Biochemical Pharmacology, Vol. 57, pp. 775–783, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(98)00352-9

Interaction of the Transactivating Protein HIV-1 Tat with Sulphated Polysaccharides

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ABSTRACT. Endogenous sulphated polysaccharides such as heparin have been shown to inhibit the infectivity of HIV-1 *in vitro*. However, these naturally occurring polymers, due to extensive microheterogeneity within their structure, are difficult to characterise accurately. In contrast, dextrin can be chemically sulphated to produce a series of compounds sulphated in the 2-, 3-, or 6- position, or in all 3 positions, and the use of these compounds provides an opportunity to investigate the anti-HIV-1 activity of sulphated polysaccharides. The mechanisms whereby sulphated polysaccharides exert their anti-HIV-1 activity have not been fully elucidated. The interaction of recombinant HIV-1 proteins with sulphated polysaccharides was investigated using a biotinylated derivative of dextrin 2-sulphate (D2S) in a solid phase binding system. D2S was found to bind strongly to HIV-1 tat (EC_{50} = 0.10 μg/mL), less strongly to CD4 (EC_{50} = 0.33 μg/mL), weakly to HIV-1 vif and gp160, and not at all to HIV-1 gp120 or p24. Other sulphated derivatives of dextrin, i.e. dextrin 3-sulphate, dextrin 6-sulphate and dextrin 2,3,6-trisulphate, as well as heparin and dextran sulphate, were also shown to bind to HIV-1 tat, whereas the unsulphated compound dextrin did not. Binding studies using a series of overlapping peptides representing the complete sequence of HIV-1 tat revealed that D2S bound most strongly to the core domain of HIV-1 tat, although there was also binding to the cysteine-rich domain; both of these regions are important for HIV-1 tat function. In assessing function, HIV-1 tat-mediated transactivation was measured using H938 cells, a cell line that contains the HIV-LTR (long terminal repeat) promoter linked to a chloramphenicol acetyltransferase gene. D2S significantly inhibited HIV-1 tat transactivation in a dose-dependent manner (IC_{50} = 0.5 μg/mL), whereas dextrin had no effect. The interaction between D2S and HIV-1 tat provides a potential mechanism of HIV-1 inhibition whereby tat is sequestered and its transactivating activity abolished, effectively inhibiting the replication cycle. BIOCHEM PHARMACOL **57**;7:775–783, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. sulphated polysaccharides; dextrin sulphate; HIV-1 tat; transactivation

It has been known for a number of years that polyanionic substances, especially sulphated polysaccharides, can prevent viruses entering cells. It is believed they do this by interfering with the virus adsorptive process [1, 2]. Sulphated polysaccharides can inhibit infection by a number of different viruses including herpes simplex virus, cytomegalovirus, and vesicular stomatitis virus [3]. In the last decade, much research has been undertaken investigating the role of sulphated polysaccharides in inhibiting HIV-1 infection. Heparin and dextran sulphate were the first sulphated polysaccharides shown to inhibit HIV-1 infectivity *in vitro* [4, 5]. Since then, many other sulphated compounds, including dextrin sulphate [6], have also been shown to inhibit HIV-1 infection. A number of studies suggest that these compounds work by binding to either viral gp120 or the cellular receptor CD4 (for review see [7]). It is possible that not all sulphated polysaccharides share a common mechanism of action, and that controversies may, in part, be explained by structural differences between the various sulphated polysaccharides. Certainly, the various dextrin sulphate analogues, dextran sulphate, and heparin all differ in their polysaccharide backbones, as well as the degree and position of sulphation.

Nevertheless, there are other proteins synthesised by the HIV-1 virion that are equally important for viral replication and integrity [8]. Since many of these HIV-1 viral proteins have been purified and much is known about their structure and function, it is important to investigate whether they too could be potential targets for sulphated polysaccharides. One of the more important virus proteins that has been characterised and one that appears to be essential for virus replication is HIV-1 tat. The HIV-1 tat gene product is unusual in that, although a transcription factor, it can be released from cells and has autocrine and paracrine activity [9]. Exogenous HIV-1 tat appears to have the ability to enter the cell and translocate to the nucleus in an active form, where it may stimulate the transcriptional activity of the HIV-LTR† [10]. HIV-1 tat can also transactivate some cellular genes [11, 12]. This may prime

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Received 22 June 1998; accepted 22 September 1998.

† *Abbreviations:* CAT, chloramphenicol acetyltransferase; D2S, dextrin 2-sulphate; D3S, dextrin 3-sulphate; D6S, dextrin 6-sulphate; D236S, dextrin 2,3,6-trisulphate; LTR, long terminal repeat; and B-D2S, biotinylated D2S.

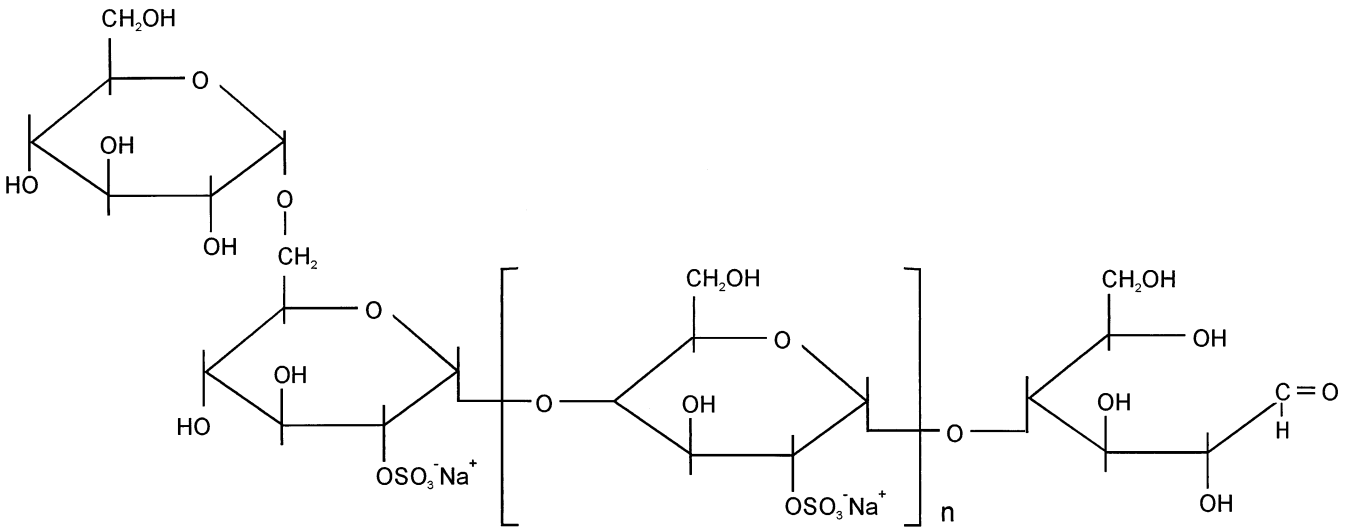

FIG. 1. Structure of D2S. D2S is produced from the enzymic hydrolysis (β-amylase) of starch. This enzyme is an exoamylase that sequentially cleaves α-1,4-linked maltose moieties from the non-reducing terminal of starch resulting in a non-hydrolysable α-1,6-linkage at the non-reducing end. The product of enzymic hydrolysis (limit dextrin) is chemically sulphated to produce D2S, represented here as an essentially linear α-1,4-linked glucose polymer containing a terminal reducing sugar and an α-1,6-linked branch point. The median molecular weight of D2S is 9 kDa, which represents N = 30 glucose units.

cells to become permissive for infection and could have implications for both HIV-1 pathogenesis and treatment. HIV-1 tat is a polypeptide containing between 86–102 amino acids depending on viral strain; the region between amino acids 49–57 is a basic, arginine-rich domain which is important in both transactivation [13] and stability of the protein [14]. HIV-1 tat also has potent angiogenic effects *in vitro* [15] and has been implicated in Kaposi sarcoma, a cancer that is common among AIDS patients [16, 17].

Endogenous sulphated polysaccharides have been shown to modulate HIV-1 tat activity *in vitro*. Indeed, cell surface glycosaminoglycans are important in the cellular uptake of HIV-1 tat; for example, uptake is significantly reduced in Chinese hamster ovary cell mutants defective in glycosaminoglycan synthesis [18]. Furthermore, transactivation of cellular genes by HIV-1 tat is markedly attenuated in the presence of heparin [19, 20]. Although endogenous sulphated polysaccharides such as heparin have anti-HIV-1 activity *in vitro*, the inherent heterogeneity in the pattern of sulphation throughout the molecule, in terms of variability in the position and degree of sulphation [21], presents difficulties when characterising the mechanism of action of these compounds. There is a need to study sulphated polysaccharides with a more defined structure, in terms of the position and degree of sulphation.

Dextrin is essentially a linear α-1,4-linked glucose polymer with a relatively low level (<5%) of branching. It can be sulphated chemically at either the 2, 3, or 6 position, resulting in mono-, di- or trisulphated compounds. D2S, with a median molecular weight of 9 kDa, contains one sulphate group per glucose moiety (Fig. 1) and is a potent *in vitro* inhibitor of both laboratory-adapted and primary HIV-1 viral isolates, with IC_{95} values of 6.25–12.5 μg/mL and 50–100 μg/mL, respectively [6]. It has previously been shown that D2S binds to the surface of activated T-cells and interacts with histones that are present on the plasma membrane [22], but the relevance of this interaction to HIV-1 infection is not clear. Early clinical studies have indicated that D2S treatment can result in a reduction of viral load [23]; whether D2S acts directly on components of the virus or through an interaction with the host cell has not been determined. In this paper, the interaction of D2S, a model sulphated polysaccharide, with proteins associated with the HIV-1 virion has been investigated to determine whether any of these components may be potential targets for therapeutic intervention.

MATERIALS AND METHODS

Materials

Dextrin, D2S, D3S, D6S, and D236S were from ML Laboratories. Heparin (15 kDa) was from Leo Laboratories. Dextran sulphate (8 kDa) and streptavidin peroxidase were purchased from Sigma Chemical Company. Pyridine borane complex and ethylene glycol were from Aldrich Chemical Company. The G-25 gel filtration resin was from Pharmacia. Biotin hydrazide was purchased from Pierce. Microtitre plates were from Dynatech. The CAT enzyme ELISA kit was purchased from Boehringer. All other chemicals were from Merck-BDH and were of AnalaR grade or best equivalent.

The H938 cell line expressing the HIV-LTR-CAT construct, recombinant soluble CD4, the HIV-1 proteins tat, vif, gp120, gp160, and p24, and the overlapping HIV-1 tat peptides were from the MRC AIDS Directed Programme, National Institute of Biological Standards and Control

(Potters Bar, UK). The recombinant HIV-1 proteins gp120 and gp160 were expressed in insect cells, and vif, tat and p24 were expressed in *Escherichia coli*.

Synthesis of Dextrin and Its Sulphated Derivatives

Limit dextrin is a polydispersed polymer produced by β-amylase hydrolysis of starch. D2S, D3S, D6S, and D236S were all synthesised from purified limit dextrin by chemical sulphation. Each compound was characterised by sulphate analysis, ^{13}C NMR, Fourier transformed infra red spectroscopy, and mass spectrometry [6]. D2S, D3S, and D6S are sulphated predominantly at the 2, 3, and 6 positions of the glucan ring, respectively. In each case, the degree of sulphation has been determined to be approximately one sulphate group per glucan moiety. D236S is sulphated at the 2, 3, and 6 positions of the glucan ring. The median molecular weight of dextrin, D2S, D3S, D6S, and D236S are approximately 6, 9, 9, 9, and 15 kDa, respectively.

Dextran Sulphate and Heparin

Dextran is an α-1,6-linked linear, glucose polymer containing approximately 5% branching through α-1,3-linkages [24]. The size of the dextran polymer can vary, and hence the sulphated derivatives of dextran that are produced have variable molecular weights. The compound used in this study contains 2.3 sulphate groups per glucose moiety and has an approximate molecular weight of 8 kDa (Sigma). Heparin was of clinical grade quality (Leo Laboratories) and had a molecular weight of approximately 15 kDa. The level of sulphation in heparin is about 2.7 sulphate groups per disaccharide unit.

Cell Line

The H938 cell line contains stably integrated, silent copies of the HIV-1 LTR promoter linked to the CAT gene. It was generated by infection of H9 cells with a vector containing the HIV-1-LTR-CAT gene construct and, in the presence of HIV-1 tat, produces high levels of CAT [25].

Synthesis of Biotinylated D2S

B-D2S was synthesised via periodate oxidation of D2S; the conditions of periodate oxidation were adapted from Stults *et al.* [26]. A sample containing 185 nmol of D2S was dissolved in 485 μL of distilled water and then mixed at room temperature for 1 hr with 485 μL of 100 mM sodium metaperiodate. Any unreacted sodium metaperiodate was quenched by the addition of 30 μL of 16 M ethylene glycol for 15 min at room temperature. Separation of the oxidised D2S from other reaction products was by gel filtration using a Sephadex G-25 column (2 cm i.d. × 20 cm). The column was equilibrated with 0.2 M sodium phosphate buffer, pH 7.0. The mixture was loaded onto the column and eluted at a flow rate of 1.5 mL/min. Oxidised D2S, eluted in the void volume of the column, was then incubated with 5 μmol of biotin hydrazide for 30 min at room temperature. Reductive amination was achieved by the addition of 25 μL of 8 M pyridine borane and the sample was left overnight at room temperature. Purification of the biotinylated product was by gel filtration under the same conditions as described above. The incorporation of biotin hydrazide into D2S was confirmed by UV absorbance spectroscopy (Hewlett Packard 8452A Diode Array spectrophotometer) over the wavelength range 200–300 nm and reactivity with streptavidin peroxidase (data not shown).

Measurement of the Binding of B-D2S to Macromolecules

The wells of microtitre plates were coated with 2 μg/mL of purified, recombinant HIV-1 tat, gp120, gp160, vif, p24 or recombinant soluble CD4 in PBS and incubated at 37° for 1 hr. Non-specific binding sites were blocked by the addition of 2% (w/v) BSA in PBS for 1 hr at 37°. The plates were then washed 3 times in PBS and incubated with 0.01–30 μg/mL of B-D2S in PBS for 1 hr at 37°. The plates were then washed 3 times in PBS containing 0.05% (v/v) Tween 20 and then once in PBS alone. Streptavidin peroxidase was added at a concentration of 250 ng/mL in PBS and the plates were incubated for 1 hr at 37°. After washing 3 times in PBS containing 0.05% (v/v) Tween 20 and then once in PBS alone, 100 μL of 2.8 mM *o*-phenylenediamine hydrochloride, 4.4 mM H_2O_2 dissolved in 0.05 M citric acid/0.1 M Na_2HPO_4 buffer, pH 5.0 was added to each well and incubated at room temperature for 10–15 min. The absorbance at 490 nm was measured using a Biorad model 3550 microtitre plate reader. From the curves produced and by plotting the concentration of B-D2S against the absorbance measured at 490 nm, an EC_{50} value (effective concentration that produced 50% of the maximum binding of B-D2S) was calculated using the 1st site ligand-binding model (hyperbolic Michaelis–Menten equation) on Fig. P 6.0 software, Fig. P. Software Corporation.

Competition between B-D2S and Various Sulphated Polysaccharides to HIV-1 Tat

This procedure was similar to that described above except that 0–30 μg/mL of competitor was mixed with 0.2 μg/mL of B-D2S prior to addition to HIV-1 tat-coated and BSA-blocked microtitre plate wells. Concentrations of B-D2S were chosen that gave about 50–75% of maximum binding to HIV-1 tat. Competition was evident as a decrease in absorbance at 490 nm in the presence of the test compounds. From the curves produced and by plotting the concentration of competitor against the absorbance measured at 490 nm, an IC_{50} value (inhibitory concentration resulting in a 50% decrease in B-D2S binding) was calculated for each compound tested.

Peptide Synthesis

The peptide RRQRRRPPQGSQTHQVSLSKC was synthesised according to procedures described previously [27], using a Gem semiautomated peptide synthesiser (Novabiochem). Synthesis was carried out by *N*-α-9-fluorenylmethoxylcarbonyl solid phase chemistry using an insoluble Polyhipe support, which consisted of polydimethylacrylamide functionalised with ethylenediamine, norleucine, and the acid labile linker 4-hydroxymethylphenoxyacetic acid and *N*-α-9-fluorenylmethoxylcarbonyl-protected amino acids. The cysteine residue was incorporated into the C-terminus of the peptide to allow conjugation of the peptide to carrier protein, BSA, in a specific orientation.

Overlapping HIV-1 Tat Peptides

The overlapping HIV-1 tat peptides used in this study were derived from the LAI strain of HIV-1 and were as follows: peptide 1, EPVDPRLEPWKHPGSQPKTA (1–20); peptide 2, KHPGSQPKTACTTCYCKKCC (11–30); peptide 3, CTTCYCKKCCFHCQVCFTTK (21–40); peptide 4, FH CQVCFTTKALGISYGRKK (31–50); peptide 5, ALGI SYGRKKRRQRRRPPQG (41–60); peptide 6, RRQRRRP PQGSQTHQVSLSK (51–70); peptide 7, SQTHQVSL SKQPTSQPRGDP (61–80); and peptide 8, QPTSQPRGDPTGPKE (71–85).

Binding of B-D2S to HIV-1 Tat Peptides

The binding of B-D2S to the overlapping peptides was investigated using a modified version of the solid phase binding system based on the method of Ball *et al.* [28]. Here, 50 μg/mL of BSA in PBS was added to the wells of microtitre plates for 1 hr at 37°. Reactive groups on BSA were generated by the addition of 1% (v/v) glutaraldehyde in PBS for 1 hr at 37°. The microtitre plates were washed twice in PBS and then 10 μg/mL of each peptide was added to the wells for 1 hr at 37°. Binding of the peptides occurs through the amine groups of the peptides and the carbonyl group of glutaraldehyde. The plates were washed twice in PBS, and unreacted groups were blocked by the addition of 1 M glycine for 30 min at 37°. Blocking of non-specific sites was achieved with 2% (v/v) BSA in PBS for 1 hr at 37°. Incubation of B-D2S with the peptides and the measurement of binding was then determined as described above.

Transactivation Assay

H938 cells containing the HIV-1 LTR promoter linked to the CAT gene [25] were resuspended in RPMI 1640 medium containing 20 mM HEPES, 10% (v/v) foetal bovine serum, 250 IU/mL penicillin, 250 μg/mL streptomycin, 2 mM L-glutamine, and 100 μM chloroquine. The cells were incubated for 72 hr in a 24-well plate at a density of 0.3×10^5/mL in the presence of either 2 μg/mL of recombinant HIV-1 tat protein alone or in the presence of 10 μg/mL of dextrin or 0.01–10 μg/mL of D2S. At the end of the incubation period, the cell number and cell viability for each treatment was determined by 0.2% (w/v) trypan blue exclusion and was approximately 0.15×10^6 cells and >90% viability, respectively. The cells were pelleted and washed in ice-cold PBS, a process that was repeated twice. CAT was released from the cytoplasm by lysing the cells by incubation in 3-(*N*-morpholino)propanesulfonic acid lysis buffer (Boehringer) for 30 min at room temperature and pelleting the cell nuclei by centrifugation in a benchtop minifuge for 5 min. The amount of CAT in the cytoplasm was determined by enzyme-linked immunoabsorbent assay using purified CAT standard as described in the manufacturer's instructions (Boehringer). A standard curve representing absorbance versus 0–500 pg/mL of purified CAT was linear ($y = 0.00474x + 0.03162$ and $r^2 = 0.999$).

RESULTS

Binding of B-D2S to Recombinant HIV-1 Proteins

The solid phase binding system was used to investigate the interaction of B-D2S with relevant recombinant HIV-1 proteins and CD4 in an attempt to establish potential targets for D2S. Amongst the proteins studied, the binding of B-D2S to HIV-1 tat was clearly the strongest, with an EC_{50} of 0.10 ± 0.02 μg/mL (N = 5) (Fig. 2). Furthermore, when HIV-1 tat was denatured by boiling for 10 min or treating with 8 M urea, there was no effect on B-D2S binding (data not shown). There was also some evidence of binding to recombinant soluble CD4 (EC_{50} = 0.33 ± 0.02 μg/mL, N = 3), although the maximum amount bound was decreased and the affinity of the interaction was less compared with HIV-1 tat (Fig. 2). Of the other proteins tested, there was only a poor interaction of HIV-1 vif and gp160 with B-D2S and no binding to p24 or gp120 (Fig. 2).

Competition Studies with Sulphated Polysaccharides

D3S, D6S, D236S, heparin, and dextran sulphate were able to compete with B-D2S for binding to HIV-1 tat, whilst unsulphated dextrin could not (Fig. 3). Furthermore, IC_{50} values were calculated and statistical analysis of the data revealed that all of the sulphated polysaccharides were significantly better competitors than D2S (Table 1).

Binding of B-D2S to Overlapping HIV-1 Tat Peptides

Overlapping peptides spanning the entire HIV-1 tat protein were used in binding studies in an attempt to locate the region of the protein where D2S bound. No binding of B-D2S to peptides 1, 3, 6, 7, or 8 was found (Fig. 4). There was some binding of B-D2S to peptide 2, but the strongest interaction was with peptides 4 and 5 (Fig. 4). Interestingly, there was also no binding of B-D2S to the highly basic peptide 6 (Fig. 4). Peptide 6 was also synthesised, containing an additional cysteine residue at the C-terminus, allowing a carrier protein, BSA, to be coupled to the

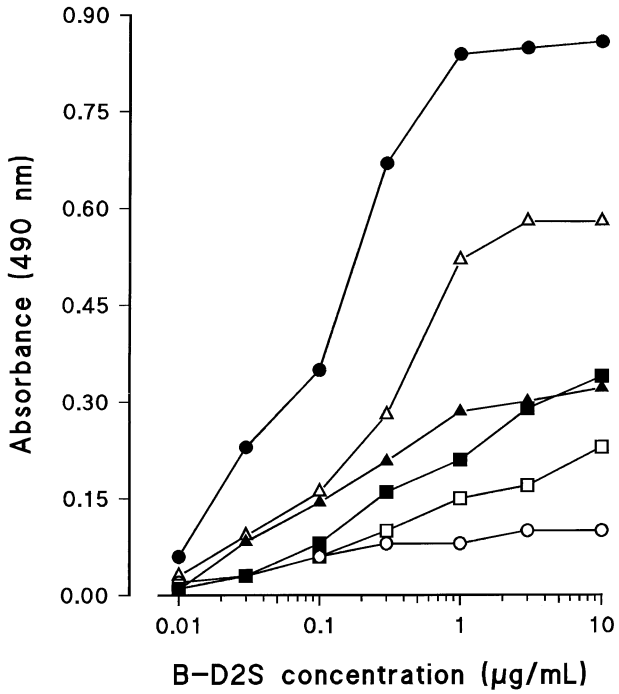

FIG. 2. Binding of B-D2S to recombinant HIV-1 proteins and CD4. Microtitre plate wells were coated with 2 μg/mL of purified recombinant HIV-1 tat (●), recombinant soluble CD4 (△), recombinant HIV-1 vif (▲), gp120 (□), gp160 (■), and p24 (○) in PBS, blocked by addition of 2% (w/v) BSA and then incubated with 0.01–10 μg/mL of B-D2S. The level of B-D2S binding to BSA was similar to that shown for p24 and for clarity is also represented as (○). Binding of B-D2S to each of the proteins, represented as the absorbance at 490 nm, was determined as described in the Methods section. Each point is the mean of duplicate measurements and the data shown are representative of 3 experiments with similar results, except for HIV-1 tat which is representative of 5 experiments.

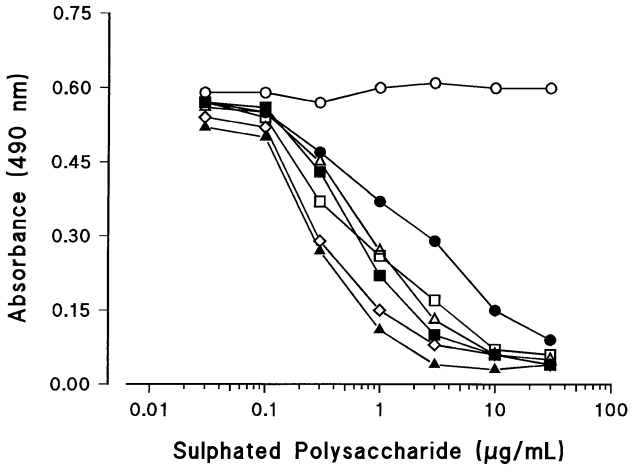

FIG. 3. Competition between B-D2S and sulphated polysaccharides to HIV-1 tat. Microtitre plate wells were coated with 2 μg/mL of recombinant HIV-1 tat and incubated with 0.03–30 μg/mL solutions of dextrin (○), D2S (●), D3S (□), D6S (■), D236S (△), 8 kDa dextran sulphate (◇), or heparin (▲) in PBS, each containing 0.2 μg/mL of B-D2S. Binding of B-D2S to HIV-1 tat, represented as the absorbance at 490 nm, was determined as described in the Methods section. Each point is the mean of duplicate measurements and the data shown are representative of 3 experiments with similar results.

C-terminus of the peptide. There was no binding of B-D2S to either the peptide alone or when coupled to BSA (data not shown).

Inhibition of HIV-1 Tat Transactivating Activity

A reporter gene system was employed to investigate the effect of D2S on HIV-1 tat transactivation *in vitro*. The addition of exogenous HIV-1 tat to H938 cells increased production of CAT protein. D2S was able to significantly inhibit HIV-1 tat transactivation in a dose-dependent manner with an IC_{50} value of 0.5 μg/mL (Fig. 5). Dextrin had no effect on HIV-1 tat transactivation (Fig. 5).

DISCUSSION

The identification of proteins that are potential targets for sulphated polysaccharides such as D2S was investigated using a solid phase binding system. The results show that D2S did not bind to the capsid protein, p24, or to the envelope glycoprotein, gp120. It bound weakly to HIV-1 vif, a putative cysteine protease important in viral infectivity [8] and to envelope glycoprotein gp160, which is the precursor of gp120. Moderate binding was found with recombinant soluble CD4, but the strongest interaction was with HIV-1 tat.

A number of studies have shown that sulphated polysaccharides including dextran sulphate and heparin inhibit HIV-1 *in vitro* [7]; in many cases it has been suggested that this is achieved by interfering with the interaction between the T-cell determinant CD4 and viral gp120. The role of the T-cell determinant CD4 in HIV-1 infection has been established for over a decade. The interaction of the virus envelope gp120 with CD4 is the initial step in HIV-1 virion binding and therefore the whole infective process

TABLE 1. Comparison of IC_{50} values of sulphated polysaccharides determined from competition with B-D2S for binding to HIV-1 tat

Compound	IC_{50} (μg/mL ± SEM)	*P* value
Dextrin (6 kDa)	No competition	NA
D2S (9 kDa)	3.00 ± 0.47	—
D3S (9 kDa)	0.90 ± 0.05	0.010
D6S (9 kDa)	0.60 ± 0.05	0.007
D236S (15 kDa)	0.63 ± 0.03	0.007
Heparin (15 kDa)	0.43 ± 0.07	0.006
Dextran sulphate (8 kDa)	0.50 ± 0.05	0.006

Recombinant HIV-1 tat was coated onto microtitre plate wells and incubated with 0.03–30 μg/mL of each sulphated polysaccharide containing 0.2 μg/mL B-D2S. Competition for binding to HIV-1 tat was determined and IC_{50} values calculated as described in the Methods section. Student's *t*-test analysis comparing D2S with other competitors was undertaken. Each IC_{50} value is the mean ± SEM determined from 3 separate experiments.

NA, statistical analysis not applicable.

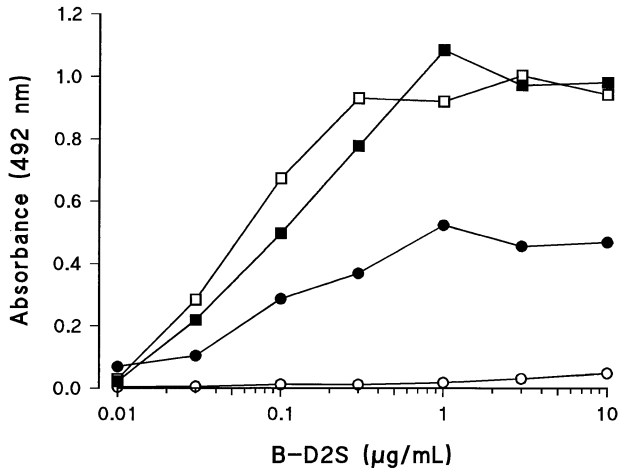

FIG. 4. Binding of B-D2S to peptides coupled to activated BSA. Microtitre plate wells were coated with 50 μg/mL of BSA that was then activated with 1% (v/v) glutaraldehyde in PBS, and HIV-1 tat overlapping peptides (10 μg/mL) were added and covalently coupled. The binding of 0.01–10 μg/mL of B-D2S to each of the peptides was determined as described in the Methods section. The data for B-D2S binding to peptide 4, FHCQVCFTTKALGISYGRKK (□), peptide 5, ALGISYGRKKRRQRRRPPQG (■), and peptide 2, KHPGSQPKTACTTCYCKKCC (●) are shown. Similar results were found for B-D2S binding to peptide 1, EPVDPRLEPWKHPGSQPKTA, peptide 3, CTTCYCKKCCFHCQVCFTTK, peptide 6, RRQRRRPPQGSQTHQVSLSK, peptide 7, SQTHQVSLSKQPTSQPRGDP, and peptide 8, QPTSQPRGDPTGPKE, which for clarity, are represented by (○). The values shown are means of duplicate determinations and are representative of 3 experiments with similar results.

[29, 30]. Both dextran sulphate [31] and heparin [32] have been shown to interact with CD4. Using a solid phase binding system, D2S was shown to bind to recombinant CD4 with an EC_{50} = 0.33 μg/mL. The binding of D2S to CD4 offers a potential mechanism by which D2S can inhibit HIV-1 *in vitro*, and this observation is also in agreement with the prevailing opinion in the literature regarding the mechanism of action of this class of compound. However, the binding of D2S to CD4 cannot be solely responsible for the anti-HIV-1 activity of D2S *in vitro*, since HIV-1 is able to infect CD4 negative cells and D2S can also inhibit this process [33]. Furthermore, D2S potentiated the inhibitory effect of recombinant soluble CD4, resulting in a 10–100 fold decrease in the amount of recombinant soluble CD4 required to achieve inhibition of HIV-1 infectivity [33], suggesting that D2S was interacting through a non-CD4 mechanism.

In addition, heparin and dextran sulphate as well as other sulphated polysaccharides have been shown to bind HIV-1 gp120 [34]. HIV-1 gp120 contains a domain termed the V3 loop, which has a small basic region that is thought to be important for the binding of polyanionic compounds. This has been supported by Callahan *et al.* [35] who showed that dextran sulphate could inhibit the binding of specific antibodies to the V3 region of gp120. The lack of binding of D2S to gp120 in the present study indicates that D2S cannot inhibit HIV-1 infection through such a mechanism.

HIV-1 tat is essential for efficient viral replication [36, 37]. Of the proteins studied, D2S bound most avidly to HIV-1 tat, with an EC_{50} = 0.10 μg/mL, this binding being approximately 3-fold that of D25 to CD4. The binding of other sulphated polysaccharides to tat was determined in a solid phase competition binding system. Unsulphated dextrin was unable to compete with B-D2S for binding to HIV-1 tat. Interestingly, analogues of D2S as well as other sulphated polysaccharides such as heparin and dextran sulphate were significantly better competitors than D2S. There is some indication that the level of interaction between HIV-1 tat and sulphated polysaccharide may be dependent on the position of the sulphate moiety within the compound as well as the degree of sulphation. D6S, a monosulphated dextrin, is a much better competitor than D2S and has a similar affinity for HIV-1 tat as the polysulphated compounds D236S, dextran sulphate, and heparin. Interestingly, heparin is also sulphated in the C-6

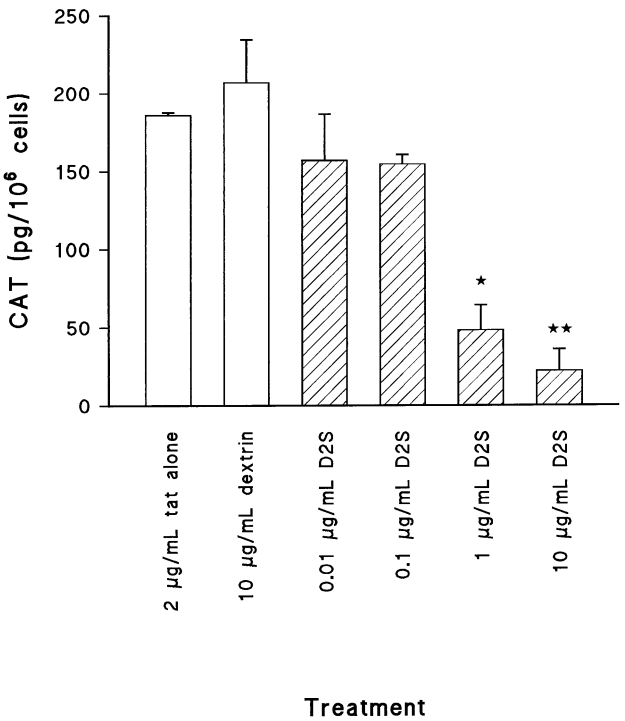

FIG. 5. Effect of dextrin and D2S on HIV-1 tat transactivation. H938 cells at a density of 0.3 × 10^5/mL were incubated with either 2 μg/mL of HIV-1 tat only, 2 μg/mL of HIV-1 tat with 10 μg/mL dextrin, or 2 μg/mL of HIV-1 tat with 0.01, 0.1, 1, or 10 μg/mL of D2S for 72 hr in RPMI 1640 medium as described in the Methods section. Transactivation was quantified by the amount of CAT produced. There was a significant inhibition of CAT production with 1 μg/mL of D2S (*P = 0.008, Student's *t*-test) and 10 μg/mL of D2S (P = 0.004, Student's *t*-test) compared to dextrin alone. The level of endogenous activation was 20 pg/10^6 cells and has been subtracted from each set of data. The values shown are the means ± SEM of triplicate determinations and are representative of 2 experiments.**

position of its constituent glucosamine sugars. This may be important for binding, although heparin is also sulphated in other positions. Even though the various sulphated polysaccharides used here could interact with HIV-1 tat, the therapeutic use of most compounds is limited, either because of their inherent anticoagulation activity or reduced ability to inhibit HIV-1 infectivity. For instance, D236S can inhibit HIV-1 infectivity *in vitro* but compromises blood coagulation [6]. Similarly, heparin and dextran sulphate inhibit HIV-1 infectivity *in vitro* [4, 5] but have a severe effect on blood clotting [38]. In contrast, D3S and D6S have only a moderate effect on blood homeostasis but D3S is a poor inhibitor of HIV-1 infectivity *in vitro* [6].

HIV-1 tat is encoded by two exons and is translated from multiply spliced 2-kilobase mRNAs [39]. HIV-1 tat promotes transcription of the viral genome by binding to the transactivation responsive element located at the 5′ end of viral mRNAs [36, 37]. By comparing sequences of tat proteins of different lentiviruses, HIV-1 tat has been divided into five structural domains termed N-terminal (Pro-rich), cysteine-rich, core, basic (Arg-rich), and C-terminal [40]. The protein comprising amino acid residues 1–72 is encoded by the first exon, is conserved in all viral isolates, and possesses full transactivating activity [41, 42].

Denaturation of HIV-1 tat did not affect the binding of D2S. Thus, it should be possible to define the regions of HIV-1 tat protein that are important for D2S binding by measuring the interaction with a series of overlapping peptides representing the complete sequence of the protein. Some binding was found to the peptide comprising amino acids 11–30 (KHPGSQPKTACTTCYCKKCC). This contains the cysteine-rich region that is essential for tat function [43] and mediates the formation of metal-linked dimers *in vitro* [44]. However, most of the D2S binding was associated with the overlapping peptides comprising amino acids 31–50 (FHCQVCFTTKALGISYGRKK) and amino acids 41–60 (ALGISYGRKKRRQRRRPPQG); importantly, these peptides contain both the core and basic regions of HIV-1 tat. Surprisingly, there was no binding to the peptide containing amino acids 51–70 (RRQRRRPPQGSQTHQVSLSK), even though this also contains the strongly basic, arginine-rich domain. Prior to the current study, the binding of sulphated polysaccharides to HIV-1 tat was believed to be mediated through an ionic interaction between the basic region in tat and the sulphate moieties in compounds such as heparin. This was inferred by Rusnati *et al.* [20] who showed that HIV-1 tat bound to heparin but not the unsulphated analogue, K5. The NMR structure of HIV-1 tat has been established [45], and it reveals that amino acids 31–50 in HIV-1 tat are part of a protruding loop, and as such may be accessible to various polyanionic compounds. However, the requirement of other properties such as polysaccharide length or secondary structure may also be important, since the same authors showed that sulphated compounds such as dermatan sulphate and chondroitin sulphate did not bind HIV-1 tat. Although the basic domain is essential for nuclear and nucleolar localisation [14, 46, 47] and hence for transactivation, Kashanchi *et al.* [48] showed that mutations in amino acids within the core domain, a stretch of 12 amino acids between the cysteine-rich domain and the basic domain that is conserved in all viral isolates, can abolish tat activity. Interestingly, it is this region to which D2S predominantly binds.

The carboxyl terminal region is encoded by the second exon and contains amino acids 72–86, although it is not required for transactivation or replication of HIV-1. However, this region contains the Arg-Gly-Asp (RGD) motif that is also present in extracellular proteins such as fibronectin and vitronectin and mediates cell adhesion and binding of extracellular tat to integrin receptors expressed by activated endothelial cells, CD4 T-cells, and other cell types [16, 49–51]. Interestingly, D2S did not bind to any of the peptides that contained this motif.

D2S was able to significantly inhibit HIV-1 tat transactivation in a dose-responsive manner, whereas dextrin was without effect. The IC_{50} value of approximately 0.5 μg/mL is similar to that reported for heparin [20]. D2S is a large, highly charged polymer that would not be expected to enter cells. Therefore, in the experiments described here, it is likely that D2S binds to HIV-1 tat extracellularly and sequesters the protein, thus inhibiting its activity by preventing it from entering cells.

Extracellular tat can be taken up by cells and subsequently activates HIV-1 gene expression in infected cells [9, 52–54]. Furthermore, extracellular tat has the ability to enter uninfected cells and transactivate endogenous genes, including the production of various cytokines and their receptors [55–59], although the mechanism of uptake, transport to the nucleus, and biological activity in uninfected cells remains unclear. HIV-1 tat also has potent angiogenic activity *in vitro* [15] and *in vivo* [60, 61]. HIV-1 tat has been shown to bind to integrin receptors [16, 50] and to the receptor Flk-1/KDR, whose natural ligand is vascular endothelial growth factor [62]. Extracellular tat can also promote growth, migration, invasion, and adhesion of Kaposi sarcoma-derived endothelial spindle cells and endothelial tumour cell lines [60, 63]. The effect of HIV-1 tat on Kaposi sarcoma-derived cells is evident with picomolar concentrations of the protein; however, transactivation requires about 1000 times more HIV-1 tat. Since D2S can attenuate the transactivating activity of HIV-1 tat, it is possible that the angiogenic properties of HIV-1 tat may also be influenced by the compound.

HIV-1 tat exerts its transactivating activity in an autocrine and paracrine manner. It can enter HIV-1-infected cells and up-regulate HIV-1 replication; tat can also be taken up by uninfected cells, where it switches on cellular genes and propagates a permissive cellular environment for HIV-1 infection. Clearly, compounds or treatments that sequester HIV-1 tat offer a therapeutic opportunity for the treatment of HIV-1 infection. In support of this, studies have shown that antibodies against HIV-1 tat inhibit virus replication *in vitro* [64], and it has been reported that HIV-1

positive haemophiliacs who have a high titre of HIV-1 tat antibodies have a significantly reduced viral load [65]. In conclusion, the ability of D2S to sequester HIV-1 tat indicates that this compound may be effective in the treatment of HIV-1 by interfering with viral replication and subsequent disease progression.

This work was funded by ML Laboratories, Liverpool, UK. The authors are grateful to the National Institute for Biological Standards and Control, Potters Bar, U.K. for providing recombinant HIV-1 proteins, recombinant soluble CD4, HIV-1 tat peptides, and the H938 cell line.

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