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A highly lipophilic sulfated tetrasaccharide glycoside related to muparfostat (PI-88) exhibits virucidal activity against herpes simplex virus

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ABSTRACT

Although sulfated polysaccharides potently inhibit the infectivity of herpes simplex virus (HSV) and human immunodeficiency virus in cultured cells, these compounds fail to show protective effects in humans, most likely due to their poor virucidal activity. Herein we report on sulfated oligosaccharide glycosides related to muparfostat (formerly known as PI-88) and their assessment for anti-HSV activity. Chemical modifications based on the introduction of specific hydrophobic groups at the reducing end of a sulfated oligosaccharide chain enhanced the compound's capability to inhibit the infection of cells by HSV-1 and HSV-2 and abrogated the cell-to-cell transmission of HSV-2. Furthermore, modification with a highly lipophilic cholestanyl group provided a compound with virucidal activity against HSV. This glycoside targeted the viral particle and, to a lesser degree, the cell, and exhibited an antiviral mode of action typical for sulfated polysaccharides and virucides, i.e., interference with the virus attachment to cells and irreversible inactivation of virus infectivity, respectively. The virucidal activity was decreased in the presence of human cervical secretions suggesting that higher doses of this glycoside might be needed for in vivo application. Altogether, the sulfated oligosaccharide-cholestanyl glycoside exhibits potent anti-HSV activity and is, therefore, a good candidate for development as a virucide.

1. Introduction

The use of mimetics of cellular receptors for viruses for the prevention and/or treatment of viral infections is a promising approach that has already resulted in the development of novel drugs against influenza virus (Von Itzstein et al., 1993; for a recent review, see Von Itzstein, 2007). Another well known example of this approach are mimetics of cell surface heparan sulfate (HS) (for reviews, see McCarthy et al., 2005; Vaheri, 1964; Witvrouw and De Clercq, 1997), a molecule that serves as an initial receptor for many different viruses including herpes simplex virus (HSV) (WuDunn and Spear, 1989). HS mimetics such as sulfated polysaccharides and other polysulfonated compounds target the virus attachment/entry components, thus preventing adherence of viral particles to cells. Although sulfated polysaccharides are known to be potent inhibitors of infectivity of HSV, human immunodeficiency virus (HIV), and other HS-binding viruses in cultured cells (Vaheri, 1964; Witvrouw and De Clercq, 1997), these compounds failed to show antiviral effects in humans. In particular, intravaginal application of cellulose sulfate (Van der Wijgert and Shattock, 2007) or carrageenan (Cohen, 2008) provided women no protection against HIV while oral dextran sulfate failed to show therapeutic effect in HIV-infected individuals (Abrams et al., 1989). A possible cause for the discrepancy between the potent antiviral activity of sulfated polysaccharides in cell cultures and the lack of such activity in humans could be the fact that these compounds are hydrophilic, negatively charged polymers and therefore their multiple electrostatic binding to viral particles is relatively weak, reversible and non-virucidal (Neyts and De Clercq, 1995). In typical virucidal assays the continuous presence of sulfated polysaccharide is frequently required to block infectivity of viral particles for cultured cells, and a simple dilution of the virus-polysaccharide mixture may dissociate complexes and release infectious virus (Vaheri, 1964). Indeed, this is known to be the case with both cellulose sulfate (Cheshenko et al., 2004) and carrageenan (Carlucci et al., 1999) but not with PRO2000 (Cheshenko et al., 2004), a polymer of naph-

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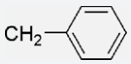
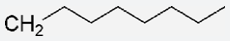
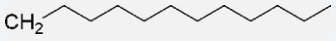
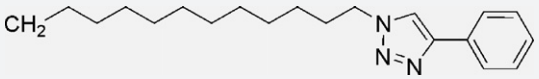
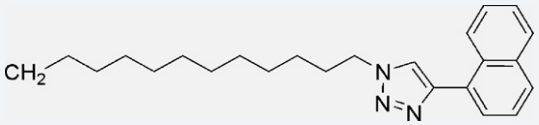
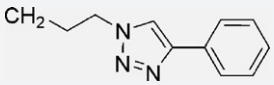
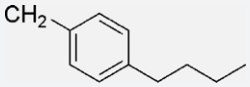
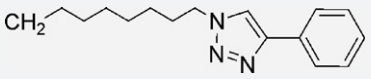
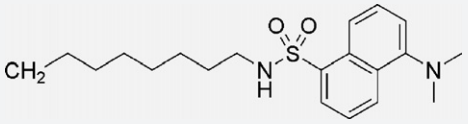
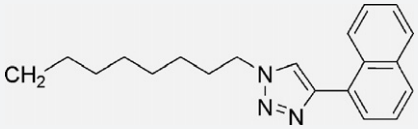
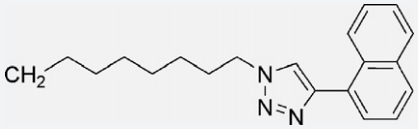
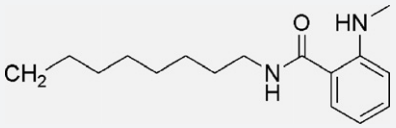
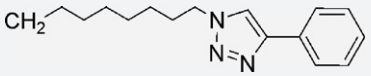
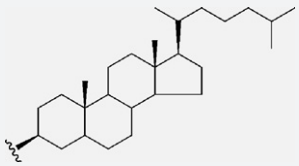
Table 1
Anti-HSV activity of test compounds found in a screening assay.

Compound	Structure of glycoside		Residual infectivity (%)[a]	
	Oligosaccharide component Manα(1 → 3)/Manα(1 → 2) (no. of residues)	Aglycone component	HSV-1	HSV-2
1	Tetrasaccharide		11	9
2	Tetrasaccharide		14	10
3	Pentasaccharide		2	0.7
4	Pentasaccharide		7	0.2
5	Pentasaccharide		0	0
6	Pentasaccharide		17	9
7	Tetrasaccharide		18	9
8	Tetrasaccharide		12	7
9	Tetrasaccharide		4	0
10	Pentasaccharide		19	4
11	Tetrasaccharide		13	5
12	Tetrasaccharide		17	8
13	Pentasaccharide		8	4
14	Tetrasaccharide		0	0
Muparfostat (PI-88)	Di- to hexa-saccharides	None	5	3

[a] Percentage of a number of viral plaques found with drug-treated virus (100 μg/ml) relative to mock-treated controls.

thalenesulfonic acid which was recently reported to provide some protection for women against HIV (Cohen, 2009). This observation suggests that when the backbone of a sulfonated polymer is made up of relatively lipophilic groups instead of sugar residues its virucidal properties could be amplified.

We previously reported that the sulfated oligosaccharide mixture muparfostat (previously known as PI-88) (Nyberg et al., 2004) and the disulfated cyclitol DSC-3 (Ekblad et al., 2006) exhibited potent anti-HSV activities in cultured cells. However, DSC-3 but not muparfostat displayed virus-inactivating (virucidal) activity. This observation, suggesting that specific hydrophobic/aromatic groups in DSC-3 could induce/potentiate the virucidal properties of sulfated compounds, encouraged us to assess the antiviral activity of several lipophilic conjugates of sulfated oligosaccharides related to muparfostat. Our studies demonstrated that, unlike muparfostat, one of these compounds exhibited virucidal activity.

2. Materials and methods

2.1. Cells and viruses

African green monkey kidney (GMK AH1) cells (Günalp, 1965) were cultured in Eagle's minimum essential medium (EMEM) supplemented with 2% calf serum, 0.05% Primaton RL substance (Kraft Inc., Norwich, NY) and antibiotics. The HSV-1 strains used were KOS321, a plaque-purified isolate of wild-type strain KOS (Holland et al., 1983), and clinical isolate 2762 derived from biopsy of encephalitis case (Bergström and Lycke, 1990). The HSV-2 strains used were 333 (Duff and Rapp, 1971), and clinical isolate VF1181 originating from cerebrospinal fluid of a patient suffering from meningitis (Bergström and Lycke, 1990).

2.2. Compounds

Test compounds were all prepared by modifications of the reducing end of Man$\alpha(1\rightarrow3)$/Man$\alpha(1\rightarrow2)$ tetra- or pentasaccharides with various groups (Table 1) as described previously for similar muparfostat-related compounds (Karoli et al., 2005).

All new compounds were characterized by ^{13}C and/or ^{1}H NMR spectroscopy and were of ≥95% purity as determined by capillary electrophoresis and/or HPLC. All compounds tested were readily soluble in water and their stocks (10 mg/ml) were stored at −20 °C. Full details of the synthesis and characterization of the test compounds will be published elsewhere.

2.3. Virus purification and assay of virus binding to cells

The extracellular, methyl-[^{3}H]-thymidine labeled HSV virions were purified by centrifugation through a three-step discontinuous sucrose gradient as described (Karger and Mettenleiter, 1993; Trybala et al., 2000). The effects of test compounds on the binding of purified virions to GMK AH1 cells at 4 °C was assayed as described previously (Nyberg et al., 2004). Briefly, the cells were washed with PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$) and serial fivefold dilutions of test compound in the same buffer and purified virus were added at 4 °C. The cells were incubated under moderate agitation for 2 h at 4 °C. Subsequently the cells were washed three times with PBS, lysed with 0.2 ml of PBS containing 5% SDS, and finally transferred to scintillation vials for quantification of radioactivity.

2.4. Viral plaque assays

The effect of test compounds on viral infectivity in cultured cells was tested in the plaque number-reduction assay and the plaque size-reduction assay (Nyberg et al., 2004). Briefly, for the plaque number-reduction assay, serial fivefold dilutions of test compound and ~100–200 plaque-forming units (PFU) were incubated for 15 min at room temperature prior to the addition to cells and during 1 h period of virus infection of cells at 37 °C. Subsequently, the cells were washed with EMEM and overlaid with 1% methylcellulose solution in EMEM. The plaques were visualized by staining with 1% crystal violet solution after 2 (HSV-2) or 3 (HSV-1) days of incubation at 37 °C. The concentration of the test compound that inhibited the number of viral plaques by 50% (IC_{50}) was interpolated from the dose-response curves. When the compounds were screened for anti-HSV activity, mixtures of 200 PFU of the virus and the test compound (100 μg/ml) in serum-free EMEM were incubated for 10 min at room temperature before addition to cells and during the entire period of viral infection of cells and the development of viral plaques.

In the plaque size-reduction assay, the compounds were added to cells (in methylcellulose overlay medium) after a 2 h period of virus infection of cells in the absence of the inhibitor. After 2–3 days of incubation at 37 °C, the viral plaques were visualized by staining the cells with a 1% solution of crystal violet. For each compound tested, the images of twenty neighboring plaques were captured using a Leica DC300 digital camera attached to a Leitz-Wetzlar Diavert microscope. The plaque area was measured by using IM500 image software (Leica).

2.5. Time-of-addition assay

The test compound (10 μg/ml) was incubated with GMK AH1 cells for 2 h at 37 °C either prior to, during or after 2 h period of cell infection with ~100–200 PFU of HSV-1 or HSV-2. The cells were washed once or thrice following each period of incubation with the test compound or the virus, and then overlaid with methylcellulose solution. The rest of the procedure was as described under the viral plaque number-reduction assay in Section 2.4.

2.6. Virus-inactivation assay

Approximately 2×10^5 PFU of HSV-1 KOS321 or HSV-2 333 strain and specific concentrations of the test compound in 200 μl of serum-free EMEM were mixed and incubated at 37 °C for 15 min. The mixtures were diluted to the non-inhibitory concentrations of the test compound (1:500 or 1:1000), and then subjected to the infectious titer determination as described under the viral plaque number-reduction assay.

To evaluate the virus-inactivating activity of test compounds at low pH or in the presence of cervical secretions, the test compounds and the virus (HSV-2 333) were diluted in a pH 4.5 buffer or were added to a preparation of cervical secretions. Two hundred microliter portions of the virus-compound mixture in low pH buffer or the virus-compound-cervical secretion mixture were processed in the virus-inactivation assay as described above. Cervical secretions were prepared from cervical swabs obtained from three different individuals. The swabs were rinsed with 1.5 ml of distilled water and the obtained fluids centrifuged at $5000\times g$ for 10 min. The supernatant was collected and stored at −20 °C.

2.7. Cytotoxicity assay

The assay was performed in GMK AH1 cells that had been seeded in 96 well cluster plates and reached approximately 80–90% confluence at day 2 of culture. The cells were washed with EMEM and incubated for 24 h at 37 °C with 100 μl of serial twofold dilutions of the test compound in serum-free EMEM. The effect of the test compound on cell viability was measured by using the tetrazolium-

based CellTiter96 assay according to the Manufacturer's protocol (Promega, Madison, WI).

2.8. Electron microscopy

GMK AH1 cells growing on a Melinex polyester film (Agar Scientific Ltd., Stansted, UK) were infected with HSV-2 333 strain at a multiplicity of infection of 0.1. After 24 h of infection at 37 °C, the culture medium was removed and a fresh Eagle's medium containing compound **14** (50 μg/ml) was added and incubated with cells for 30 min at 37 °C. The medium was then collected and the cells gently washed once with Eagle's medium. The cells were fixed with 2.5% glutaraldehyde solution in Eagle's medium for 30 min at 37 °C, and then thrice washed with 0.05 M Tris–HCl buffer (pH 7.4) supplemented with 2 mM $CaCl_2$. The cells were then processed for electron microscopy as described by Widéhn and Kindblom (1990). For each prepared sample, at least ten images were captured and evaluated independently by two investigators.

3. Results

3.1. Sulfated oligosaccharide glycosides demonstrate improved anti-HSV activity

In our previous studies we found that, although both muparfostat and DSC-3 can inhibit HSV infection of cells through interference with the virus attachment and/or cell-entry steps, only DSC-3 exhibited virus-inactivating activities (Nyberg et al., 2004; Ekblad et al., 2006). Because DSC-3 is more hydrophobic than muparfostat due to the presence of hydrophobic/aromatic residues attached to its cyclitol core, we sought to examine the antiviral activity of sulfated tetra- and pentasaccharide glycosides prepared by attachment of specific hydrophobic/aromatic groups to the reducing end of a muparfostat oligosaccharide chain (Table 1). We had also previously tested the antiviral activity of a smaller, earlier series of compounds, including the pentasaccharide analogues of compounds **1** and **2**, and showed that they had similar activity to muparfostat against HSV-1 (Karoli et al., 2005). In this study, preliminary screening of compounds **1**–**14** at a concentration of 100 μg/ml revealed that in contrast to the parent compound, compounds **5** and **14** completely inhibited HSV-1 and HSV-2 infectivity, while **3** showed near-complete reduction of infectivity of these viruses (Table 1). The specific lipophilic modifications in the active analogues were dodecyl (**3**), 12-(4-naphthalen-1-yl-[1,2,3]triazol-1-yl)dodecyl (**5**) or cholestanyl (**14**) attached to the reducing end of a muparfostat tetra- or pentasaccharide chain (Table 1).

The compounds that showed the most pronounced anti-HSV activities in the screening assay, i.e., **3**, **5**, and **14** were selected for detailed investigation of their effect on different biological activities of HSV. The influence of these compounds on HSV infectivity and on viability of GMK AH1 cells is shown in Fig. 1A and B. The concentrations of these compounds that reduced by 50% both the HSV infectivity (IC_{50}) and the viability of GMK AH1 (CC_{50}), as wells the calculated values of selectivity index (CC_{50}/IC_{50}) are summarized in Table 2. Compared with muparfostat, the compounds were more or equally efficient at inhibiting infection of cells by HSV-1 and HSV-2. Compound **14** appeared to be more cytotoxic than muparfostat, however, its selectivity index value was still favorable for both HSV-1 and HSV-2. The cytotoxic activity of compound **14** remained unchanged when incubated with GMK AH1 cells for 24, 48 or 72 h. In addition to GMK AH1 cells we also tested the cytotoxic activity of **14** in human HaCaT keratinocytes (Boukamp et al., 1988), i.e. cells targeted by HSV in humans. The CC_{50} value was

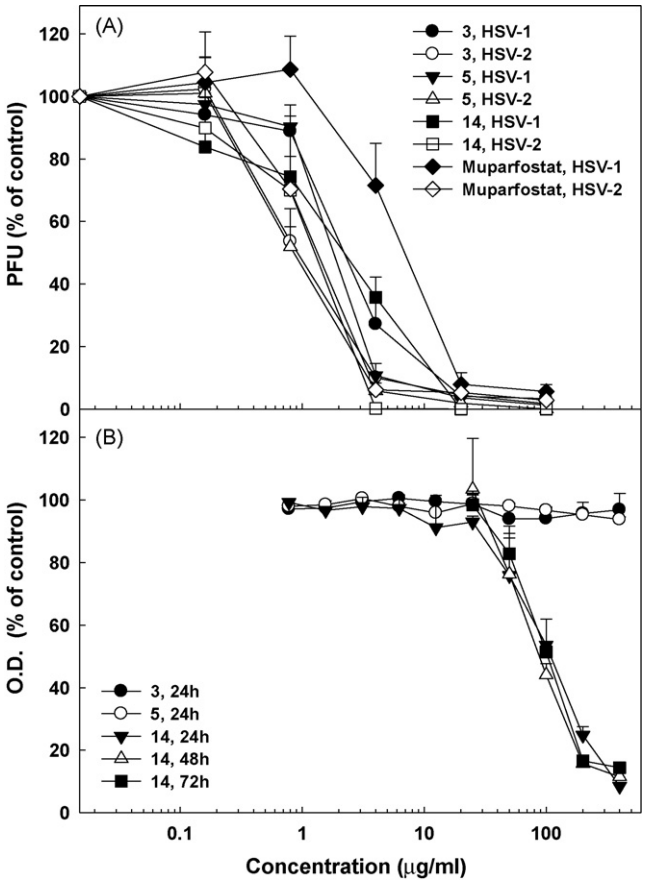


Fig. 1. Effect of test compounds on HSV infectivity and on the viability of GMK AH1 cells. Test compounds (A) at specific concentrations were incubated with ~200 plaque-forming units (PFU) of HSV for 15 min prior to and during 2 h period of virus infection of GMK AH1 cells. The results are expressed as a percentage of the number of viral PFU found with drug-treated virions relative to mock-treated controls. In cytotoxicity assay (B), test compounds at specific concentrations were incubated for 24 h at 37 °C with GMK AH cells, prior to the addition of tetrazolium-based reagent (Promega, Madison, WI). Cytotoxicity of compound **14** was also tested after 48 and 72 h of incubation of this compound (25–400 μg/ml) with cells. Values shown are means of four determinations from two separate experiments.

80 μg/ml when measured after 72 h of incubation of compound **14** with HaCaT cells (data not shown).

Since muparfostat is known to efficiently interfere with the cell-to-cell spread of HSV-1 and HSV-2 (Nyberg et al., 2004), we tested the effect of the new compounds on lateral spread of the virus in cultured cells measured as a reduction in the size of viral plaques. To exclude possible cytotoxicity, all compounds were tested at concentrations at least 10-fold lower than their respective CC_{50} values (Table 2). In contrast to muparfostat, all compounds (at 10 μg/ml) completely inhibited the development of HSV-2 strain 333 plaques

Table 2
Antiviral activity and cytotoxicity of sulfated oligosaccharide glycosides.

Compound	Cytotoxicity (CC_{50})[a]	IC_{50} (selectivity index CC_{50}/IC_{50})[b]	
		HSV-1	HSV-2
Muparfostat	>400	7.0 (>57)	1.1 (>364)
3	>400	2.1 (>190)	0.9 (>444)
5	>400	1.8 (>222)	0.9 (>444)
14	110	2.1 (52)	1.1 (100)

[a] Concentration of test compound (μg/ml) that reduced GMK AH1 cell viability by 50%.
[b] Concentration of a test compound that reduced the number of HSV plaques in GMK AH1 cells by 50%. In parentheses are the values of selectivity index.

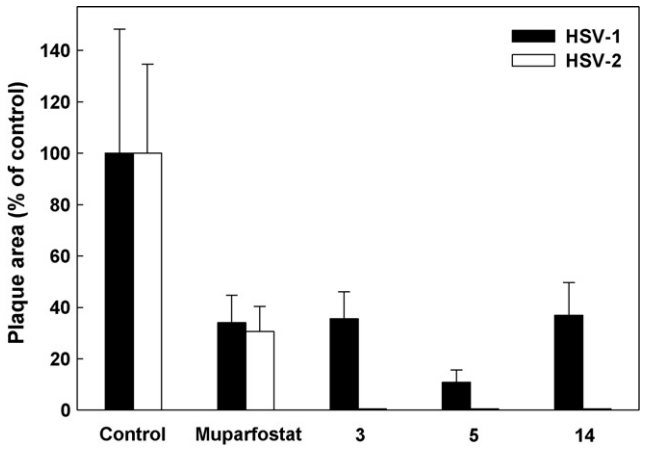


Fig. 2. Effect of test compounds on the cell-to-cell transmission of HSV. GMK AH1 cells were infected with ~100–200 plaque-forming units (PFU) of either HSV-1 or HSV-2, and then overlaid with Eagle's medium supplemented with 1% methylcellulose and 10 μg/ml of specific test compound. The results are expressed as a percentage of the average area of viral plaques developed in drug-treated cells relative to mock-treated controls. Images of twenty neighboring viral plaques captured with digital camera were subjected to area determinations using the IM500 software.

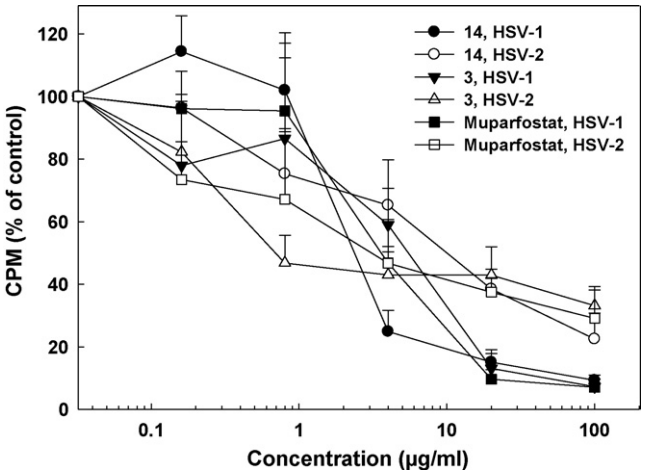


Fig. 3. Effect of test compounds on the binding of HSV virions to GMK AH1 cells. Test compounds at specific concentrations were incubated at 4 °C with methyl-[^{3}H]-thymidine labeled HSV-1 or HSV-2 during a 2 h period of virus adsorption to GMK AH1 cells. The results are expressed as a percentage of attached viral cpm found with compound-treated virions relative to mock-treated controls. Values shown are means of four determinations from two separate experiments.

(Fig. 2). With respect to HSV-1 KOS, compounds **3** and **14** demonstrated similar plaque size-reducing activity to muparfostat while **5** displayed improved activity. We also tested the effect of compound **14** on the cell-to-cell spread activity of clinical isolates of HSV. Similar to what was found with strains 333 of HSV-2 and KOS of HSV-1, the development of plaques by HSV-2 isolate VF1181 was abrogated, while that of HSV-1 isolate 2762 was reduced partially by compound **14**, i.e., the mean area of 20 plaques of HSV-1 isolate 2762 was 30.2% ± 7.9 of the plaque size developed in the absence of **14** (data not shown). These results demonstrate that compound **14** affected the cell-to-cell spread activity in the HSV-type specific manner.

3.2. Mechanism of anti-HSV activity of sulfated oligosaccharide glycosides

Sulfated oligo- and polysaccharides, including muparfostat (Nyberg et al., 2004), exert their anti-HSV activity mainly through inhibition of the virus attachment to susceptible cells. To examine whether the new compounds exhibit a similar mode of activity, we tested the effect of compounds **3** and **14** on binding of purified radiolabeled HSV-1 and HSV-2 to cells. Both compounds inhibited virus attachment to cells (Fig. 3). This suggests that like muparfostat, these compounds act at least in part through interference with the virus attachment to cells.

To further clarify which step of HSV infection of cells is targeted by the compounds, we tested the effect of **14** on HSV infectivity in the standard "time-of-addition" experiment. The compound (10 μg/ml) was added to GMK AH1 cell at different time points relative to the cell infection with the virus (Fig. 4). Presence of **14** during the virus infection of cells completely inhibited HSV-1 or HSV-2 infectivity, while addition of **14** after infection of cells had only a marginal effect on these viruses. Incubation of cells with **14** for 2 h, followed by removal of compound and extensive cell washing prior to the addition of virus resulted in substantial reduction of infectivity of HSV-2 (Fig. 4). These results suggest that this compound preferentially targets HSV particles and prevents their infection of cells. This compound can also target the cell (Figs. 1B and 4) and interfere with HSV-2 infectivity.

To identify the HSV-2 component(s) targeted by compound **14**, we attempted to prepare viral variants resistant to this drug. Two different approaches were undertaken. First, preincubation of 2×10^5 PFU of HSV-2 333 strain with 10 μg of **14** for 15 min, followed by the drug presence during and after infection of cells, resulted in complete abrogation of virus infectivity and no selection, in two further blind passages, of HSV-2 variants resistant to **14**. Second, preincubation of 2×10^5 PFU of HSV-2 333 strain with low doses (2 or 4 μg) of **14** for 15 min, followed by 5 serial passages in GMK AH1 cells in the presence of the same drug concentrations, did result in the virus survival, which, however, in the plaque reduction assay appeared to be as sensitive to **14** as the original HSV-2 333 strain (data not shown). The difficulties in the development of HSV-2 variants resistant to **14** suggest that this drug may target more than one viral and/or cellular component. We also attempted to prepare variants of HSV-2 resistant to compound **3** which like **14** inhibited the cell-to-cell spread activity completely (Fig. 2) but unlike **14** possessed little or no virucidal properties (Table 3). Preincubation of 4×10^5 PFU of HSV-2 333 strain with 20 μg of compound **3** for 15 min, followed by the drug presence

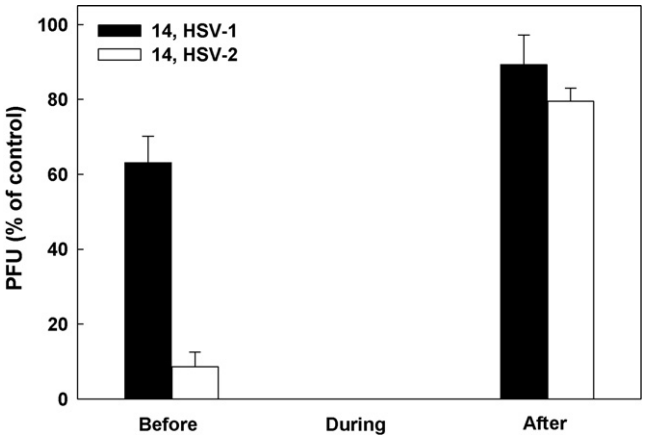


Fig. 4. Anti-HSV activity of compound **14** added to GMK AH1 cells at different time points relative to virus infection. The drug (10 μg/ml) was added to and incubated for 2 h with cells either "before", "during" or "after" 2 h period of cell infection with ~100–200 plaque-forming units (PFU) of HSV-1 or HSV-2. Three separate experiments, each in duplicate wells, were performed. The results are expressed as a percentage of a number of viral plaques found in drug-treated cells or virus relative to mock-treated controls.

Table 3
Virus-inactivating activities of sulfated oligosaccharide glycosides[a].

Virus	Compound concentration (μg/ml)	Compound		
		Muparfostat	**3**	**14**
HSV-1	100	100.3	83.8	0
	10	108.0	79.4	0
	1	99.8	83.9	88.6
HSV-2	100	107.7	68.1	0
	10	102.9	97.5	0.3
	1	95.1	98.6	120.3

[a] Approximately 2×10^5 PFU of respective virus were co-incubated with test compound or the diluent medium (control) for 15 min at 37 °C (water bath) prior to dilution of the mixtures 1:500 or 1:1000 and viral plaque titration. The results are expressed as a percentage of the number of viral plaques detected with the compound-treated virus relative to mock-treated controls.

during and after infection of cells, resulted in survival of a few tiny viral plaques which, however, disappeared in two further blind passages. This suggests that drug **3** may like compound **14** target more than one viral and/or cellular component.

3.3. Compound *14* exhibits virucidal activity

We were interested in whether or not the inhibition of virus infectivity by the new compounds was associated with inactivation of the virus particles. Some sulfated polysaccharides that target viral particles can inhibit the virus binding/entry steps without inactivating the virions (Vaheri, 1964). Accordingly, approximately 10^5 of viral PFU were incubated with each compound and then assayed for residual viral infectivity upon dilution of the mixture to the non-inhibitory concentrations of the compounds. As expected, muparfostat did not inactivate HSV particles; however, compound **14** completely or near completely inactivated HSV-1 and HSV-2 particles at ≥10 μg/ml (Table 3), i.e. at concentrations >10-fold lower than its 50% cytotoxic value (CC_{50}; Table 2). Given the possible genital application of **14**, we tested the effect of low pH or cervical secretions on inactivation of HSV-2 infectivity by this compound. This activity of **14** was to some extent inhibited by low pH, and to a greater extent by the presence of cervical secretions (compare data presented in Table 4 with those of Table 3 showing the native level of the inactivating activity of **14**). These inhibitory effects of low pH and cervical secretions were overcome when a higher concentration of the compound was used.

To investigate whether interaction of **14** with HSV-2 causes any morphological alterations of the viral particle, the HSV-2 virions on the surface of infected GMK AH1 cells were treated with **14** and then observed under electron microscope. No gross morphological changes were observed in HSV-2 virions treated with **14** (Fig. 5). However, there was a tendency towards more frequent virion-to-virion linking at the cell surface in **14**- than the mock-treated preparation of infected cells. In particular, in control preparation the number of the virion-to-virion bridges per 258 virus particles counted was 32 (12.4%), while the corresponding values in compound **14** treated preparation were 128 bridges per 271 (47.2%) virions counted.

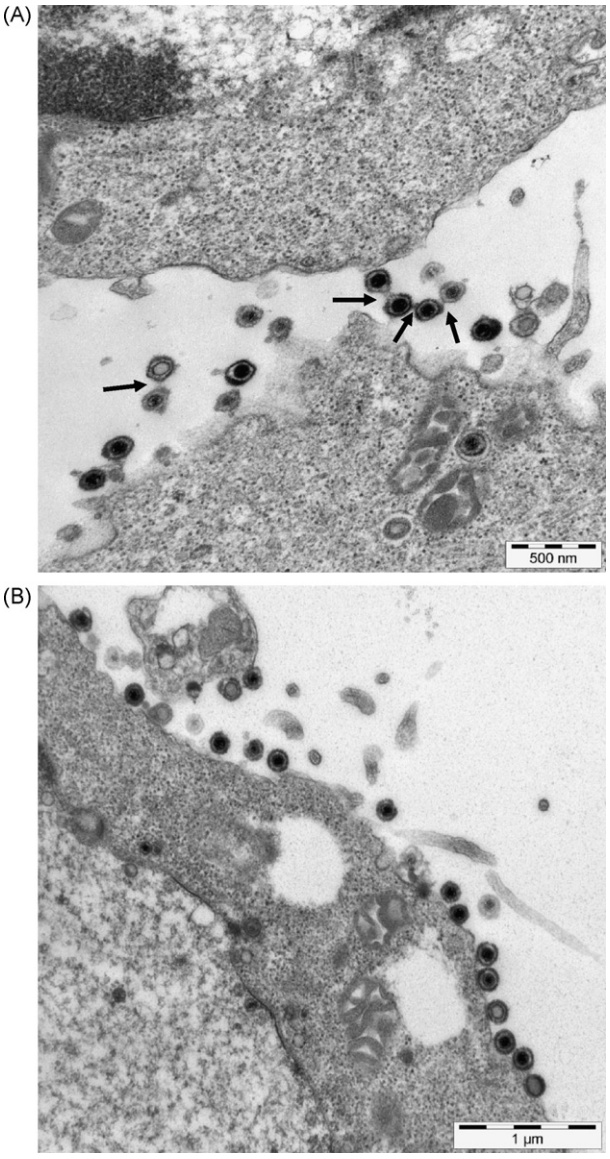


Fig. 5. Electron micrographs of the virus-infected GMK AH1 cells subjected to treatment with compound **14**. The cells were infected with HSV-2 strain 333 for 24 h, and then the virus particles on the cell surface treated for 30 min at 37 °C with compound **14** (50 μg/ml) (A) or mock-treated (B). The virion-to-virion linking is indicated by arrows. Two separate experiments were performed, and >20 images were captured for each group.

Table 4
The virus-inactivating activities of muparfostat and compound **14** in low pH media or in the presence of cervical secretions[a].

Virus	Compound concentration (μg/ml)	Compound			
		Muparfostat		**14**	
		Low pH[b]	CS[c]	Low pH[b]	CS[c]
HSV-2	100	111.5	90.3	0.0	0.3
	10	110.8	98.3	6.8	78.5
	1	101.1	88.4	93.7	82.6

[a] Approximately 2×10^5 PFU of respective virus were co-incubated with test compound or the diluent medium (control) for 15 min at 37 °C (water bath) prior to dilution of the mixtures 1:500 or 1:1000 and viral plaque titration. The results are expressed as a percentage of the number of viral plaques detected with the compound-treated virus relative to mock-treated controls.
[b] The pH value during the virus-compound incubation was 4.5.
[c] Cervical secretions diluted 1:2.2 was present during the 15 min period of the virus-compound incubation.

4. Discussion

One strategy to prevent acquisition of some viral infections in humans is topical application of virucidal compounds that can permanently inactivate viral particles. Sulfated polysaccharides as mimetics of cellular glycosaminoglycan receptors for a number of viruses including HIV, HSV and others (Vaheri, 1964; Witvrouw

and De Clercq, 1997), represent a group of viral inhibitors suitable for application as topical virucides. The vast majority of sulfated oligo/polysaccharides act by reversible binding to the viral attachment proteins, an interaction that blocks virus attachment to cells but causes no permanent inactivation of viral particles (Neyts and De Clercq, 1995). Thus, one cannot exclude that sulfated polysaccharides cellulose sulfate or carrageenan failed to protect women against HIV infection (Van der Wijgert and Shattock, 2007; Cohen, 2008) due to their poor virucidal activity manifested as incomplete inactivation of viral infectivity in virucidal assay (Cheshenko et al., 2004; Carlucci et al., 1999).

In this work attempts were made to prepare modified sulfated oligosaccharides with improved virucidal activity. We have found that compound **14**, a cholestanyl glycoside of the sulfated tetrasaccharide sequence found in muparfostat [Manα(1 → 3)-Manα(1 → 3)-Manα(1 → 2)-Man], selectively inactivated HSV particles. This compound exhibited activities characteristic for both specific heparan sulfate mimetics and virucides. In particular, **14** inhibited the virus binding to cells in a similar fashion to muparfostat or other sulfated oligo/polysaccharides, suggesting a similar mechanism of action, i.e., interference with the heparan sulfate-virus attachment protein interaction. However, unlike muparfostat and other sulfated oligo/polysaccharides, this compound also exhibited virus-inactivating properties, a feature typical for virucidal compounds.

Compound **14** is composed of sulfated oligosaccharide conjugated to a lipophilic component. Similar structural design has been exploited in preparation of the sulfated sialyl lipid NMSO3, a potent inhibitor of respiratory syncytial virus infectivity in cultured cells and in experimental animals (Kimura et al., 2000). It is likely that while the sulfated oligosaccharide chain of **14** can bind to and block the virus attachment proteins, the cholestanyl group may insert into and destabilize the viral lipid envelope, thus irreversibly inactivating the virus particle. The virucidal activity of **14** was slightly affected by a low pH buffer, and the presence of cervical secretions reduced this activity by approximately 10-fold. This observation is not surprising since it has been reported that the virucidal activity of anionic surfactants decrease in protein-rich solutions (Piret et al., 2002; Isaacs et al., 2004). Thus, one cannot exclude that anionic and lipophilic glycoside **14** may bind tightly to some cationic and hydrophobic components present in cervical secretions. Anyhow, these results indicate that higher concentration of **14** might be necessary to inactivate the viral particles in humans as compared to a laboratory assay. It should be emphasized that although **14** was capable of inactivating HSV virions at non-cytotoxic concentrations, induction of this activity was accompanied by an increase in its cytotoxicity. This suggests that **14** can adversely affect the cell, although at much higher concentrations than those required for virucidal activity. Indeed, the results of the time-of-addition experiment indicated that **14** can bind to the cell plasma membrane, even at non-cytotoxic concentrations, an event that resulted in substantial blockage of infection of cells by HSV-2. Our ongoing experiments suggest that the decrease of anti-HSV activity of compound **14** observed in the presence of cervical secretions is paralleled by a similar decrease in its cytotoxicity so the selective index remained unchanged. Furthermore, we are now testing a number of analogs of compound 14, the aim being to search for a compound with improved selectivity index and reduced neutralization by human cervical secretions.

Apart from virucidal activity, compound **14** and two other lipophilic analogues (**3** and **5**) interfered with the cell-to-cell transmission of the virus in the HSV-type specific manner where the cell-to-cell spread of HSV-2 was abrogated while that of HSV-1 was modestly reduced, similarly to the activity of muparfostat. This HSV-type specific difference was also found to occur in clinical isolates of HSV-1 and HSV-2. We reported previously (Nyberg et al., 2004) that the low molecular mass and extensively sulfated muparfostat oligosaccharides potently reduce the cell-to-cell spread activity of HSV. Herein, in addition to compound **14** we identified several lipophilic glycosides as much more efficient inhibitors than muparfostat of HSV-2 lateral spread. We have as yet no explanation of why HSV-2 lateral spread is more sensitive than that of HSV-1 to the glycosides tested.

Although both HSV-1 and HSV-2 variants resistant to muparfostat were easily selected and appeared to carry mutations abolishing expression of viral mucin-like proteins (Adamiak et al., 2007; Ekblad et al., 2007), we failed to isolate HSV-2 variants resistant to **14** and to another lipophilic compound **3**. Inability of **14** and **3** to generate variants of HSV-2 is likely to be due to their overwhelming selective pressure as compound **14** exhibited virucidal activity; it reduced HSV-2 attachment to cells, and abrogated the cell-to-cell spread of this virus. The latter two activities were also demonstrated by compound **3**. It is likely that these compounds targeted several different components of HSV-2, and simultaneous presence of the drug escape mutations in all these putative components was required for selection of resistant variants.

Another cause for the reported poor performance of sulfated polysaccharides in humans could be an unexpected interaction of these compounds with epithelial mucus. Dextran sulfate polysaccharide, a potent antiviral compound (Witvrouw and De Clercq, 1997) and a known inducer of experimental colitis in mice (Kim and Berstad, 1992) was recently found to produce holes in physiologically impermeable intestinal inner mucus layer, thus permitting free access of bacteria and other pathogens to susceptible cells (Johansson, 2009). These observations further emphasize the need for structural modifications of sulfated polysaccharides to induce/potentiate their virucidal properties or to alter their interactions with mucus.

In conclusion, we have identified the sulfated cholestanyl tetrasaccharide **14**, which in addition to greatly improved anti-HSV-2 activities compared with muparfostat, also exhibited the virus-inactivating activity and abrogated the virus spread from infected to normal cells. These features suggest that this compound has a potential to be used in humans both for prevention of acquisition of HSV-2 infection and for topical treatment of the virus-induced lesions.

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