrombin time (data not shown). Therefore, we selected the doses of 4 mg/kg/day and 1 mg/kg/day to investigate the in vivo anti-HIV efficacy of Y-ART-3 in hu-PBL-SCID mice.

Hu-PBL-SCID mice may be considered as a useful small animal model in AIDS research.

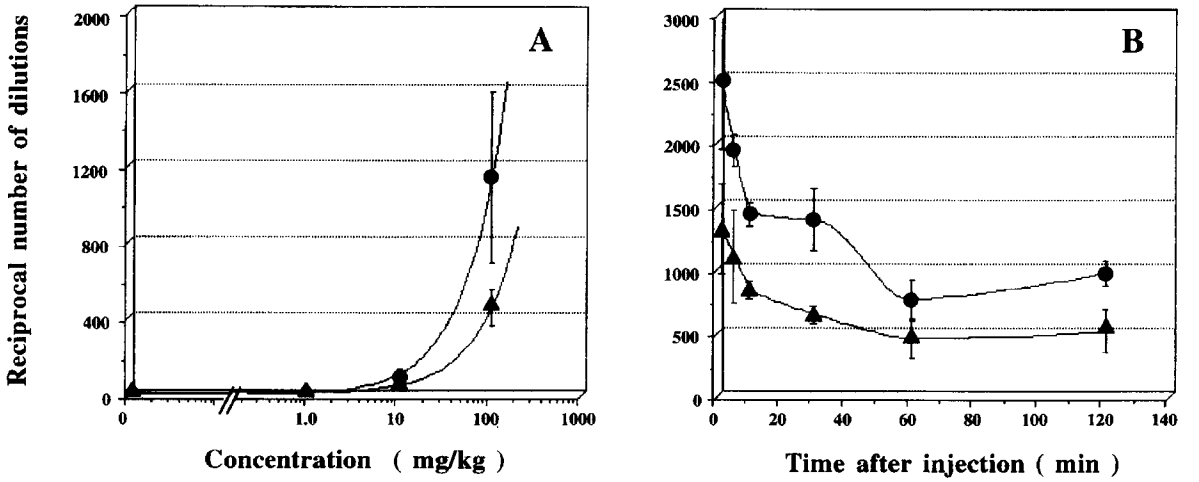

Fig. 5. Anti-HIV activity of mouse serum obtained from Y-ART-3 injected mice. (A) various amounts of Y-ART-3 (100, 10, 1 mg/kg) or PBS were injected into mice and blood was collected at 30 min after injection. (B) 100 mg/kg of Y-ART-3 was injected into mice and blood was collected at various times after injection. Each point indicates the reciprocal number of dilutions of the serum for 50% inhibition (●) or 90% inhibition (▲) for HIV-1-induced cytopathogenicity. Data represent mean values and standard errors for three mice.

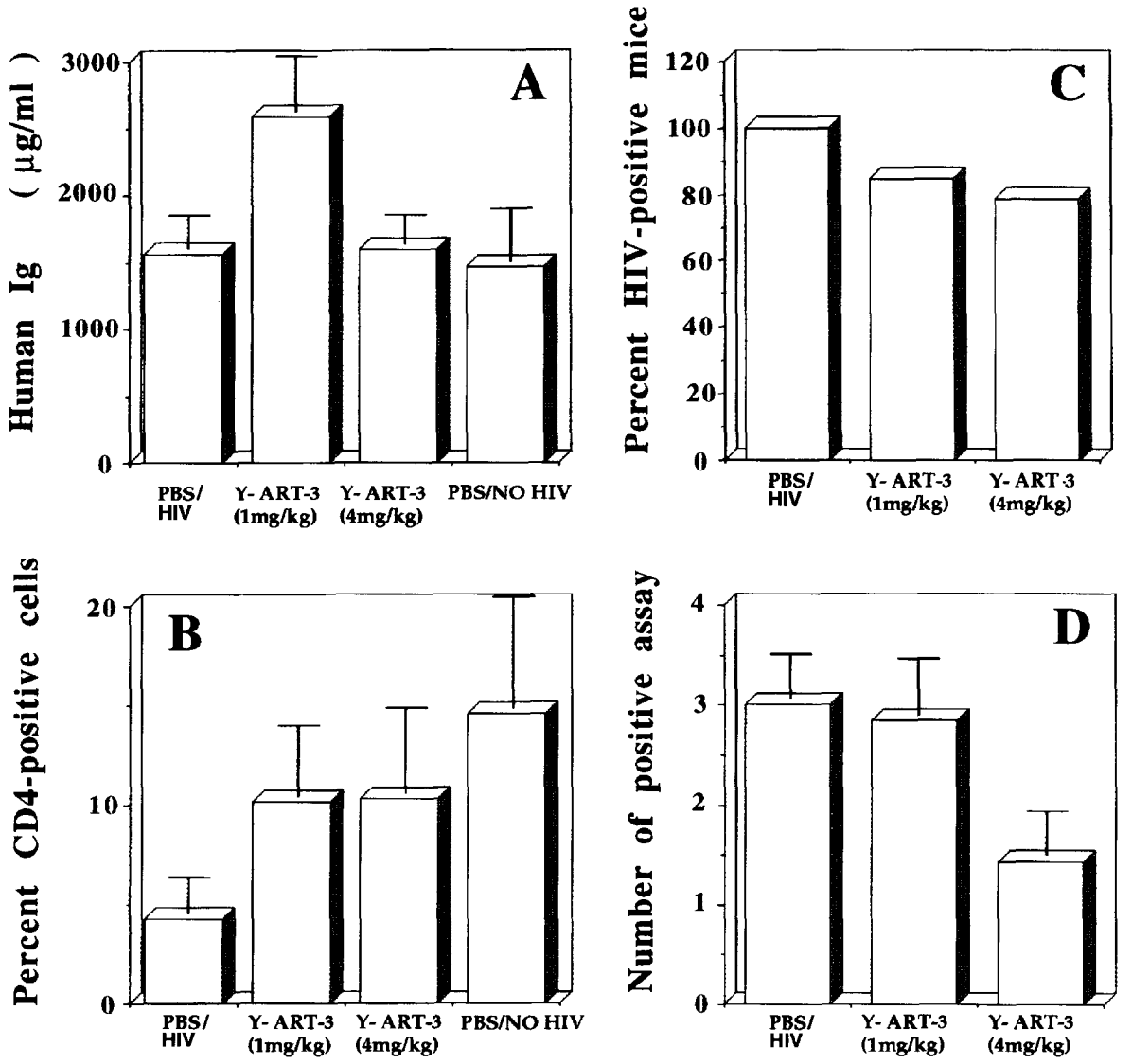

Fig. 6. Evaluation of the effect of Y-ART-3 on reduction of HIV infection of hu-PBL-SCID mice. (A) Means and standard error of the mean for human Ig levels at sacrifice of hu-PBL-SCID mice from each treatment group. (B) Means and standard error of the mean for CD4-positive cells among peritoneal wash cells in hu-PBL-SCID mice from each treatment group. (C) Percentages of hu-PBL-SCID mice from which HIV-1 could be recovered in each HIV-infected treatment group. (D) Means and standard error of the mean for the 'number of positive assays' in hu-PBL-SCID mice from each treatment group.

These mice have been infected with HIV-1 either by injection of cell-free virus or virus-infected T lymphoblasts. HIV-1 can be recovered from the peritoneal cavity, spleen, peripheral blood and lymph nodes of infected mice for at least 16 weeks post-infection. As few as ten tissue culture infectious doses of HIV suffice to infect hu-PBL-SCID mice. Infection of mice with various strains of HIV-1, including Bru, IIIB, MN, SF2 and SF13, has been confirmed. HIV infection can result in alteration of human immunoglobulin levels and CD4-positive T cell numbers. The establishment

Table 4
Anti-HIV-1 effects of Y-ART-3 in hu-PBL-SCID mice

Treatment	Sex	No. of animals	p24[a]		HLA PCR[b]		HIV PCR[b]		No of positive assays[c]
			Spleen	P. Wash	Spleen	P. Wash	Spleen	P. Wash	
Sacrificed 4 weeks after PBL grafting									
PBS	F	4	3/4	2/4	4/4	3/4*	0/4	3/4	1, 3, 3, 1
	M	3	3/3	2/3	3/3	3/3	1/3	2/3	4, 2, 2
Y-ART-3	F	3	2/3	2/3	3/3	3/3	3/3	2/3	4, 2, 4
(1 mg/kg)	M	4	3/4	2/4	4/4	3/4	3/4	2/4	4, 2, 0, 4
Y-ART-3	F	4	0/4	0/4	4/4	4/4	0/4	2/4	1, 0, 0, 1
(4 mg/kg)	M	3	1/3	0/3	3/3	2/3*	0/3	3/3	2, 1, 1
PBS	F	2	0/2	0/2	0/2	0/2	0/2	0/2	0, 0
(no HIV)	M	2	0/2	0/2	0/2	0/2	0/2	0/2	0, 0
Sacrificed 5 weeks after PBL grafting									
PBS	F	3	3/3	3/3	3/3	3/3	2/3	3/3	4, 4, 3
	M	3	3/3	3/3	3/3	3/3	3/3	3/3	4, 4, 4
Y-ART-3	F	3	2/3	2/3	3/3	3/3	1/3	2/3	4, 3, 0
(1 mg/kg)	M	3	3/3	3/3	3/3	3/3	1/3	3/3	3, 4, 3
Y-ART-3	F	3	2/3	3/3	3/3	3/3	1/3	2/3	4, 1, 3
(4 mg/kg)	M	4	3/4	1/4	4/4	3/4*	0/4	2/4	0, 1, 2, 3
PBS	F	2	0/2	0/2	0/2	0/2	0/2	0/2	0, 0
(no HIV)	M	2	0/2	0/2	0/2	0/2	0/2	0/2	0, 0

[a]p24 results are recorded as number of positive animal samples/number of experimental animals.
[b]All PCR data are recorded as positive, OD > 0.4 and negative, OD < 0.4.
*Experiments included not enough sample animal.
[c]Number of positive assays was calculated as the sum of the numbers of postive results for p24 co-cultures or HIV PCR for both peritoneal wash (P. Wash) and spleen cells.

of the hu-PBL-SCID model for HIV infection allows the evaluation of candidate antiviral compounds in vivo (Mosier et al., 1991; Aldrovandi et al., 1993).

Our present results show that HIV replication was significantly suppressed by Y-ART-3 at 4 mg/kg, as revealed by PCR and p24 assays of co-cultures of spleen and peritoneal wash cells. At 2 and 3 weeks after HIV inoculation (4 and 5 weeks after PBL grafting, respectively), animals from each of the experimental groups were sacrificed, and samples prepared for human Ig determination, analysis of HIV infection by means of p24 assays and PCR amplification of HIV gene sequences, and analysis of CD4 and CD8 positive human cells by flow cytometry. We also examined the human HLA DQ alpha sequences by PCR to confirm the presence of human DNA, thereby validating the negative results for HIV PCR.

As can be seen, HIV PCR results do not always agree with p24 co-culture results. Discrepancies were usually seen in cases where the p24 assay result was positive, while the HIV PCR result was negative. This is probably due to differences in sensitivity reflecting the ways in which the two assays were performed. In the co-culture assay, one-fourth of the cells from the spleen and peritoneal wash were added to cultures containing stimulated human lymphocytes, and fed weekly with additional fresh stimulated human lymphocytes for 4 weeks. This is probably adequate to detect a single infectious virion, due to the considerable viral replication that is possible. The PCR assay uses a fraction of the DNA recovered from one-fourth of the cells, so that sampling effects could result in a negative PCR/positive p24 result in spite of the amplification of the PCR.

The detection of human Ig and CD4-positive human cells at similar levels in Y-ART-3-treated hu-PBL-SCID mice and PBS-treated control hu-PBL-SCID mice not infected with HIV suggests that Y-ART-3 did not overtly interfere with the engrafted human cells. The results indicate that the proportion of mice found to be infected with HIV was not significantly different in each treatment group. However, a semi-quantitative measure of HIV detection (the number of positive assays) showed a significant effect of Y-ART-3 at 4 mg/kg. Further, in vivo evaluation of higher concentrations and statistical analysis are underway to assess the efficacy and clinical usefulness this compound. Y-ART-3 seems to be a good candidate for further investigation for the possible treatment of HIV infection.

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