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DIFFERENTIAL ANTIVIRAL ACTIVITY OF DERIVATIZED DEXTRANS

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Abstract—The antiviral activity of water-soluble dextrans derivatized with varying percentages of carboxymethyl, benzylamide, and sulfonate groups was evaluated. Several of the polymers exhibited potent antiviral activity against a variety of enveloped viruses, but not against non-enveloped viruses, and only when present during virus adsorption. The mechanism of activity against retroviruses [i.e. human immunodeficiency virus (HIV)] and herpes viruses (i.e. human cytomegalovirus) could be ascribed to inhibition of virus binding to the cells. An absolute requirement for anti-HSV activity appeared to be a sufficiently high percentage of benzylamide and benzylamide sulfonate groups. This did not, however, apply for human cytomegalovirus, respiratory syncytial virus, and HIV. The sensitivity of the latter viruses appeared to be influenced by factors other than the global chemical composition, which leads us to assume that physical factors such as the distribution and sequence of the substituents on the sugar backbone play an important role in the antiviral activity of the derivatized dextrans.

Key words: herpes virus; cytomegalovirus; antiviral; HIV; heparan sulfate; derivatized dextrans

Dextran [poly(α,1-6)glucose] macromolecular chains to which various substituents (i.e., carboxymethyl, benzylamide, and sulfonate groups) have been attached were synthesized. These chemical groups were selected to mimic the hydroxyl, carboxylate, and sulfate groups on the polysaccharide chains of heparin. We have demonstrated before that dextrans bearing more "simple" sulfonate groups without sulfamide (–CO–NH–) methylene ($-CH_2$) and benzyl (–ψ–) arms have no anticoagulant heparin-like activity. To obtain heparin-like dextrans, the substituent benzylamide sulfonate is essential. After we made these observations, we found that some of the derivatized dextrans have anticomplementary properties, antiproliferative effects on smooth muscle cell growth, anti-adhesive capacity on *Staphylococcus aureus*, etc. [1–8]. Like heparin, they also form a complex with the fragment C3b derived from the cleavage of protein C3. These capacities depend, as for anticoagulant activity, on the overall composition in carboxylate and sulfonate groups.

We and others have reported: (a) that the cell surface constituent heparan sulfate is involved in the initial binding of HSV,¶ type 1 (HSV-1) and type 2 (HSV-2) and human cytomegalovirus (HCMV) to the host cell; (b) that heparin, as well as several sulfated and carboxylated polymers, prevents the binding of these viruses to the cells; (c) that dextran sulfate prevents the interaction of human immunodeficiency virus (HIV) with the host cell via binding to the V3 loop of gp120; and (d) that dextran sulfate and other polyanionic polymers inhibit the fusion of influenza A virus with the host cell and both the binding and fusion of respiratory syncytial virus (RSV) with the host cell [9–12]. In the present study we wanted to evaluate which factors are required to construct dextrans with antiviral (i.e., anti-HSV-1, -HSV-2, -HCMV, -RSV, -VSV, -HIV-1, and -HIV-2) activity.

Since some polyanionic compounds are able to (a) prevent the binding of HSV as well as that of HIV to the host cell, (b) prevent the binding of HIV-infected lymphocytes to epithelial cells as demonstrated in an *in vitro* model [13], and (c) prevent infection with HSV-2 in mice intravaginally infected with this virus [14], this class of compounds could be of some value in formulating vaginal gel(s) to prevent infection by HSV and HIV of those women who cannot ensure that their sexual partners use condoms [15].

MATERIALS AND METHODS

Compounds

Water-soluble dextran derivatives were prepared as previously described (1) from dextran T40 (MW: 43,900 g/mol; MN: 26,200 g/mol) obtained from Pharmacia-LKB (St. Quentin en Y., France). Carboxymethyl dextrans were synthesized from native dextran (D) by substituting glucosyl units with carboxymethyl groups (CM). In a second step, benzylamine was coupled to

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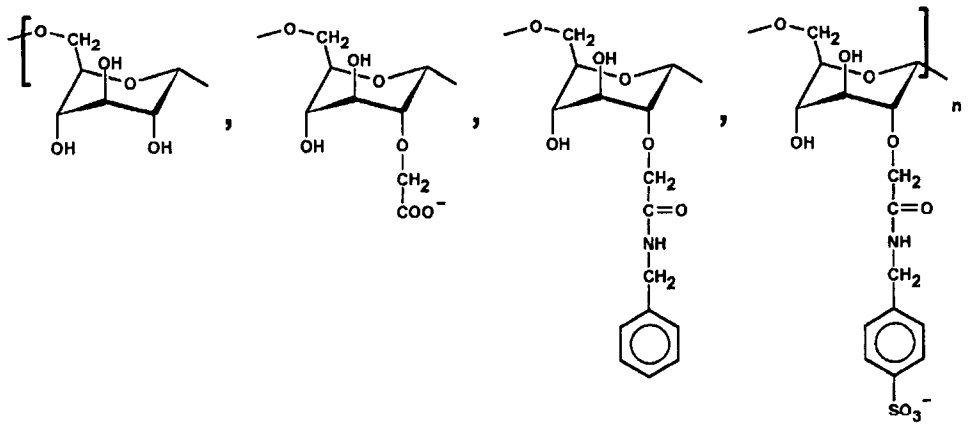
¶ Abbreviations: $CCID_{50}$, cell culture infective dose 50%; CMD, carboxymethyl dextran: CMDB, carboxymethylbenzylamide dextran; CMDBS, carboxymethylbenzylamide sulfonated dextran; HCMV, human cytomegalovirus; HEL, human embryonic lung cells; HIV, human immunodeficiency virus; HSV, herpes simplex virus; IEA, immediate early antigen; MCC, minimum cytotoxic concentration; MDCK, Madin-Darbey canine kidney; PFU, plaque forming units; RSV, respiratory syncytial virus; VSV, vesicular stomatitis virus.

carboxylic groups to form benzylamide units (B). Finally, benzylamide aromatic rings were sulfonated (S). The chemical representation of these synthetic polysaccharides is shown in Fig. 1. Changes in chemical conditions resulted in the formation of different globally substituted macromolecules (Table 1). Thus, native dextran (T40), one carboxymethyldextran (CMD), four carboxymethylbenzylamide dextrans (CMDB), and twelve carboxymethylbenzylamide sulfonated dextrans (CMDBS) were tested. One derivatized dextran (#13) was fractionated by preparative gel filtration on a 5 × 40 cm Sephacryl S 300 column (Pharmacia-LKB) at a flow rate of 1 ml/min. The chromatographic molecular weights of derivatized dextran fractions were determined by analytical high-performance steric exclusion chromatography in 0.2 M sodium chloride using a Licrospher Si 500 Diol column (Merck-Clevenot, Nogent sur Marne, France) calibrated with sulfonated polystyrene standards (Polymer Laboratories Ltd., Montlucon, France). Five fractions (#31–35) of molecular weight ranging from 4,000 to 180,000 g/mol were prepared. The chemical compositions of these fractions were similar to that of the unfractionated derivative (#13). All the sodium salts of the derivatives were ultrafiltrated on a YM2 Diaflo membrane (1,000 MW cut-off; Amicon Co., Danvers, MA, USA) and lyophilized. The global chemical compositions characterized by acidimetric titrations and by elemental analysis are reported in Table 1. Dextran sulfate (MW: 5,000) was purchased from Sigma (La Verpilliere, France). Aurintricarboxylic acid (ATA) (MW 422.25) was obtained from Aldrich Chemical Co. (Brussels, Belgium).

Viruses

The virus strains used were as follows: herpes simplex virus type 1 (HSV-1 strain KOS) and type 2 (HSV-2 strain G), thymidine kinase-deficient (TK^-) HSV-1 (B2006) (see ref. [6]), cytomegalovirus [strain Davis (ATCC VR-807), and strain AD-169 (ATCC VR-538)]; murine CMV [Smith strain (ATCC VR-194)], vesicular stomatitis virus, Coxsackie B4 and poliovirus type 1, parainfluenza virus type 3 (ATCC VR-93), reovirus type 1 (ATCC VR-230); Sindbis virus and Semliki forest virus (ATCC VR-67); Punta Toro virus (ATCC VR-559) and Yellow fever virus (stock prepared after one passage of the vaccine strain Stamaril® in Vero cells); influenza A virus [strain Ishikawa/222/82 (H3N2)] and influenza B virus (strain Singapore/222/79); respiratory syncytial



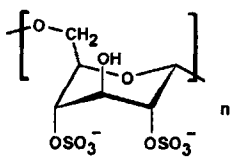


Fig. 1. Structural formulae of the derivatized dextrans. Dextran derivatives (D, CMD, CMDB, CMDBS) were synthesized at different rates by substituting step-by-step the native T40 Dextran with carboxymethyl (CM), carboxymethyl benzylamide (B), and carboxymethyl benzylamide sulfonate (S) groups. Dextran sulfate (DS) was directly substituted from dextran with sulfate groups.

Table 1. Composition of the derivatized dextrans

Compound	% D*	% CM	% B	% S
T40				
6	100	0	0	0
CMD				
5	22	78	0	0
CMDB				
20	0	60	45	0
23	0	83	34	0
29	6	59	35	0
42	0	90	24	0
CMDBS				
1	1	84	3	12
2	0	82	23	13
9	8	73	12	7
11	4	42	42	11
12	37	41	0	22
13	0	58	19	26
17	47	17	0	36
30	0	82	3	15
31 (MW 180,000)	0	58	19	26
32 (MW 50,000)	0	58	19	26
33 (MW 20,000)	0	58	19	26
34 (MW 8,000)	0	58	19	26
35 (MW 4,000)	0	58	19	26
39	0	114	2	16
40	0	81	10	13
43	0	95	2	11
DS (MW: 5,000)	0	0	0	230†

* D: non-substituted glycosyl units on dextran; CM: carboxymethyl; B: carboxymethyl benzylamide; S: carboxymethyl benzylamide sulfonate.
† Sulfate instead of sulfonate.

virus (strain Long); arena virus (Junin and Tacaribe); and human immunodeficiency virus [HIV-1, (HTLV-III_B) and HIV-2 (LAV-2_{ROD}) [12, 17].

Antiviral assays

In all assays the compounds were added to the cell cultures before infection. For all viruses (except for HCMV, influenza virus, RSV, and HIV), confluent cultures of human embryonic skin-muscle (E_6SM), HeLa, or Vero cells in microtiter trays were inoculated with virus at 100 times the $CCID_{50}$ (50% cell culture infective dose) per well. Virus-induced cytopathicity was recorded at 1–2 days post infection (p.i.) for VSV; at 2 days for Coxsackie virus, Semliki forest virus, and polio virus; at 2–3 days for HSV-1, HSV-2, TK^- HSV-1, Sindbis virus; at 5 days for reovirus and Punta Toro virus; and at 7–8 days for Yellow fever virus. For the anti-HCMV assay, human embryonic lung (HEL) fibroblasts in microtiter trays were infected with 100 PFU (plaque-forming units) of HCMV per well in the presence of compounds. Virus-induced cytopathicity was recorded 7 days p.i. Anti-myxovirus was assessed on MDCK cells (influenza virus type A and B) or Hela (RSV) cells infected with 20 $CCID_{50}$ of virus. Evaluation of the anti-HIV activity of the compounds in MT4 cells was assessed as described previously [19]. Briefly, MT-4 cells (3×10^5 cells/ml) in 96-well microtiter plates were infected with HIV-1 or HIV-2 at 100 $CCID_{50}$ per cup in the presence of compounds, and the effect on virus-induced cytopathicity was recorded by means of the MTT method.

Cytotoxicity assay

The 50% cytotoxic concentration (CC_{50}) of the compounds was assessed by means of the MTT method as described previously [8]. Alternatively, the minimum cytotoxic concentration (MCC) of the compounds was recorded [i.e., the minimal concentration required to cause a microscopically detectable alteration in the morphology of confluent (stationary) cells].

Determination of HCMV-induced immediate early antigens (IEA) in HEL cell cultures

Different concentrations of the appropriate compounds were added to confluent HEL cell monolayers in 8-well tissue culture chamber slides (Nunc, Naperville, IL), which were then infected with HCMV at a multiplicity of infection (MOI) of 0.2. Monoclonal antibody (mAb) E13 (Biosoft, Paris, France) directed against immediate early antigen (IEA) was used to monitor HCMV immediate early antigen expression at 24 hours post infection, as described previously [11]. Quantitation of IEA expression by immunofluorescence (IF) microscopy was performed by counting IEA positive cells in 15 to 30 microscopic fields.

Giant cell formation

MOLT-4 (clone 8) cells (1.8×10^6 cells/ml) were co-cultured with persistently infected HUT-78/HTLV-III_B or HUT-78/LAV-2_{ROD} cells (2×10^5 cells/ml) in microtiter tray wells containing various concentrations of the test compounds. After 24 h co-cultivation, the

number of giant cells (syncytia) was recorded microscopically and analysed by flow cytometry, as described previously [19, 20].

Inhibition of gp120 binding

The inhibitory effects of the test compounds on HIV adsorption were measured by an indirect immunofluorescence flow cytometric method modified from Schols *et al.* [25, 29]. Briefly, MT-4 cells were exposed to recombinant HIV-1 gp120 (1.25 μg/ml; Intracel) in the presence or absence of the test compounds. After a 30-min incubation period, cells were washed and gp120 attached to the cells was stained with an anti-HIV-1 gp120 (mAb) and a FITC-labeled secondary antibody, and analyzed by flow cytometry. The inhibitory index for gp120 binding to MT-4 cells (II_{VB}) was calculated according to the following formula:

$$II_{VB} = 1 - (MF_{vc} - MF_{cc})/(MF_V - MF_c),$$

where MF_{vc} is the mean fluorescence of HIV-1 recombinant gp120-loaded cells exposed to a given concentration of the compound in inoculated cells; MF_{cc} is the mean fluorescence for the control cells (not loaded with recombinant gp120) exposed to the compound, MF_v is the mean fluorescence for the HIV-1 recombinant gp120-treated cells (not exposed to any compound); and MF_c is the mean fluorescence for the control cells (not loaded with HIV-1 recombinant gp120 and not exposed to any compound). If $II_{VB} = 1$, there is total inhibition of gp120 binding; if $II_{VB} = 0$, there is no inhibition of gp120 binding. All compounds were evaluated at a concentration of 25 μg/ml.

Binding of HIV to MT4 cells

Briefly, MT4 cells were incubated with HIV-1 in the presence or absence of compounds (final concentration 25 μg/ml) upon which the cells were incubated for 30 min at 37°C, and washed twice to remove unbound virus. Virus was detected by incubating with a high-titered polyclonal antiserum derived from an AIDS patient; cells were then incubated with an FITC-labelled rabbit anti-human antibody, after which they were fixed with 0.37% formaldehyde and analyzed by flow cytometry, as described previously [21].

Binding of radiolabeled HCMV to HEL cells

[*Methyl*-^{3}H]dThd-labeled HCMV was prepared as described previously [11]. Confluent HEL cell cultures grown in 96-well plates were treated with 50 μl of the test compounds at 110, 55, 22.5, 4.5, and 0.8 μg/ml for 1 to 5 min at room temperature. Five μl of radiolabeled virus suspension (~10^8 pfu/ml and 5×10^5–10^6 cpm/ml) was added to the cell cultures for an additional 60 min at 37°C. The incubation medium was removed and the cells rinsed four times with 200 μl PBS. Cells were detached by trypsinization and then spotted onto GF/C filters (Whatman). Radioactivity was determined in a toluene-based scintillant.

Penetration of HCMV in HEL cells

Confluent HEL cell cultures in chamberslides were cooled to 4°C and were then infected with HCMV at a MOI of 0.5. After a 2-hour adsorption period at 4°C, unbound virus was removed by washing with cold medium, after which cold dilutions of the different compounds were added. Thereafter, the temperature was raised to 37°C. The expression of IEA was determined 24 h later.

CD4 Immunofluorescence assay

Briefly, MT-4 cells (200,000 cells/100 μl PBS) were incubated for 10–20 sec at room temperature with or without test compound at 25 μg/ml. The cells were then stained with optimal concentrations of the monoclonal antibodies OKT4A-FITC (Ortho Diagnostics) 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. 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 mAb binding inhibition was calculated according to the following formula:

$$II_{CD4} = 1 - (MF_{CD4x} - MF_c)/(MF_{CD4} - MF_c),$$

where MF_{CD4x} is the *MF* for the cells incubated with test compound and OKT4A mAb, and MF_c is the mean channel fluorescence of the cells incubated with Simultest control. MF_{CD4} is the mean channel fluorescence (*MF*) for the cells incubated only with OKT4A mAb. The mean channel fluorescence was determined by the Consort 30 program (Becton Dickinson). Analysis of the effect on binding of other cell surface markers was performed in a similar way, except that the cells (MT4 and superantigen stimulated peripheral blood lymphocytes) were preincubated for 60 min (at 4°C) with the compounds.

In an additional set of experiments, MT4 cells (2×10^5 cells/100 μl PBS) were incubated with or without rgp120 (5 μg/ml; American Biotechnologies) and with or without compound #5 (10 μg/ml) and compound #13 (5 μg/ml) in a total volume of 200 μl. The compounds and rgp120 were added simultaneously to the cells. After a 30-min incubation period at 4°C, the cells were stained with anti-Leu3a-PE (Becton Dickinson) for 20 min at 4°C, after which they were washed once in PBS, resuspended in 0.5 ml of 1% paraformaldehyde in PBS and analyzed by flow cytometry.

Glycoprotein gp120 immunofluorescence assay

Normal HUT-78 and HIV-1-infected HUT-78 (HUT-78/HIV-1) cells were washed twice, incubated with the compounds at 25 μg/ml at 20°C for 15–20 min, 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 and an FITC-labeled secondary antibody, and were then analyzed by flow cytometry as described previously [25]. The inhibitory index for anti-gp120 mAb binding inhibition (II_{gp120}) was calculated according to the formula:

$$II_{gp120} = 1 - (MF_{gp120x} - MF_c)/(MF_{gp120} - MF_c),$$

where 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. MF_{gp120} is the mean channel fluorescence (*MF*) for the cells incubated only with anti-

gp120 mAb. All data represent mean values ± *SD* ($n = 2$).

RESULTS

Antiviral activity of the derivatized dextrans

The derivatized dextrans were evaluated for their inhibitory effect on the cytopathicity of a wide array of viruses (Table 2). When present during the virus adsorption period, several of the polymers showed potent activity against HCMV, RSV, HIV-1, HIV-2, HSV-1, and HSV-2, with HCMV, RSV and HIV being more sensitive to the polymers than VSV and HSV. When added after virus adsorption, the compounds lost significant, if not all, antiviral activity (data not shown). A requirement for anti-HSV activity appeared to be a sufficiently high percentage of benzylamide and benzylamide sulfonate groups (compounds #2, #11, #13, #31–35). Polymers (a) without benzylamide sulfonate groups (compounds #20, #23, #29, #42) or (b) with a relatively high degree of benzylamide sulfonate substituents but a low number of benzylamide groups (compounds #1, #12, #17, #30, #39, #43), irrespective of the number of carboxymethyl groups, proved inactive against herpes simplex viruses. Native dextran (compound #6) or dextran bearing only carboxylic units (compound #5) elicited no antiviral effects.

The requirement for sufficient benzylamide sulfonate and benzylamide groups did not apply to activity against HCMV, RSV, and HIV. The sensitivity of these latter viruses appeared to be influenced by factors other than the global chemical composition. For example, although the composition of compounds #20 and #42 does not differ much from that of compounds #23 and #29, and the composition of compounds #39 and #43 is very similar to the composition of compound #1, there is a marked difference in the susceptibility of HCMV, HSV, RSV, and HIV to these polymers; (a) compounds #23 and #29 show anti-HCMV, anti-RSV, and anti-HIV (but no anti-HSV) activity, whereas (b) compounds #39 and #43 show no or only weak anti-HCMV, anti-HIV, and anti-RSV activity. On the other hand (c), compound #1 has a pronounced anti-HCMV and anti-HIV effect. These findings lead us to assume that not only the global chemical composition, but also the spatial distribution of the substituents may play an important role in their inhibitory effects, at least against some viruses.

Other conformational factors may also play a prominent role in the antiviral activity of the compounds, as suggested by the following observation. Compound #13 was fractionated in different molecular weight forms (compounds #31–35). As can be deduced from Table 2, the higher the molecular weight, the greater the activity against RSV, HCMV, and HSV, whereas the molecular weight had virtually no influence on the anti-HIV activity of compound #13.

Those compounds that proved most active against HCMV and HIV also demonstrated some moderate activity against influenza virus type A, Punta Toro virus, Yellow fever virus, Tacaribe virus, and Junin virus, with

Table 2. Activity of the derivatized dextrans against some enveloped viruses

	EC_{50} (μg/ml)*							
Compound	HCMV	HSV-1 (KOS)	HSV-2 (G)	HSV-1 (TK^-)	RSV	VSV	HIV-1	HIV-2
T40								
6	>100	>200	>200	>200	>200	>200	>250	>250
CMD								
5	>100	>200	>200	>200	>200	>200	>250	>250
CMDB								
20	≥100	>200	>200	>200	>200	>200	136	>250
23	2.4	>200	>200	>200	6	>200	0.2	0.2
29	8.0	>200	>200	>200	4	70	0.3	0.1
42	≥100	>200	>200	>200	200	>200	103	28
CMDBS								
1	8.0	>200	>200	>200	70	150	2.2	2.9
2	0.6	7	7	7	4	7	0.2	0.1
9	50	>200	>200	>200	40	150	1.1	4.6
11	1.5	7	7	7	4	7	0.7	0.6
12	6.5	>200	>200	>200	20	≥20	4.0	1.6
13	6.6	10	7	20	—	70	0.4	0.3
17	6.2	>200	>200	>200	20	150	1.5	0.2
30	70	>200	>200	>200	100	150	4.9	5.1
31	0.7	7	7	20	1.6	15	0.1	0.8
32	0.7	20	7	7	4	55	0.3	0.4
33	1.5	70	7	70	13	200	0.3	0.7
34	5.2	150	150	150	60	>200	0.5	0.7
35	12	35	70	20	100	≥20	2.8	4.3
39	≥100	>200	>200	>200	70	>200	15.7	21.6
40	50	>200	>200	>200	>200	>200	7.4	2.7
43	≥100	>200	>200	>200	>200	>200	11.7	38
DS (MW 5,000)	0.2	0.4	2	2	0.1	0.06	0.4	0.1

* Fifty percent effective concentration, or concentration required to reduce virus-induced CPE by 50%. All data are mean values for at least two separate experiments.

EC_{50} values ranging from 20–40 μg/ml for compounds #1, #2, #13, and #11 against Punta Toro; 40–85 μg/ml for compounds #2, #31, #32 against the arenaviruses TCV and JV; and 20–70 μg/ml for compounds #11 and #13 influenza A virus. None of the polymers inhibited infection of the cells by influenza B, picornaviruses (polio-1 or Coxsackie B4), parainfluenza-3 virus, reovirus-1, or Semliki forest virus. Also, none of the compounds altered normal cell morphology at concentrations up to 500 μg/ml or inhibited the growth of MT-4 cells at concentrations up to 250 μg/ml (data not shown).

Mechanism of antiviral action of the derivatized dextrans

Those compounds that inhibited HCMV-induced cytopathicity (CPE) formation also inhibited the adsorption of radioactive HCMV particles with EC_{50} values comparable to the EC_{50} values for inhibition of HCMV immediate early antigen expression and the EC_{50} values for inhibition of CPE formation (Table 3). Taken together, these data suggest that the compounds act by preventing the binding of HCMV to the host cell. When the compounds and dextran sulfate were added after HCMV was allowed to bind at 4°C to HEL cell cultures, no inhibition of IEA expression was noted, indicating that the sulfated polymers do not affect the penetration of HCMV in the cell.

Those compounds that completely inhibited HIV cytopathicity at a concentration of 25 μg/ml also inhibited, at this concentration, HIV-1-induced giant cell formation (Table 4) and the binding of recombinant gp120 to $CD4^+$ cells (Table 5). This assay mimics HIV gp120 interaction with the CD4 receptor, and is a model for evaluating compounds that inhibit HIV binding to its receptor. These data were further corroborated by the observation that compounds that efficiently prevented the interaction of rgp120 with CD4 also prevented the binding of virus to the CD4 positive cells. When compared to dextran sulfate (mol. wt 5000), which has been repeatedly shown to result in a complete inhibition of HIV binding to the MT4 cells, compounds #2, #13, #23, #29, and #31 resulted in respectively 110 ± 16, 112 ± 14, 86 ± 25, 63 ± 16, and 85 ± 26% inhibition of binding. Compounds devoid of anti-HIV activity, such as compounds #5, #6, and #43, and without effect in the rgp120/CD4 assay also did not inhibit virus binding (i.e., 0% inhibition of virus binding for all three compounds). At a concentration of 25 μg/ml, the compounds with anti-HIV activity also prevented the binding of anti-gp120 mAb binding to the V3 region of HIV-1 gp120, whereas this was not the case with an inactive compound (i.e., compound #5). These results were further corroborated in the following experiment (Fig. 2) in which the interference of compounds on the rgp120-CD4 interaction was studied. An inactive compound (e.g., compound #5) did not prevent the interaction of rgp120 with the CD4 receptor on MT4 cells, whereas an active anti-HIV compound (e.g., compound #13) prevented rgp120 binding to CD4. The assay was based on the binding of an anti-CD4 mAb to the CD4 receptor at the cell surface. On the histogram (Fig. 2), the binding of anti-CD4 mAb to the CD4 receptor was prevented when rgp120 was added. In the presence of compound #13 (25 μg/ml), however, rgp120 was almost unable to bind to CD4; conversely presence of compound #5 did not prevent CD4 staining, which indicates that rgp120 in the presence of this compound was able to bind to the CD4 receptor.

Table 4. Effect of selected compounds on HIV-1- and HIV-2-induced syncytium formation

Compound	Giant cell formation IC_{50} (μg/ml)	
	HIV-1 (HTLV-III)	HIV-2 ($LAV\text{-}1_{ROD}$)
2	6	20
11	8	50
13	6	50
29	30	250
31	8	250
32	6	150
33	6	40
AZT	>1	>1
DS (MW 5,000)	4	0.08
ATA	3.6	0.8

* Fifty percent inhibitory concentration, based on the inhibition of giant cell formation between persistently HIV-1- or HIV-2-infected HUT-78 cells and uninfected MOLT-4 (clone 8) cells.

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

Table 3. Inhibitory effect of a selected series of compounds on several parameters of HCMV infectivity

Compound	EC_{50} (μg/mL)*			% Binding† (compared to control)
	CPE	IEA	Binding	
2	0.6	2.1 + 0.9	4.6 ± 4.3	10 ± 10
5	>100	>50	>100	118 ± 18
13	6.6	2.2 ± 1.9	—	6.3 ± 8.9
31	0.7	1.0 ± 0.8	8.0 ± 6.2	2.0 ± 2.8
32	0.7	4.1 ± 0.3	2.9 ± 2.4	0.6 ± 0.06
39	≥100	—	—	78 ± 6.2
43	≥100	>50	>100	82 ± 32

* Concentration required to reduce HCMV-induced CPE (as measured at 7 days p.i.) or IEA expression (as measured at 24 hours p.i.) or binding of radiolabeled virus by 50%.

† Percentage binding of radiolabeled HCMV particles to HEL cells in the presence of 100 μg/ml of the compound.

All data are mean values for at least 2–3 separate determinations.

Table 5. Inhibitory effects of selected compounds on the interaction of gp120 with CD4$^+$ cells, and on the interaction of OKT4A mAb with gp120 and CD4

Compounds	gp120-cellular CD4 binding II_{VB}	II_{gp120}†	II_{CD4}‡
2	0.55	0.94 ± 0.05	0.0
5	0.0	0.0	—
13	0.65	0.71 ± 0.01	0.0
23	0.44	0.76 ± 0.06	0.0
29	0.25	0.38 ± 0.05	0.0
31	0.46	0.71 ± 0.06	0.0
DS 5,000	0.94	0.93 ± 0.08	0.0

* Inhibitory index for virus binding to MT-4 cells (II_{VB}) (see Materials and Methods).
† Inhibitory index for binding of anti-gp120 mAb to HIV-1 gp120 (see Materials and Methods).
‡ Inhibitory index for binding of OKT4A mAb binding to MT-4$^+$ cells (see Materials and Methods).

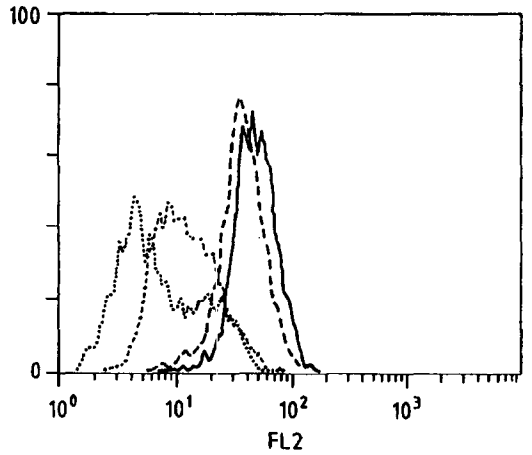


Fig. 2. Flow cytometric analysis of MT4 cells stained with an anti-CD4 mAb (———), stained with an anti-CD4 mAb in the presence of rgp120 (·····), stained with an anti-CD4 mAb in the presence of rgp120 and compound 13 (- - -), or stained with an anti-CD4 mAb in the presence of rgp120 and compound #5 (. . . .).

No significant effect of the compounds was detected on the interference with the binding of an mAb directed against the HIV recognition domain of the CD4 receptor on CD4$^+$ cells, nor with a selection of other haematopoietic cell surface molecules [CD2 (LFA-2); CD3, CD8 (MHC class I receptor); CD11b (C3bi receptor), CD25 (IL-2 receptor), CD28, CD31 (PECAM-1), CD54 (ICAM-1), CD49d (VLA-α4 chain), CD58 (LFA-3), HLA-DR, and CD45 RO] on either MT4 cells or superantigen-activated peripheral blood lymphocytes (data not shown).

DISCUSSION

Several water-soluble dextrans derivatized with varying percentages of carboxymethyl, benzylamide, and sulfonate groups were evaluated for their inhibitory effects on a wide array of viruses. Several of the polymers showed potent antiviral activity, but only against enveloped viruses, and only when present at the time of virus adsorption.

The mechanism of action of the compounds against HCMV and HIV can be attributed to an inhibition of virus-cell binding. For HCMV this is evident: (a) from the fact that the compounds need to be present during the virus adsorption period to achieve their antiviral activity; and (b) from the observation that the compounds that show anti-HCMV activity also inhibit virus-specific immediate early antigen expression and binding of radiolabeled viruses to the host cell. Those compounds that are devoid of antiviral activity are unable to do so. No effect on HCMV penetration was noted with the compounds displaying potent anti-HCMV activity. The mechanism of anti-HIV action of the active compounds may also be attributed to inhibition of virus-cell binding; those compounds that inhibit virus-induced cytopathic effect, inhibit the binding of HIV to CD4$^+$ cells, block the binding of recombinant gp120 to the CD4 receptor by shielding off the V3 loop of HIV-1 gp120. However, they do not interfere with the interaction of anti-CD4 mAb with the CD4 receptor expressed on MT-4 cells, nor with other surface molecules on human lymphocytes.

We and others have shown that the initial site of interaction of herpes viruses (such as HSV and HCMV) with the host cell is cell surface heparan sulfate [9, 11, 23, 29], and postulated that heparin and other sulfated and carboxylated polymers block this interaction by binding to positively charged sites on the virion envelope that are necessary for attachment to the anionic heparan sulfate proteoglycan constituent of the cell surface. Similarly, Schols *et al.* [22] demonstrated that several polysulfates and poly(hydroxy)carboxylates owe their antiviral effect to shielding off the positively charged V3 loop of gp120, thus preventing an efficient interaction of HIV with the host cell. The mechanism of anti-HSV, anti-HCMV, and anti-HIV activity of the polymers described here thus appears very similar to that of the sulfated polymers.

In general, HCMV, RSV, and HIV appeared more sensitive to several of the derivatized polymers than HSV. As a rule, only those compounds that contain a sufficiently high combined percentage of benzylamide and benzylamide sulfonate groups elicit anti-HSV activity. Inhibition of HCMV, HSV, and RSV infection of the host cells by different molecular weight (*MW*) forms of compound #13 (i.e., compounds #31–35) was found to be dependent on molecular size (i.e., the higher the *MW*, the more potent the compound), whereas such a corre-

lation was not observed for HIV. Although the molecular weight and the nature of the substituents play an important role in the antiviral activity of the tested compounds, other factors must be involved as well. For example, the composition of (a) compounds #1 and #43, and (b) of compounds #23 and #42 are comparable; yet compounds #42 and #43 have no activity against HCMV or little activity against HIV, whereas compounds #1 and #23 inhibit the replication of both viruses. Conformational factors related to the distribution and sequence of the substituents on the sugar backbone may play an important role in their antiviral effect. In fact, similar observations were made earlier when the anti-HCMV effect of dextran sulfate, co-polymers of acrylic acid with vinyl alcohol sulfate (PAVAS), and sulfated cyclodextrins were compared [11]. Although the molecular weight and the degree of sulfation of these compounds is very similar, PAVAS is some 10-fold more potent than dextran sulfate, which in turn is 20-fold more active than the sulfated cyclodextrins. The (a) differential sensitivity of viruses such as HCMV and HIV to the derivatized dextrans and (b) the differential susceptibility of HCMV and HIV to compounds with an almost identical chemical composition suggests that the distribution of the substituents may influence the shape and/or flexibility of the polymers. This is at least as important for antiviral activity as the anionic character of the polymers.

In conclusion, several derivatized dextrans were found to exert a potent and selective inhibitory effect on some enveloped viruses such as HCMV, HSV-1, HSV-2, RSV, HIV-1, and HIV-2. The compounds inhibit the first step in the replicative cycle of HCMV and HIV (i.e., virus adsorption to the cells), probably by direct interference with viral envelope glycoproteins for herpes viruses, and with the gp120 V3 loop for HIV. Conformational factors related to the sequence and distribution of the polyanionic substituents may also play an important role in the antiviral effects of these molecules.

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REFERENCES

1. Mauzac M and Jozefonvicz J, Anticoagulant activity of dextran derivatives. Part I: Synthesis and characterization. *Biomaterials* **5**: 301–304, 1984.
2. Mauzac M, Maillet F, Jozefonvicz J and Kazatchkine MD, Anticomplementary activity of dextran derivatives. *Biomaterials* **6**: 61–63, 1985.
3. Crepon B, Maillet F, Kazatchkine MD and Jozefonvicz J, Molecular weight dependency of the acquired anticomplementary and anticoagulant activities of specifically substituted dextrans. *Biomaterials* **8**: 248–253, 1987.
4. Avramoglou T and Jozefonvicz J, Derivatized dextran inhibition of smooth muscle cells proliferation. *J Biomater Sci Polymer Edn* **3**: 149–154, 1992.
5. Letourneur D, Logeart D, Avramoglou T and Jozefonvicz J, Antiproliferative capacity of synthetic dextrans on smooth muscle cell growth: The model of derivatized dextrans as heparin-like polymers. *J Biomater Sci Polymer Edn* **4**: 431–444, 1993.
6. Vaudaux P, Avramoglou T, Letourneur D, Lew DP and Jozefonvicz J, Inhibition by heparin and derivatized dextrans of *Staphylococcus aureus* adhesion to fibronectin-coated biomaterials. *J Biomater Sci Polymer End* **4**: 89–97, 1992.
7. Tardieu M, Gamby C, Avramoglou T, Jozefonvicz J and Barritault D, Derivatized dextrans mimic heparin as stabilizers, potentiators, and protectors of acidic or basic FGF. *J Cell Physiol* **150**: 194–203, 1992.
8. Letourneur D, Champion J, Slaoui F and Jozefonvicz J, *In vitro* stimulation of human endothelial cells by derivatized dextrans. *In Vitro Cell Dev Biol* **29**: 27–72, 1993.
9. Wu-Dunn D and Spear PG, Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. *J Virol* **63**: 52–58, 1989.
10. Hosoya M, Balzarini J, Shigeta S and De Clercq E, Differential inhibitory effects of sulfated polysaccharides and polymers on the replication of various myxoviruses and retroviruses, depending on the composition of the target amino acid sequences of the viral envelope glycoprotein. *Antimicrob Agents Chemother* **35**: 2515–2520, 1991.
11. Neyts J, Snoeck R, Schols D, Balzarini J, Esko JD, Van Schepdael A and De Clercq E, Sulfated polymers inhibit the interaction of human cytomegalovirus with cell surface heparan sulfate. *Virology* **189**: 48–58, 1992.
12. Ikeda S, Neyts J, Verma S, Wickramasinghe A, Mohan P and De Clercq E, *In vitro* and *in vivo* inhibition of ortho- and paramyxovirus infections by a new class of sulfonic acid polymers interacting with virus-cell binding and/or fusion. *Antimicrob Agents Chemother* **38**: 256–259, 1994.
13. Pearce-Pratt R and Phillips DM, Studies on the adhesion of lymfocytic cells: Implications for sexual transmission of human immunodeficiencyvirus. *Biol Reprod* **48**: 431–445, 1993.
14. Neyts J and De Clercq E, Effect of polyanionic compounds on intracutaneous and intravaginal herpes virus infection in mice: Impact on the search for vaginal microbicides. *J Acquir Immun Defic Syndr Hum Retrovirol*, in press.
15. Elias CJ and Heise LL, Challenges for the development of female controlled vaginal microbicides. *AIDS* **8**: 1–9, 1994.
16. De Clercq E, Descamps J, Verhelst G, Walker RT, Jones AS, Torrence PF and Shugar D, Comparative efficacy of antiherpes drugs against different strains of herpes simplex virus. *J Infect Dis* **141**: 563–574, 1980.
17. Schols D, De Clercq E, Balzarini J, Baba M, Witvrouw M, Hosoya M, Andrei G, Snoeck R, Neyts J, Pauwels R, Nagy M, Györgi-Edelényi J, Machovich R, Horvath I, Löw M and Görög S, Sulphated polymers are potent and selective inhibitors of various enveloped viruses, including herpes simplex virus, cytomegalovirus, vesicular stomatitis virus, respiratory syncytial virus, and toga-, and arena- and retroviruses. *Antiviral Chem Chemother* **1**: 233–240, 1990.
18. Pauwels R, Balzarini J, Baba M, Snoeck R, Schols D, Herdewijn P, Desmyter J and De Clercq E, Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. *J Virol Methods* **20**: 309–321, 1988.
19. Baba M, Schols D, Pauwels R, Nakashima H and De Clercq E, Sulfated polysaccharides as potent inhibitors of HIV-induced syncytium formation: A new strategy toward AIDS chemotherapy. *J Acquir Immun Defic Syndr* **3**: 493–499, 1990.
20. Schols D, Pauwels R, Baba M, Desmyter J and De Clercq E, Syncytium formation and destruction of bystander CD4+ cells cocultured with T cells persistently infected with human immunodeficiency virus as demonstrated by flow cytometry. *J Gen Virol* **70**: 2397–2408, 1989.
21. Schols D, Baba M, Pauwels R and De Clercq E, Flow

cytometric method to demonstrate whether anti-HIV agents inhibit virion binding to T4 cells. *J Acquire Immun Defic Syndr* **2:** 10–15, 1989.

22. Schols D, Wutzler P, Klöcking R, Helbig B and De Clercq E, Selective inhibitory activity of polyhydroxycarboxylates derived from phenolic compounds against human immunodeficiency virus replication. *J Acquir Immun Defic Syndr* **4:** 677–684, 1991.
23. Kari B and Gehrz R, A human cytomegalovirus glycoprotein designated gC-II is a major heparin-binding component of the viral envelope. *J Virol* **66:** 1761–1764, 1992.
24. Neyts J, Snoeck R, Wutzler P, Cushman M, Klöcking R, Helbig B, Wang P and De Clercq E, Poly(hydroxy)-carboxylates as selective inhibitors of cytomegalovirus and herpes simplex virus replication. *Antiviral Chem Chemother* **3:** 215–222, 1992.
25. Schols D, Pauwels R, Desmyter J, De Clercq E, Dextran sulphate and other polyanionic anti-HIV compounds specifically interact with viral gp120 glycoprotein expressed by T cells persistently infected HIV-1. *Virology* **174:** 556–561, 1990.