Antiviral activity of low-MW dextran sulphate (derived from dextran MW 1000) compared to dextran sulphate samples of higher MW

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Summary

Dextran sulphate inhibits the replication of enveloped viruses (such as retro-, herpes-, toga, arena-, rhabdo-, orthomyxo- and paramyxoviruses), but is inactive against non-enveloped viruses (such as polio, Coxsackie and reovirus). Within the molecular weight (MW) range of 10000–50000, not much variation was observed in the antiviral potencies of different dextran sulphate (DS) samples, irrespective of the virus examined. However, in contrast with the higher MW samples, the low MW DS sample (prepared from dextran with a MW of 1000) was virtually inactive against herpes simplex virus type 1 and type 2, vesicular stomatitis virus, vaccinia virus, influenza A virus, respiratory syncytial virus and togaviruses (Sindbis, Semliki Forest). It was 10–20-fold less active than the higher MW samples against cytomegalovirus and arenaviruses (Junin, Tacaribe). The inhibitory potency of the 1000 MW DS sample against human immunodeficiency virus (HIV) varied considerably depending on the virus strain and cell type. When examined in MT-4 cells, the 1000 MW DS sample was 7000-, 1000-, 200- or 10-fold more inhibitory to HIV-1_{HE} than HIV-2_{EHO}, HTLV-III_B, HTLV-III_{RF} and LAV-2_{ROD}, respectively. In CEM cells, however, HIV-1_{HE} was less sensitive to the inhibitory effect of the 1000 MW DS sample than HIV-2_{EHO}, equally sensitive as HTLV-III_B and fivefold more sensitive than LAV-2_{ROD}.

Introduction

Several sulphated polysaccharides (i.e. dextran sulphate, heparin, pentosan polysulphate, mannan sulphate) are highly inhibitory to the replication of human immunodeficiency virus (HIV) *in vitro* (Ueno and Kuno, 1987; Ito *et al.*, 1987, 1989; Baba *et al.*, 1988a,b,c, 1990c; Mitsuya *et*

al., 1988; De Clercq, 1989). A sugar backbone is not strictly needed for antiviral activity, since sulphated polymers containing a vinylalcohol backbone are also efficient anti-HIV agents (Baba *et al.*, 1990a; Schols *et al.*, 1990b). Cyclical sulphated polysaccharides such as sulphated cyclodextrins are also inhibitory to the *in vitro* replication of HIV (Anand *et al.*, 1990; Schols *et al.*, 1991). The mechanism of antiviral action of these compounds can be attributed to the inhibition of virus adsorption, as has been demonstrated by Baba *et al.* (1988a,b,c, 1990a,c), Mitsuya *et al.* (1988), Nakashima *et al.* (1989) and Schols *et al.* (1989a).

Sulphated polysaccharides are also inhibitory to various DNA and RNA viruses other than HIV and, as a rule, their antiviral activity is limited to enveloped viruses (Baba *et al.*, 1988c; Schols *et al.*, 1990b). We have now evaluated various dextran sulphate (DS) samples, prepared from dextran fractions with molecular weights (MW) ranging from 1000 to 500000, for their activity against a wide variety of DNA and RNA viruses. The antiviral activity of the low MW DS samples, and in particular the 1000 MW sample, differed markedly from the antiviral activity of the higher MW samples. In some anti-HIV assay systems the 1000 MW DS sample was even more active than the higher MW DS samples. Irrespective of their MW, all dextran sulphate samples seem to interfere with the HIV adsorption process, as demonstrated by an indirect immunofluorescence-laser flow cytofluorographic method (Schols *et al.*, 1989a). They also show a similar pharmacokinetic profile following intravenous administration to rabbits.

Results

Activity against retroviruses

When various dextran sulphate (DS) samples prepared from dextran fractions with MW varying from 1000 to 500000 were examined for their inhibitory effect on the cytopathicity of HIV-1 (HTLV-III_B) in MT-4 cells (Fig. 1) or of HIV-1 (HTLV-III_B) antigen expression in CEM cells (Fig. 2), a marked increase in anti-HIV-1 activity was observed when the MW increased from 1000 to 5000 or 10000. At higher MW (10000–500000), the activity of DS against HTLV-III_B tended to level off. A similar correlation was found between the MW of the DS samples and their

Fig. 1. Inhibitory effects of DS samples of different MW (as indicated in the x-axis) on HIV-1- [HTLV-III_B (—■—), HTLV-III_{RF} (---□---), HIV-1-HE (—○—)] and HIV-2- [LAV-2_{ROD} (·····●·····), HIV-2_{EHO} (---□---)] induced cytopathicity in MT-4 cells. The IC_{50} corresponds to the concentration required to inhibit cytopathicity of HIV in MT-4 cells by 50%.

inhibitory effect on giant cell formation between HUT-78/HTLV-III_B and MOLT-4 cells (Fig. 3). Also, with respect to the anti-HTLV-III_{RF} and anti-HIV-2_{EHO} activity, a similar inhibition pattern was observed as for HTLV-III_B (Figs 1, 2 and 3), except for HIV-2_{EHO} antigen expression in CEM cells (Fig. 2). In the latter case, the 1000 MW DS sample proved more inhibitory than the higher MW samples.

Furthermore, the 1000 MW sample was also more active than the higher MW samples against HIV-1_{HE} and LAV-2_{ROD}, regardless of the assay system used [viral cytopathicity (Fig. 1), viral antigen expression (Fig. 2) or giant cell formation (Fig. 3)]. Also, the activity of the DS samples against HIV-1_{HE} and LAV-2_{ROD} tended to level off when the MW increased from 10000 to 500 000 (Figs 1, 2

Fig. 2. Inhibitory effects of DS samples of different MW (as indicated in the x-axis) on HIV-1- [HTLV-III_B (—■—), HTLV-III_{RF} (---□---), HIV-1-HE (—○—)] and HIV-2- [LAV-2_{ROD} (·····●·····), HIV-2_{EHO} (---□---)] antigen expression in CEM cells. The IC_{50} corresponds to the concentration required to inhibit HIV antigen expression in CEM cells by 50%.

and 3). As a rule, the DS samples were 10–50-fold more inhibitory to HIV-induced cytopathicity in MT-4 cells (Fig. 1) than giant cell formation between HIV-infected HUT-78 cells and uninfected MOLT-4 cells (Fig. 3). Against simian immunodeficiency virus (SIV) (strain SIV_{MAC251}) in MT-4 cells, all DS samples tested (i.e. DS 1000, DS 5000, DS 40000, DS 500000) proved antivirally effective within the concentration range of 2–12 $\mu g/ml$ (Table 1). They were inhibitory to MSV-induced transformation of murine C3H/3T3 cells only at concentrations exceeding 10 $\mu g/ml$. None of the DS samples tested impaired the viability of mock-infected MT-4 cells at concentrations up to 2.5 mg/ml (data not shown).

Fig. 3. Inhibitory effects of DS samples of different MW (as indicated in the x-axis) on HIV-1- [HTLV-III_B (—■—), HTLV-III_{RF} (---□---)] and HIV-2- [LAV-2_{ROD} (·····●·····), HIV-2_{EHO} (---□---)] induced giant cell formation. The IC_{50} corresponds to the concentration required to inhibit by 50% giant cell formation following co-cultivation of persistently HIV-infected HUT-78 cells with MOLT-4 cells. Attempts to obtain a persistently HIV-1 HE-infected HUT-78 cell line with syncytium-inducing capacity were unsuccessful.

As previously demonstrated with radiolabelled virus particles (Baba *et al.*, 1988a; Mitsuya *et al.*, 1988), a radioimmunoassay (Nakashima *et al.*, 1989) and a flow cytometric method (Schols *et al.*, 1989a), dextran sulphate blocks HIV-1 binding to MT-4 cells. Using this flow cytometric procedure, we found that all DS samples listed in Table 1 blocked HIV-1 (HTLV-III_B) or HIV-2 (LAV-2_{ROD}) adsorption to MT-4 cells at a concentration of 25 $\mu g/ml$ (data not shown). For all DS samples evaluated, the inhibition of virus binding was estimated at 91–98%.

Activity against other RNA viruses

When the dextran sulphates were examined for their inhibitory effects on the replication of RNA viruses other than retroviruses, the compounds were found to be active against enveloped viruses [togaviruses (Sindbis, Semliki Forest),

Table 1. Inhibitory effects of DS samples of different MW on the replication of retroviruses.

Compound	IC ₅₀ (μg/ml) ^a											
	HIV-1				HIV-2				SIV		MSV	
	HTLV-III _B		HTLV-III _{RF}		HIV-1 _{NE}		LAV-2 _{ROD}		HIV-2 _{BO}		SIB _{MAC251}	
	MT-4	CEM	MT-4	CEM	MT-4	CEM	MT-4	CEM	MT-4	CEM	MT-4	C3H/3T3
DS 1000 K	9.9 ± 6.2	0.5 ± 0.3	1.8 ± 1.6	33.8 ± 16	0.01 ± 0.006	0.2 ± 0.1	0.2 ± 0.1	1.0 ± 0.091	73 ± 59	0.08 ± 0.06	10.0 ± 1.3	272 ± 8
DS 1000 Na	7.1 ± 2.1	—	0.8 ± 0.7	—	0.01 ± 0.002	—	0.1 ± 0.07	—	19 ± 12	—	9.8 ± 0.3	480 ± 20
DS 1500	2.9 ± 1.6	0.1 ± 0.06	0.1 ± 0.06	36 ± 14	0.03 ± 0.01	0.5 ± 0.2	0.1 ± 0.02	1.0 ± 0.01	49 ± 57	0.2 ± 0.1	—	40 ± 24
DS 3000	0.7 ± 0.4	—	0.04 ± 0.002	—	0.1 ± 0.02	—	0.3 ± 0.3	—	2.3 ± 0.4	—	—	10 ± 4
DS 5000	0.5 ± 0.2	0.03 ± 0.007	0.07 ± 0.009	1.1 ± 0.07	0.08 ± 0.05	0.9 ± 0.3	0.3 ± 0.1	1.2 ± 0.02	3.6 ± 2.9	0.4 ± 0.4	9.8 ± 1.7	9.3 ± 4.7
DS 10 000	0.2 ± 0.02	0.2 ± 0.05	0.18 ± 0.04	0.9 ± 0.1	0.6 ± 0.005	2.9 ± 0.4	1.2 ± 0.6	2.6 ± 0.2	6.6 ± 2.5	3.8 ± 0.4	—	27 ± 24
DS 40 000	0.4 ± 0.1	0.7 ± 0.2	0.7 ± 0.1	0.9 ± 0.01	0.8 ± 0.02	2.9 ± 0.4	2.3 ± 1.4	2.0 ± 0.4	3.2 ± 1.8	2.6 ± 1.3	2.0 ± 0.01	321 ± 179
DS 70 000	0.6 ± 0.1	—	0.8 ± 0.2	1.1 ± 0.04	1.8 ± 0.6	4.5 ± 0.5	3.9 ± 0.6	—	4.3 ± 2.7	—	—	303 ± 198
DS 110 000	0.6 ± 0.1	—	0.8 ± 0.1	—	2.3 ± 0.005	—	4.1 ± 0.8	—	3.9 ± 2.2	—	—	743 ± 68
DS 500 000	0.6 ± 0.1	0.9 ± 0.02	0.7 ± 0.08	0.9 ± 0.03	1.0 ± 0.1	3.7 ± 0.7	4.1 ± 0.6	3.8 ± 0.2	3.3 ± 1.6	2.4 ± 1.1	2.0 ± 0.2	326 ± 99
DS 5000 (Ueno)	0.6 ± 0.4	0.1 ± 0.08	0.06 ± 0.04	—	0.03 ± 0.01	—	0.2 ± 0.08	1.5 ± 0.5	3.3 ± 3.7	0.2 ± 0.03	9.8 ± 2.0	40 ± 16
DS 5000 (Sigma)	0.4 ± 0.3	0.1 ± 0.07	0.06 ± 0.02	4.2 ± 3.3	0.08 ± 0.02	0.6 ± 0.4	0.1 ± 0.05	0.9 ± 0.2	5.5 ± 6.3	0.2 ± 0.04	11.9 ± 2.1	14 ± 6

a. 50% inhibitory concentration. Antiviral assays as described in the Materials and Experimental procedures.

All data represent mean values for at least two separate experiments.

Table 2. Inhibitory effects of DS samples of different MW on the replication of RNA viruses other than retroviruses.

Compound	IC ₅₀ (µg/ml) ^a									
	Enveloped viruses							Non-enveloped viruses		
	Sindbis virus	Semliki forest virus	Junin virus	Tacaribe virus	VSV	Influenza A virus	RSV	Polio virus	Coxsackie virus	Reo virus
	Vero	Vero	Vero	Vero	HeLa	MDCK	HeLa	HeLa	HeLa	Vero
DS 1000 K	>400	>400	1.1	1.6	110	—	—	>400	>400	>400
DS 1000 Na	300	>400	0.2	0.2	70	200	12	>400	>400	>400
DS 1500	>400	>400	—	—	70	200	1.4	>400	>400	>400
DS 3000	185	100	0.2	0.4	4.5	42	0.4	>400	>400	>400
DS 5000	150	120	0.3	0.4	2	5.8	0.2	>400	>400	>400
DS 10 000	15	14	0.2	0.3	0.7	1.4	0.1	>400	>400	>400
DS 40 000	2	14	0.04	0.2	0.2	1.0	0.1	>400	>400	>400
DS 70 000	14	14	0.1	0.2	0.5	1.4	0.1	>400	>400	>400
DS 110 000	2	20	—	—	0.7	—	—	>400	>400	>400
DS 500 000	7	20	—	—	0.7	—	—	>400	>400	>400
DS 5000 (Ueno)	100	400	0.5	1.1	7	8	0.1	>400	>400	>400
DS 5000 (Sigma)	175	175	2.0	5.0	6	18	0.6	>400	>400	>400

a. 50% inhibitory concentration. Antiviral assays as described in the Materials and Experimental procedures.

All data represent mean values for at least two separate experiments.

arena- (Junin, Tacaribe), rhabdo- (vesicular stomatitis), orthomyxo- (influenza A), paramyxo- (respiratory syncytial) (Table 2). Except for the arenaviruses Junin and Tacaribe, the 1000- and 1500 MW DS samples were much less active than the higher MW samples against Sindbis virus, Semliki Forest virus, vesicular stomatitis virus, influenza A virus and respiratory syncytial virus, but their activity against Junin virus and Tacaribe virus was only slightly reduced as compared to that of the higher MW samples. As a rule, the antiviral activity increased with increasing MW, with an optimum at MW 40 000 (Table 2). None of the DS samples proved active against influenza B virus, measles virus, parainfluenza-3 virus (data not shown) and to the non-enveloped viruses polio, Coxsackie and reo (Table 2).

Activity against DNA viruses

When the dextran sulphates were examined for their inhibitory effects on the replication of DNA viruses (Table 3), all compounds were inhibitory to the herpesviruses [herpes simplex virus type 1 (HSV-1), thymidine kinase deficient (TK⁻) HSV-1, HSV-2 and cytomegalovirus (CMV)] and to vaccinia virus (VV). The 1000 and 1500 MW DS samples were much less active than the higher MW DS samples against HSV-1, TK⁻ HSV-1, HSV-2 and VV, but they were almost as active as the higher MW samples against CMV. As for the RNA viruses (Table 2), the optimal activity against DNA viruses was observed with 40 000 MW DS.

The IC₅₀ (50% inhibitory concentration) values of the DS 5000 MW sample for both RNA viruses (Table 2) and DNA viruses (Table 3) were comparable to those of the two reference samples obtained from Ueno Fine Chemicals Industry Ltd (Hyogo, Japan) and Sigma Chemical Co. (St Louis, MO), respectively. None of the dextran sulphates impaired the viability of primary rabbit kidney (PRK), Vero, HeLa or human embryonic lung (HEL) cells at concentrations up to 400 µg/ml (data not shown).

Pharmacokinetics in rabbits

When the DS samples with MW 1000, 1500, 3000, 5000 or 10 000 were administered intravenously (i.v.) to rabbits at a dose of 0.5 g/kg, DS concentrations of about 2.3 mg/ml were found in the serum 7.5 min after injection (Fig. 4). At 2 h after injection, the serum DS concentration was about 300 µg/ml. At 8 h after injection, the concentration had fallen below the minimal detectable levels (i.e. 70–170 µg/ml). The serum DS concentrations declined biphasically, with an initial half-life of approximately 25–60 min. When evaluated under the same experimental conditions, the half-life of the Sigma DS (MW 5000) was approximately 30–70 min.

Discussion

Within the MW range of 3000–500 000, no marked differences in the anti-HIV-1 or anti-HIV-2 activity of DS were observed. However, when the MW decreased to 1000,

Fig. 4. Compound concentrations in the serum following i.v. injection of DS samples of different MW [DS 1000 (—■—); DS 1500 (—□—); DS 3000 (—○—); DS 5000 (·····●·····); DS 10 000 (---□---)] at a dose of 0.5 g/kg to rabbits.

significant differences in the activity of DS against the different HIV strains were noted. The 1000 MW DS sample was 7000-, 1000- or 200-fold more inhibitory to HIV-1_{HE} than HIV-2_{CHO}, HTLV-III_B and HTLV-III_{RF}, respectively. Hartman *et al.* (1990) reported that DS with a MW less than 2300 had no anti-HTLV-III_B effect. In their experiments they exposed ATH8 cells to HTLV-III_B at 2.8×10^4 times the 50% cell culture infective dose (CCID₅₀). This high multiplicity of infection (MOI) may have made it difficult to demonstrate an antiviral effect with DS, since, as demonstrated by Nakashima *et al.* (1989), the anti-HTLV-III_B activity of DS decreases when the MOI increases.

Our findings indicate that with lowering the MW of dextran sulphate, its activity against HIV-1_{HE} and LAV-2_{ROD} increases (Figs 1 and 2). Thus, low MW DS demonstrates a marked virus strain dependence in its anti-HIV activity. A strain dependence in the anti-HIV activity of DS has also been described by Busso and Resnick (1990). From our data, it is evident that (i) the variability between various HIV strains in their susceptibility to DS increases with decreasing the MW of DS, and (ii) some HIV-1 as well as HIV-2 strains may prove more susceptible to the 1000 MW DS than DS samples of higher MW.

DS has been shown to block the attachment of HIV particles to T lymphocytes (Mitsuya *et al.*, 1988; Baba *et al.*, 1988b; Schols *et al.*, 1989a). DS does not interfere with the binding of OKT4/Leu-3a monoclonal antibodies (mAb) to the cells (Mitsuya *et al.*, 1988; Schols *et al.*, 1989a, 1990a; Lederman *et al.*, 1989), although DS has been postulated to interact with the CD4 molecule (Lederman *et al.*, 1989; Parish *et al.*, 1990). Lederman *et al.* (1989) developed a solid-phase assay to study the influence of DS on the interaction of recombinant (r) forms of CD4 and gp120 in the absence of any other cellular or viral components. The interaction of rCD4 with gp120 was inhibited by DS but not dextran. Pretreatment experiments indicated that DS interfered predominantly with rCD4. Parish *et al.* (1990) demonstrated that DS MW 500 000, but not DS MW 5000 inhibited mAb G19-2 binding to the CD4 receptor. However, mAb G19-2 fails to block HIV infection. It is surprising, therefore, that DS, in spite of its inhibitory action on HIV adsorption to CD4⁺ cells, does not affect the cell binding of anti-CD4 mAb known to interact with the

Table 3. Inhibitory effects of DS samples of different MW on the replication of enveloped DNA viruses.

Compound	IC ₅₀ (μg/ml) ^a						
	HSV-1 (KOS)	TK ⁻ HSV-1 (B2006)	TK ⁻ HSV-1 (VMW-1837)	HSV-2 (G)	CMV (AD-169)	CMV (Davis)	VV
	PRK	PRK	PRK	PRK	HEL	HEL	PRK
DS 1000 K	110	45	95	45	2	3	300
DS 1000 Na	110	110	110	95	2.5	3	275
DS 1500	10	20	10	7	—	—	400
DS 3000	4.5	2	4.5	2	0.7	0.8	—
DS 5000	2	2	2	1.4	0.7	0.7	80
DS 10 000	2	1.4	2	1.4	0.6	0.5	10
DS 40 000	2	0.7	1.4	0.3	0.3	0.3	4.5
DS 70 000	2	0.9	2.4	0.6	0.6	0.6	20
DS 110 000	1	1.4	2	0.7	0.1	0.3	20
DS 500 000	2	2	2	0.7	0.1	0.3	40
DS 5000 (Ueno)	7	7	7	2	0.7	0.5	70
DS 5000 (Sigma)	5	6	7	3	2	0.6	100

a. 50% inhibitory concentration. Antiviral assays as described in the Materials and Experimental procedures.

All data represent mean values for at least two separate experiments.

gp120 binding site. Possibly, DS binds to CD4 in such a way that it leaves the external surface of CD4 exposed to mAb but blocks an epitope involved in gp120 binding.

It has been shown clearly that DS interacts with the V3 region of gp120 glycoprotein, and this may explain why DS inhibits HIV binding to the cells as well as fusion of HIV-infected cells with uninfected CD4⁺ cells (Schols *et al.*, 1990a). The molecular basis for the differential inhibitory effects of DS samples of different MW on different HIV strains and other enveloped viruses remains to be resolved. As the anti-HIV activity of the low MW samples of DS is dependent on both the virus strain and cell type, it is obvious that these samples are able to recognize subtle differences in the interaction of the gp120 glycoproteins of different HIV strains with the CD4 receptor. As the MW of DS decreases it may be able to probe certain differences in the gp120 structure of different HIV strains which go unnoticed for the higher MW DS samples. In this sense, the 1000 MW DS could be considered as a marker for specific structural features of the gp120 molecule. It would seem imperative to investigate the molecular basis that underlies the differential effects of the 1000 MW DS on different HIV strains.

An important issue concerns the potential clinical usefulness of DS in the treatment of patients with acquired immunodeficiency syndrome (AIDS) or AIDS-related complex (ARC). The pharmacokinetics of DS samples of MW ranging from 1000 to 10 000 following i.v. injection to rabbits did not show significant differences. For all DS samples the initial half-life could be estimated at 25–60 min. These data are in agreement with the results of Hartman *et al.* (1990) who found, following high-performance liquid chromatography (HPLC) analysis of plasma from rats injected i.v. with DS, an initial plasma half-life of about 30 min.

DS exhibits anti-HIV activity at a concentration which is well below the anticoagulant threshold: i.e. DS 5000 inhibits HIV-1 (HTLV-III_B) at a concentration of 0.5 µg/ml, which corresponds to 0.01 heparin units/ml. For DS 1000, the difference between the concentrations required to inhibit the most sensitive virus strains (HIV-1_{HE} and LAV-2_{ROD}; IC₅₀ 0.01 and 0.1 µg/ml, respectively) and the anticoagulant concentration (1 heparin unit being 515 µg) exceed even 50 000–5000-fold. The low MW DS samples may have real clinical utility in the chemoprophylaxis or -therapy of AIDS or ARC, provided the HIV strains involved in the pathogenesis of the disease has a DS-sensitivity pattern similar to that of HIV-1_{HE} and LAV-2_{ROD}.

Materials and Experimental procedures

Compounds

DS samples were prepared specially from dextran fractions with MW of 1000, 1500, 3000, 5000, 10 000, 40 000, 70 000, 110 000

and 500 000 by the Company Pfeifer & Langen, Dormagen, Germany. DS samples (MW 5000) obtained from Sigma Chemical Co. and Ueno Fine Chemicals Industry Ltd (lot 548) served as reference samples.

Cells

MT-4 cells (Miyoshi *et al.*, 1982), CEM cells (Foley *et al.*, 1965) and MOLT-4 cells (clone 8) (Kikukawa *et al.*, 1986) were grown in RPMI-1640 medium supplemented with 10% heat-inactivated foetal calf serum (FCS), 2 mM L-glutamine, 0.1% sodium bicarbonate and 20 µg/ml gentamicin. All other cell cultures [Vero, HeLa, PRK, MDCK (Madin-Darby canine kidney) and murine C3H/3T3 embryo fibroblast cells], except for HEL cells, were maintained in Eagle's minimum essential medium (MEM) supplemented with 10% FCS, 2 mM L-glutamine, 0.1% sodium bicarbonate. HEL fibroblast cells were kept in MEM supplemented with 10% FCS, 2 mM L-glutamine and 0.02% sodium bicarbonate.

Viruses

The origin of the virus stock was as described previously: HSV-1 (KOS strain), HSV-2 (G strain), TK⁻ HSV-1 (B2006 strain) (De Clercq *et al.*, 1980); TK⁻ HSV-1 (VMW-1837) (Vinckier *et al.*, 1987); Moloney murine sarcoma virus (MSV) (De Clercq and Merigan, 1971); VV, vesicular stomatitis virus (VSV), Coxsackie virus type B4, poliovirus type 1 and Sindbis virus (De Clercq *et al.*, 1975); influenza A virus A/Ishikawa/7/82 (strain H3N2), influenza B virus (B/Singapore/222/79 strain) (Shigeta *et al.*, 1988); measles virus (Sugiyama strain) (Hosoya *et al.*, 1989); respiratory syncytial virus (RSV) (Long strain) (Kawana *et al.*, 1987); HIV-1 (HTLV-III_B and HTLV-III_{RF} strains) (Popovic *et al.*, 1984); HIV-1 (HIV-1_{HE} strain, which represents a clinical isolate from a Belgian patient with AIDS) [for HIV-1_{HE}, virus stocks were prepared from the supernatant fluid of MT-4 cells which had been infected with the supernatant of the 5th passage of co-cultures of the patient's peripheral blood cells with cord blood lymphocytes (at 1:1 ratio)], HIV-2 (LAV-2_{ROD} strain) (Barré-Sinoussi *et al.*, 1983) and HIV-2 (HIV-2_{HO} strain) (Rey *et al.*, 1989), as obtained from the culture supernatant of HIV-1- or HIV-2-infected cell lines (HUT-78/HTLV-III_B, HUT-78/HTLV-III_{RF}, MT-4/HE, MT-4/LAV-2_{ROD} or CEM/HIV-2_{HO}, respectively) (Pauwels *et al.*, 1987; Schols *et al.*, 1989b); SIV (strain SIV_{MAC251}) was originally isolated by Daniel *et al.* (1987) and obtained from Dr C. Bruck (Smith Kline-RIT, Rixensart, Belgium). SIV virus stocks were prepared from the supernatants of SIV-infected MT-4 cells; reovirus type 1 (ATCC VR-230), CMV [Davis strain (ATCC VR-807) and AD-169 strain (ATCC VR-539)], parainfluenza virus type 3 (ATCC VR-93), Semliki Forest virus (ATCC VR-67), as obtained from the American Type Culture Collection (Rockville, MD); Junin virus and Tacaribe virus were provided by Dr C.E. Coto (Buenos Aires, Argentina).

Antiviral assays

For HSV, VV, VSV, Coxsackie, polio, parainfluenza-3, 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 PRK) cell cultures in 96-well microtitre trays were exposed to

100 CCID₅₀ of virus per well in the presence of varying concentrations of the test compounds diluted in Eagle's MEM supplemented with 3% FCS, 2 mM L-glutamine and 0.1% sodium bicarbonate. For the antiviral assays with Junin and Tacaribe, MEM supplemented with 2% FCS, 2 mM L-glutamine and 0.02% sodium bicarbonate was used. The cells were further incubated at 37°C until virus-induced cytopathicity was completed in the control (virus-infected untreated) cell cultures.

The inhibitory effects of the test compounds on HIV-1, HIV-2 and SIV replication were monitored by the inhibition of virus-induced cytopathicity in MT-4 cells, as described by Pauwels *et al.* (1988) and Balzarini *et al.* (1989b), respectively.

For influenza virus, measles virus and RSV, confluent cell cultures (Vero, MDCK and HeLa cells) in microtitre trays were inoculated with 20 CCID₅₀ 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 cytopathicity, as described previously (Hosoya *et al.*, 1989; Shigeta *et al.*, 1988; Kawana *et al.*, 1987).

Anti-HIV activity was also determined by monitoring viral antigen expression in CEM cells cultured in the presence of various concentrations of the test compounds. CEM cells (2×10^5 cells per ml) were infected with HIV at a 200 CCID₅₀ for all virus strains, except for LAV-2_{ROD} which was used at 100 CCID₅₀. Four days after infection with HIV-1_{HE}, LAV-2_{ROD} or HIV-2_{CHO}, 6 days after infection with HTLV-III_{RF} and 7 days after infection with HTLV-III_B, expression of HIV antigens was revealed by an indirect immunofluorescence procedure using polyclonal antibodies. The percentage of antigen-positive cells was determined by FACS analysis.

For the anti-CMV assays, HEL fibroblasts were infected with 100 PFU (plaque-forming units) of virus. Compounds and virus were added at the same time 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).

For the anti-MSV assays, murine C3H/3T3 embryo fibroblast cells were infected with 80 focus-forming units of MSV and transformation of the cell cultures was scored microscopically at Day 6 after infection, as described by Balzarini *et al.* (1989a).

Virus-induced cytopathicity was recorded at 1 or 2 days post-infection for VSV; at 2 days for Coxsackie virus, Semliki Forest virus and poliovirus; at 2 or 3 days for HSV-1 (KOS), HSV-2 (G), HSV-1 TK⁻ (B2006), HSV-1 TK⁻ (VMW-1837), VV and Sindbis virus; at 3 days for measles virus, influenza A and B virus and RSV; at 4 or 5 days for parainfluenza-3 virus; at 5 days for Junin virus; Tacaribe virus, HIV and SIV; and at 6–7 days for CMV, reovirus and MSV.

Giant cell formation

MOLT-4 (clone 8) cells (1×10^6 cells/ml) were cultured with an equal number of HUT-78/HIV-1 or HUT-78/HIV-2 cells in microtitre tray wells containing various concentrations of the test compounds. After a 24-h co-cultivation period, the number of giant cells was recorded microscopically, as described previously (Baba *et al.*, 1990b).

Virus adsorption assay

The inhibitory effect of the test compounds on virus adsorption

was measured by an indirect immunofluorescence laser flow cytofluorographic method, which had been specifically designed for this purpose (Schols *et al.*, 1989a). Briefly, MT-4 cells were exposed to HIV-1 and HIV-2 virions, which had been concentrated from the supernatant of HUT-78/HTLV-III_B or HUT-78/LAV-2_{ROD} cells, respectively, in the presence or absence of the test compounds. The compounds were added to 10–20 seconds before virus was added. The cells were processed for indirect immunofluorescence using a polyclonal antibody to HIV-1 or HIV-2 and analysed for cell-bound HIV virions by laser flow cytofluorography.

Bioassay

Serum concentrations of the compounds (following i.v. administration to rabbits) were determined by a bioassay, as described by Witvrouw *et al.* (1990). The rabbits received an i.v. injection (in the ear vein) of the dextran sulphates at a dose of 0.5 g/kg. At different times after injection, the blood was collected from the rabbit's other ear and kept at 4°C overnight, after which the serum was collected. The serum samples were stored at -20°C until assayed. The compound concentrations in the serum were determined by measuring the inhibitory effects of the serum samples on LAV-2_{ROD}-induced cytopathicity in MT-4 cells.

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