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Activity of a sulfated polysaccharide extracted from the red seaweed *Aghardhiella tenera* against human immunodeficiency virus and other enveloped viruses

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Summary

A galactan sulfate (GS) was isolated from an aqueous extract of the red seaweed *Aghardhiella tenera* and partially purified. GS inhibited the cytopathic effect of human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) in MT-4 cells at concentrations 10-fold higher than those required for the inhibition by dextran sulfate (MW 5000) of the cytopathic effect of HIV-1 and HIV-2 (50% inhibitory concentrations: 0.5 and 0.05 $\mu g\,ml^{-1}$, respectively). GS suppressed syncytium formation between MOLT-4 cells and persistently HIV-1- or HIV-2-infected HUT-78 cells at concentrations higher than 5 $\mu g\,ml^{-1}$. Like dextran sulfate (DS) and aurintricarboxylic acid (ATA), GS inhibited the binding of HIV-1 to the cells and the binding of anti-gp120 mAb to HIV-1 gp120. Like DS and ATA, GS proved active not only against HIV-1 and HIV-2 but also against other enveloped viruses, i.e. herpes-, toga-, arena-, myxo- and rhabdoviruses. GS represents a natural polysaccharide with broad-spectrum activity against a number of important viral pathogens.

Introduction

The acquired immunodeficiency syndrome (AIDS) is a pandemic immunosuppressive disease caused by the depletion of CD4 positive lymphocytes ($CD4^+$ T-cells). The causative agent of AIDS is the human immunodeficiency virus type 1 (HIV-1) (Barré-Sinoussi *et al.*, 1983).

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Sulfated polysaccharides such as heparin and dextran sulfate are potent inhibitors of the replication of HIV-1 *in vitro* (Ito *et al.*, 1987). Their mechanism of action has been associated with their capacity to block the adsorption of HIV virions to the $CD4^+$ T-cell membrane (Baba *et al.*, 1988a,b; Mitsuya *et al.*, 1988; Nakashima *et al.*, 1989; Schols *et al.*, 1989a). They specifically interact with the viral gp120 glycoprotein and inhibit HIV-induced syncytium formation (Baba *et al.*, 1990), which is assumed to play an important role in the depletion of $CD4^+$ T-lymphocytes in AIDS patients (Haseltine, 1988). Sulfated polysaccharides, and polysulfates in general, are inhibitory not only to HIV but also to other enveloped viruses including herpes simplex virus, cytomegalovirus, influenza A virus, vesicular stomatitis virus, respiratory syncytial virus and various toga-, arena- and retroviruses (Baba *et al.*, 1988c; Schols *et al.*, 1990a; Witvrouw *et al.*, 1991, 1992).

We have initiated an investigation of the antiviral activity of Venezuelan medicinal plants. Extracts from a diverse group of plant families have been tested against HIV-1 *in vitro* (Chang and Yeung, 1988), and some extracts, namely these from *Arctium lappa* L., *Viola yedoensis* and *Prunella vulgaris*, were shown to demonstrate anti-HIV activity (Tan *et al.*, 1991). Also, natural substances such as lignin and tannin were found to inhibit HIV replication (Unten *et al.*, 1991). Anti-HIV activity has also been reported for a series of natural polysaccharides extracted from various sources (Nakashima *et al.*, 1987; Hirabayashi *et al.*, 1989).

The present study deals with the antiviral properties of GS, a galactan sulfate extracted from the red seaweed *Aghardhiella tenera*, its activity spectrum and its mechanism of antiviral action.

Results

Anti-HIV activity

When GS was examined for its inhibitory effect on the cytopathic effect of HIV-1 ($HTLV\text{-}III_B$) and HIV-2 ($LAV\text{-}2_{ROD}$) (Table 1), the compound was found to provide protection at a concentration 10-fold higher than the concentration at which dextran sulfate (DS) protected the cells (50% inhibitory concentration: approximately 0.5 and 0.05

Table 1. Inhibitory effects of GS, ATA and DS on HIV-1- and HIV-2-induced cytopathic effect and cell viability

Compounds	Virus cytopathicity IC_{50}[a] ($\mu g\ ml^{-1}$)		Cytotoxicity CC_{50}[b] ($\mu g\ ml^{-1}$)
	HIV-1	HIV-2	
GS	0.6 ± 0.4	0.5 ± 0.3	>250
ATA	0.2 ± 0.03	0.8 ± 0.5	>250
DS	0.05 ± 0.02	0.04 ± 0.02	>250

[a]50% inhibitory concentration, based on the inhibition of HIV-induced cytopathic effect in MT-4 cells.
[b]50% Cytotoxic concentration, based on the reduction of viability of mock-infected MT-4 cells.

$\mu g\ ml^{-1}$, respectively). The protective activity shown by GS against HIV-1/HIV-2 was comparable to that of aurintricarboxylic acid (ATA) (Table 1). GS suppressed syncytium (giant cell) formation (induced by either HIV-1 or HIV-2) at concentrations higher than 5 $\mu g\ ml^{-1}$. None of GS, ATA and DS reduced the viability of mock-infected MT-4 cells, as monitored by the MTT method (Pauwels *et al.*, 1988) at concentrations up to 250 $\mu g\ ml^{-1}$ (Table 1).

Mechanism of anti-HIV activity

Using a p24 ELISA assay, we demonstrated that GS blocks the binding of HIV-1 to the MT-4 cells (50% inhibitory concentration: approximately 40 $\mu g\ ml^{-1}$). Following the same procedures, we found that ATA (50% inhibitory concentration: 2 $\mu g\ ml^{-1}$) and DS (50% inhibitory concentration: <0.2 $\mu g\ ml^{-1}$) achieved a 20- to at least 200-fold stronger inhibition of virus–cell binding than did GS (Table 2). This confirms previous results obtained for DS and ATA by a flow cytometric (FACS) method (Schols *et al.*, 1989a).

When GS, ATA and DS were evaluated for their ability to inhibit the binding of OKT4A mAb to the CD4 receptor of MT-4 cells (which are OKT4A$^+$ for more than 99%), GS and DS were found not to influence the binding of OKT4A mAb, even at concentrations that were higher than those required for complete protection of MT-4 cells against HIV-1 cytopathic effect (Table 3). In contrast, ATA effected a marked, concentration-dependent reduction of OKT4A mAb binding: i.e. at a concentration of 25 $\mu g\ ml^{-1}$, ATA achieved a 90% inhibition of OKT4A staining by mAb, even if the cells had been in contact with the compound for only 10–20 s (Table 3).

Aurintricarboxylic acid and dextran sulfate have been shown previously to interact directly with the HIV envelope glycoprotein gp120 (Schols *et al.*, 1990b). The viral gp120 is involved in both virus–cell binding and giant cell formation (Skinner *et al.*, 1988). When GS, ATA and DS were examined for their ability to interfere with the binding of anti-gp120 mAb (recognizing the V3 region of gp120) to persistently HIV-1-infected HUT-78 cells, all three compounds were found to inhibit the binding of anti-gp120 (Table 4). At a concentration of 25 $\mu g\ ml^{-1}$, GS, ATA and DS inhibited anti-gp120 mAb binding by 96, 100 and 84%, respectively.

Activity against DNA and RNA viruses other than HIV

When GS was examined for its inhibitory effect on the replication of DNA viruses, it proved markedly inhibitory to HSV-1, HSV-2, TK$^-$ HSV-1, CMV and VV (Table 5). The IC_{50} of GS for HSV-1 and VV was 3- to 30-fold lower than the IC_{50} of ATA and DS for these viruses. When GS was examined for its inhibitory effect on the replication of RNA viruses other than HIV-1 or HIV-2 (Table 5), the compound was found to be particularly active against Sindbis virus, influenza virus A, RSV and VSV. GS inhibited these viruses at a concentration that was equal to or lower than the concentration at which DS or ATA inhibited these viruses. None of GS, ATA and DS was active against influenza virus B, poliovirus-1, Coxsackie-B4 virus, reovirus-1 or arenaviruses (Junin, Tacaribe) at concentrations that were significantly lower than the cytotoxicity threshold. GS did not cause a microscopically detectable change in the morphology of HeLa or MDCK cells at concentrations up to 400 $\mu g\ ml^{-1}$. The concentration required to cause an alteration of E_6SM, Vero and HEL cell morphology was ≥100 $\mu g\ ml^{-1}$.

Discussion

Our studies indicate that GS, like other sulfated polysaccharides (e.g. dextran sulfate) (Baba *et al.*, 1988c;

Table 2. Inhibitory effects of GS, ATA and DS on the binding of HIV-1 to MT-4 cells

Compound	Concentration ($\mu g\ ml^{-1}$)	Inhibition of binding (% of control)
GS	125	63
	25	44
	5	9
ATA	25	88
	5	85
	1	29
DS	25	97
	5	75
	1	69
	0.2	57

The inhibitory effect on the binding of HIV-1 to MT-4 cells was determined using a p24 ELISA assay, as described in 'Materials and Experimental procedures'.

Table 3. Effects of GS, ATA and DS on the binding of OKT4A mAb to MT-4 cells, as detected by FACS analysis

Compound	Concentration ($\mu g\ ml^{-1}$)	Mean channel fluorescence	% CD4$^+$ cells	IICD4
GS	200	89.8	99.7	0.02
	100	89.2	99.8	0.02
ATA	25	31.6	1.1	0.90
	5	35.5	4.4	0.84
	1	80.2	98.7	0.16
DS	25	89.2	99.8	0.02
Simultest control			25.3	0.3
OKT4A-leu3a mAb			90.8	99.9

MT-4 cells [200 000 cells ($100\ \mu l^{-1}$) RPMI without serum] were incubated with the compounds at the indicated concentrations at 20°C for 10–20 s, further stained and analysed as described in the 'Materials and Experimental procedures'. Measurements were based on the percentage of cells showing fluorescence intensity greater than the control cells stained with normal mouse IgG1-FITC-Phycoerythrin (PE)-labelled IgG_{2a} (Simultest Control) to monitor non-specific immunoglobulin labelling. The inhibitory index for OKT4A mAb binding inhibition (II_{CD4}) was calculated according to the following formula: $II_{CD4} = 1 - (MF_{CD4X} - MF_C/MF_{CD4} - MF_C)$, where MF_{CD4} is the mean channel fluorescence (MF) for the cells incubated only with OKT4A mAb, MF_{CD4X} is the MF for the cells incubated with test compound, and OKT4A mAb and MF_C are the MFs of the cells incubated with IgG1-FITC and IgG2-PE (Simultest immune control). The MF was determined by the Consort 30 program (Becton-Dickinson).

Witvrouw *et al.*, 1991) and sulfated polymers (e.g. polyvinylalcohol sulfate and polyacetal polysulfate) (Schols *et al.*, 1990a; Witvrouw *et al.*, 1992), has a broad-spectrum antiviral activity against a variety of enveloped viruses (Tables 1 and 5). GS is particularly active against HIV and HSV. It inhibits the replication of these viruses at a concentration of about 0.5–1.0 $\mu g\ ml^{-1}$. GS is not inhibitory to MT-4 and E_6SM cell proliferation at concentrations up to 250 and 100 $\mu g\ ml^{-1}$, respectively. Thus the selectivity index of GS for HIV (in MT-4 cells) and HSV (in E_6SM cells) can be estimated to be >350 and >150, respectively. The marked activity of GS against both HIV and HSV may be of particular utility in AIDS patients where recurrent HSV infections may often complicate and even enhance the underlying HIV disease (Tremblay *et al.*, 1989).

The antiviral activity spectrum of GS is somewhat different from that of dextran sulfate, in that this compound is more active than dextran sulfate against parainfluenza virus type 3, Semliki forest virus, Sindbis virus, influenza A virus, RSV, VV and HSV-1, but less active against HIV-1, HIV-2 and CMV. In fact, the activity spectrum of GS is comparable to that of ATA.

The mechanism of the anti-HIV action of GS may be attributed to the inhibition of the binding of virus to the cells (Table 2). The relative potencies of GS, ATA and DS in achieving 50% inhibition of virus binding correspond to their relative potencies in inhibiting HIV-1 cytopathic effect in MT-4 cells; namely, GS is effective at an approximately 5- to 10-fold higher concentration than is ATA or DS. The inhibition of virus binding by GS can be ascribed to a direct interaction of the compound with the HIV envelope glycoprotein (gp120) (Table 4). Pretreatment of the HIV particles for 1 h at 37 °C with GS did not influence the cytopathic effect of HIV (data not shown). The inhibitory effects of GS on enveloped viruses other than HIV, such as HSV, RSV, VSV and influenza A virus, may also be attributed to inhibition of virus binding to the host cells.

Seaweeds are a rich source of proteins in the diet of oriental people. Few side-effects and a low cost price may be expected for antiviral agents extracted from natural sources. In this respect, the identification in red seaweed of a component with anti-HIV and -HSV activity may be of particular importance. Whether GS has any clinical potential, i.e. in the systemic and/or topical therapy or prophylaxis of HIV or HSV infections, remains to be determined.

In conclusion, our results suggest that a natural polysaccharide product is a potent inhibitor of HIV and

Table 4. Effect of GS, ATA and DS on the binding of anti-gp120 mAb to persistently HIV-1-infected HUT-78 cells, as detected by FACS analysis

Compound	Concentration ($\mu g\ ml^{-1}$)	Mean channel fluorescence	% gp120$^+$ cells	IIgp120
GS	25	29.2	2.7	0.96
	5	33.1	10.7	0.79
	1	52.7	70.3	0.15
ATA	25	26.5	1.6	1.00
	5	28.0	3.0	0.95
	1	41.3	35.8	0.52
	0.2	53.9	75.6	0.11
DS	25	31.6	8.7	0.84
	5	34.9	16.7	0.73
	1	40.3	30.1	0.55
	0.2	41.3	35.1	0.52
RaM-IgG(ab')$_2$-FITC		26.6	1.3	
anti-gp120 mAb + RaM-IgG-F(ab')$_2$-FITC		57.2	80.7	

HIV-1-infected HUT-78 cells [200 000 cells ($100\ \mu l^{-1}$) RPMI without serum] were washed twice, incubated with the compounds at the indicated concentrations at 20 °C for 20 min, stained and analysed as described in 'Materials and Experimental procedures'. The inhibitory index for anti-gp120 mAb binding inhibition (IIgp120) was calculated according to the formula $IIgp120 = 1 - (MF_{gp120X} - MF_C/MF_{gp120} - MF_C)$, where MF_{gp120} is the mean channel fluorescence (MF) for the cells incubated with anti-gp120 mAb at concentration *X*, MF_{gp120X} is the MF for the cells incubated with test compound and anti-gp120 mAb, and MF_C is the MF of the cells incubated with RaM-IgG-F(ab')$_2$-FITC to determine non-specific fluorescence. MF was determined by the Consort 30 program (Becton-Dickinson).

Table 5. Inhibitory effects of GS, ATA and DS on the replication of DNA and RNA viruses (other than HIV)

Viruses	IC_{50}[a] ($\mu g\,ml^{-1}$)		
	GS	ATA	DS
DNA viruses			
Herpes simplex virus type 1 (HSV-1) (strain KOS)	0.7	5.5	2.0
TK$^-$ HSV-1 (strain B2006)	0.7	0.7	1.0
TK$^-$ HSV-1 (strain VMW-1837)	3.0	4.5	1.4
Herpes simplex virus type 2 (HSV-2) (strain G)	2.5	2.0	1.4
Cytomegalovirus (CMV) (strain Davis)	4.0	0.7	0.4
Vaccinia virus	7	70	200
RNA viruses			
Sindbis virus	11	20	150
Semliki forest virus	85	150	>400
Junin virus	>100	10	8
Tacaribe virus	>100	10	8.4
Parainfluenza virus type 3	55	40	>400
Influenza A virus	4	–	14
Influenza B virus	>200	–	>200
Respiratory syncytial virus (RSV)	4	–	14
Vesicular stomatitis virus (VSV)	14	110	14
Polio virus type 1	>200	>200	>400
Reo virus type 1	>100	>200	300
Coxsackie virus type B4	>200	>200	>200

[a]50% inhibitory concentration, based on the inhibition of HSV-1, TK$^-$ HSV-1, HSV-2 or VV in E_6SM cells; CMV in HEL cells; Sindbis virus, Semliki forest virus, Junin virus, Tacaribe virus, parainfluenza virus and reovirus in Vero cells; RSV, VSV, poliovirus and Coxsackie virus in HeLa cells; and influenza A and B viruses in MDCK cells. All data represent mean values for at least two separate experiments.

HSV infections *in vitro*. The *in vivo* efficacy and bioavailability of GS, and its toxicity profile upon both systemic and topical administration, are subjects for further study.

Materials and Experimental procedures

Compounds

Dextran sulfate (DS) (MW 5000) was purchased from the Sigma Chemical Co. (St Louis, MO, USA). Aurintricarboxylic acid (ATA) (MW 422.35) was obtained from the Aldrich Chemical Co. (Brussels, Belgium). GS, a galactan sulfate (DiNinno and McCandless, 1978; Taylor and Buchanan-Smith, 1992), was isolated by Dr Natale Vittori Bianchini (Biotecnología Epsilon C.A., Caracas, Venezuela).

The polysaccharide was extracted and purified from the Venezuelan red seaweed *Agardhiella tenera*, isolated from the Mochima National Park on the eastern coast of Venzuela. After harvesting, the alga was air-dried for transport to the laboratory, then washed in cold running water (30 min), drained, and heated (2 h) in boiling water, before blending (Waring Blender) and further heating (2 h).

After filtration, the polysaccharide was precipitated by addition of propan-2-ol (2–3 volumes), washed (2 volumes, 90% v/v water-propan-2-ol), dried at 60 °C, pulverized, and converted into the potassium salt by stirring the recovered powder at 25 °C for 12 h in 25 parts by weight of water-propan-2-ol (50% v/v) containing 14% by weight of potassium chloride.

The product was recovered on a Buchner filter and treated again three times, using a fresh solution each time, before it was redissolved in water; the solution was clarified by filtration, and the polysaccharide was reprecipitated with propan-2-ol as before. Finally, the polysaccharide was washed repeatedly with water-propan-2-ol (three times in 60% v/v and twice in 90% v/v) until the washing gave a negative test for chloride, then dried at 60 °C and ground to powder. The sulphate content was measured according to the procedure described by Therno (1971). The sulphate content was 28% of dry weight.

Cells

MT-4 cells (Miyoshi *et al.*, 1982) and MOLT-4 cells (clone 8) (Kikukawa *et al.*, 1986) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 0.1% sodium bicarbonate and 20 $\mu g\,ml^{-1}$ gentamicin. All other cell cultures [Vero, HeLa, E_6SM and MDCK (Madin-Darby canine kidney)], except for HEL cells, were maintained in Eagle's minimum essential medium (MEM) supplemented with 10% FCS, 2 mM L-glutamine and 0.1% sodium bicarbonate. HEL (human embryonic lung) fibroblasts cells were kept in MEM supplemented with 10% FCS, 2 mM L-glutamine and 0.02% sodium bicarbonate.

Viruses

The virus stocks were prepared as described previously: herpes simplex virus type 1 (HSV-1) (strain KOS), HSV-2 (strain G), thymidine kinase-deficient (TK$^-$) HSV-1 (strain B2006) (De Clercq *et al.*, 1980), TK$^-$ HSV-1 (strain VMW-1837) (Vinckier *et al.*, 1987), vaccinia virus (VV), vesicular stomatitis virus (VSV), Coxsackie virus type B4, poliovirus type 1 and Sindbis virus (De Clercq *et al.*, 1975); influenza virus A (strain A/Ishikawa/7/82 H3N2); influenza virus B (strain B/Singapore/222/79) (Shigeta *et al.*, 1988); respiratory syncytial virus (RSV) (strain Long) (Kawana *et al.*, 1987); human immunodeficiency virus type 1 (HIV-1) (strain HTLV-III_B/LAI) (Popovic *et al.*, 1984), HIV-2 (strain LAV-2_{ROD}) (Clavel *et al.*, 1986), as obtained from the culture supernatant of HIV-1- or HIV-2-infected cell lines (HUT-78/HTLV-III_B, MT-4/LAV-2_{ROD}, respectively) (Pauwels *et al.*, 1987; Schols *et al.*, 1989b); parainfluenza virus type 3 (ATCC VR-93), reovirus type 1 (ATCC VR-230), cytomegalovirus (CMV) [strain Davis (ATCC VR-807)], Semliki forest virus (ATCC VR-67), as obtained from the American Type Culture Collection (Rockville, MD, USA). Junin virus and Tacaribe virus were provided by Dr C.E. Coto (Buenos Aires, Argentina).

Antiviral activity assays

For HSV, VV, VSV, Coxsackie, polio, parainfluenza, Sindbis, Semliki forest, Junin, Tacaribe and reovirus, the assay procedures have been described previously (De Clercq *et al.*, 1975, 1980, 1986; Andrei and De Clercq, 1990). Confluent (Vero, HeLa or E_6SM) cell cultures in 96-well microtitre trays were exposed to 100 $CCID_{50}$ (1 $CCID_{50}$ being the infective dose for 50% of the cell cultures) of virus per well in the presence of varying concentrations of the test compounds diluted in Eagle's

minimum essential medium (MEM) supplemented with 3% FCS, 2 mM L-glutamine and 0.1% sodium bicarbonate. The cells were further incubated at 37 °C until virus-induced cytopathic effect was completed in the control (virus-infected untreated) cell cultures.

The inhibitory effects of the test compounds on HIV-1 and HIV-2 replication were monitored by the inhibition of virus-induced cytopathic effect in MT-4 cells. Briefly, MT-4 cells were suspended at 3×10^5 cells ml^{-1} and infected with HIV-1 or HIV-2 at 100 $CCID_{50}$ ml^{-1}. Immediately after infection, 100 μl of the cell suspension was brought into each well of a flat-bottomed microtitre tray containing various concentrations of the test compounds. After 5 days incubation at 37 °C, the number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method, as previously described (Pauwels *et al.*, 1988).

For influenza virus and RSV, confluent cell cultures (MDCK and HeLa, respectively) in microtitre trays were inoculated with 20 $CCID_{50}$ of the virus per well. The cells were further incubated in the presence of various concentrations of the test compounds and monitored for virus-induced cytopathic effect as described previously (Kawana *et al.*, 1987; Shigeta *et al.*, 1988).

For the anti-CMV assays, human embryonic lung (HEL) fibroblasts were infected with 100 plaque forming units (pfu) of virus. Compounds and virus were added simultaneously, and the cells were further incubated at 37 °C in MEM supplemented with 2% FCS, 2 mM L-glutamine and 0.02% sodium bicarbonate. Virus plaque formation was monitored as described previously (Snoeck *et al.*, 1988).

Virus-induced cytopathic effect was recorded at 1 or 2 days post-infection p.i. for VSV; at 2 days p.i. for Coxsackie virus, Semliki forest virus and poliovirus; at 2 or 3 days p.i. for HSV-1 (KOS), HSV-2 (G), TK^- HSV-1 (VMW-1837 and B2006), VV and Sindbis virus; at 3 days p.i. for influenza A and B viruses and respiratory syncytial virus; at 4 to 5 days p.i. for parainfluenza virus; at 5 days p.i. for Junin virus, Tacaribe virus, HIV-1 and HIV-2; and at 6 to 7 days p.i. for CMV and reovirus.

Giant cell syncytium formation

MOLT-4 (clone 8) cells (1.8×10^6 cells ml^{-1}) were cultured with persistently HIV-1- or HIV-2-infected HUT-78 cells (2×10^5 cells ml^{-1}) in microtitre tray wells containing various concentrations of the test compounds. After a 24-h cocultivation period, the number of giant cells (syncytia) was recorded microscopically, as described previouly (Baba *et al.*, 1990).

Virus adsorption assay

The inhibitory effects of the test compounds on virus adsorption were measured in parallel using two techniques. First, an indirect immunofluorescence-laser flow cytofluorographic method has been specifically designed for this purpose (Schols *et al.*, 1989a). Briefly, MT-4 cells were exposed to HIV-1 virions, which had been concentrated from the supernatant of HIV-1-infected MT-4 cells in the presence or absence of the test compounds. The compounds were added 10–20 s before the virus was added. The cells were processed for indirect immunofluorescence using a polyclonal antibody to HIV-1, and analysed for cell-bound HIV-1 virions by laser flow cytofluorography. In a second assay, MT-4 cells (5×10^5 cells per tube) were incubated with HIV-1 (corresponding to 100 ng of p24) in the presence or absence of the test compound. After a 2-h incubation period at 37 °C, cells were extensively washed with phosphate buffered saline (PBS) to remove unadsorbed virus particles. The cells were then lysed with PBS containing 0.5% Nonidet-P40. The amount of antigen was determined by an enzyme-linked immunosorbent assay (ELISA) (DuPont).

CD4 immunofluorescence assay

CD4 expression was determined by FACSTAR (Becton-Dickinson, Erembodegem, Belgium) analysis, as described previously (Schols *et al.*, 1989b). Briefly, MT-4 cells were incubated for 10–20 s at room temperature in PBS in the absence of serum with or without test compound. The cells were then stained with optimal concentrations of the monoclonal antibodies OKT4A-FITC (Ortho Diagnostics) and Simultest immune monitoring kit control [FITC-labelled IgG_1-Phycoerythrin (PE)-labelled IgG_{2a}] (Becton-Dickinson) for 20 min at 4 °C, washed once in PBS, and fixed in 0.5 ml of 0.5% paraformaldehyde in PBS.

Glycoprotein gp120 immunofluorescence assay

Normal HUT-78 and HIV-1-infected HUT-78 (HUT-78/HIV-1) cells were grown in RPMI 1640 supplemented with 10% heat-inactivated FCS, 100 IU ml^{-1} of penicillin G, and 20 μg ml^{-1} of gentamicin. HUT-78/HIV-1 cells (200 000 cells) in 100 μl of RPMI with 10% FCS were washed twice in RPMI with 10% FCS, incubated with the compounds at various concentrations at 20°C for 15–20 min in RPMI, washed twice with RPMI to remove residual compound, stained with anti-gp120 mAb (9284, DuPont de Nemours, Brussels, Belgium) for 50 min at 37 °C, washed twice in PBS, incubated with FITC-conjugated $F(ab')_2$ fragments of rabbit anti-mouse immunoglobulin antibody [RaM-IgG-$F(ab')_2$-FITC] (Prosan, Ghent, Belgium) for 50 min at 37 °C, washed once in PBS, resuspended in 0.5 ml of 0.5% paraformaldehyde in PBS, and analysed by flow cytometry as described previously (Schols *et al.*, 1990b). The threshold of positivity for green fluorescence intensity was arbitrarily established on the basis of HIV-1-infected HUT-78 cells incubated solely with RaM-IgG-$F(ab')_2$-FITC.

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