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The low molecular weight heparan sulfate-mimetic, PI-88, inhibits cell-to-cell spread of herpes simplex virus

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Abstract

Although a number of sulfated polysaccharides have been shown to inhibit infection of cells by herpes simplex virus (HSV), little is known about their effects on the cell-to-cell spread of the virus. These compounds act by inhibiting the virus binding to cells, and their antiviral potencies usually increase with increasing molecular weight and sulfation density. We report that the low molecular weight HS-mimetic, PI-88, which is a mixture of highly sulfated mannose-containing di- to hexa-saccharides, inhibited HSV infection of cells and cell-to-cell spread of HSV-1 and HSV-2. Compared to a relatively large heparin polysaccharide, PI-88 demonstrated weaker inhibition of HSV infectivity but more efficient reduction of cell-to-cell spread of HSV. A tetrasaccharide fraction of PI-88 was the minimum fragment necessary to inhibit HSV-1 infectivity, while a trisaccharide was sufficient to reduce cell-to-cell spread. A reduction in HSV lateral spread was also observed in cells incubated with another low molecular weight compound, pentosan polysulfate but not with much larger polysaccharide chondroitin sulfate E. Some differences as regards the effects of PI-88, heparin, protamine, poly-L-lysine and sodium chlorate on intercellular spread of HSV-1 and HSV-2 were found. We conclude that structurally different sulfated oligosaccharides are preferred for inhibition of HSV infectivity and the cell-to-cell spread. The latter was efficiently inhibited by a relatively small but densely sulfated PI-88 oligosaccharide, very likely due to the capability of the compound to access the narrow intercellular space.

Keywords: Herpes simplex virus; Cell-to-cell spread; PI-88; Heparan sulfate

1. Introduction

Heparan sulfate (HS) chains provide binding sites for initial interactions with cells of many viruses including herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) (WuDunn and Spear, 1989). This property of the virus is manifested by both wild-type and laboratory strains (Terhune et al., 1998; Trybala et al., 2002), and in HSV-1 appears to be a complex process. In addition to the attachment step, virus entry into cells can also be promoted by HS (Shukla et al., 1999) or other receptor molecules such as TNF receptor-like protein or nectin (Montgomery et al., 1996; Geraghty et al., 1998). The HS-binding glycoproteins B and C (gB and gC) are the major components of the HSV envelope that mediate virus attachment to cells (Herold et al., 1991, 1994; Gerber et al., 1995), whereas glycoprotein D, which in the case of HSV-1 also interact with HS (Shukla et al., 1999), promotes, along with gB and gH-gL heterodimer, the viral entry step (Cai et al., 1987; Ligas and Johnson, 1988; Forrester et al., 1992). The same viral glycoproteins and the same cellular receptors that mediate HSV entry via the apical surface of the cell are thought to promote cell-to-cell spread, i.e., the virus movement across the narrow space between infected and adjacent uninfected cell (Cai et al., 1987; Ligas and Johnson, 1988; Forrester et al., 1992; Shieh and Spear, 1994; Cocchi et al., 2000; Roller and Rauch, 1998). In addition, the heterodimers of HSV glycoproteins E and I were shown to promote cell-to-cell spread by sorting nascent virions to epithelial cell junctions (Johnson et al., 2001). Efficient cell-to-cell spread of the virus is of vital importance for productive infection of humans, and is, therefore, an attractive target for antiviral intervention.

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Because the virus-HS interaction relies on multiple electrostatic associations between basic amino acid residues of the HS-binding site of viral gC/gB and negatively charged sulfate/carboxylate groups of the HS chain, a number of sulfated polysaccharides have been reported to interfere with HSV infection of cells (Vaheri, 1964). These include heparin (Vaheri, 1964; Grossman and Thonnard-Neumann, 1985) and several other HS-mimetics such as modified heparins (Herold et al., 1996), fucoidans (Lee et al., 2001; Ponce et al., 2003), carrageenans (Gonzalez et al., 1987; Zacharopoulos and Phillips, 1997), sulfated galactans (Di Caro et al., 1999; Duarte et al., 2001), dextran sulfate and pentosan polysulfate (Baba et al., 1988), xylogalactans (Damonte et al., 1996), mannan sulfate (Ito et al., 1989), calcium spirulan (Lee et al., 2001) and others. These compounds demonstrate the most pronounced activity against free virions when present during the phase of virus attachment to and infection of cells (for review, see Witvrouw and De Clercq, 1997). Furthermore, their antiviral potencies usually increase with increasing (i) degree of sulfation and (ii) size of oligosaccharide chain (Witvrouw and De Clercq, 1997). The former feature increases the likelihood of successful recognition of oligosaccharide by the HS-binding domain of a virus attachment protein, whereas the latter enables simultaneous interaction of oligosaccharide with numerous copies of the virus attachment protein and perhaps crosslinking the virions. In contrast, inhibition of HSV during its cell-to-cell spread requires compound penetration into a narrow intercellular space, hence, the size of the molecule might be a limiting factor.

The anti-cancer drug, PI-88, is a mixture of highly sulfated mannose containing oligosaccharides, the major components being a penta- and tetrasaccharide, although di-, tri- and hexasaccharides are also present (Ferro et al., 2002). PI-88 is prepared by hydrolysis of the extracellular phosphomannan polysaccharide of the yeast *Pichia holstii*, to yield a phosphorylated oligosaccharide fraction (PM_5), which is subsequently chemically sulfated (Parish et al., 1999; Ferro et al., 2001; Yu et al., 2002). The antitumor efficacy of PI-88 is based on its ability to inhibit tumor metastasis by inhibiting the degradation of HS chains in healthy tissues by tumor cell heparanase, and its ability to inhibit angiogenesis by interfering with the interaction between HS chains and angiogenic growth factors (Parish et al., 1999). PI-88 has successfully undergone Phase I and Ib trials in healthy volunteers and in cancer patients, with low toxicity being demonstrated. Several Phase II trials are currently in progress to establish the efficacy of PI-88 in patients with malignancies (Ferro and Don, 2003).

In this study, PI-88 and heparin were compared for their abilities to inhibit HSV infectivity and cell-to-cell spread of progeny virus. Heparin, whose oligosaccharide chain is on average six times larger than PI-88, was a better inhibitor of HSV infectivity than PI-88. In contrast, PI-88 more efficiently reduced cell-to-cell spread of the virus than heparin. These results indicate that low molecular weight HS-mimetics such as PI-88 might be preferred for an efficient inhibition of intercellular transmission of HSV.

2. Materials and methods

2.1. Cells, viruses and PI-88 agent

African green monkey kidney (GMK AH1) epithelial cells (Gunalp, 1965) were cultured in Eagle's minimum essential medium (EMEM) supplemented with 2% calf serum, 0.05% Primaton RT substance (Kraft Inc., Norwich, Conn. USA) and antibiotics. The HSV strains used were HSV-1 KOS321, a plaque purified isolate of wild-type strain KOS (Holland et al., 1984), HSV-1 KOS gC-null variant $gC^{-}39$ (Holland et al., 1983), HSV-1 gC-negative variant MP (Hoggan and Roizman, 1959), HSV-2 strain 333 (Duff and Rapp, 1971), and its gC-negative derivative designated HSV-2 gCneg1 (Trybala et al., 2000). The preparation and compositional analysis of PI-88 (Yu et al., 2002) and its completely non-sulfated precursor PM_5 (Ferro et al., 2001, 2002) were carried out as previously described. The preparation and characterization of dephosphorylated PI-88 (DPI-88) and the sulfated penta-, tetra- and trisaccharides (SM_5, SM_4 and SM_3) were carried out as previously described (Cochran et al., 2003). The structures of the oligosaccharides used in this study are given in Fig. 1. Chondroitin sulfate E from squid cartilage (Seikagaku, Japan) and pentosan polysulfate (Sigma, MO, USA) were also used.

2.2. Purification of viral particles and viral glycoproteins

GMK AH1 cells were infected with HSV at a multiplicity of 3–5, and then incubated in EMEM supplemented with 25 μCi/ml methyl-3*H*-thymidine (Amersham Pharmacia Biotech, Little Chalfont, UK; specific activity 25 Ci/mmol) for 40 h at 37 °C. The media were clarified by centrifugation at 1000 × *g* for 25 min, and then by centrifugation at 5000 × *g* for 7 min. Extracellular virus was pelleted from the resulting supernatant by centrifugation at 22,000 × *g* for 2 h. The pellet was covered with PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and left

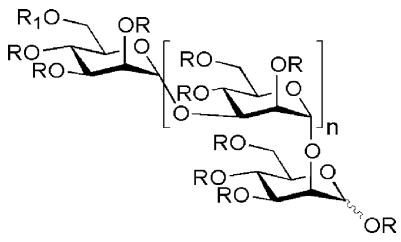

Fig. 1. The structure of PI-88 and other oligosaccharides used in this study. PI-88: R = SO_3Na or H, R_1 = PO_3Na_2, *n* = 0–4; PM_5: R = H, R_1 = PO_3Na_2, *n* = 0–4; DPI-88: R = R_1 = SO_3Na or H, *n* = 0–4; SM_5: R = R_1 = SO_3Na or H, *n* = 3; SM_4: R = R_1 = SO_3Na or H, *n* = 2; SM_3: R = R_1 = SO_3Na or H, *n* = 1.

overnight at 4 °C. The virus was purified from the pelleted material by centrifugation through a three-step discontinuous sucrose gradient (Karger and Mettenleiter, 1993). The viral glycoproteins gC and gB were purified from lysates of extracellular virus particles and virus-infected cells by immunoaffinity chromatography, as previously described (Trybala et al., 2000).

2.3. Viral plaque assays

GMK AH1 cells were seeded in 6-well cluster plates and confluent dense monolayers that developed after 3 days of culture were used. For plaque number-reduction assay, 1 ml volumes of serial dilutions of PI-88 or heparin in EMEM were mixed with approximately 200 PFU of HSV-1 or HSV-2 and incubated for 15 min at room temperature prior to the addition to cells. After incubation of the virus-PI88 mixture with cells for 1 h at 37 °C, the cells were washed with 2 ml of EMEM and overlaid with 3 ml of EMEM containing 1% methylcellulose, 2% fetal calf serum and antibiotics. The plaques were stained with crystal violet solution after 2 (HSV-2) or 3 (HSV-1) days of incubation at 37 °C. The concentration of PI-88 that inhibited the number of viral plaques by 50% (IC_{50}) was interpolated from the dose-response curves. For plaque size-reduction assay, approximately 200 PFU of HSV-1 or HSV-2 were added to and incubated with cells for 2 h at 37 °C. Subsequently, the cells were washed with 2 ml of EMEM and overlaid with 3 ml of EMEM containing 1% methylcellulose or 0.5% pooled human γ-globulin (Aventis Behring, Marburg, Germany), and one of the following compounds, i.e., 0.16–100 μg/ml PI-88, 0.16–100 μg/ml heparin, 50 μM poly-L-lysine (Sigma, St. Louis, MO), 50 μg/ml protamine (Pharmacia, Stockholm, Sweden) or 20 μM sodium chlorate (Sigma). After 2–3 days of incubation at 37 °C, the viral plaques were visualized by immunostaining with pooled human γ-globulin as primary and peroxidase-conjugated $F(ab')_2$ fragment goat anti-human IgG (Jackson, West Grove, PA) as secondary antibody. The images of 20 neighboring plaques per each compound tested were captured using a Leica DC 300 digital camera attached to a Leitz-Wetzlar Diavert microscope. The area of each plaque was determined by using IM 1000 image software (Leica).

2.4. Binding of purified virions and viral glycoproteins to cells

Confluent monolayers of GMK AH1 cells in 24 well plates were washed with PBS-A (PBS supplemented with 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$) and then pretreated with PBS-A containing 1% bovine serum albumin for 1 h at room temperature. Serial tenfold dilutions of PI-88 in PBS-A were mixed with purified ^{3}H-thymidine labeled HSV-1 or HSV-2 virions and incubated for 15 min at room temperature. The cells were washed once with PBS-A and 240 μl of the virus-PI88 mixture added and incubated with the cells under moderate agitation for 2 h at 4 °C. Subsequently, the cells were washed three times with PBS-A, lysed with 0.2 ml of PBS-A containing 5% SDS, and finally transferred to scintillation vials for quantification of radioactivity. The effect of PI-88 on the binding of viral gC and gB to GMK AH1 cells was tested as described previously (Lycke et al., 1991). Briefly, purified gC and gB were preincubated for 15 min at 4 °C with tenfold increasing concentrations of PI-88. Then, the mixtures were transferred to cells growing in 96-well plates, and left for attachment for 1 h at 4 °C. Bound glycoproteins were detected by an ELISA-based procedure.

2.5. Cytotoxicity assay

GMK AH1 cells that were seeded in 24-well cluster plates and had reached approximately 80–90% confluence at day 2 of culture were incubated for 24 h at 37 °C with 0.5 ml of serial twofold dilutions of PI-88 in EMEM. Subsequently, 2.5 μCi of methyl-^{3}H-thymidine were added and incubated with cells for 2 h at 37 °C. The cells were then washed three times with 1 ml of EMEM and lysed with 5% SDS solution for quantification of radioactivity.

3. Results

3.1. PI-88 reduces cell-to-cell spread of HSV

We sought to examine whether PI-88 would interfere with HSV infection of cells and subsequent cell-to-cell spread of progeny viruses. The structure of PI-88 is shown in Fig. 1. The major constituents of PI-88 are penta- and tetrasaccharides that represent approximately 60 and 30% of the mixture, respectively. The remaining components are di-, tri- and hexasaccharides (Ferro et al., 2002). The average degree of sulfation of each PI-88 monosaccharide unit ranges from 2.9 to 3.1, which is twice that of heparin. Thus, PI-88 has a significantly shorter chain length and a higher degree of sulfation than most species of HS.

Incubation of GMK AH1 cells for 24 h in the presence of PI-88 at concentrations of up to 1 mg/ml exerted no significant cytotoxicity as evaluated by a ^{3}H-thymidine incorporation assay (Fig. 2A) and microscopic observation of the cell morphology (data not shown). The effect of PI-88 on HSV infectivity was assayed by incubating the virus with the compound for 15 min before addition to the GMK AH1 cells and during the 1 h period of virus attachment to and infection of cells. The results expressed as a reduction in the number of viral plaques developed are shown in Fig. 2A and Table 1 (IC_{50} values). The effect of PI-88 on cell-to-cell spread of HSV was investigated by adding the compound to cells after their infection with HSV and leaving it on the monolayer of cells throughout the entire period of the development of viral plaques (Fig. 2B). To ensure that the virus spread occurred only via the cell-to-cell transmission, the overlay medium was supplemented with methylcellulose or pooled

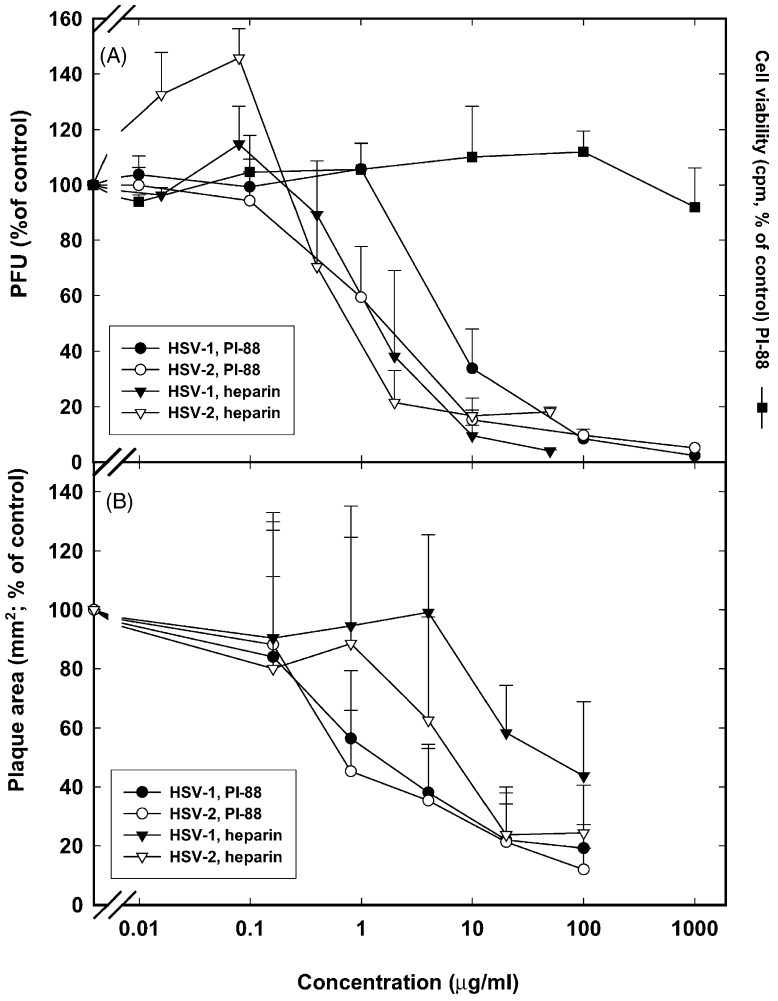

Fig. 2. Interference of PI-88 with HSV infectivity and cell-to-cell spread. Panel A: PI-88 or heparin at different concentrations were incubated with approximately 200 PFU of HSV-1 KOS321 or HSV-2 333 for 15 min prior to and during 1 h period of virus infection of GMK AH1 cells. The results are expressed as a percentage of the number of viral PFU found with drug-treated virions relative to mock-treated controls. Values shown are means of four determinations from two separate experiments. The square symbols show the effect of different concentrations of PI-88 on the viability of GMK AH1 cells as measured by incorporation of ^{3}H-thymidine by PI-88-treated cells relative to mock-treated controls. Panel B: The cells were infected with approximately 200 PFU of either HSV-1 KOS321 or HSV-2 333, and then overlaid with EMEM supplemented with 1% methylcellulose and different concentrations of PI-88 or heparin. 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 20 viral plaques per each dilution of the compounds were captured and subjected to area determinations using the IM1000 software. The average area of HSV-1 plaques in control wells was $0.21\,mm^2 \pm 0.05$ (PI-88 experiment) and $0.19\,mm^2 \pm 0.07$ (heparin experiment). The corresponding values for HSV-2 were $0.35\,mm^2 \pm 0.22$ and $0.28\,mm^2 \pm 0.14$, respectively.

Table 1
Effect of PI-88 on HSV infectivity[a] and size[b] of HSV plaques

Compound	Molecular weight (kDa; average)	Sulfate groups per saccharide (average)	Assay	IC_{50} (μg/ml)	
				HSV-1	HSV-2
PI-88	2.4	3	Infectivity	6	2
			Plaque size	2	0.7
Heparin	15	1.4	Infectivity	1	0.8
			Plaque size	50	7
Pentosan polysulfate	3	2	Plaque size	12	3
Chondroitin sulfate E	70	1	Plaque size	>100	>100

[a] Plaque number reduction assay.
[b] Plaque size reduction assay.

human γ-globulin. The results expressed as a reduction in the area of viral plaques formed are shown in Fig. 2B and Table 1 (IC_{50} values). Heparin appeared to a better inhibitor than PI-88 of HSV infectivity, whereas PI-88 more efficiently than heparin reduced the cell-to-cell spread of HSV. These data suggest that the low molecular weight and/or extensive sulfation might be important structural determinants of the compound ability to interfere with intercellular virus transmission. Therefore, another pair of structurally different compounds, i.e., a relatively small but extensively sulfated pentosan polysulfate and a much larger chondroitin sulfate E polysaccharide (Table 1) were compared for their effects on the virus spread. Pentosan polysulfate has been reported to be a good inhibitor of HSV infectivity (Baba et al., 1988), while chondroitin sulfate E potency upon HSV infection of cells seems to be one of the most pronounced among polysaccharide compounds (IC_{50} of 10–30 ng/ml in GMK AH1 cells; Bergstrom et al., unpublished data). Pentosan polysulfate but not chondroitin sulfate E reduced the area of HSV-1 and HSV-2 plaques when examined at a concentration range of 0.016–100 μg/ml (Table 1). In addition to GMK AH1 cells, PI-88 also exerted an inhibitory effect on the size of HSV-1 and HSV-2 plaques formed in human oral SVpgC2a keratinocytes, mink lung Mu1Lu cells, but not in mouse L cell fibroblasts (data not shown).

3.2. Structural features of PI-88 required for antiviral activity

The effect of fractionated PI-88 oligosaccharides on HSV-1 infectivity and cell-to-cell spread of the virus is shown in Fig. 3A–D, respectively. The inhibition of HSV infectivity by PI-88 was dependent upon the presence of anionic charge and the size of the oligosaccharide components of PI-88 (Fig. 3). Replacement of the 6-*O*-phosphate group in PI-88 by a sulfate group (DPI-88) did not alter the

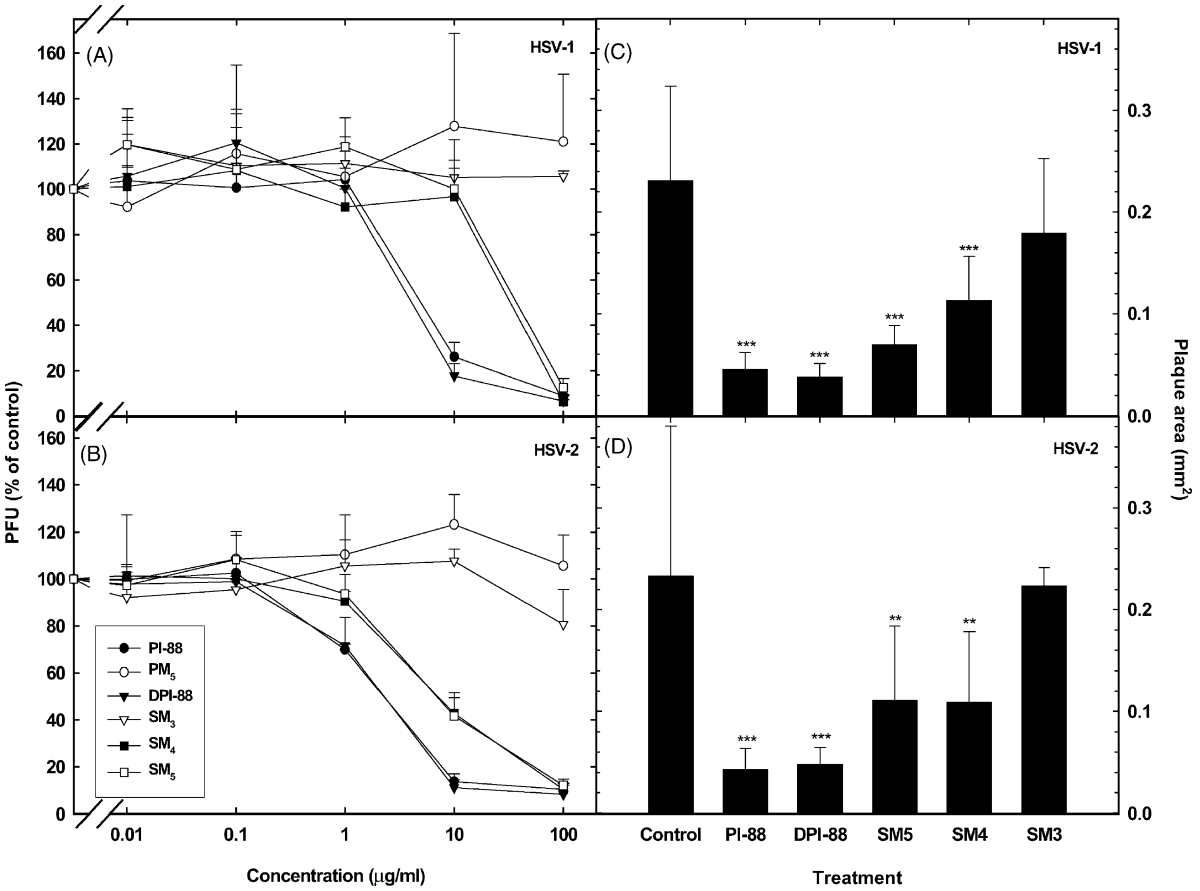

Fig. 3. Effect of PI-88, PM_5 and DPI-88 preparations and size-fractionated DPI-88 oligosaccharides on HSV infectivity in GMK AH1 cells. Different concentrations of native PI-88, its non-sulfated precursor (PM_5) and a non-phosphorylated preparation (DPI-88) as well as tri- (SM_3), tetra- (SM_4), and pentasaccharide (SM_5) fractions of DPI-88 were incubated with approximately 200 PFU of HSV-1 KOS321 (Panel A) or HSV-2 333 (Panel B) for 15 min prior to and during 1 h period of virus infection of GMK AH1 cells. The results are expressed as a percentage of the number of viral PFU formed in cells infected with the oligosaccharide-treated virus relative to mock-treated controls. Values shown are means of four determinations from two separate experiments. Panels C and D: PI-88 or derivative compounds (each at 100 μg/ml) were added to cells after their infection with the virus and incubated with cells throughout the entire period of the development of viral plaques. The results are expressed as a percentage of the average areas of 20 viral plaques developed in drug-treated cells relative to mock-treated controls. Statistically significant differences at P values of <0.01 (**) and <0.005 (***). Two separate experiments were carried out for each compound. For further explanations, see legend to Fig. 2B.

anti-HSV-1 (Fig. 3A) and anti-HSV-2 (Fig. 3B) potency of PI-88. However, PM_5, the non-sulfated precursor of PI-88, demonstrated no anti-HSV activity. Fig. 3A and B also depicts the anti-HSV activity of the individual oligosaccharide components of the DPI-88 mixture, which were fractionated according to their size. The penta- (SM_5) and tetrasacharide (SM_4) fractions of DPI-88 were the most active components, however, their antiviral (and especially their anti-HSV-1) activities were less than that of the native compound. These results indicate that the simultaneous presence of tetra-, penta- and perhaps hexasaccharide (a minor DPI-88 fraction not tested in this experiment) components might be required for expression of the full antiviral activity of DPI-88. This interpretation can be extended to PI-88, as the only structural difference between PI-88 and DPI-88 (sulfate-for-phosphate substitution) did not alter their anti-HSV activities (see above). In the cell-to-cell spread assay, the PI-88 precursor/derivative fractions were only tested at a concentration of 100 μg/ml. Most of the compounds that inhibited HSV-1 infectivity (Fig. 3A and B) also reduced cell-to-cell spread of HSV-1 (Fig. 3C) and HSV-2 (Fig. 3D). Note that the trisaccharide preparation at 100 μg/ml did not inhibit HSV-1 infectivity (Fig. 3A), but somewhat reduced ($P = 0.058$) the cell-to-cell spread of this virus (Fig. 3C).

3.3. Mechanism of antiviral activity of PI-88

The inhibitory effect of PI-88 upon HSV infectivity occurred when the compound was present during the phase of viral attachment to and subsequent infection of the cell (see Fig. 2A). In contrast, incubation of PI-88 with cells for 3 h either prior to, or after the virus adsorption step, had no effect on the number of HSV-1 and HSV-2 plaques formed (data not shown). The effect of PI-88 on the binding to cells of purified radiolabeled HSV-1 and HSV-2 particles or isolated HSV-1 attachment components gC and gB is shown in Fig. 4A and B, respectively. Presence of PI-88 during the attachment phase reduced the binding of both the virus particles and viral proteins to GMK AH1 cells. PI-88 also reduced the size of HSV gC-negative variants: HSV-1 gC$^-$39, HSV-1 MP, and HSV-2 gC-neg1 (data not shown) in which gB is thought to mediate attachment functions. These results suggest that PI-88 inhibits HSV infection of cells through interfering with the binding of the viral attachment glycoproteins gC and gB to the cells.

Several other compounds, which are known to inhibit HSV infectivity through interference with the HSV-HS interaction, were also tested for their effect on the cell-to-cell spread of HSV-1 (Fig. 5A) and HSV-2 (Fig. 5B). As already observed (see Fig. 2B), heparin (100 μg/ml) was a weaker inhibitor than PI-88 of HSV-1 cell-to-cell (Fig. 5A) spread. Note that although some plaque-size heterogeneity was observed with the HSV-2 333 strain, all of these plaque variants were affected by PI-88. Heparin, although being a poorer inhibitor of HSV-2 lateral spread than of HSV-2

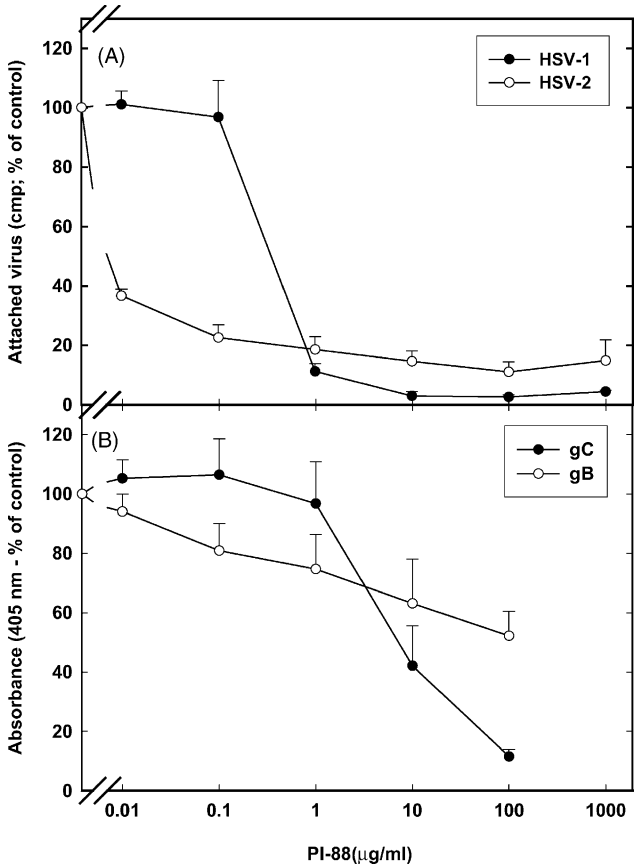

Fig. 4. Interference of PI-88 with the binding to cells of HSV-1 virions and HSV-1 glycoproteins. PI-88 at different concentrations was incubated with ^{3}H-thymidine labeled HSV-1 virions (Panel A) or with isolated HSV-1 glycoproteins gB and gC (Panel B) for 15 min prior to and during 1 h period of virus adsorption to GMK AH1 cells. The results are expressed as a percentage of attached viral cpm, or absorbance of attached viral glycoproteins found with PI-88-treated virions or proteins relative to mock-treated controls. Values shown are means of four determinations from two separate experiments.

infectivity (for IC_{50} values, see Table 1), at the concentration used in this experiment (100 μg/ml) reduced the size of HSV-2 plaques (Fig. 5B). Since the molecular weight of heparin chains ranges from 5 to 25 kDa in commercial preparations (Lindahl et al., 1994), it cannot be excluded that the low molecular weight fragments of heparin chain were responsible for the observed reduction in HSV spread. A reduced size of HSV-1 plaques was also observed in cells incubated in medium supplemented with polycationic compounds poly-L-lysine (50 μM) and protamine (50 μg/ml) (Fig. 5) although the inhibitory effects were less that those exerted by PI-88. In contrast protamine had only a marginal effect on the size of HSV-2 plaques but moderate reduction was found with poly-L-lysine. When used at relatively low concentrations both poly-L-lysine (5 μM) and protamine (5 μg/ml) had only a marginal effect on HSV-1 spread. In addition, sodium chlorate, an inhibitor of glycosaminoglycan sulfation, was added to GMK AH1 cells after their infection with HSV and left on monolayers of cells throughout the en-

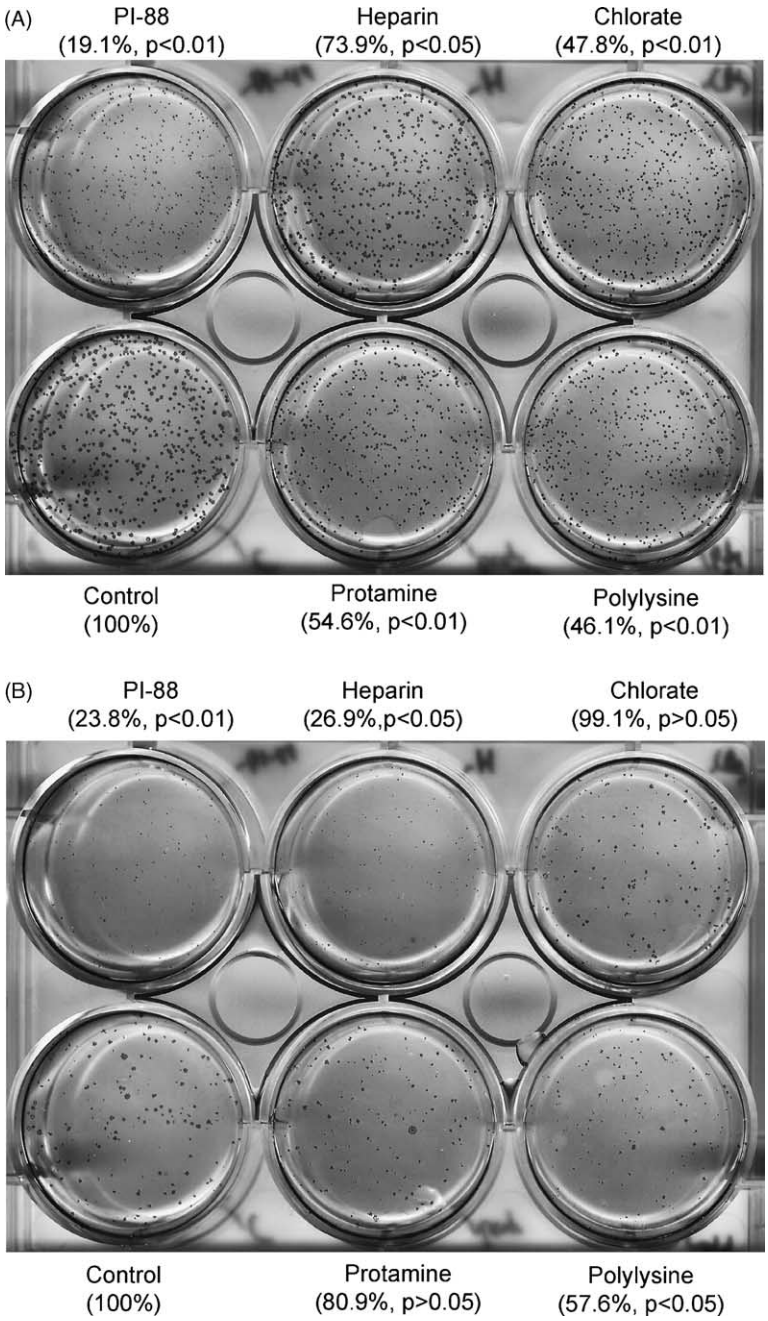

Fig. 5. Effects of PI-88, heparin, sodium chlorate, protamine or poly-L-lysine on the size of HSV plaques in GMK AH1 cells. The cells were infected with approximately 200 PFU of either HSV-1 KOS321 (Panel A), HSV-2 333 (Panel B), and then overlaid with EMEM supplemented with 1% methylcellulose and PI-88 (100 μg/ml), heparin (100 μg/ml), sodium chlorate (20 mM), protamine (50 μg/ml) or poly-L-lysine (50 μM). After incubation for 3 days at 37 °C, the viral plaques were visualized by immunostaining. In parentheses are the percentages of the average area of 20 plaques measured in drug-treated cells relative to mock-treated controls. Two to three separate experiments were carried out for each compound. For further explanations, see legend to Fig. 2B.

tire period of development of viral plaques. Sodium chlorate (20 mM) was added to complete, sulfate-containing EMEM, conditions which have been reported to inhibit 6-*O*-, and 2-*O*-sulfation of HS chains (Safaiyan et al., 1999). As seen in Fig. 5, HSV-1 but not HSV-2 formed smaller plaques in cells treated with sodium chlorate than in mock-treated control cells. These results indicate that the cell-to-cell spread of the virus could be targeted by some polycationic and polyanionic compounds, and that HSV-1 and HSV-2 differed in this regard.

4. Discussion

HSV infects the cells of stratified squamous epithelium that form epidermis and mucosa at different anatomical sites

including oral and genital regions. A rapid cell-to-cell spread of the virus is of vital importance in avoiding the developing immune response and thus in establishing productive primary or recurrent infections in humans. Our experiments in cultured cells revealed that the cell-to-cell spread of HSV can be targeted by PI-88 sulfated oligosaccharide. Although the inhibition of HSV infection of cells by sulfated polysaccharides is a long known phenomenon (Takemoto and Fabisch, 1964; Vaheri, 1964), little is known about the effect of these compounds on intercellular transmission of HSV. Aguilar et al. (1999) reported that the polysulfonated compound, suramin, interfered with the cell-to-cell spread of HSV-1. Because the same viral proteins and the same cellular receptors that promote virus attachment to and entry via the apical surface of cells are thought to mediate intercellular transmission of the virus (Cai et al., 1987; Ligas and Johnson, 1988; Forrester et al., 1992; Shieh and Spear, 1994; Cocchi et al., 2000; Roller and Rauch, 1998), sulfated polysaccharides should theoretically interfere with both these activities. However, our data demonstrate that the high molecular weight polysaccharide compounds, heparin (∼15 kDa) and chondroitin sulfate E (∼70 kDa), which are known to be potent inhibitors of HSV infectivity, reduced cell-to-cell spread of the virus inefficiently. In contrast, the low molecular weight compounds, PI-88 (∼2.4 kDa) and pentosan polysulfate (∼3 kDa) demonstrated substantial reduction of intercellular transmission of HSV. Thus, it is likely that the high molecular weight of the sulfated polysaccharide, which is a required feature for efficient inhibition of viral infectivity, might limit accession of a compound to the narrow intercellular space, and thereby limit its interference with the cell-to-cell transmission of the virus. However, one cannot exclude that in addition to the molecular weight, the extensive sulfation of PI-88 and pentosan polysulfate oligosaccharides might contribute to their anti-HSV spread activities. The low molecular weight (3.4 kDa) and densely sulfated dextran sulfate derivative was reported to inhibit cytopathic effect of several viruses including HSV (for review, see Witvrouw and De Clercq, 1997). Carlucci et al. (1997) reported that the relatively small but highly sulfated seaweed galactan (∼2.8 kDa) inhibited infection of cells by HSV-1 and HSV-2. It would be of interest to determine a possible contribution of anti-spread effects of these compounds to the inhibition of viral cytopathicity or their ability to reduce the size of viral plaques.

The mechanism of inhibition of HSV infectivity by PI-88, i.e., its interference with the binding of viral attachment proteins gC and gB to cell surface glycosaminoglycans was similar to that reported earlier for heparin (WuDunn and Spear, 1989; Svennerholm et al., 1991; Trybala et al., 2002) and other sulfated polysaccharides (Witvrouw and De Clercq, 1997). It is likely that the inhibition of cell-to-cell spread of HSV by PI-88 is based on a similar mechanism. This assumption stems from our data that several other putative inhibitors of virus-HS interaction or inhibitors of HS chain O-sulfation such as protamine, poly-L-lysine (Vaheri, 1964; Langeland et al., 1987) or sodium chlorate (Safaiyan et al., 1999), reduced, like PI-88, intercellular spread of HSV. Nonetheless, differential sensitivities of HSV-1 spread and infectivity of this virus to the trisaccharide fraction of PI-88 suggested that the virus-HS interaction might be somehow different in these two phenomena.

Certain structural features of sulfated polysaccharide such as the high molecular weight, hydrophilicity and negative charge load are known to adversely affect stability, bioavailability, and penetration of these compounds into the solid tissues (e.g., Lorentsen et al., 1989; Artmann et al., 1990). Consequently, a potential antimicrobial application of sulfated polysaccharides has so far been limited to topical prevention of the host-to-host expansion of certain sexually transmitted agents (Neyts and De Clercq, 1995; Zacharopoulos and Phillips, 1997). Our observation that PI-88 reduced HSV spread also in monolayer cultures of human oral keratinocytes, i.e., in cells relevant for an in vivo HSV-1 infection, could extend potential application of this class of sulfated carbohydrate compounds to topical treatment of HSV lesions. In this respect, it is noteworthy that penetration of topically applied heparin into human epidermis was dependent upon the molecular size of the preparation used. The low molecular weight fraction demonstrated greater penetration into the epidermal tissue than native heparin (Betz et al., 2001). In the light of these data it would be of interest to determine whether relatively small but densely sulfated oligosaccharides such as PI-88 could penetrate into the deep layers of human mucosal or epidermal membranes, i.e., into the sites of preferential infection with HSV.

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