Anti-herpes simplex virus activities of two novel disulphated cyclitols

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By screening a library of sulphated compounds of low molecular weight, we have found that several cyclitol derivatives, each modified with two sulphate groups in addition to pyrrole and various aromatic moieties, inhibited infectivity of herpes simplex virus (HSV) at concentrations approximately 100 times lower than those toxic for cultured cells. These disulphated cyclitols interfered with HSV-1 attachment to cells, and efficiently reduced the cell-to-cell spread of the virus. This effect is most likely due to their low molecular weight and associated with the compounds’ capability to access the narrow intercellular spaces. Furthermore, these disulphated cyclitols also inactivated infectivity of HSV. However, the virus-inactivating activities of these compounds were to some extent diminished in the presence of human cervical secretions or other protein-rich solutions suggesting that disulphated cyclitols may have some features of surfactant-type virucides. In conclusion, this new class of anti-HSV compounds offers potential for further development.

Keywords: antiviral potential, disulphated cyclitols, herpes simplex virus,

Introduction

The potential of sulphated polysaccharides and polysulphonated compounds to inhibit infectivity of herpes simplex virus (HSV) and other heparan sulphate-binding viruses has long been known (Takemoto & Liebhaber, 1961; Vaheri, 1964). In spite of this, possible antiviral application of these compounds is limited to their development as microbicides in topical vaginal formulations to protect against infections with herpes simplex virus type 2 (HSV-2), human immunodeficiency virus and other sexually transmitted pathogens (Neyts & De Clercq, 1995). One reason for this is the fact that the development of sulphated polysaccharides as candidate drugs was based on a premise that their antiviral activities usually increase with increasing size of the polysaccharide chain and the degree of sulphation. However, the high molecular weight and extreme negative charge load of the developed compounds have made them unsuitable for oral or systemic administration given their poor stability, bioavailability and tissue-penetrating activity (Artmann et al., 1990; Lorentsen et al., 1989). Furthermore, the antiviral activity of sulphated polysaccharides seems to rely on their weak and reversible interaction with viral components; since prevention of virus attachment to cells requires the continuous presence of the compound, a simple dilution of the virus–drug mixture removes the antiviral effect (Vaheri, 1964). It is not unlikely that the virus–polysaccharide complex could dissociate in the presence of heparan sulphate-binding proteins of non-viral origin such as those present in different body fluids. This possibility may explain a frequently observed discrepancy between the potent antiviral activity of certain sulphated polysaccharides in cultured cells and their poor virus-protective capability in experimental animals (Vaheri, 1964; Neyts & De Clercq, 1995) or lack of antiviral effects in humans (Abrams et al., 1989). However, polysulphates which show high affinity for viral particles such as co-polymers of acrylic acid and vinyl alcohol sulphate (PAVAS; Neyts & De Clercq, 1995) or lambda carrageenans (Zacharopoulos & Phillips, 1997) conferred satisfactory protection to mice against intravaginally administered HSV-2.

Besides sulphated polysaccharides, some sulphated surfactants such as sodium lauryl sulphate (SLS) or docusate have been reported to possess anti-HSV activity (Piret et al., 2002; Gong et al., 2001). These compounds inactivate viral infectivity by denaturing viral proteins and/or disrupting viral membranes. However, sulphated surfactants exhibit low selectivity towards virus particles
and therefore may also target cellular membranes causing cytotoxicity.

We have recently reported that in contrast to conventional sulphated polysaccharides, the low molecular weight heparan sulphate mimetic PI-88 efficiently reduced the cell-to-cell spread of HSV in cultured cells (Nyberg et al., 2004). In addition, PI-88 has been reported to increase survival of animals in murine models of Dengue and encephalitic flavivirus disease (Lee et al., 2006). These results encouraged us to screen for anti-HSV activity of a library of cyclitol-based compounds. These compounds were of low molecular weight and most of them possessed only two sulphate groups per molecule. Our studies identified anti-HSV activity in three different cyclitol derivatives, which exhibited functional features typical for both surfactant-type virucides and sulphated polysaccharides.

Materials and methods

Compounds

A mini-library of 125 compounds which was previously synthesized to search for inhibitors of protein-heparan sulphate interactions was used. The test compounds 1–10, selected for structure-function relationship analysis (Table 1), were all prepared from (1R, 4S, 5S, 6R)-3-bromo-4,5-O-isopropylidene-7-oxabicyclo[4.1.0]hept-2-ene-4,5-diol, which is readily obtained by standard transformations following microbial dihydroxylation of bromobenzene. The aryl groups attached to the cyclohexenyl double bond within compounds 1–4 and 6–10 were introduced by Suzuki–Miyaura cross-coupling of the relevent cyclohexenyl bromide with the appropriate arylboronic acid derivative. All new compounds were vigorously washed out with water giving colourless or slightly coloured solutions.

Cells, viruses and clinical specimens

African green monkey kidney (GMK AH1) cells (Gunalp, 1965) were cultured in Eagle's minimum essential medium (EMEM) supplemented with 2% fetal calf serum (FCS), 0.05% primatom substance (Kraft Inc., Norwich, CT, USA), 100 U of penicillin per ml and 100 µg/ml of streptomycin. The cells were seeded in cluster plates and used for experiments when they had reached confluence. The virus strains used were HSV-1 KOS321 (Holland et al., 1984) and HSV-2 333 (Duff & Rapp, 1971).

Swabs of human cervical secretions were immersed in 1 ml of phosphate buffer solution (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄ and 1.5 mM KH₂PO₄) and

Viral plaque assay

Mixtures of serial fivefold dilutions of a test compound and 200 plaque forming units (PFU) of HSV in serum-free EMEM were incubated for 10 min at room temperature prior to the addition to GMK AH1 cells. After 2 h of viral infection at 37°C, the cells were washed with EMEM and overlaid with EMEM containing 1% methylcellulose, 2% FCS and antibiotics. After 3 days of incubation at 37°C, the viral plaques were visualized by staining with crystal violet. Alternatively, the test compounds were incubated with GMK AH1 cells for 2 h at 37°C either prior to or after infection with the virus. For pilot screening of large numbers of compounds, the mixtures of virus and compound (100 µM) were incubated for 10 min at room temperature before addition to the cells. The compounds were incubated with cells throughout the entire period of plaque development.

For the plaque size reduction assay, the test compounds were added to GMK AH1 cells 2 h after their infection with 200 PFU of the virus and incubated with cells for the entire period of plaque development (Nyberg et al., 2004). The images of 20 plaques per each concentration of compound were captured using a DC300 digital camera (Leica, Heerbrugg, Switzerland) attached to a Diavert microscope (Leitz, Wetzlar, Germany). The area of each plaque was determined by using IMS500 image software (Leica, Cambridge, UK).

Virus purification

Extracellular HSV particles were purified by the method noted in Karger & Mettenleiter (1993) with some modifications. Briefly, GMK AH1 cells were infected with HSV at a multiplicity of 3 PFU per cell. After 1–2 h of incubation at 37°C, 20 µCi/ml of methyl-[3H]thymidine (Amersham Pharmacia Biotech, Uppsala, Sweden) was added and the cells were incubated for 48 h at 37°C. All subsequent steps of the procedure were carried out at 4°C. The cells and the infectious medium were harvested and centrifuged for 15 min at 1,000×g. The supernatant medium was further clarified by centrifugation for 7 min at 5,000×g, and then centrifuged for 2 h at 22,000×g. The resulting viral pellet was stored overnight at 4°C and resuspended in PBS. The virus suspension was loaded on top of a discontinuous sucrose gradient consisting of 2 ml each of 50, 40 and 30% sucrose in 10 mM Tris-HCl, 1 mM EDTA at pH 7.4, and centrifuged for 2 h at 20,000 rpm (SW28.1 rotor, Beckman Coulter, Fullerton, CA, USA). The 40/50% interphase band was aspirated, diluted in PBS and centrifuged for 90 min at 19,000 rpm (SW28.1 rotor). The resulting viral pellet was washed twice with PBS, then dissolved in the same buffer.
Table 1. Anti-HSV activities of cyclitol-based compounds as established in a screening assay

<table>
<thead>
<tr>
<th>Compound</th>
<th>HSV-1</th>
<th>HSV-2</th>
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<tbody>
<tr>
<td>DSC1</td>
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<td>DSC2</td>
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<td>52</td>
</tr>
<tr>
<td>10</td>
<td>111</td>
<td>57</td>
</tr>
</tbody>
</table>

*Results are expressed as a percentage of the number of viral plaques developed by the virus treated with 100µM solution of a compound relative to mock-treated controls. DSC, disulphated cyclitol.
and stored at 4°C. The specific activity (counts per minute [cpm]/PFU) was 9.6x10^3 for HSV-1 KOS321 and 3.1x10^3 for HSV-2 333 strain.

**Virus attachment assay**
Stocks of test compounds were diluted in serum-free EMEM and mixed with purified radiolabelled HSV-1 or HSV-2. The mixture was incubated for 15 min at 37°C prior to the addition to cells. After a 2 h period of virus attachment at 37°C, the cells were washed three times with EMEM and lysed with 5% SDS solution in PBS. The lysates were transferred to scintillation vials for radioactivity quantification. In similar experiments carried out at 4°C, the step of pre-incubating the virus-compound mixture at 37°C was omitted and these two components were simultaneously added to cells.

**Assay of virus entry into cells**
The assay was carried out as described by Schnipper et al., (1980) with some modifications. Briefly, mixtures of test compounds (100µM) and radiolabelled virus in serum-free EMEM were pre-incubated for 15 min at 37°C prior to the addition to GMK AH1 cells and subsequent incubation for 3 h at 37°C. After twice washing with PBS, the cells in some wells were harvested for radioactivity quantification as in the virus attachment assay. The rest of the cells were incubated with warm citrate-buffered saline (pH 3) for 1 min, then washed twice with PBS and treated with pronase (200µg/ml; Sigma) for 15 min at 37°C. The cells were harvested and washed twice with PBS by centrifugation at 250xg for 5 min. Half of the volume of sedimented cells was mixed with 5% SDS and subjected to quantification of radioactivity, whereas the remaining cells were lysed with 1% NP40 solution in hypotonic phosphate buffer and centrifuged for 10 min at 800xg to sediment the nuclei. The sedimented fraction was lysed with 5% SDS and subjected to quantification of radioactivity. In some experiments, the step of pre-incubation of the virus-compound mixture at 37°C was omitted. After the virus attachment period of 1 h at 4°C, the compounds were added to and incubated with cells for 3 h at 37°C.

**Virus inactivation assay**
Mixtures of test compound (0.16–100µM) and 4x10^4 PFU of the virus in serum-free EMEM were co-incubated for 15 min at 37°C in a water bath prior to the dilution of the mixture to non-inhibitory concentrations of compound (1:100 and/or 1:500), and the virus assayed for residual infectivity in GMK AH1 cells.

**Antiviral activity of disulphated cyclitols (DSC) compounds in the presence of cervical secretions or other protein-rich solutions**
Equal volumes (50 µl) of 100 µM solution of a test compound and serial 10-fold dilutions of a sample of human cervical secretions were mixed and incubated for 5 min at room temperature prior to the addition of 200 PFU of HSV-2 333 strain in 100 µl of serum-free EMEM. Additional incubation was then carried out for 15 min at 37°C and the mixtures then transferred to GMK AH1 cells. The rest of the procedure was conducted as described under the viral plaque assay. The effect of bovine serum albumin (BSA) or FCS on inactivation of HSV by the test compounds was assayed in a similar manner.

**Cytotoxicity assay**
GMK AH1 cells that were seeded in 96-well plates and had reached approximately 90% confluence at day 2 of culture were incubated with serial twofold dilutions of a test compound in serum-free EMEM for 24 or 72 h at 37°C. The cell viability was measured using CellTiter 96 Aqueous One Solution Reagent kit (Promega, Madison, WI, USA) according to the manufacturer's protocol.

**Results**

**Identification of anti-HSV compounds**
A mini-library of 125 compounds characterized by their relatively low molecular weight (300–1700 Da), reduced hydrophilicity relative to conventional sulphated polysaccharides and (in most cases) the presence of only one or two sulphate groups, have been screened for anti-HSV activity. Thus, 100µM solutions of each of these compounds were incubated with approximately 200 PFU of HSV-1 or HSV-2 for 10 min at room temperature, then added to GMK AH1 cells and left on the cell monolayer throughout the entire period of viral plaque development. Three structurally-related cyclitol derivatives designated as disulphated cyclitols (DSC1, DSC2, and DSC3) exhibited anti-HSV activities (Table 1). Besides the presence of two sulphate groups, pyrrole and benzyl groups attached to the central cyclitol core are the common structural features of these compounds. However, phenanthrene, anthracene, and indole groups attached to C1 are specific for DSC1, DSC2 and DSC3, respectively. To facilitate the structure-function relationship analysis, seven other compounds of this mini-library that are structurally-related to DSC1–3 are also included in Table 1. Compound 4, which lacks an O-sulphate group at C6 of the cyclitol core, possessed lesser anti-HSV-1 potential than DSC3. Similarly, substitution of bromine (compound 5), phenyl (6), 4-chlorophenyl (7), 1-naphthyl (8), 2-naphthyl (9) or 3,4-ethylenedioxyphenyl (10) for the respective phenanthryl/anthracyl/indoyl group of DSC1–3 greatly decreased or abolished the anti-HSV activity of these compounds. These results suggest that modification of the cyclitol core with two cis-oriented sulphate groups and the specific aromatic group of phenanthrene, anthracene or indole are important for anti-HSV
Anti-HSV activities of disulphated cyclitols

However, further variants of DSC1–3 are needed to complete the structure-function relationship analysis.

**Mechanism of anti-HSV activity of DSC1–3**

To investigate whether DSC1–3 exert their antiviral activities by targeting the virus particle or the cell, the compounds were either incubated with the virus prior to the addition of the mixture to cells, or added to and incubated with cells at different time points relative to the HSV infection. Co-incubation of these compounds with the virus, but not with the cells completely abolished HSV-1 and HSV-2 infectivity (Figure 1) suggesting that the viral particle is the site of antiviral activity of DSC1 (Figure 1) and the other compounds (data not shown).

Incubation of HSV with different concentrations of DSC1–3 for 15 min prior to the addition to GMK AH1 cells, and during a 2 h period of virus infection of cells, revealed that DSC3 was the most potent inhibitor of viral infectivity followed by DSC1 and DSC2 (Figure 2A). The 50% inhibition concentration (IC$_{50}$) of HSV-1 and HSV-2 infectivity by DSC3 occurred at 1.4 and 0.46 μM.

**Figure 1. The virus particle-targeted activity of DSC1**

The compound (100 μM) was either incubated for 15 min at 37°C with 200 plaque forming units (PFU) of herpes simplex virus (HSV)-1 or HSV-2 prior to the addition of the mixture to cells (disulphated cyclitol 1 + virus), incubated with cells for 2 h prior to (DSC1 + cells) or after (virus + cells) infection with respective virus. The number of PFU is expressed as a percentage of the average number of viral plaques formed in the absence of inhibitor. Values shown are the means of four determinations from two separate experiments.

**Figure 2. The effect of DSC compounds on viral infectivity and viability of GMK AH1 cells**

(A) Mixtures of serial fivefold dilutions of respective compounds and 200 plaque forming units (PFU) of herpes simplex virus (HSV)-1 or HSV-2 were incubated for 10 min at room temperature before addition to African green monkey kidney (GMK AH1) cells. The average molecular weight of 15 kDa was accepted for heparin (8 μM corresponds to 100 μg/ml). The results are expressed as a percentage of the average number of viral plaques formed by drug-treated virus relative to mock treated controls. Values shown are the means of four determinations from two separate experiments. (B) GMK AH1 cells were incubated for 24 or 72 h at 37°C with respective compounds. The cell viability was then measured by using the tetrazolium-based Promega’s CellTiter 96 kit. Values shown are the means of four determinations from two separate experiments. DSC, disulphated cyclitol; SLS, sodium lauryl sulphate.
respectively. The corresponding values for DSC1 were 3.7 \mu M for HSV-2 and 4.2 \mu M for HSV-1. In contrast, DSC2 inhibited HSV-1 and HSV-2 infectivity with relatively high IC_{50} values of 42.7 and 30 \mu M, respectively. Given these results, our further investigations focused on DSC3 and DSC1. Concentrations of DSC1 and DSC3 that reduced the GMK AH1 cells viability by 50% were \sim 400 \mu M (Figure 2B), thus exceeding the IC_{50} values by approximately 100 times. For comparative purposes, we also tested the effect of sulphated surfactant SLS or sulphated polysaccharide heparin on HSV-2 infectivity. These compounds act, like DSC1-3, by targeting the virus particles (Vaheri, 1964; Piret et al., 2002). Under the conditions of this experiment, SLS exhibited anti-HSV-2 potency comparable to that of DSC2 (Figure 2A), whereas antiviral activity of heparin occurred at substantially lower concentrations than those found with DSC3. Note that heparin, in contrast to DSC compounds and SLS, did not completely neutralize HSV infectivity even at the highest concentration tested. The concentration of SLS that reduced the GMK AH1 cell viability by 50% was 120 \mu M, whereas heparin at the highest concentration tested, that is, 32 \mu M (400 \mu g/ml) was not toxic for these cells (Figure 2B).

In addition to the inhibitory effects on HSV infectivity, DSC1 and DSC3 interfered with the cell-to-cell spread of the virus, which was manifested as a reduction in size of HSV-1 and HSV-2 plaques developed in the presence of these compounds in the overlay medium (Figure 3A and B). Note that DSC3, although being a more potent inhibitor of viral infectivity (Figure 2A), caused a lesser reduction than DSC1 in cell-to-cell spread of the virus (Figure 3).

Given that conventional sulphated polysaccharides act by interfering with the virus’ attachment to cells (Witvrouw & De Clercq, 1997), DSC1 and DSC3 were tested for their ability to inhibit the binding of purified radiolabelled HSV-1 and HSV-2 to cells. The virus was treated with these compounds for 15 min at 37°C prior to the addition to cells or added simultaneously with DSC1 or DSC3 to cells without any pre-treatment. These compounds inhibited HSV-1, and to much lesser extent, HSV-2 attachment to cells (Figure 4A). However, the inhibitory effect was observed at higher concentrations than those required for interference with the virus infectivity (compare Figures 2A and 4A). Note that the pre-treatment of virus with DSC3 at 37°C was not required for the inhibition of its attachment to cells (Figure 4A), which suggests that the blockade of viral attachment component(s) rather than the disintegration of viral particles by DSC3 was responsible for the observed effects. To further trace the steps of viral invasion of cells in which the compound-treated virus may show defect, purified radiolabelled HSV-1 virions were treated with DSC1 or DSC3 prior to their incubation with cells for 3 h at 37°C. The amount of viral cpm associated with intact cells (Figure 4B; attachment); the low pHi- and pronase-treated cells (Figure 4B; entry 1), and the NP40 resistant nuclear fraction (Figure 4B; entry 2) were quantified, and related to controls. The decreased amounts of HSV-1 label in all these fractions suggest that DSC1 and DSC3 impaired the capability of the virus to attach to cells and to enter the cell or the nucleus; however, the reduced cell- and nuclear-entry of HSV-1 might be a consequence of impairment in the virus binding to cells. To discriminate between DSC effects on virus binding and cell-entry steps,
HSV-1 was adsorbed to cells for 1 h at 4°C prior to the addition of DSC3 and its incubation with cells for 3 h at 37°C. Under these conditions, DSC1 decreased the amount of labelled virus in the low pH- and pronase-treated cells by 36.1% (data not shown) suggesting that this compound could interfere with HSV-1 entry into the cells.

**Figure 4. The effect of DSC compounds on HSV attachment to and penetration into cells**

(A) Mixtures of disulphated cyclitol (DSC) compounds and purified radiolabelled herpes simplex virus (HSV)-1 or HSV-2 were incubated for 15 min at 37°C prior to the addition to cells. After the virus adsorption period of 2 h at 37°C, the cells were washed and then lysed with 5% SDS to quantify radioactivity. In other experiments, DSC3 and HSV-1 (DSC3, HSV-1, 4°C) or HSV-2 (DSC3, HSV-2, 4°C) were simultaneously added to and incubated with cells for 2 h at 4°C. Values shown are the means of four determinations from two separate experiments. (B) Mixtures of DSC compounds (100 µM) and radiolabelled HSV-1 were incubated for 15 min at 37°C prior to the addition to and incubation with African green monkey kidney cells for 3 h at 37°C. The cells in some wells were harvested by lysis with SDS and subjected to quantification of radioactivity (attachment column). The cells in remaining wells were washed with citrate-buffered saline (pH 3), then treated with pronase and half of the volume of detached cells were lysed to quantify 3H label (entry 1). The remaining cells were treated with 1% NP40 solution to quantify radioactivity in the nuclear fraction (entry 2). The results are expressed as a percentage of the average number of counts per minute (CPM) found with DSC-treated virus relative to mock-treated controls. Values shown are the means of four determinations from two separate experiments.

HSV-1 was also included in this investigation. Experiments based on the co-incubation of 4×10⁴ PFU of the virus with the respective compound, followed by the dilution of the mixture to non-inhibitory compound concentrations revealed that these compounds (especially DSC3) and SLS caused complete or near-complete inactivation of HSV infectivity (Table 2). However, this effect occurred at higher concentrations of compound than those required for the inhibition of HSV infectivity (compare Table 2 and Figure 2A). In contrast, DSC1 and heparin exhibited only moderate effects on inactivation of infectivity of HSV-2 particles. Note that HSV-2 was more resistant than HSV-1 to inactivation by DSC1 or DSC3.

Inactivation of HSV infectivity by DSC1 and DSC3

Given that antiviral activity of conventional sulphated polysaccharides relies on reversible blockade of viral components rather than permanent inactivation of viral particles, we have tested the DSC compounds for possible virus-inactivating activities. Heparin and SLS were also included in this investigation. Experiments based on the co-incubation of 4×10⁴ PFU of the virus with the respective compound, followed by the dilution of the mixture to non-inhibitory compound concentrations revealed that these compounds (especially DSC3) and SLS caused complete or near-complete inactivation of HSV infectivity (Table 2). However, this effect occurred at higher concentrations of compound than those required for the inhibition of HSV infectivity (compare Table 2 and Figure 2A). In contrast, DSC1 and heparin exhibited only moderate effects on inactivation of infectivity of HSV-2 particles. Note that HSV-2 was more resistant than HSV-1 to inactivation by DSC1 or DSC3.
Table 2. Inactivation of HSV infectivity by DSC compounds*

<table>
<thead>
<tr>
<th>Compound concentration (µM)</th>
<th>DSC1</th>
<th>DSC3</th>
<th>Heparin</th>
<th>SLS</th>
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<tbody>
<tr>
<td></td>
<td>HSV-1</td>
<td>HSV-2</td>
<td>HSV-1</td>
<td>HSV-2</td>
</tr>
<tr>
<td>100</td>
<td>0.8±1.1</td>
<td>36.0±4.2</td>
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<td>15.2±1</td>
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<tr>
<td>20</td>
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<td>4</td>
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<tr>
<td>0.8</td>
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<td>86.5±7.0</td>
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<tr>
<td>0.16</td>
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<td>94.1±6.7</td>
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<td>97.9±5.2</td>
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*Approximately 4×10^4 plaque forming units of the virus were co-incubated for 15 min at 37°C with specific compound. The concentration of heparin is expressed in µg/ml (100 µg/ml corresponds to 8 U/mL). Results are expressed as a percentage of residual virus infectivity detected with the compound-treated virus relative to mock-treated controls. Values shown are means of four determinations from two separate experiments. DSC, disulphated cyclitol; HSV, herpes simplex virus; SLS, sodium lauryl sulphate.

Given that the virus-inactivating or virucidal activities of anionic surfactants are known to be diminished in protein-rich solutions (Piret et al., 2002; Isaacs et al., 2004), the antiviral potential of DSC1 and DSC3 in the presence of human cervical secretions, BSA or FCS was investigated. A sample of human cervical secretions employed in this study (see Material and methods section) exhibited by itself only a little intrinsic anti-HSV-2 activity reducing the viral infectivity by approximately 12% (data not shown). Residual HSV-2 infectivity in the presence of this sample and 25 µM of DSC3 and DSC1 (final concentration) was 4.9 and 39.9%, respectively (Table 3), indicating that human cervical secretions can to some extent neutralize the anti-HSV-2 activities of DSC compounds. This effect was ablated in diluted samples of cervical secretions (Table 3) suggesting that the higher concentrations of DSC1 and DSC3 than used in this experiment would be needed to overcome the neutralizing effects of cervical secretions. DSC1 and DSC3 also exhibited decreased anti-HSV-2 activities when incubated in the presence of BSA or FCS (Table 3).

Discussion

In this study, we have screened a library of sulphated compounds, which in contrast to conventional sulphated polysaccharides, were of low molecular weight, non-polymeric in nature, possessed fewer sulphate groups per molecule and were less hydrophilic due to attachment of aromatic/hydrophobic residues to the cyclitol core. This approach led to the identification of two novel anti-HSV compounds DSC1 and DSC3. Structural differences between DSC compounds and sulphated polysaccharides were the likely molecular basis for some of the novel biological activities of DSC. We found that DSC exhibited certain functional features typical for both sulphated polysaccharides and sulphated surfactants. In particular, both DSC1 and DSC3 completely neutralized, like the anionic surfactant SLS, infectivity of free viral particles in GMK AH1 cells. In contrast, sulphated polysaccharide heparin did not reach the IC90/95 values even at the highest concentration tested. DSC compounds also reduced transmission of progeny virions from an infected to normal cell (cell-to-cell spread). Although the molecular basis of DSC interference with cell-to-cell spread of HSV requires further clarification, it is likely that the relatively low molecular weight of DSC1 (666 Da) and DSC3 (605 Da) permitted their access to the narrow intercellular space. In contrast, although the high molecular weight of conventional sulphated polysaccharides is a required feature for inhibition

Table 3. Anti-HSV-2 activities of DSC compounds in protein-rich solutions*

<table>
<thead>
<tr>
<th>Sample</th>
<th>DSC1</th>
<th>DSC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cervical secretions (dilution factor)</td>
<td>39.9±3.7</td>
<td>4.9±5.1</td>
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<tr>
<td>BSA (%)</td>
<td>87.1±7.6</td>
<td>58.7±6.3</td>
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<tr>
<td>FCS (%)</td>
<td>42.3±8.7</td>
<td>5.8±4.0</td>
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*Sulphated cyclitol 1(DSC1) or DSC3 was co-incubated with specific protein-rich solution before addition of 200 plaque forming units of herpes simplex virus (HSV)-2. Results are expressed as a percentage of the residual HSV-2 infectivity detected in virus incubated with DSC compound and cervical secretion or bovine serum albumin (BSA) or fetal calf serum (FCS) relative to the virus treated with specific protein-rich sample only. Values shown are means of four determinations from two separate experiments.
of infectivity of free virus particles (Vitvrouw & De Clercq, 1997), it was also shown to adversely affect the capability of a polymer to penetrate into epidermal tissue (Betz et al., 2001) or to interfere with the cell-to-cell spread of HSV (Nyberg et al., 2004).

In contrast to sulphated polysaccharides that act by interfering with the virus binding to cells, the antiviral activity of DSC compounds could only in part be explained by the interference with virus attachment to cells, which was mainly observed with HSV-1 and occurred at higher DSC concentrations than those required for inhibition of viral infectivity. However DSC also inactivated viral particles similarly to sulphated surfactants. HSV inactivation by surfactant-based microbicides is known to be diminished in protein-rich solutions (Piret et al., 2002; Isaacs et al., 2004). It is noteworthy that inactivation of HSV by anionic surfactants may not always be a consequence of the disruption of viral particles. For example, the virus attachment to cells, an activity that requires intact integrity of viral particles, was found to be unaltered in HSV pre-treated with SLS (Piret et al., 2002). In contrast, we observed that DSC3 inhibited virus attachment to cells when these two components were simultaneously added to cells at 4°C, that is, in the absence of prior HSV-1 treatment with the virus-inactivating agent at 37°C. This suggests that interaction of DSC3 with the attachment component(s) of HSV-1 may in part contribute to its virus-inactivating activity.

Given the fact that certain anionic surfactants such as docucate or SLS are regarded as candidate microbicides in topical vaginal formulations to prevent infection with HSV and other sexually transmitted pathogens (Gong et al., 2001; Piret et al., 2002), modulation of their virus-inactivating activities in the presence of human cervical secretions seems to be of importance. Cervicovaginal lavage samples have been recently reported to possess intrinsic anti-HSV-2 activities very likely due to the presence of neutrophil defensin-type peptides (John et al., 2005). These protective activities varied considerably in samples collected from different individuals and were greatly diminished in secretions of subjects infected with human immunodeficiency virus (John et al., 2005). Under conditions employed in our experiments, human cervical secretions caused partial neutralization of anti-HSV-2 activity of DSC1, whereas DSC3 preserved most of its antiviral activity in the presence of this body fluid. It is also important to note that DSC1 and DSC3, although being neutralized to some extent by protein-rich solutions, inhibited HSV infectivity at substantially lower concentrations than those toxic for cultured cells. In contrast, virucidal effects of anionic surfactants were observed at only slightly lower concentrations than those causing cytotoxicity (Gong et al., 2001). Because surfactants act by disrupting viral/cellular membranes and/or denaturing/dissociating proteins, one can speculate that the lack of extended fatty acid-like hydrophobic domains in DSC1 and DSC3 as compared to anionic surfactants may contribute to their limited cellular toxicity. This functional feature of DSC1 and DSC3 together with their virus-inactivating properties and potential to reduce the cell-to-cell spread of HSV, suggest that these compounds could be regarded as promising candidates for development as anti-HSV agents.

Acknowledgements

This work was supported by grants from the Mizutani Foundation for Glycoscience, the Swedish Research Council, the Sahlgren’s University Hospital Läkarutbildningsavtal, and the Scandinavian Society for Antimicrobial Chemotherapy.

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Received 16 January 2006, accepted 20 March 2006