Antiviral Research, 18 (1992) 139–150

AVR 00551

Sulfonic acid polymers as a new class of human immunodeficiency virus inhibitors

Prem Mohan[a], Dominique Schols[b], Masanori Baba[c] and Erik De Clercq[b]

[a]*Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, Chicago, USA,* [b]*Rega Institute for Medical Research, Katholieke Universiteit, Leuven, Belgium and* [c]*Department of Microbiology, Fukushima Medical College, Fukushima, Japan*

(Received 20 September 1991; accepted 14 November 1991)

Summary

Four sulfonic acid polymers [poly(4-styrenesulfonic acid)(PSS), poly(anetholesulfonic acid)(PAS), poly(vinylsulfonic acid)(PVS), poly(2-acrylamido-2-methyl-1-propanesulfonic acid)(PAMPS)] have been found to inhibit the cytopathicity of HIV-1 and HIV-2 in MT-4 cells at concentrations that are not toxic to the host cells. The sulfonic acid polymers also inhibited syncytium formation in co-cultures of MOLT-4 cells with HIV-1- or HIV-2-infected HUT-78 cells. They also inhibited binding of anti-gp120 mAb to HIV-1 gp120 and blocked adsorption of HIV-1 virions to MT-4 cells. PSS and PAS, but not PVS and PAMPS, interfered with the binding of OKT4A/Leu3a to the CD4 receptor. The anti-HIV activity of these polyanionic compounds can be ascribed to inhibition of the gp120-CD4 interaction. Sulfonic acid polymers represent a lead of anti-HIV compounds that warrant further evaluation of their therapeutic potential.

Sulfonic acid polymer; Human immunodeficiency virus; Glycoprotein gp120; CD4 receptor; Syncytium formation

Introduction

Acquired immunodeficiency syndrome (AIDS) is caused by a pathogenic human retrovirus (Barré-Sinoussi et al., 1983; Gallo et al., 1984; Levy et al.,

Correspondence to: P. Mohan, Department of Medicinal Chemistry and Pharmacognosy (M/C 781), College of Pharmacy, University of Illinois at Chicago, Box 6998, Chicago, IL 60680, USA.

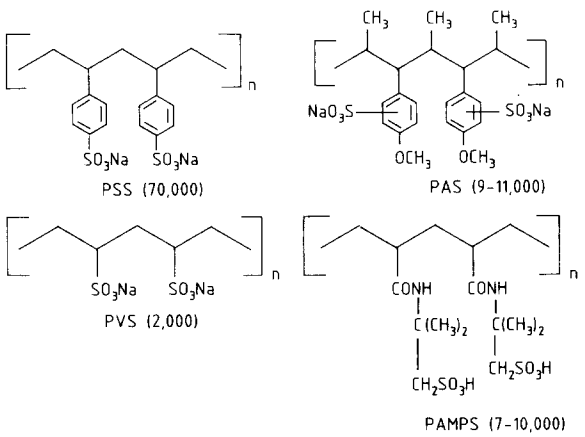

Fig. 1. Sulfonic acid polymers evaluated for anti-HIV activity.

1984), human immunodeficiency virus (HIV), and still remains a formidable challenge to antiviral chemotherapy. Several compounds have been found to interact with specific events within the viral replicative cycle and yield promise as potential anti-AIDS drugs (for a review see De Clercq, 1991). Previous studies have demonstrated the anti-HIV activity of a variety of napthalene-disulfonic acids (Mohan et al., 1991a,b). We have examined the anti-HIV properties of a series of polysulfonates (Fig. 1) and found that these polyanions achieve a highly potent and selective inhibition of HIV replication.

Materials and Methods

Chemicals

Poly(4-styrenesulfonic acid) sodium salt (PSS) (MW = 70,000) and aurintricarboxylic acid (ATA) were purchased from Aldrich (Milwaukee, WI) and poly(anetholesulfonic acid) sodium salt (PAS) (MW = 9–11,000) were purchased from Sigma (St. Louis, MO). Poly(vinylsulfonic acid) sodium salt (PVS) (MW = 2000) was purchased from Polysciences Inc. (Warrington, PA), and poly(2-acrylamido-2-methyl-1-propanesulfonic acid) (PAMPS) (MW = 7–10,000) was purchased from Monomer-Polymer and Dajac Labs Inc. (Trevose, PA). Dextran sulfate (DS) (MW = 5000) and suramin (MW = 1429) were obtained from Sigma and Bayer AG (Wuppertal, Germany), respectively.

Cells

MT-4 (Miyoshi et al., 1982) and MOLT-4 (clone No. 8) (Kikukawa et al., 1986) cells were used in the anti-HIV assays and MOLT-4 cells and persistently HIV-infected HUT-78 cells were used in the giant cell formation assay. The cell

lines were grown and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin G, and 20 μg/ml gentamicin (culture medium).

Viruses

HIV-1_{IIIB} (Popovic et al., 1984) and HIV-2_{ROD} (Barré-Sinoussi et al., 1983) were prepared from the supernatant fluid of HUT-78/HIV-1_{IIIB} and HUT-78/HIV-2_{ROD}, i.e. HUT-78 cells persistently infected with HIV-1_{IIIB} or HIV-2_{ROD}, respectively.

Antiviral assays

Inhibitory effects of the compounds on HIV-1 and HIV-2 replication were monitored by the inhibition of virus-induced cytopathicity in MT-4 cells, as previously described (Pauwels et al., 1987). Briefly, MT-4 cells were suspended at 3×10^5 cells per ml and infected with HIV at 100 times the 50% cell culture infective dose per ml. Immediately after infection, 100 μl of the cell suspension was brought into each well of a flat-bottomed microtiter tray containing various concentrations of the test compound. After 5 days of 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). Cytotoxicity of the compounds for mock-infected MT-4 cells was also assessed by the MTT method.

Giant cell formation assay

MOLT-4 cells (clone 8) were co-cultured with an equal number of HUT-78/HIV-1_{IIIB} or HUT-78/HIV-2_{ROD} cells in microtiter tray wells containing various concentrations of the test compounds. After 24 h of co-cultivation, cells were analyzed by microscopic evaluation or laser flow cytofluorography, as described previously (Schols et al., 1989b, 1990b; Baba et al., 1990a).

Glycoprotein gp120 immunofluorescence assay

Normal HUT-78 cells and HIV-1-infected HUT-78 cells (HUT-78/HIV-1) were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 IU/ml of penicillin G, and 20 μg/ml 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 with 10% FCS, washed twice with RPMI to remove residual compound, stained with anti-gp120 mAb (9284, DuPont de Nemours, Brussels, Belgium) for 45 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 45–50 min at 37°C, washed twice in PBS, resuspended in 0.5 ml of 0.5% paraformaldehyde in PBS, and analyzed by flow cytometry, as described previously (Schols et al., 1990a).

CD4 immunofluorescence assay

CD4 expression was determined by FACSTAR (Becton-Dickinson) analysis, as described previously (Schols et al., 1989c). Briefly, MT-4 cells were incubated for various times at room temperature in PBS in the absence or presence of serum with or without test compound. The cells were then stained with optimal concentrations of the monoclonal antibodies OKT4A-FITC (Ortho Diagnostics) or anti-leu3a-PE and Simultest immune monitoring kit control (FITC-labeled IgG_1 and PE-labeled IgG_2) (Becton-Dickinson) for 20 min at 4°C, washed once in PBS, and fixed in 0.5 ml of 0.5% paraformaldehyde in PBS.

Virus binding assay

The inhibitory effects of the compounds on HIV-1 binding to MT-4 cells were determined by adding a concentrated HIV-1 stock to the cells (200,000 cells/100 μl RPMI with 10% FCS), upon which the cells were incubated for 30 min at 37°C and washed twice in PBS to remove unbound virus. The compounds were added 10–20 s before the virus addition. After the 30-min incubation period, a high-titer polyclonal antibody derived from a patient with AIDS-related complex (diluted 1/100 in PBS) was added, and after another 30 min incubation at room temperature, the cells were washed again twice with PBS. The cells were then incubated with FITC-conjugated $F(ab')_2$ fragments of rabbit anti-human immunoglobulin antibody [RaH-IgG-$F(ab')_2$-FITC] (Prosan, Ghent, Belgium) for 30 min at room temperature, washed once in PBS, resuspended in 0.5 ml of 0.5% paraformaldehyde in PBS, and analyzed by flow cytometry, as described previously (Schols et al., 1989a).

Reverse transcriptase (RT) assay

Purified recombinant HIV-1 RT was obtained from MicroGeneSys Inc. (West Haven, CT). The assay was performed at 37°C for 30 min with 50 μl reaction mixture containing 50 mM Tris-HCl (pH 8.4), 2 mM dithiotreitol, 100 mM KCl, 10 mM $MgCl_2$, 0.1% Triton X-100, 1 μCi [*methyl*-^{3}H]dTTP (30 Ci/mmol), 0.01 A_{260} unit of poly(A)·oligo(dT), test compound, and 0.05 U of enzyme. The reaction was terminated by adding 200 μl of 5% trichloroacetic acid, and the precipitated material was analyzed for radioactivity.

Results

When the sulfonated polymers were evaluated for their inhibitory effect on the cytopathicity of HIV-1 in MT-4 cells, the compounds showed a 50% antivirally effective concentration (EC_{50}) of 1 to 6 μg/ml, whereas the 50% cytotoxic concentration (CC_{50}) was >500 μg/ml for PSS, PAMPS and PVS and 300 μg/ml for PAS (Table 1). Based on the ratio of CC_{50} to EC_{50}, their selectivity index varied from 100 to >625. For HIV-2 similar EC_{50} values were obtained as for HIV-1. Also, the EC_{50} values of the compounds for HIV-1 in MT-4 cells (Table 1) were comparable to those obtained in MOLT-4 cells (data not shown). The sulfonated polymers were also inhibitory to HIV-1- and HIV-2-induced syncytium (giant cell) formation at EC_{50} values similar to those that were inhibitory to HIV-1 and HIV-2 replication, except for PVS (EC_{50}: 30–50 μg/ml) (Table 1).

Using a flow cytometric method (Schols et al., 1989a), we have previously demonstrated that sulfated polysaccharides (Schols et al., 1989a), sulfated polymers (Baba et al., 1990a; Schols et al., 1990c), sulfated cyclodextrins (Schols et al., 1991a) and the hexasulfonated compound suramin (Schols et al., 1989a) block HIV binding to MT-4 cells. These observations apparently extend to the sulfonated polymers PSS, PAS, PVS and PAMPS (Table 1). At a

TABLE 1

Inhibitory effects of the sulfonic acid polymers on HIV-1- and HIV-2-induced cytopathicity, HIV-1- and HIV-2-induced giant cell formation, cell viability and HIV-1 binding to the cells

Compound	Virus cytopathicity EC_{50}[a] (μg/ml)		Giant cell formation EC_{50}[b] (μg/ml)		Cytotoxicity CC_{50}[c] (μg/ml)	Virus-cell binding II_{VB}[d]
	HIV-1	HIV-2	HIV-1	HIV-2		
PSS	6	7	3	4	>500	0.74
PAS	3	7	1	1	300	0.48
PVS	3	1	50	30	>500	0.52
PAMPS	1	3	1	2	>500	0.38
DS	1	0.1	20	18	>500	0.90

[a] 50% Effective concentration, required to achieve 50% protection against induced cytopathicity in MT-4 cells.

[b] 50% Effective concentration, achieving 50% inhibition of giant cell formation between persistently HIV-1- or HIV-2-infected HUT-78 cells and uninfected MOLT-4 cells.

[c] 50% Cytotoxic concentration, corresponding to 50% reduction of the viability of mock-infected MT-4 cells.

[d] The inhibitory index for virus binding to MT-4 cells (II_{VB}) was calculated according to the following formula: $II_{VB} = 1 - (MF_{VC} - MF_{CC})/(MF_V - MF_C)$, whereby MF_{VC} is the mean fluorescence (MF) with a given concentration of the compound for cells exposed to HIV-1, MF_{CC} is the mean fluorescence for the control cells (not exposed to HIV-1) treated with compound, MF_V is the mean fluorescence for the HIV-1-exposed cells (not treated with any compound), and MF_C is the mean fluorescence for the control cells (neither exposed to HIV-1 nor treated with any compound). If $II_{VB} = 1$, there is total inhibition of virus binding; if $II_{VB} = 0$, there is no inhibition of virus binding. All compounds were evaluated at a concentration of 20 μg/ml.

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

concentration of 20 μg/ml, all compounds proved effective in inhibiting HIV-1 binding to MT-4 cells (II_{VB}: 0.38–0.74).

As the sulfonated polymers interfered with HIV adsorption to the cells, they were further evaluated for their ability to inhibit the binding of OKT4A/Leu3a mAb to the CD4 receptor of MT-4 cells. Exposure of MT-4 cells for a few seconds to PSS or PAS resulted in a dose-dependent loss of the staining of OKT4A/Leu3a mAb. Akin to ATA, PSS and PAS strongly interact with the CD4 receptor at a concentration of 20 μg/ml (Table 2). In contrast with PSS and PAS, the two other polysulfonates PVS and PAMPS did not interact with CD4 even at a concentration of 100 μg/ml.

Addition of 10% FCS (as normally present in the culture medium used in the antiviral assays) strongly neutralized the interaction of PSS and PAS with the CD4 receptor. In the presence of 10% FCS, PSS, PAS and ATA had no effect

TABLE 2

Inhibitory effects of the sulfonic acid polymers on the binding of OKT4A/Leu3a mAb to MT-4 cells, as detected by FACS analysis

Compound	Concentration (μg/ml)	Mean channel fluorescence	$CD4^+$ cells (%)	II_{CD4}
PSS	20	3.7	1.1	0.96
	4	3.7	1.1	0.96
	0.8	5.5	6.8	0.84
PAS	20	4.5	2.0	0.90
	4	6.8	18.3	0.76
	0.8	13.8	96.6	0.32
PVS	100	18.7	99.6	0
	20	18.7	99.8	0
	4	18.8	99.7	0
PAMPS	100	18.9	99.7	0
	20	18.8	99.7	0
	4	18.8	99.6	0
ATA	20	3.3	0.6	0.98
	4	3.4	0.9	0.97
	0.8	8.5	43.9	0.65
Simultest control		3.0	0.3	
OKT4A/Leu3a mAb		18.8	99.6	

MT-4 cells (200,000 cells/100 μl PBS) were incubated with the compounds at the indicated concentrations at 20°C for 10–20 s, further stained and analyzed, as described in Materials and Methods. Measurements were based on the percentage of cells showing fluorescence intensity greater than the control cells stained with normal mouse IgG_1-FITC and IgG_2-PE (Simultest control) to monitor nonspecific immunoglobulin labeling. The inhibitory index for OKT4A/Leu3a mAb binding inhibition (II_{CD4}) was calculated according to the following formula: $II_{CD4} = 1 - (MF_{CD4X} - MF_C)/MF_{CD4} - MF_C)$, whereby MF_{CD4} is the mean channel fluorescence (MF) for the cells incubated only with OKT4A/Leu3a mAb, MF_{CD4X} is the MF for the cells incubated with test compound and OKT4A/Leu3a mAb and MF_C is the mean channel fluorescence of the cells incubated with IgG_1-FITC and IgG_2-PE. The mean channel fluorescence was determined by the Consort 30 program (Becton-Dickinson).

TABLE 3

Inhibitory effects of the sulfonic acid polymers on the binding of OKT4A/Leu3a mAb to MT-4 cells in the presence of 10% FCS, as detected by FACS analysis

Compound	Concentration (μg/ml)	Mean channel fluorescence	$CD4^+$ cells (%)	II_{CD4}
PSS	100	3.4	1.2	0.93
	20	13.6	97.3	0.08
PAS	100	7.4	26.8	0.60
	20	14.7	98.0	0
PVS	100	14.8	98.1	0
	20	14.9	98.2	0
PAMPS	100	14.9	98.2	0
	20	14.4	98.3	0
ATA	100	7.0	21.3	0.63
	20	14.3	97.4	0.02
Simultest control		2.6	0.4	
OKT4A/Leu3a mAb		14.6	97.8	

MT-4 cells (200,000 cells/100 μl RPMI with 10% FCS) were incubated with the compounds at the indicated concentrations, stained and analyzed as described in the footnote to Table 2.

on OKT4A/Leu3a mAb binding to the CD4 receptor at a concentration of 20 μg/ml (Table 3), which contrasts with the marked inhibitory effect of these compounds (at 20 μg/ml) on OKT4A/Leu3a mAb binding in the absence of serum (Table 2). The sulfonated polymers (at 100 μg/ml) had no effect on the binding of anti-Leu1 mAb, anti-Leu2a mAb, anti-Leu4 mAb and anti-HLA-DR mAb (Becton-Dickinson), used at optimal concentrations, to the MT-4 cells in the absence of serum (data not shown).

We also examined whether the compounds directly interacted with the viral gp120 glycoprotein which is involved in HIV binding to the cells and syncytium formation. To this end, we used persistently HIV-1-infected HUT-78 cells and a specific anti-gp120 mAb recognizing the gp120 V3 fusion region (Skinner et al., 1988) which plays an important role in HIV fusion to the cells and syncytium formation. All sulfonated polymers had a marked inhibitory effect (50% inhibitory concentration: 0.8–4 μg/ml) on the binding of the anti-gp120 mAb, even if the compounds had been exposed to the cells for only 15 min at room temperature (Table 4).

When the compounds were examined for their inhibitory effects on recombinant HIV-1 RT activity they exhibited a concentration-dependent inhibition. In particular, PSS and PAS were found to be highly potent inhibitors of HIV-1 RT. Their 50% inhibitory concentrations (IC_{50}) were 0.06 and 0.04 μg/ml, respectively (Table 5). These values are significantly lower than the IC_{50} (9.7 μg/ml) of suramin, a well-known inhibitor of retroviral RT (De Clercq, 1979).

TABLE 4

Inhibitory effects of the sulfonic acid polymers on the binding of anti-gp120 mAb to persistently HIV-1-infected HUT-78 cells, as detected by FACS analysis

Compound	Concentration (μg/ml)	Mean channel fluorescence	gp120$^+$ cells (%)	II_{gp120}
PSS	100	3.5	2.2	1.00
	20	4.0	3.6	0.95
	4	6.3	20.8	0.64
	0.8	10.9	71.5	0.07
	0.16	11.6	71.3	0.00
PAS	100	3.5	2.4	1.00
	20	4.2	3.5	0.91
	4	5.9	20.1	0.69
	0.8	10.5	70.2	0.10
	0.16	11.4	72.6	0.00
PVS	100	4.5	4.9	0.87
	20	5.3	8.3	0.77
	4	5.6	14.4	0.73
	0.8	8.9	51.4	0.31
	0.16	10.2	67.4	0.10
PAMPS	100	4.2	3.1	0.91
	20	4.5	5.8	0.87
	4	5.1	10.3	0.79
	0.8	8.6	52.3	0.35
	0.16	11.1	74.6	0.02
ATA	100	3.5	2.2	1.00
	20	4.9	7.3	0.82
	4	6.3	23.6	0.64
	0.8	10.4	57.4	0.11
	0.16	10.7	70.4	0.08
RaM-IgG-F(ab')$_2$-FITC		3.5	1.8	
anti-gp120 mAb + RaM-IgG-F(ab')$_2$-FITC		11.3	73.5	

HIV-1-infected HUT-78 cells (200,000 cells/100 μl RPMI with 10% FCS) were washed twice, incubated with the compounds at the indicated concentrations at 20°C for 15 min, stained and analyzed as described in Materials and Methods. The inhibitory index for anti-gp120 mAb binding inhibition (II_{gp120}) was calculated according to the formula: $II_{gp120} = 1 - (MF_{gp120X} - MF_C / MF_{gp120} - MF_C)$, whereby MF_{gp120} is the mean channel fluorescence (MF) for the cells incubated only with anti-gp120 mAb, MF_{gp120X} is the MF for the cells incubated with test compound and anti-gp120 mAb and MF_C is the mean channel fluorescence of the cells incubated with RaM-IgG-F(ab')$_2$-FITC. Mean channel fluorescence was determined by the Consort 30 program (Becton-Dickinson).

Discussion

The sulfonic acid polymers PSS, PAS, PVS and PAMPS (Fig. 1) were found to inhibit HIV-1 and HIV-2 replication in vitro (Table 1). Except for PVS (MW: 2000), there was a close correlation between the inhibitory effects of the compounds [PAS, PSS and PAMPS (MW: 7000–70,000)] on HIV-induced cytopathicity and their inhibitory effects on HIV-induced giant cell formation

TABLE 5

Inhibitory effects of sulfonic acid polymers on HIV-1 reverse transcriptase

Compound	[% Inhibition]						IC_{50}[a] (μg/ml)
	[Concentration (μg/ml)]						
	100	20	4	0.8	0.16	0.032	
PSS			96	94	90	25	0.06
PAS			98	97	90	43	0.04
PVS	97	92	71	27	5	–	1.9
PAMPS	91	77	65	25	6	–	2.4
Suramin	99	65	31	13	2	–	9.7

[a]50% Inhibitory concentration. Purified recombinant HIV-1 reverse transcriptase was used in this experiment. All data represent mean values for at least two experiments.

(Table 1). This suggests that a sufficiently high molecular weight is a more stringent requirement for inhibition of giant cell formation than for inhibition of cytopathicity.

There was close correlation between the anti-HIV activity of the test compounds and their inhibitory effects on anti-gp120 mAb binding. The EC_{50} of the compounds for inhibition of HIV cytopathicity ranged from 0.8 to 7.3 μg/ml (Table 1). They showed comparable IC_{50} (0.8–4 μg/ml) in the anti-gp120 mAb assay (Table 4).

On the other hand, the results obtained with the test compounds in the CD4 assay (Table 2) and the RT assay (Table 5) did not correlate with the data obtained for anti-HIV activity. PSS and PAS were the only compounds that were active in the CD4 assay (Table 2), and, interestingly, they were also the most active in the RT assay (Table 5). Yet, they were not more active than PVS and PAMPS against HIV replication (Table 1). Moreover, in the presence of 10% FCS (antiviral assay conditions), PSS and PAS did not efficiently react with the CD4 receptor (Table 3).

The sulfonic acid functionality is present in several agents possessing anti-HIV activity. These include suramin (Mitsuya et al., 1984) and its analogs (Jentsch et al., 1987), certain dyes (Balzarini et al., 1986; Baba et al., 1988; Schinazi et al., 1990), natural products (Suzuki et al., 1989; Gustafson et al., 1989) and naphthalenedisulfonic acid derivatives (Mohan et al., 1991a,b).

Polysulfates (i.e., dextran sulfate and pentosan polysulfate), polysulfonates (i.e., Evans blue and suramin) and polycarboxylates (i.e., ATA) represent three different classes of polyanionic substances, which are all endowed with marked anti-HIV activity. All of these compounds inhibit HIV binding to the cells. Except for ATA (Schols et al., 1989c; Cushman et al., 1991), polyhydroxycarboxylates (Schols et al., 1991b), and two of the sulfonated polymers described here [PSS and PAS (Table 3)], these compounds do not interfere with the binding of OKT4A/Leu3a mAb to the cells (Schols et al., 1989a,c, 1990a). However, all the compounds interfere with the binding of anti-gp120 mAb to HIV-1-infected cells.

The practical utility of polyanionic substances in the treatment of AIDS remains to be proven. The oral bioavailability of dextran sulfate is extremely low (Lorentsen et al., 1989), and therefore oral dextran sulfate is unlikely to provide a significant anti-HIV effect in vivo (Hartman et al., 1990). This problem may be solved by administering the drug parenterally. However, polysulfates may also be apt to rapid degradation in vivo. In this respect, the polysulfonates may be advantageous over the polysulfates as they are relatively inert to degradation (Williams, 1959). Aromatic sulfonic acids are excreted largely unchanged in the urine, with negligible desulfonation (Batten, 1979). Studies of linear or branched alkyl benzene sulfonates in the rat showed no detectable desulfonation (Michael, 1968). Of some concern is the known ability of some of these agents, for example PVS, to possess anticoagulant properties (Reynolds, 1989). It remains to be determined if it is possible, as with the sulfated polysaccharides (Baba et al., 1990b), to dissociate the anti-HIV-1 activity of the polysulfonates from their anticoagulant potential. Studies to optimize the anti-HIV specificity of these agents are in progress.

In conclusion, the sulfonic acid polymers can be considered as potent in vitro anti-HIV agents which owe their antiviral activity to an inhibition of virus adsorption/fusion to the cells, resulting from the interaction of the compounds with the viral glycoprotein gp120.

Acknowledgements

We thank S. Mori (Fukushima Medical College) and H. Azijn (Rega Institute) for excellent technical assistance. P. Mohan is a Scholar (No. 700065-5-RF) of the American Foundation for AIDS Research. This work was supported in part by a grant from the Ministry of Education of Japan, by the Basic AIDS Programme of the European Community, and by grants from the Belgian Fonds voor Geneeskundig Wetenschappelijk Onderzoek and the Belgian Geconcerteerde Onderzoeksacties. D. Schols is a Janssen Research Fellow. We thank Christiane Callebaut and Dominique Brabants for their fine editorial help.

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