Anti-herpes simplex virus activities of sulfomannan oligosaccharide PI-88 and disulfated cyclitols

Maria Ekblad

Department of Clinical Virology
Göteborg University
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Abstract

Herpes simplex virus (HSV) initiates invasion of human cells by binding to the cell surface heparan sulfate (HS) glycosaminoglycan chains. This step is mediated by the viral envelope glycoproteins gC and/or gB. Sulfated polysaccharides are compounds that mimic the structure of HS chains, and therefore are capable of inhibiting HSV attachment to and subsequent infection of cells. However the high molecular weight and associated with it poor tissue-penetrating activity have limited potential antiviral application of sulfated polysaccharides in humans. Here we found that the HS mimetic PI-88, a sulfomannan oligosaccharide of low molecular weight, efficiently reduced, in contrast to conventional sulfated polysaccharides, the cell-to-cell spread of HSV. Analogues of PI-88 with chemical modifications based on the introduction of specific hydrophobic/aromatic group(s) at the reducing end of PI-88 oligosaccharide chain showed enhanced capability to inhibit infection of cells and the cell-to-cell transmission of HSV and respiratory syncytial virus (RSV). One of these analogues (denoted 536), prepared by modification of PI-88 with cholestanol group, exhibited in contrast to the parental compound an HSV-inactivating activity. Furthermore, several disulfated cyclitols, identified by screening for an anti-HSV activity of a large number of low molecular weight sulfated compounds, efficiently reduced the cell-to-cell spread of HSV and demonstrated an HSV-inactivating activity.

The second aim of this thesis was to elucidate the molecular basis for viral resistance to PI-88. Variants of HSV type 1 (HSV-1) and type 2 (HSV-2), selected for by virus propagation in cultured cells in the presence of PI-88 were analysed. Many of these variants had a low infectious titer, indicative of a profound impairment in biological activities of the virus in response to continuous pressure from the drug. These variants were substantially resistant to PI-88 presence during their initial infection of cells and/or their cell-to-cell spread. Nucleotide sequence analysis revealed that PI-88 targeted predominantly the viral envelope glycoproteins that comprise mucin-like region(s), i.e., glycoprotein gC of HSV-1 and glycoprotein gG of HSV-2. The deletion of the mucin-like region of HSV-1 gC (amino acids 33-116) or the deletion of whole HSV-2 gG provided the virus with selective advantage to attach to and to infect cells in the presence of PI-88.

In conclusion, we have identified several novel antiviral compounds. One of these compounds, the PI-88 analogue 536, seems to be an attractive candidate for the development of a topical virucide for prevention of genital HSV infections in humans. We have also identified a novel biological function of HSV-2 gG, i.e., its targeting by sulfated oligosaccharides, which suggests involvement of this protein in HSV-2 attachment to cells or in modulation of this step.

Keywords: herpes simplex virus, antiviral drugs, viral glycoproteins, heparan sulfate mimetic, PI-88

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List of papers

This thesis is based on the following papers:

I. **Nyberg K, Ekblad M, Bergström T, Freeman C, Parish CR, Ferro V, Trybala E.** The low molecular weight heparan sulfate mimetic, PI-88, inhibits cell-to-cell spread of herpes simplex virus. Antiviral Research, 2004, 63:15-24.

II. **Ekblad M, Adamiak B, Bergefall K, Nenonen H, Roth A, Bergström T, Ferro V, Trybala E.** Molecular basis for resistance of herpes simplex virus type 1 mutants to the sulfated oligosaccharide inhibitor PI-88. Virology, in revision.

III. **Adamiak B, Ekblad M, Bergström T, Ferro V, Trybala E.** Analysis of herpes simplex virus type 2 variants resistant to the sulfated oligosaccharide inhibitor PI-88 identifies viral glycoprotein G as the major target of drug activity. In manuscript.

IV. **Ekblad M, Bergström T, Banwell MG, Bonnet M, Renner J, Ferro V, Trybala E.** Anti-herpes simplex virus activities of two novel disulphated cyclitols. Antiviral Chemistry and Chemotherapy, 2006, 17:97-106.

V. **Ekblad M, Andrighetti-Fröhner CR, Bergström T, Banwell MG, Renner J, Kreipl A, Ferro V, Trybala E.** Analogues of sulfated oligosaccharide PI-88 and disulfated cyclitol DSC3 exhibit potent anti-herpes simplex virus and anti-respiratory syncytial virus activities. In manuscript.

Contents

Abbreviations

a.a.	Amino acid
CMV	Cytomegalovirus
CPE	Cytopathic effect
CS	Chondroitin sulfate
DSC	Disulfated cyclitol
EBV	Epstein-Barr virus
EMEM	Eagle´s minimum essential medium
gC, gB, gD, gG	Glycoprotein C, B, D, G
GlcA	Glucuronic acid
GlcNAc	*N*-acetylated glucosamine
GlcNS	*N*-sulfated glucosamine
GMK AH1	African green monkey kidney AH1 cells
HIV	Human immunodeficiency virus
HS	Heparan sulfate
HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2
HVEM	Herpes virus entry mediator
IdoA	Iduronic acid
mgG-2	Mature part of gG-2
PFU	Plaque forming unit
RSV	Respiratory syncytial virus
RT	Room temperature
sgG-2	Secreted part of gG-2
SLS	Sodium lauryl sulfate
TK	Thymidine kinase
UL31	Unique long region 31
VZV	Varicella zoster virus

Introduction

Herpes simplex virus infections

Herpes simplex virus (HSV) belongs to the *Herpesviridae* family which is a group of large DNA viruses divided into three subfamilies of alpha-, beta- and gammaherpesvirinae. The human alphaherpesviruses are HSV type 1 (HSV-1), HSV type 2 (HSV-2) and varicella zoster virus (VZV); betaherpesviruses include among other members human cytomegalovirus (CMV) and human herpesviruses 6 and 7, while typical gammaherpesviruses are Epstein-Barr virus (EBV) and human herpesvirus 8.

HSV infects the cells of stratified epithelium forming skin and mucosa most commonly in oral and genital regions. Viral infection may lead to development of lesions in the form of cold sores and genital ulcers. Following infection of keratinocytes, HSV infects the sensory nerve endings and through the retrograde axonal transport reaches the local ganglia where it is protected from the host immune response thus causing a life-long latent infection. The neuron-hidden virus can periodically reactivate and cause ulcers at the site of primary infection. The stimuli that trigger reactivation of the virus include mechanical injuries, UV light exposure, stress, hormonal influences, certain microbial infections and others. Although HSV-1 is predominantly associated with oral while HSV-2 with genital infection, both subtypes can infect cells at either site, even though reactivation of HSV-1 is more frequent in the oral whereas HSV-2 in the genital area (Lafferty et al., 1987). HSV can also infect the epithelial cells of the eye causing keratitis (HSV-1) or enter the CNS to cause encephalitis (HSV-1) or meningitis (HSV-2). HSV-1 is the most common cause of viral encephalitis in a non-epidemic setting with estimated frequency of 1 per 250000-500000 annually. Without antiviral treatment the mortality rate in HSV-1 encephalitis is over 70% (Whitley and Gnann, 2002). In immunocompromised individuals, the virus reactivations are usually more frequent and symptomatic. The HSV disease in these subjects usually has a chronic character with severe ulcers also occurring at sites other than oral and genital regions. HSV can also cause a disseminated disease in immunocompromised patients and neonates, with several organs affected including liver, lungs and brain. This form of disease is associated with high mortality/morbidity of affected individuals (Levin et al., 2004; Sacks et al., 2004).

HSV is transmitted via close contact. HSV-1 is often acquired during childhood while HSV-2 is mainly a sexually transmitted pathogen. Both HSV types can also be transmitted vertically *in utero* or during the delivery, with the greatest risk in women suffering from a primary infection. In Sweden, the occurrence of neonatal HSV disease is approximately 1 per 15000 live births (Sacks et al., 2004).

HSV infections are common in the human population. Serological surveys indicate that the prevalence of HSV infection significantly may vary in different social and ethnic groups. In Sweden, approximately 70% and 25% of the adult population are infected with HSV-1 and HSV-2, respectively (Tunbäck 2004). In children, the seroprevalence of HSV infections was approximately 24% in 1-4 years old individuals and 37% in 15-19 years old teenagers (Tunbäck et al., 2003). Interestingly, only a minor part of HSV infected individuals suffer from recurrent outbreaks of the virus, as approximately 9-25% of HSV-2 seropositive subjects have had a symptomatic infection. HSV can cause recurrent lesions even in persons with high antibody titers (Corey and Spear, 1986). Asymptomatic shedding of HSV may occur frequently, and seems to be a major source of spreading the disease (Koelle and Wald, 2000).

Furthermore, genital HSV-2 infection was reported to be associated with an increased risk of acquiring HIV infection (Corey et al., 2004).

Herpes simplex virus particle

The HSV particle (Fig. 1) is approximately 150 nm in diameter. It consists of a double stranded linear DNA that theoretically can code for approximately 80 proteins. The viral DNA is enclosed in an icosahedral capsid composed of 162 capsomers. Outside of the capsid is the tegument, a protein layer which in turn is surrounded by a lipid envelope with glycoprotein spikes. HSV encodes at least 11 different kinds of glycoproteins which mediate various steps in the virus entry into and egress from the cells as well as protect the virus particle by evading immune response of the host. A list of HSV-encoded glycoproteins and their functions is shown in table 1.

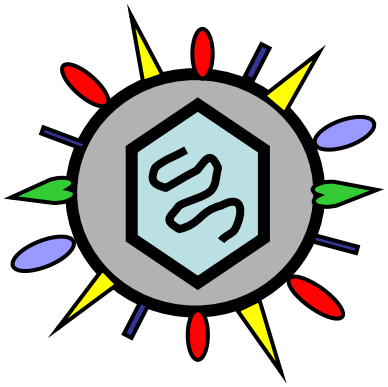

Fig. 1. The schematic structure of the HSV particle

Table 1. HSV glycoproteins and their biological functions

Glycoprotein	**Function**	**Essential for growth in cell culture**
gB	Cell attachment/entry/fusion	yes
gC	Cell attachment/immune evasion	no
gD	Triggering cell entry of the virus	yes
gE	Cell-to-cell spread/immune evasion	no
gG	Cell entry through the apical surface (HSV-1)	no
gH	Cell entry/fusion	yes
gI	Cell-to-cell spread	no
gJ	Infected cell apoptosis inhibitor	no
gK	Cell egress	no
gL	Cell entry/fusion	yes
gM	Virus particle assembly	no

Herpes simplex virus infection of cells

HSV infection of cell is a multistep phenomenon initiated by the attachment of the virion to the cell surface, where viral glycoproteins gC and/or gB mediate virus binding to heparan sulfate (HS) or chondroitin sulfate (CS) proteoglycans on the cell surface (Fig. 2). After this

initial event, another viral glycoprotein gD binds to one of the following receptors: herpes virus entry mediator (HVEM; both HSV subtypes), nectin-1 (both subtypes), nectin-2 (HSV-2) or 3-O-sulfated HS (HSV-1). This interaction possibly triggers conformational changes in viral gB and gH/gL components thus initializing fusion between the viral lipid envelope and cell plasma membrane with the insertion of viral nucleocapsid into the cytoplasm as a consequence (reviewed by Spear, 2004). Alternative pathways of HSV entry into the cell can occur in some cell types where endocytosed HSV fuses with the endosomal membrane instead of the cell plasma membrane (Nicola et al., 2003). The released nucleocapsid is transported to the nucleus where replication and transcription of viral DNA takes place.

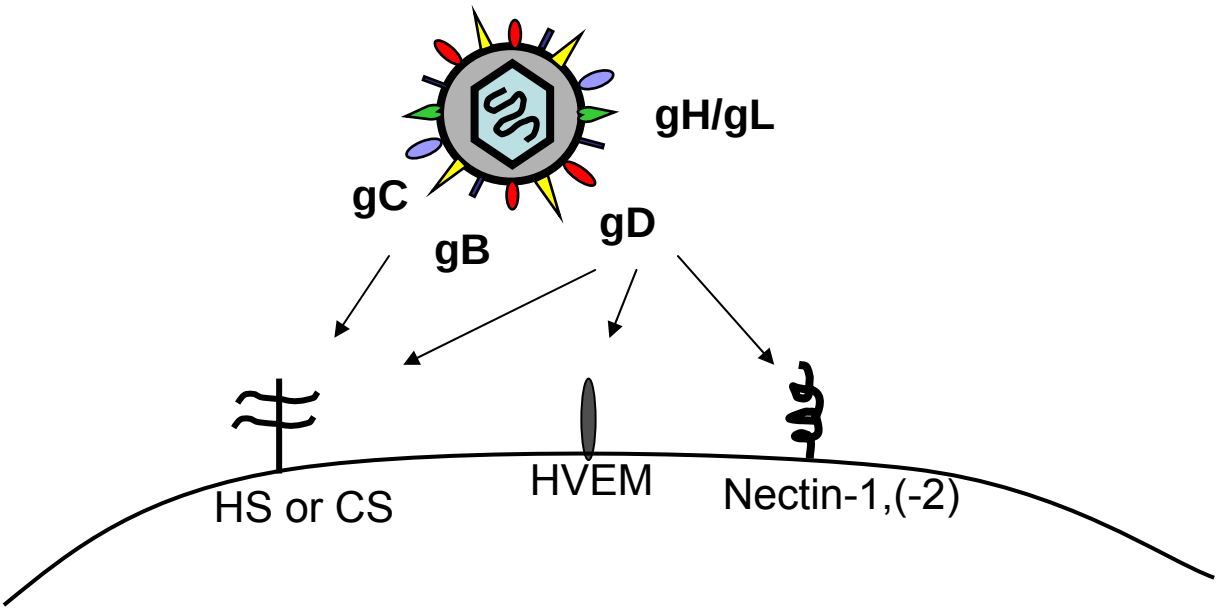

Fig. 2. Attachment of HSV to cell surface molecules includes initial binding (gC-mediated) and stable binding (gD-mediated)

The earliest viral proteins to be expressed in a cell are immediate early gene products including DNA binding proteins that promote DNA synthesis and transcription of early viral genes. Early gene products are mostly enzymes including DNA-dependent DNA polymerase and enzymes that induce cell cycle arrest (ICP0 and ICP27) thus inhibiting synthesis of cellular DNA. These inhibitory effects are amplified by activity of the virion host shut off proteins which inhibit synthesis of cellular proteins by disrupting the polysomes and degrading cellular mRNA. Other enzymes encoded by the viral genome are deoxyribonuclease, thymidine kinase, ribonucleotide reductase and protease. Transcription of late genes, which code for structural proteins of the virus particle, is triggered by genome replication.

The newly formed viral DNA in the nucleus is encapsulated in a protein shell forming the nucleocapsid. The nucleocapsid buds through the nuclear membrane with the primary envelopment at the inner nuclear membrane and its deenvelopment at the outer nuclear membrane. The UL31 and UL34 proteins and possibly UL11 component of HSV are involved in this process. However none of the HSV glycoproteins essential for the virus-cell fusion activity, i.e. gB, gD, gH/gL, are required for the capsid deenvelopment at the outer nuclear membrane. Instead contribution to this event of gK, UL20 and UL48 has been suggested (Mettenleiter 2002; Skepper et al., 2001). The naked nucleocapsid released into the cytoplasm is covered with the tegument proteins UL36 and UL37 via their binding to the major capsid protein UL19 (VP5). This tegument coated capsid interacts with the cytosolic domains of the

viral glycoproteins which have been processed and assembled in the trans golgi network (TGN). In particular, the cytosolic domain of gE and in some cases gM and other glycoproteins interact with UL11 and the UL49 tegument protein (Farnsworth et al., 2003; 2007; Mettenleiter, 2002). Following this event, the nucleocapsids buds into TGN vesicle thus receiving its final lipid envelope with fully processed glycoproteins. Finally the virus particle is released from the cell by fusion between TGN/endosomal vesicle membrane and the cell plasma membrane. The cytoplasmic domain of gE is directing the assembly of virions into compartments of the TGN/endosomes that are delivered to lateral cell junctions by interacting with Ap-1 clathrin adapters. Owing to this, the virus takes advantage of the cellular sorting machinery to deliver certain viral proteins to such specific sites in the plasma membrane. In these sites, the viral receptors such as nectin are concentrated and the virus neutralizing antibodies are absent, so the virions can spread rapidly from an infected to the adherent non-infected cell (Johnson et al., 2001; Johnson and Huber 2002).

HSV can productively infect a variety of different cells provided that the viral entry receptors are expressed. For example, the African green monkey kidney (GMK AH1) cells, which are extensively used in our laboratory, are sensitive to HSV and are known to produce, upon infection, a large amount of virus progeny. The typical form of HSV-induced cytopathic effect in cultured cells including GMK AH1 cells is roundening and detachment of infected cells, a change known as island or plaque formation in the cell monolayer (Fig. 3A). Some HSV-induced plaques may have another appearance, a syncytial phenotype, where several infected cells fuse together to form a multinucleated giant cell (Fig. 3B). Mutations in certain viral glycoproteins are known to induce this type of cytopathic effect (see below). The cell death is the usual fate of HSV-infected cells. However certain cell types can survive HSV invasion exemplified by neurons where the virus infection has a latent character and the only viral components detected are the latency-associated transcripts.

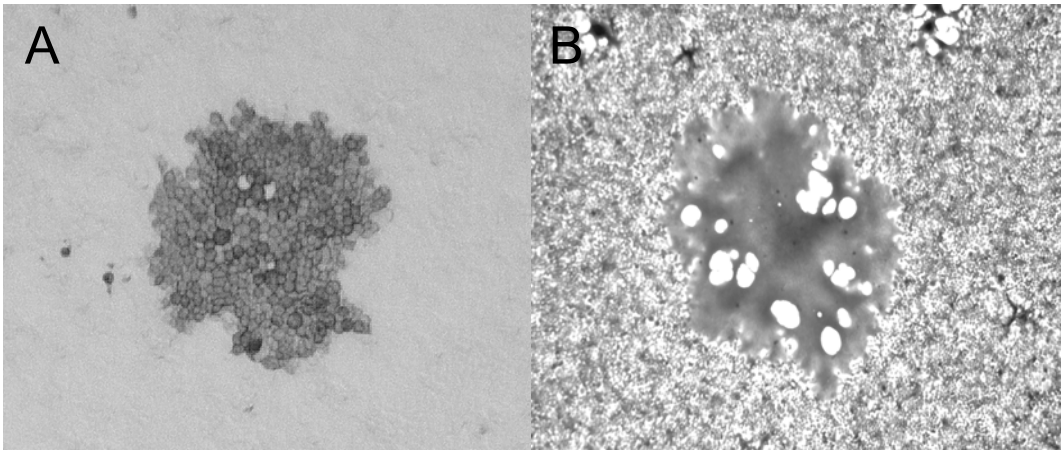

Fig. 3. HSV plaque morphology A: "normal" B: syncytial

Viral glycoproteins

Some of the HSV glycoproteins that are associated with the antiviral activity of compounds investigated in this thesis are described in more details.

Glycoprotein B

HSV gB (designated gB1 and gB2 for HSV-1 and HSV-2 respectively) is required for the virus entry into the cell, an event that relies on the fusion between the lipids of the virion envelope and the cell plasma membrane. gB binds to cell surface HS receptor thus mediating HSV attachment to cells in the absence of gC1. However, the protein is not essential for the virus adsorption to cells in the presence of gC1 (Herold et al., 1994; Cai et al., 1988). gB is thought to be the major attachment protein of HSV-2 (Herold et al., 1996; Cheshenko and Herold 2002). It is possible that gB may bind to another molecule on the cell surface, since soluble gB1 was able to block the virus entry into cells lacking HS or CS (Bender et al., 2005). This hypothetical receptor can be associated with the lipid rafts moiety of the cell membrane (Bender et al., 2003). gB1 consists of 904 a.a., and approximately 85% of the sequence is homologous to its HSV-2 counterpart. Most of the variability between gB1 and gB2 is seen in a lysine rich region (a.a. 68-76), which is responsible for binding to HS, but not essential for the fusogenic activity of gB (Laquerre et al., 1998; Shukla and Spear 2001). The cytoplasmic domain of gB comprising of 109 a.a., is longer than those of other HSV glycoproteins. Mutations in the cytoplasmic domain of gB are known to induce a syncytial phenotype of the viral plaques (Cai et al., 1988). Deletions of parts of the cytoplasmic tail of gB either increased the cell fusing activity (≤41 a.a.) or eliminated it (≥48 a.a.) depending on the number of a.a. deleted. Moreover, certain mutations in the gB2 cytoplasmic tail increase or decrease the fusion activity. Exactly how mutations in the cytoplasmic tail of gB trigger the virus fusogenic activity is not known. Certain alterations may lead to an increased cell surface expression of gB2, which might explain the increased fusogenic activity (Beitia Ortiz de Zarate et al., 2004; Fan et al., 2002; Ruel et al., 2006). It is likely that the cytoplasmic tail of gB2 may contain a domain that is inhibitory to the cell-cell fusion activity (Zago and Spear, 2003), or that mutations in the cytoplasmic tail may change the conformation of distal portions of the protein.

The crystal structure of gB1 was revealed by Heldwein et al. (2006) (Fig. 4). It occurs as a trimer with each of the monomers divided in five distinctive domains: I base, II middle, III core, IV crown, and V arm. Domain I exhibited structural similarity to the pleckstrinhomology (PH) domains found in certain phospholipid-binding cytoplasmic proteins. Specific hydrophobic/aromatic a.a. from this domain were found to be important for fusogenic activity of gB suggesting that this protein is the potential fusogen (Hannah et al., 2007; Heldwein et al. 2006).

Glycoprotein C

gC1 is a principal attachment protein of HSV-1 that mediates the virus binding to cell surface HS (Herold et al., 1991; Trybala et al., 1993) or CS (Banfield et al., 1995; Mårdberg et al., 2002). gC2 also binds to HS (Trybala et al., 2000) but is not regarded as essential for HSV-2 attachment to cells (Gerber et al., 1995; Herold et al., 1996). Although gC is not essential for HSV growth in cell culture, clinical isolates of HSV are almost always gC-proficient (Liljeqvist et al., 1999). gC is a type 1 membrane glycoprotein that contains 511 (gC1) or 480 (gC2) a.a. and exhibits 65% identity in primary a.a. sequence. gC1 has nine sites for N-linked oligosaccharides and numerous sites for O-linked glycans. The latter are clustered at the N terminal part of the protein, which makes this region structurally similar to mucins. The mucin-like region is not present in gC2 (Rux et al., 1996). The eight cysteins in gC1 form 4 disulfide bonds (Rux et al., 1996). Clusters of basic and hydrophobic a.a. located between residues 129 and 160 of gC1 (Mårdberg et al., 2001; 2002; Trybala et al., 1994) as well as the

mucin-like region of this protein (a.a. 33-123) (Tal-Singer et al., 1995) were identified as important for HSV-1 attachment to cell surface HS/CS. By binding to the third component of complement (C3b), gC also acts in the virus evasion of immune response of the host since the gC-C3b interaction inhibit further activation of the complement cascade (Friedman et al., 1996; Fries et al., 1986).

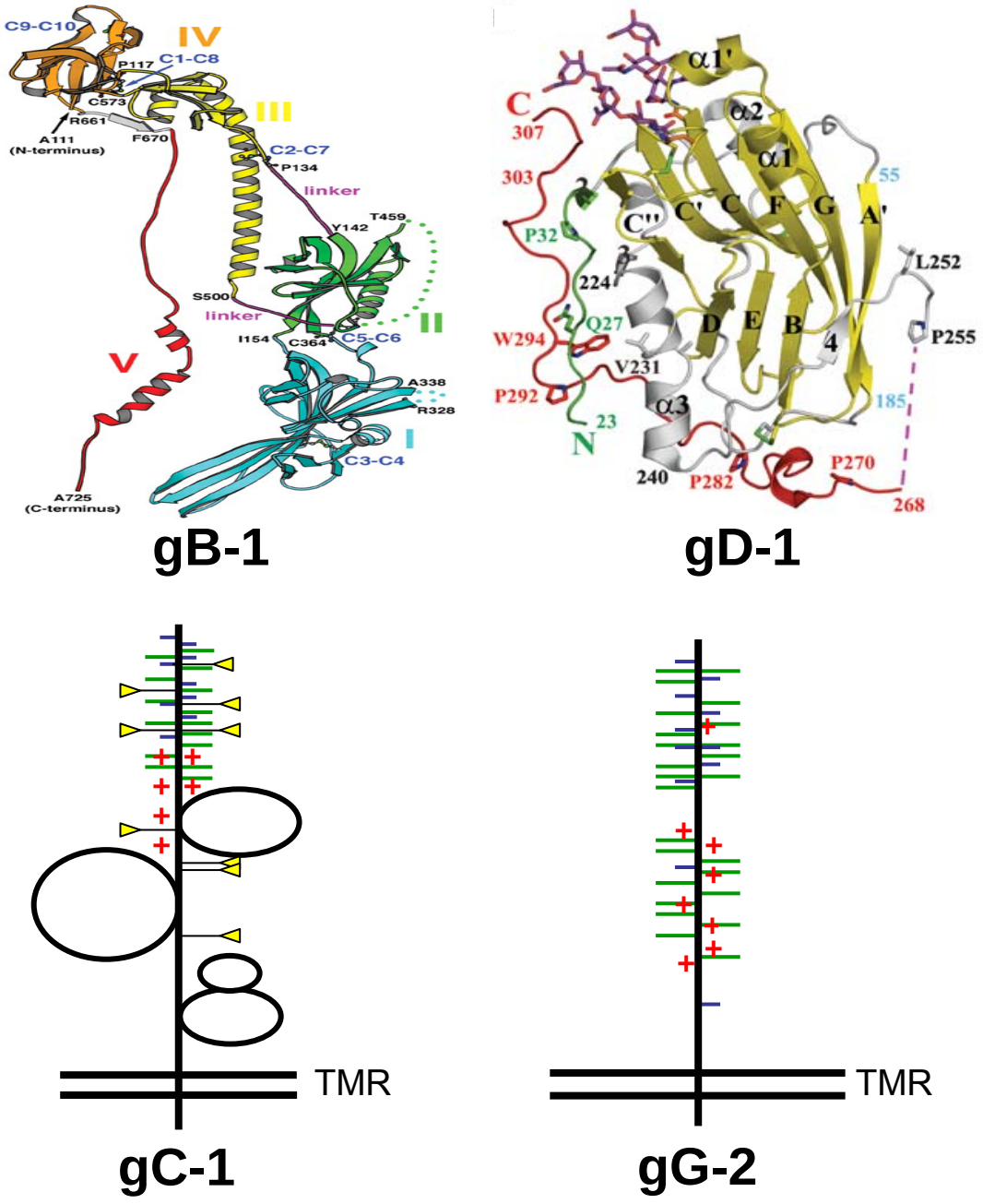

Fig. 4. Crystal structures of gB1 (Heldwein et al., 2006) and gD1 (Krummenacher et al., 2005) (reproduced with permission from AAAS and Macmillan Publishers Ltd: EMBO Journal), and cartoon structures of gC1 and mgG2, TMR = transmembrane region; the O-linked and the N-linked oligosaccharides are in green and yellow respectively. The blue/red symbols denote charged a.a. in the protein backbone.

Glycoprotein D

gD1 consists of 369 a.a. residues and contains three N-linked oligosaccharides and O-linked glycans. gD2 contains one a.a. less than gD1 (Spear et al., 2006; Watson et al., 1982). gD is essential to trigger HSV entry into the cells. It interacts with three different cellular receptors: herpesvirus entry mediator (HVEM) a member of the tumour necrosis factor receptor superfamily (Montgomery et al., 1996; Whitbeck et al., 1997), nectin-1and -2, cell adhesion molecules localized to the cadherin-based adherens junctions related to the poliovirus receptor CD155 (Geraghty et al., 1998; Mendelsohn et al., 1989; Warner et al., 1998), and specific 3-O-sulfotransferase II-IV-modified HS that shows affinity for gD1 only (Shukla et al., 1999).

The crystal structure of gD has been determined by Carfi et al. (2001) and Krummenacher et al. (2005) (Fig. 4). It appears that gD has a central Ig-like domain with the C-terminus wrapping around the core of the protein and anchoring near the N-terminus. This locked conformation of gD, made by close positioning of N-terminal residues 25-27 and 289-306 at the C-terminal segment is believed to be opened/activated by HVEM or nectin-1 binding. The activated gD may interact with other glycoprotein such as gB and gH/gL to trigger membrane fusion (Krummenacher et al., 2005, Spear et al., 2000, 2006). The distal portion of the N-terminus forms a hairpin structure which binds to HVEM and 3-O-sulfated HS. Deletion of residues 7-32 abolished binding of gD to HVEM, nectin-2 and 3-O-sulfated HS, but not to nectin-1 (Yoon et al., 2003). Moreover, specific substitutions for conserved a.a. L25 and Q27 abolished gD1 binding to HVEM and 3-O-sulfated HS (Montgomery et al., 1996; Whitbeck et al., 1997; Yoon and Spear 2004) but, interestingly, extended the receptor specificity of gD1 also to nectin-2 (a receptor for gD2) (Lopez et al., 2000; Warner et al., 1998; Yoon et al., 2003). The substitution of Q27P also enhanced binding of gD to nectin-1 but it did not affect interaction of gD2 with nectin-2 (Krummenacher et al., 1999; Yoon et al., 2003).
Residues of gD1 that are important for binding to nectin-1 are D215, R222, F223 (Cocchi et al., 2004; Manoj et al., 2004). These residues are exposed on a protein region that is hidden during the HVEM binding to gD1, suggesting that gD1 assumes two different conformations when bound to either HVEM or nectin1 (Manoj et al., 2004; Spear et al., 2006). A region different from the receptor binding sites is necessary for the gD-induced cell fusion (a.a. 262-285) (Spear et al., 2006,). Since gD is able to bind to at least three different types of receptors and mutations within gD are known to alter receptor preferences, different receptors may be targeted in different cells and natural gD polymorphisms may influence cell tropism and pathogenesis of HSV (Spear et al., 2000).

Glycoprotein G

The amino acid sequence of gG varies considerably between two types of HSV with a sequence homology of <30%. Therefore, it is not surprising that mature forms of gG1 and gG2 as well as secreted gG2 have been used as antigens in the HSV type-discriminating serology (Lee et al., 1985; 1986; Görander et al., 2003). The gG2 precursor of 699 a.a. is cleaved into a secreted amino-terminal part (sgG2) of 300 a.a. and a virion-associated, heavily O-glycosylated part known as mature protein (mgG2) (Su et al., 1987; 1993). The biological function(s) of gG are not known. It has been reported that gG1 may help entering polarized cells via the apical surface (Tran et al., 2000) while sgG2 may function in the inflammatory response as a phagocyte chemoattractant or involved in immue evasion strategies of the virus (Bellner et al., 2004). gG2 is not essential for growth in cultured cells, however the gG-

negative mutants of HSV-2 usually exhibit lower infectivity, and clinical occurrence of such mutants is infrequent (Liljeqvist et al., 1999).

Respiratory syncytial virus

Since the last paper of this thesis also concerns respiratory syncytial virus (RSV) a short description of this pathogen is included. RSV is a common cause of the lower respiratory tract infections in infants, immunocompromised individuals, and in elderly, i.e. persons with immature, inefficient or retarded immune response. It is a common pathogen that infects almost 100% of the population before the age of two. The pathogen is highly contagious with epidemics occurring every winter and spring seasons. RSV infection can lead to severe disease of respiratory tract with high mortality. It is evaluated that worldwide RSV-induced disease takes 3-5 million human lives annually.

RSV is a paramyxovirus, a family of enveloped single-stranded RNA viruses. Its 15kb genome codes for at least 11 proteins including three transmembrane surface proteins G (attachment component), F (fusion glycoprotein), SH (small hydrophobic membrane protein), and a matrix protein (M), a nucleocapsid protein (N), nucleocapsid-associated proteins (M2-1 and M2-2), phosphoprotein (P), RNA-dependent RNA polymerase (L), and two non-structural proteins (NS1 and NS2). Like HSV, RSV initiates infection of cells by binding to cell surface HS or CS, and glycoprotein G, a virus attachment component, was found to mediate this interaction (Krusat and Streckert, 1997). Interestingly, glycoprotein G of RSV possesses common structural features with gC of HSV-1 and gG of HSV-2, i.e., an extensive and clustered O-glycosylation that forms the mucin-like segments (Hallak et al., 2000; Maggon and Barik, 2004), and a presence of regions rich in positively charged a.a. adjacent to or partly overlapping with the mucin-like domains (Langedijk et al., 1996).

The human polyclonal and humanized monoclonal anti-RSV antibodies can prevent the RSV-induced disease, and their use is recommended in selected individuals prone to RSV disease. The only antiviral approved for treatment of RSV infections is an aerosol formulation of ribavirin, a non-specific and potentially teratogenic nucleoside analogue of disputed clinical benefit. Since RSV can interact with cell surface HS, mimetics of HS such as sulfated polysaccharides were demonstrated to block the virus attachment to and entry into the cultured cells (Reviewed in Maggon and Barik, 2004).

Heparan sulfate receptor

Since the candidate antiviral compounds investigated in this thesis, i.e. sulfated oligosaccharide PI-88 and disulfated cyclitols, are distant mimetics of cell surface HS receptor for HSV and RSV, a brief description of HS moiety is provided. The glycosaminoglycan chain of HS is a carbohydrate component of proteoglycans expressed on cellular plasma membranes, in extracellular matrices and in basement membranes. HS is produced by virtually all mammalian cells. The most known proteoglycans, distinguished based on their core proteins, are cell surface glypicans and syndecans (Lindahl et al., 1998) or perlecan and agrin of the extracellular matrix/basement membrane (Cole and Halfter 1996; Iozzo et al., 1994). The glycosaminoglycan chain of HS is a biologically active component of proteoglycans. Due to extreme negative charge and associated with it affinity for basic cellular proteins, HS chains are involved in many biological phenomena including cell differentiation, binding of growth factors, cell adhesion, wound healing, lipoprotein

metabolism, angiogenesis, regulation of blood coagulation, organogenesis and others (Bernfield et al., 1999; Liu and Thorp 2002; Salmivirta et al., 1996).

HS chain consists of repeating disaccharide units of glucuronic acid (GlcA) and N-acetylglucosamine (GlcNAc). The chain is attached to a serine residue of a core protein by an oligosaccharide linker of -GlcA-galactose-galactose-xylose-. The first residue added to this linker region determines whether a glucosaminoglycan (HS/heparin) or galactosaminoglycan (CS/dermatan sulfate) chain is to be synthesized. The enzyme responsible for the chain elongation is the HS copolymerase which is encoded by the EXT1 gene. This chain is modified in some regions by N-deacetylation of GlcNAc followed by its N-sulfation (GlcNS), and epimerization of GlcA to iduronic acid (IdoA). This step is followed by 2-O-sulfation of IdoA and 6-O sulfation of GlcNS, and by a less frequent 3-O-sulfation of GlcNS (Bernfield et al., 1999; Lander and Selleck, 2000; Lindahl et al., 1998; Liu and Thorp 2002; Salmivirta et al., 1996; Wei et al., 2000). Since the N-deacetylation/N-sulfation reactions have a tendency to occur in stretches of HS of different length, the mature chain has a domain-like structure with different patterns of sulfation. Hence, HS chains can provide specific binding sites for various proteins exemplified by binding of HSV-1 gC that depend on the presence of one or more 6-O-sulfate and 2-O- sulfate groups (Feyzi et al., 1997), and binding of HSV-1 gD that requires HS chain modified by 3-O-sulfotransferases isoforms II-IV (Shukla et al., 1999). In addition to HSV and RSV cell surface HS chains provide receptor sites for many other viruses including HIV, dengue virus, EBV, CMV, vaccinia virus, hepatitis C virus, human papilloma virus, VZV, adenovirus, sinbis virus, foot and mouth disease virus, pseudorabies virus, swine fever virus, and others (Liu and Thorp, 2002; Olofsson and Bergström, 2005).

Antiviral drugs; the treatment of herpesviral infections

A successful antiviral agent must selectively target the virus particle or its replication inside the cells without adverse effects on host cells. Since viruses are obligate intracellular parasites which due to the absence of an own metabolism are strictly dependent on the cellular nucleic acid- and protein-synthesising machinery, the task to develop virus-selective antiviral drugs has been difficult. In practice, every step in the infectious cell cycle of the virus i.e., its attachment to cell surface molecules, its penetration into cells, nucleic acid synthesis, translation of viral proteins, virus assembly and egress from the cell or the free virions, can be a target for antiviral drug. Some viral proteins (enzymes) or the virus replicative pathways, although similar to those existing in cells, exhibit enough viral specificity to be selectively targeted by antivirals. Most of the antiviral drugs currently approved for clinical use (Table 2) are nucleoside analogues targeting the viral DNA/RNA synthesis, the majority of which is being used in treatment of HIV infections.

Table 2. Antiviral drugs licensed for clinical use (DeClercq, 2004)

Virus	**Class**	**Drug**
HSV	Nucleoside analogue	Acyclovir/Valaciclovir, Penciclovir/Famciclovir, Idoxuridine, Trifluridine, Brivudin
CMV	Nucleoside analogue	Ganciclovir/Valganciclovir, Cidofovir
	Pyrophosphate analogue	Foscarnet
	Antisense oligodeoxynucleotide	Fomivirsen
HIV	Nucleoside reverse transcriptase inhibitor (NRTI)	Zidovudine, Didanosine, Zalcitabine, Stavudine, Lamivudine, Abacavir, Emtricitabine
	Nucleotide reverse transcriptase inhibitor (NtRTI)	Tenofovir disoproxil
	Non-nucleoside reverse transcriptase inhibitor (NNRTI)	Nevirapine, Delavirdine, Efavirenz
	Protease inhibitor (PI)	Saquinavir, Ritonavir, Indinavir, Nelfinavir, Amprenavir, Lopinavir, Atazanavir
	Entry inhibitor	Enfuvirtide
Hepatitis B virus	NRTI	Lamivudine
	NtRTI	Adefovir dipivoxil
Influenza virus	Entry inhibitor	Amantadine, Rimantadine
	Neuraminidase inhibitor	Zanamivir, Oseltamivir
Broad viral spectrum		Ribavirin, interferon-α

The breakthrough in antiviral treatment came in the late 1970s when the first specific and selective antiviral drug, the nucleoside analogue acyclovir, was introduced for treatment of HSV infections. Nowadays, the standard therapy for HSV infections is still acyclovir (Zovirax) and related nucleoside analogues valaciclovir (Valtrex), famciclovir (Famvir) and penciclovir (Vectavir, Denavir). Following uptake by the cell, acyclovir is selectively phosphorylated by the viral (HSV, VZV or EBV) thymidine kinase (TK) and subsequently triphosphorylated by cellular kinases. This selectivity renders acyclovir the low cytotoxicity since conversion to active forms takes place in infected cells only, and this drug is also a better substrate for viral than cellular DNA polymerase. Phosphorylated acyclovir acts by competition with cellular deoxyguanosine triphosphate (dGTP) for binding to the viral DNA polymerase. Given the fact that acyclovir has a higher affinity for viral DNA polymerase than its cellular competitor, this nucleoside analogue is selectively incorporated into viral DNA where it terminates the DNA chain elongation. Alterations in viral TK may lead to the generation of drug resistant variants of the virus (DeClercq, 2001).

Nucleoside analogues can be used for episodic and/or long-time suppressive treatment of HSV disease. Suppressive long-time therapy with valaciclovir is beneficial in that it also reduces the host-to-host transmission of HSV including its asymptomatic shedding, a major source of the virus spread in humans (Koelle and Wald 2000; Sacks et al., 2004).

Sulfated polysaccharides as antiviral agents

Sulfated polysaccharides are mimetics of HS chains. These compounds are known to inhibit in vitro many enveloped viruses, mostly by interfering with the virus attachment to cells. As mentioned above, HS serves as an initial receptor for binding to cells of HSV (WuDunn and Spear, 1989) and many other viruses (Liu and Thorp, 2002). The antiviral activity of sulfated polysaccharides usually increases with increasing size of polysaccharide chain and degree of its sulfation. A larger polysaccharide chain is more likely to recognize and to interact with the viral attachment protein(s), and possibly to cross-link the virions. Likewise, a highly charged molecule is more likely to efficiently interfere with electrostatic interaction between the virion and HS chain. However the high molecular weight and extreme negative charge of these compounds have limited their use as antivirals due to their poor bioavailability, stability, and tissue-penetrating activity (Betz et al., 2001; Witvrouw and DeClercq, 1997). Little is known about the effect of sulfated polysaccharides on the cell-to-cell spread of the virus. This viral activity would require a relatively small compound that could penetrate into the narrow intercellular space, hence, the size of the molecule might be a limiting factor. A number of sulfated oligo- and polysaccharides that show antiviral effects have been described. These include fucoidans (Lee et al., 2001; Ponce et al., 2003), sulfated galactans (Di Caro et al., 1999; Duarte et al., 2001), 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 (for review see Witvrouw and DeClercq, 1997).

One of the most studied sulfated polysaccharides is heparin, a molecule structurally related to HS. Heparin, a known anticoagulant drug, inhibits attachment to cells of HSV, RSV and other viruses (Krusat and Streckert, 1997; Nahmias and Kibrick, 1964; Vaheri, 1964). However, heparin does not inactivate the viral particle, an issue common for most sulfated polysaccharides as their interaction with the viral components is reversible and non-virucidal. Therefore continuous presence of these compounds at the site of action is required, a condition which may be difficult to fulfil in vivo. An example of this is polysaccharide dextran sulfate, which as a potent inhibitor of HSV infection in vitro showed no antiviral effect in a murine model of HSV infection (Neyts and De Clercq, 1995), and demonstrated no activity against HIV infection in humans (Abrams et al., 1989). However few exceptions exist, the seaweed λ-carrageenan 1T1 had an irreversible/virucidal mode of action against HSV probably due to its high affinity binding to the virion, and exhibited anti-HSV activity in laboratory mice (Carlucci et al., 2004). This compound is now being evaluated in clinical trials as a microbicide (Spieler Trager, 2003). To exhibit antiviral activity, the backbone of the sulfated molecule need not to be a sugar residue as sulfated polymers composed of polyacetyl or polyvinyl alcohol were as effective as polysaccharide chains (Neyts and DeClercq, 1995). Furthermore, increased hydrophobicity of such compounds is likely to be important for their high affinity binding to viral components.

Microbicides

Microbicides (virucides) are compounds that inactivate infectivity of viral particles by disrupting the viral lipid membranes or denaturing the viral proteins (surfactants, detergents, antimicrobial peptides) or permanently blocking the virus particle by irreversible binding to the virus attachment/entry or other components (antibodies, mimetics of viral receptors, or antimicrobial peptides). Because of these properties, microbicides can be used in topical formulations to prevent the host-to-host transmission of viral infection. One well known

example of that is the use of microbicides in prophylaxis of sexually transmitted diseases. A highly effective topical microbicide that shows no adverse effects on human tissues would greatly diminish the host-to-host transmission of these diseases. Cytotoxicity at the site of administration is a common problem with the use of surfactant/detergent type of virucide as these compounds do not discriminate between the cellular and viral membranes. For example, frequent administration in female sex workers of the detergent nonoxynol-9 increased, instead of decreasing, the risk of HIV transmission due to its cytotoxicity observed as epithelial disruptions and genital inflammation (Van Damme et al., 2002). Furthermore, a cellulose sulfate based gel has recently been withdrawn from phase III clinical trials due to an increased risk for the participants to acquire HIV infection (Editorial team 2007).

Other microbicides of the detergent/surfactant type, currently in preclinical evaluation, are sodium lauryl sulfate (SLS) and dioctyl sodium sulfosuccinate (docusate, Zorex^{TM}) SLS and docusate are anionic surfactants which act by denaturing of viral proteins and/or solubilization/dissociation of viral membrane lipids. Pretreatment of HSV-1 or HSV-2 with SLS or docusate inhibited in a concentration dependent manner the virus infection both in cell culture (Gong et al., 2001; Piret et al., 2000) and in laboratory mice (Piret et al., 2002). Furthermore, prior application of SLS on mouse skin or on vaginal epithelium prevented the development of HSV lesions and death of animals. SLS also showed inhibitory activity against HIV and HPV (Piret et al., 2000; 2002). Both SLS and docusate decreased the viability of cultured cells (Gong et al., 2001; Piret et al., 2002).

Resistance of herpes simplex virus to antiviral drugs

The development of HSV resistance to antiviral drugs used in clinics or in laboratory assays is a known phenomenon. Variants of HSV resistant to nucleoside analogues are found in approximately 0.1-0.7% of isolates from immunocompetent patients, and in 4-7% of isolates from immunocompromised patients. Relatively long virus replication in the presence of drug is usually required for the selection for/generation of the resistant variants with the drug-adaptive changes in their genome. Immunocompromised patients usually suffer from more frequent and prolonged periods of active virus replication, so the continuous treatment of these individuals can lead to the selection of the drug resistant variants (Levin et al., 2004).

Acyclovir resistant variants of HSV are usually ≥10 times less sensitive to the drug than the wild-type virus. Herpesviruses gain resistance to acyclovir by changes in the gene coding for thymidine kinase (TK): either by lacking this protein, expressing reduced levels or by changing its substrate specificity. Viruses with these types of mutations are usually resistant to all drugs dependent on TK for activation but not to the agents acting at other steps of virus replication (Levin et al., 2004; Studahl et al., 2006).

Sulfated polysaccharides are known to be poor inducers of virus resistance (Damonte et al., 1996; Trybala et al., 2002). However, HSV-1 variants resistant to sulfated polysaccharides heparin or carrageenan have been generated by virus propagation in cultured cells in the presence of these agents (Carlucci et al., 2002; Goodman and Engel 1991; Pertel and Spear, 1996). Analysis of the drug resistant variants of a virus may help to develop a new generation of antiviral drugs with improved antiviral activity. Furthermore, analysis of these variants may increase our knowledge about biological activities of viral proteins as exemplified by a novel function of HSV-2 gG described in this thesis in paper III.

Aims

- To compare anti-HSV activities of the low molecular weight HS mimetic PI-88 and heparin, the high molecular weight prototype of HS mimetics.

- To generate PI-88 resistant variants of HSV-1 and HSV-2, and to investigate molecular basis for their resistance.

- To screen libraries of low molecular weight HS mimetics for novel potential anti-HSV/RSV drugs, and to evaluate antiviral activity of eventual lead compounds and their analogues.

Material and methods

Synthesis of compounds

The two classes of compounds (Fig. 5) i.e., sulfated oligosaccharide PI-88 and its analogues, and disulfated cyclitol (DSC) and its analogues were synthesized. PI-88 was prepared by hydrolysis of the extracellular phosphomannan polysaccharide of the yeast *Pichia holstii*, to yield a phosphorylated oligosaccharide fraction (PM_5), which was subsequently chemically sulfonated (Ferro et al., 2001; Parish et al., 1999; Yu et al., 2002). The preparation and compositional analysis of non-sulfated PI-88 precursor PM_5 (Ferro et al., 2001; 2002), 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). Analogues of PI-88 were all prepared by modifications of the reducing end of PI-88 tetra-/pentasaccharide with various groups as described previously for other PI-88 derived compounds (Karoli et al., 2005).

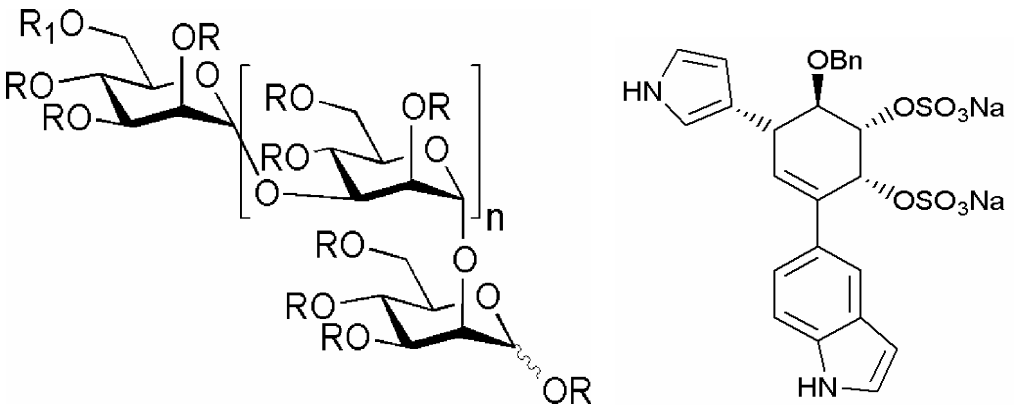

Fig. 5. Structure of PI-88 (left side): R = SO_3Na or H, R_1 = PO_3Na_2, n = 0-4, and DSC3

The DSC compounds and their analogues (mini-libraries of >125 compounds) were all prepared from (1*R*, 4*S*, 5*S*, 6*R*)-3-bromo-4,5-*O*-isopropylidene-7-oxabicyclo[4.1.0]hept-2-ene-4,5-diol, itself readily obtained by standard transformations following microbial dihydroxylation of bromobenzene. The aryl groups attached to the cyclohexenyl double bond within each of compounds 1-4 and 6-10 (paper IV), were introduced by Suzuki-Miyaura cross-coupling of the relevant cyclohexenyl bromide with the appropriate arylboronic acid derivative. All new compounds were characterized by ^{1}H NMR, ^{13}C NMR and mass spectrometric techniques. All compounds tested were readily soluble in water and their stocks (10 mM) were stored at -20^{o}C.

Chondroitin sulfate E from squid cartilage (Seikagaku, Japan), pentosan polysulfate, heparin, sodium lauryl sulfate (SLS), sodium chlorate, protamine, and poly-L-lysine (all from Sigma, Mo, USA) were also used.

Cells, viruses, and clinical specimens

African green monkey kidney (GMK AH1) epithelial cells (Gunlap, 1965) were cultivated in Eagle's minimum essential medium (EMEM) supplemented with 2% fetal calf serum, 0.05% Primaton RT substance (Kraft Inc., Norwich, USA) and 100 U/ml of penicillin and 100 μg/ml of streptomycin. Human epidermoid carcinoma (HEp-2) cells were cultured in Dulbecco´s modified EMEM (DMEM) supplemented with 10% fetal calf serum and antibiotics. Human oral SVpgC2a keratinocytes, mink lung Mu1Lu cells, and mouse L cell fibroblasts were also used (paper I). Unless otherwise stated, the cells were seeded in plastic cluster plates and used for experiments when they had reached confluence.

The virus strains and mutants used were HSV-1 KOS (ATCC, VR-1493), HSV-1 KOS321, a plaque purified isolate of wild-type strain KOS (Holland et al., 1983), HSV-1 KOS gC-null strain gC$^-$39 (Holland et al., 1984), HSV-1 gC-negative and syncytium-inducing strain MP (Hoggan and Roizman, 1959), and HSV-2 strain 333 (Duff and Rapp, 1971). The RSV strain A2 (Lewis et al., 1961) was used. The RSV stock was prepared as described by Hallak et al. (2000), and stored at -70^{o}C in the presence of 40% sucrose (Gupta et al., 1996).

Swabs of human cervical secretions were immersed in 1 ml of PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and vigorously washed out with the same buffer. The resulting solution was clarified by centrifugation and stored at -70^{o}C.

Preparation of PI-88 escape variants of HSV-1and HSV-2

The procedure used for generation of HSV-1 and HSV-2 variants resistant to PI-88 is outlined in Fig. 6. The plaque-purified HSV-1 strain KOS and HSV-2 strain 333 (uncloned) were serially (ten times) passaged in GMK AH1 cells in the presence of 100 μg/ml of PI-88 according to three different protocols (Fig. 6). (A) The virus was incubated with PI-88 for 15 min prior to and during a 2 h period of infection of GMK AH1 cells at 37^{o}C. The cells were then washed with EMEM and incubated in fresh EMEM without PI-88 until complete cytopathic effect (CPE) was observed. (C) The virus infection of cells took place at 37^{o}C for 2 h in the absence of PI-88. The cells were then washed with EMEM and incubated in EMEM supplemented with PI-88 until the development of complete CPE. (AC) The virus was incubated with PI-88 for 15 min prior to and during a 2 h period of infection of GMK AH1 cells at 37^{o}C. The cells were then washed with EMEM and incubated in EMEM supplemented with PI-88 until the development of CPE. For control purposes, HSV-1 KOSc and HSV-2 333 were passaged ten times without PI-88 (KOScp10 and 333p10 respectively). After each passage the infectious culture medium and infected cells were harvested and subjected to one freeze-thaw cycle. Following centrifugation for 10 min at 1000 x g, the supernatant medium was diluted in EMEM and used for a subsequent passage.

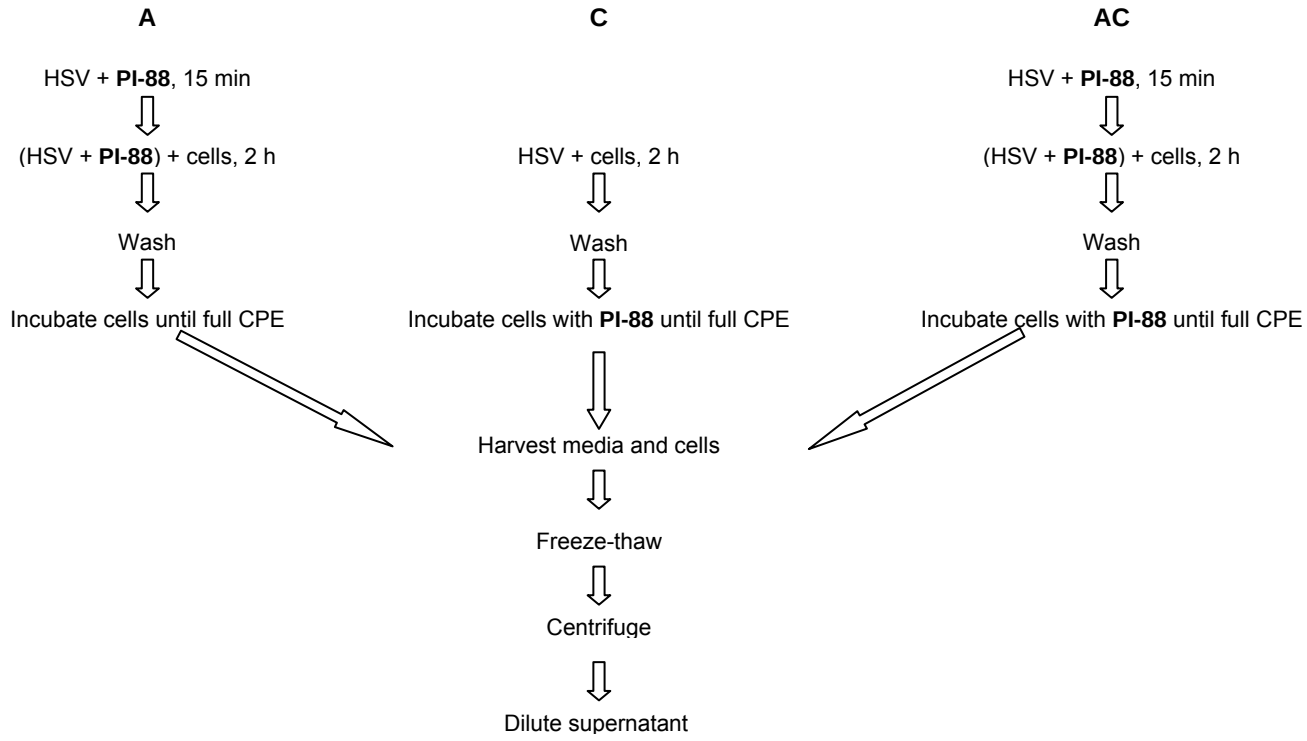

Fig. 6. Preparation of PI-88 escape variants of HSV-1 and HSV-2

Purification of viral particles and viral glycoproteins

GMK AH1 cells in roller bottles were infected with HSV at a multiplicity of 3 PFU per cell. After 1-2 h of incubation at 37°C, methyl-[^{3}H]thymidine (Amersham Pharmacia Biotech, Little Chalfont, England) was added (20 μCi/ml) 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 x g. The supernatant medium was further clarified by centrifugation for 7 min at 5,000 x g, and then centrifuged for 2 h at 22,000 x g. The resulting viral pellet was stored overnight at 4°C and resuspended in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4). 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, pH 7.4, and centrifuged for 2 h at 20,000 rpm (SW28.1 rotor, Beckman). 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, stored overnight at 4°C and resuspended in PBS supplemented with bovine serum albumin (BSA, 0.25%) and stored at -70°C (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).

Viral plaque assays

Two versions of viral plaque assays were used for evaluation of antiviral activities of candidate antiviral compounds and their analogues, i.e., the plaque number- and the plaque size-reduction assays. The evaluated plaque features in these assays are schematically shown in Fig. 7 A, B.

A. Plaque number-reduction assay (Fig. 7A). This procedure evaluates the effect of the test compound on infection of cells by the free virus particles and thereby on the number of viral plaques formed in cells as every viral particle, non-neutralized by the compound, will infect the cells and form a plaque. In particular, serial dilutions of compounds in EMEM were mixed with ~200 infectious viral particles (PFU) of HSV-1 or HSV-2 and incubated for 10-15 min at room temperature prior to the addition to cells. After incubation of the virus-compound mixture with cells for 1-2 h at 37°C, the cells were washed with EMEM and overlaid with viscous solution of methylcellulose so the virus can spread only via cell-to-cell route to form plaques. The plaques that developed after 2-3 days of incubation were stained with crystal violet. The concentration of compound that inhibited the number of viral plaques by 50% (IC_{50}) was interpolated from the dose-response curves. For screening of large numbers of compounds (libraries), the mixtures of virus and compound (100 μM) were incubated for 10 min at room temperature before addition to cells. The compounds were left on the cells during the time of plaque development.

B. Plaque size-reduction assay (Fig. 7B). In this assay the free viral particles do not directly interact with the test compound. Instead the test compound is added (in the methylcellulose overlay) after infection of cells and left on cell monolayer throughout the development of viral plaques. Hence, this assay evaluates the effect of test compound on the cell-to-cell spread of the virus measured as a reduction in the size of viral plaque. In particular, monolayer cultures of densely growing GMK AH1 cells were infected with the virus in the absence of test compound for 2 h at 37°C. Subsequently, the serum-free overlay medium, composed of EMEM supplemented with 1% methylcellulose solution or 0.5% pooled human γ-globulin (Aventis Behring, Marburg, Germany), and one of the compounds of interest, were added to and incubated with cells throughout the entire period of the development of viral plaques. After 2-3 days of incubation at 37°C, the viral plaques were visualized by crystal violet staining or immunostaining with pooled human γ-globulin as primary and peroxidase-conjugated $F(ab')_2$ fragment goat anti-human IgG (Jackson, West Grove, PA, USA) as secondary antibody. The images of twenty neighboring plaques were captured using a Leica DC 300 digital camera (Leica, Heerbrugg, Switzerland) attached to a Leitz-Wetzlar Diavert microscope (Leitz-Wetzlar, Germany). The area of each plaque was determined by using IM 1000 image software (Leica, Cambridge, UK).

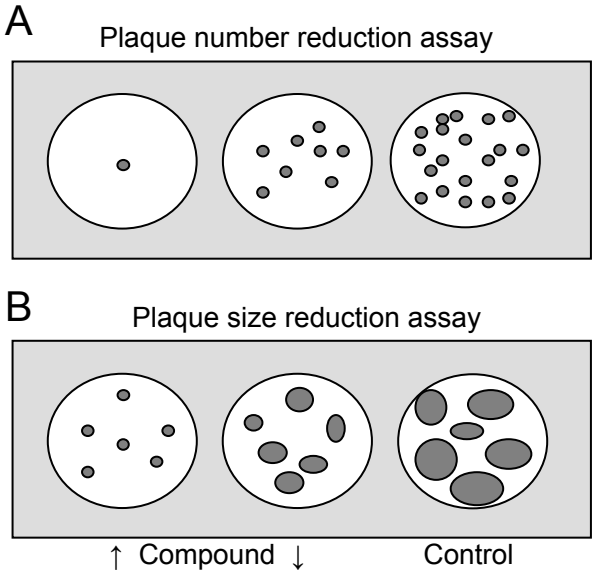

Fig. 7. Schematic appearance of viral plaques formed in (A) plaque number reduction assay and (B) plaque size reduction assay

Similar protocols were used for RSV, except that the assays were performed in HEp-2 cells and DMEM supplemented with 2% heat-inactivated fetal calf serum was used instead of EMEM.

Binding of purified virions and viral glycoproteins to cells

This assay was used to evaluate the effect of test compounds on binding to cells of purified, radiolabeled particles of HSV or isolated viral attachment proteins gB or gC. In particular, confluent monolayers of GMK AH1 cells in 24 well plates were blocked with PBS-A (PBS supplemented with 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$) containing 1% BSA for 1 h at room temperature to avoid non-specific binding. Mixtures of the compound of interest and purified ^{3}H-thymidine labelled HSV-1 or HSV-2 virions were incubated for 15 min at room temperature prior to the addition to and incubation with cells for 2 h at 4^{o}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. Alternatively the assay was carried out at 37^{o}C using EMEM as diluent medium and omitting the steps of BSA blocking and preincubation of the virus-compound mixture.

The effect of PI-88 on the binding of viral gC or gB to GMK AH1 cells was tested as described previously (Lycke et al., 1991). Briefly, purified gC or gB were preincubated for 15 min at 4^{o}C with tenfold increasing concentrations of PI-88. The mixtures were then transferred to cells growing in 96-well plates, and left for attachment for 1 h at 4^{o}C. Bound glycoproteins were detected by an ELISA-based procedure.

Assay of virus entry into cells

This assay was used to identify the step of virus infection of cells, affected by the test compound. The assay was carried out as described by Schnipper *et al.* (1980) with some modifications. Briefly, mixtures of test compounds (100 μM) and radiolabeled virus in serum-free EMEM were preincubated for 15 min at 37^{o}C prior to the addition to GMK AH1 cells and subsequent incubation for 3 h at 37^{o}C. After twice washing with PBS, the cells in some wells were harvested for quantification of radioactivity 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 (200 μg/ml; Sigma) for 15 min at 37^{o}C. The cells were harvested and washed twice with PBS by centrifugation at 250 g for 5 min. Half of the volume of sedimented cells was mixed with 5% SDS and subjected to quantification of radioactivity while the remaining cells were lysed with 1% NP40 solution in hypotonic PBS and centrifuged for 10 min at 800 g to sediment the nuclei. The sedimented fraction was lysed with 5% SDS and subjected to quantification of radioactivity.

Virus inactivation (virucidal) assay

This assay enables identification of virucidal activity associated with a compound in question. Usually a high dose of the virus (~10^5 PFU) and the test compound are mixed and incubated at 37^{o}C for 15 min. The mixtures are then diluted to the non-inhibitory concentrations of the test compound, and subjected to the infectious titer determination. If the binding of test compound to the virus particle is irreversible and virucidal the diluted virions will not infect

the cells and form plaques. In case of RSV, the assay was carried out in similar manner using DMEM supplemented with 2% heat-inactivated fetal calf serum instead of EMEM.

Antiviral activity of test compounds in the presence of cervical secretions

Given the potential application of compounds examined in this study as virucides for intravaginal administration to prevent sexually transmitted infections, this assay was used to evaluate possible neutralization of a compound antiviral potential by human cervical secretions. Equal volumes of 100 μM solution of a test compound and diluted 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. Additional incubation was then carried out for 15 min at 37^{o}C and the mixtures then transferred to GMK AH1 cells. The rest of the procedure was conducted as described under the viral plaque number reduction assay.

Cytotoxicity assays

Two assays were used to evaluate cytostatic/cytotoxic properties of test compounds. The effect of test compound on proliferation of GMK AH1 cells was tested in subconfluent monolayers of cells exposed to the test compound for 24 h at 37^{o}C. Subsequently, 2.5 μCi of *methyl*-^{3}H-thymidine were added and incubated with cells for 2 h at 37^{o}C to measure the extent of its cell-intake as an indicator of the cell-proliferating activity. Possible cytotoxic effects of test compounds were measured by using the tetrazolium-based CellTiter 96 Aqueous One Solution Reagent kit (Promega, Madison, WI) according to manufacturer's protocol.

Nucleotide sequencing

This technique was used to identify genomic alterations in HSV-1 and HSV-2 variants resistant to PI-88. Amplification and sequencing of HSV DNA fragments specific for gB, gC, gD, gE, gG, gI, gK, and UL24 gene was performed as described previously (Liljeqvist et al., 1999). The primers used in amplification and sequencing reactions were designed based on the published sequence of respective gene of HSV-1 strain 17 or HSV-2 strain HG52. Some genes "difficult" to sequence were amplified in several overlapping fragments, and then sequenced.

Marker transfer assay

The protocol and principle of this assay is outlined in Fig. 8. This assay was used to verify whether specific gene alteration found in the drug-resistant variant of HSV was indeed responsible for the resistant phenotype. To this end, purified whole DNA of parental drug-sensitive HSV (isolated according to Mettenleiter et al., 1988) was mixed with DNA of a mutated gene (amplified by PCR using Pfu Turbo polymerase and specific flanking primers). Following addition of the lipofectamine 2000 reagent, the mixture was transfected into GMK AH1 cells. Identification of recombinant viruses was based on their resistance to the drug or on differential expression of specific proteins.

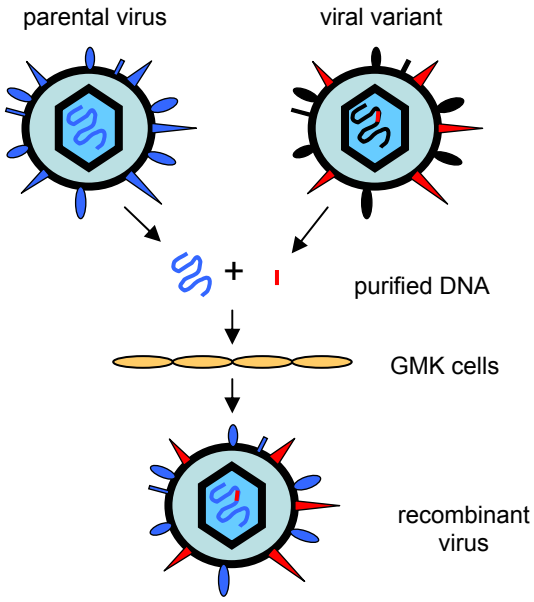

Fig. 8. Principle of the marker transfer assay

Results and discussion

The low molecular weight HS-mimetic PI-88 efficiently reduces HSV cell-to-cell spread (paper I)

We sought to search for novel inhibitors of HSV with improved antiviral activity as related to conventional sulfated polysaccharides. We paid attention to the compound ability to interfere with the cell-to-cell spread activity of the virus, a feature of great importance in pathogenesis of HSV infection in humans. In the investigation described in first paper, we compared anti-HSV activities of the relatively small HS mimetic compound PI-88 with heparin, a well known sulfated polysaccharide that exhibits antiviral properties (Nahmias and Kibrick 1964; Vaheri 1964). PI-88 is a mixture of highly sulfated mannose containing oligosaccharides, the major components being penta- (60%) and tetra- (30%) saccharides, although di-, tri- and hexasaccharides are also present (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 but more extensively sulfated than that of heparin and most species of HS. PI-88 is an anti-cancer drug currently in phase III clinical trial. Its anti-cancer properties are based on PI-88s ability to inhibit tumour metastasis by reducing the degradation of HS chains in healthy tissues by tumour cell heparanase, and by its ability to inhibit angiogenesis (Parish et al., 1999). Included in the evaluation were also other sulfated oligo/polysaccharides differing in molecular weight and the degree of sulfation, as well as the PI-88 precursor (PM5), dephosphorylated PI-88 (DPI-88) and size-fractionated PI-88 oligosaccharides (SM3-5). The effects of these compounds on HSV infectivity (the plaque number reduction assay) and HSV cell-to-cell spread (the plaque size-reduction assay) in cultures of GMK AH1 cells are summarized in table 3.

Table 3. Anti-HSV activity of PI-88 oligosaccharide and related compounds

Compound	MW kDa (average)	Sulfate groups /saccharide (average)	Plaque number reduction assay IC50 (µg/ml)		Plaque size reduction assay IC50 (µg/ml) or % of control at 100 µg/ml PI-88	
			HSV-1	HSV-2	HSV-1	HSV-2
Heparin	15	1.4	1	0.8	50	7
PI-88	2.4	3	6	2	2 (20%)	0.7 (20%)
PM5	0.9	0	>100	>100	NT	NT
DPI-88	2.4	3	5	2	17%	22%
SM3	1.6	3	>100	>100	80%	96%
SM4	2.1	3	50	8	50%	50%
SM5	2.6	3	50	8	33%	50%
Pentosan polysulfate	3	2	NT	NT	12	3
Chondroitin sulfate E	70	1	NT	NT	>100	>100

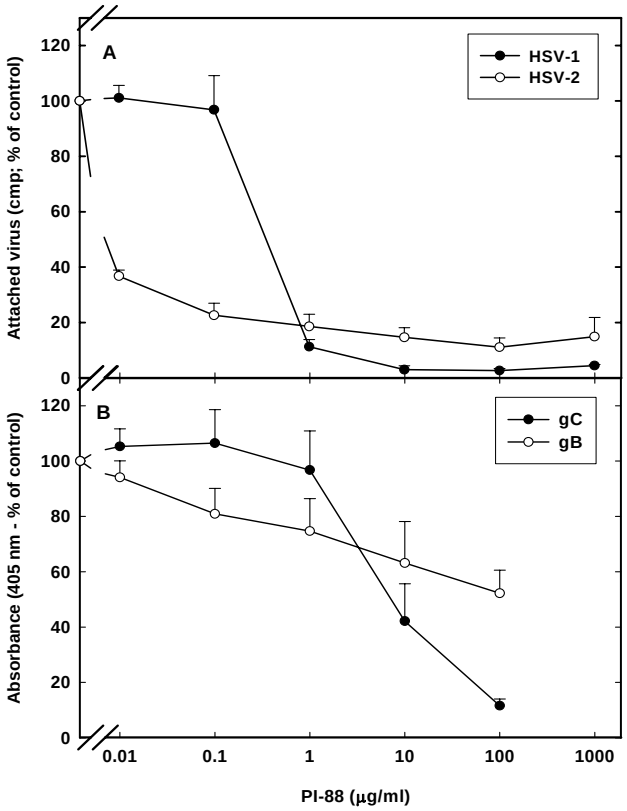

Fig. 9. The effect of PI-88 on the binding to cells of HSV virions and HSV-1 glycoproteins. PI-88 was incubated with ^{3}H-thymidine labeled HSV-1 or HSV-2 virions (A) or with isolated HSV-1 glycoproteins gB or gC (B). The results are expressed as a percentage of attached viral cpm, or absorbance of attached viral glycoproteins found with PI-88-treated virions or proteins relative to mock-treated controls.

In comparison with heparin, PI-88 was less efficient at inhibiting initial infection of cells by HSV (plaque number reduction assay) but inhibited the virus cell-to-cell spread activity (plaque size-reduction assay) more profoundly. This tendency, i.e., efficient reduction of viral spread by the low molecular weight sulfated compounds was confirmed by comparing anti-

HSV cell-to-cell spread activities of chondroitin sulfate E (high MW) and pentosan polysulfate (low MW). The latter but not the former reduced viral spread in cultured cells (Table 3), although both compounds efficiently inhibited initial infection of cells by HSV (Baba et al., 1988; Bergefall et al., 2005). The non-sulfated precursor of PI-88, PM5 had no inhibitory effect on HSV indicating that sulfate groups are essential for anti-HSV activity. In contrast, DPI-88 that lacks phosphate group at C6 of maltose, exhibited anti-HSV activity comparable to PI-88 indicating that phosphate moiety is dispensable for antiviral potency. The tetra-(SM4), penta-(SM5), and possibly hexasaccharides (not tested) fractions of PI-88 are responsible for antiviral potency of PI-88 (Table 3).

PI-88 inhibited attachment to GMK AH1 cells of purified virions of HSV-1 and HSV-2 as well as isolated attachment components gB and gC of HSV-1 (Fig. 9A,B). These results suggest that PI-88 inhibits HSV infection of cells by interfering with the binding of the viral attachment glycoproteins gC and gB to the cells, a mechanism similar to that reported earlier for heparin (Trybala et al., 2000; WuDunn and Spear 1989) and other sulfated polysaccharides (Witvrouw and DeClercq, 1997). According to the most accepted opinion, the same viral proteins and cellular receptors that promote virus attachment to and entry via the apical surface of cells are likely to mediate intercellular transmission of the virus (Cai et al., 1987; Cocchi et al., 2000; Forrester et al., 1992; Ligas and Johnson, 1988; Roller and Rauch, 1998; Shieh and Spear, 1994). Hence, sulfated saccharides should theoretically interfere with both these activities. However we demonstrated herein that the molecular weight of sulfated compound is an important limiting factor for the compound ability to interfere with the cell-to-cell spread. One can speculate that the low molecular weight compounds may in contrast to larger sulfated polysaccharides such as heparin or chondroitin sulfate E access the narrow intercellular spaces to interfere with the cell-to-cell spread activity of HSV. These results suggest that the low molecular weight sulfated oligosaccharides such as PI-88 might be preferred for an efficient inhibition of intercellular transmission of HSV. Upon completion of our studies, PI-88 was also shown to possess antimalarial activity (Adams et al., 2006) and to reduce mortality of laboratory mice infected with dengue and encephalitic flaviviruses, presumably by interfering with glycosaminoglycan-binding domains on flavivirus E protein (Lee et al., 2005).

HSV proteins comprising the mucin-like regions, i.e., HSV-1 gC and HSV-2 gG are the major targets for sulfated oligosaccharide PI-88 (papers II and III)

While investigating the effect of PI-88 on plaque size of HSV-1 (Paper I), we noticed the presence of large PI-88 resistant plaques in HSV-1 gC null/negative strains gC$^-$39 and MP. This observation encouraged us to generate *in vitro* the PI-88 resistant variants of HSV-1 (Paper II) and HSV-2 (Paper III).

Cloned HSV-1 KOS or HSV-2 333 strains were passaged 10 times in GMK AH1 cells in the presence of PI-88 to select for resistant variants. This was accomplished using three different procedures: (A) PI-88 (100µg/ml) was present during virus attachment to cells, (C) PI-88 (100µg/ml) was present during the cell-to-cell spread of the virus, or (AC) where PI-88 (100µg/ml) was present both during attachment to and the cell-to-cell spread of the virus (Fig. 6). HSV-1 KOScp10 and HSV-2 333p10 were passaged 10 times in the absence of PI-88. The viral plaque variants of HSV-1 KOS that survived ten passages in the presence of PI-88 were stained with monoclonal anti-gC-1 antibody, which revealed that 47% of AC variants, 61% of A variants and none of the C variants were gC negative. These results indicated that

the HSV-1 attachment component gC was primarily targeted by PI-88 during initial infection of cells.

Several plaque purified variants from each group were selected for further analysis in plaque number- and plaque size- reduction assays (HSV-1 in table 4; HSV-2 in table 5). Furthermore, we sought to identify the alteration(s) in viral variants responsible for PI-88 resistant phenotype based on the reasoning that the viral component(s) involved may provide, in their wild-type form, the potential binding sites for HS and for the HS-mimetic compounds. Hence, HSV-1 and HSV-2 genes coding for known HS-binding and/or syncytium inducing and other proteins gB, gC, gD, gG, and gK were sequenced.

Table 4. Features of PI-88 escape variants of HSV-1

Virus variant	Phenotype		PI-88 sensitivity		Mutations			
	gC	Syn-cytium	Plaque nr.red. assay IC_{50} (μg/ml)	Plaque size red. assay % of control (area mm^2)	gB	gC	gD	gK
KOSc	+	-	8.3	27.5 (0.205)	-	-	-	-
KOScp10	+	-	6.0	19.7 (0.094)	NT	NT	NT	NT
AC1	+	-	48	53.5 (0.255)	-	ΔP33-G116	-	-
AC2	+	-	58	67.7 (0.304)	T23N, E244K	ΔP33-G116	-	-
AC3	+	+	>100	70.6 (1.750)	R828H	ΔP33-G116	Q27P	-
AC4	+	-	47	50.6 (0.299)	-	ΔP33-G116	A5V	-
AC5	-	+/-	4.0	50.0 (0.274)	-	Δc264 frameshift	-	-
AC6	-	-	4.6	44.7 (0.250)	-	Δc264 frameshift	Q27P	-
A1	-	+	2.8	14.2 (0.278)	-	Δc365 frameshift	-	L325I
A2	+	+	3.6	15.0 (0.815)	-	R151H	-	L325I
A3	+	+	5.1	10.7 (0.338)	-	-	-	L325I
C1	+	-	7.0	53.4 (0.306)	-	R151H	-	-
C2	+	+/-	11.0	58.7 (0.262)	-	-	P288L	A285T
C3	+	-	8.5	42.9 (0.262)	-	S251N	-	-
gC-39 R6	-	-	2.4	57.6 (0.197)	T474A	NT	Q27R	-
MP R15	-	+	15	59.6 (1.801)	R770W	NT	-	-

Table 5. Features of PI-88 escape variants of HSV-2

Virus	Infectivity (PFU/ml)	Syn	PI-88 sensitivity		Mutations				
			Plaque nr. red. assay IC_{50} (μg/ml)	Plaque size red. assay % of ctr. (area mm^2)	gB	gC	gD	gG	gK
333	2.0×10^7	-	2	16.3 (0.137)	-	-	-	-	-
333p10	2.5×10^7	-	0.4	16.8 (0.179)	NT	NT	NT	NT	NT
AC1	6.0×10^5	+	>100	15.5 (1.218)	L784P	-	Q27R	trun.641aa gG-neg.	-
AC2	1.3×10^5	+	>100	51.7 (2.753)	L784P	-	-	trun.562aa gG-neg.	-
AC3	1.5×10^4	+	>100	71.2 (0.589)	N235T	-	-	trun.306aa gG-neg.	-
AC4	8.0×10^5	+	>100	22.2 (0.644)	L784P	-	-	trun.306aa gG-neg.	-
AC6	2.5×10^3	+	>100	83.1 (1.296)	L784P	-	-	trun.641aa gG-neg.	-
AC8	1.4×10^5	+	>100	72.6 (0.314)	R814Q	-	Q27R	trun.641aa gG-neg.	A79T
AC9	1.4×10^5	+/-	>100	55.3 (0.527)	-	-	-	trun.641aa gG-neg.	-
AC10	6.4×10^5	+	1.8	79.6 (0.397)	L784P	-	-	-	-
A2	3.1×10^5	+	0.5	2.7 (0.562)	L784P	L198P	-	-	-
A4	1.5×10^6	-	0.7	8.1 (0.307)	-	-	-	-	-
A6	4.4×10^5	+	0.7	1.8 (0.208)	L784P	L198P	M148I	-	-
A9	5.1×10^3	+	38	3.0 (0.749)	Y345H L784P	-	-	trun.304aa gG-neg.	-
C1	6.9×10^5	+	1.2	NT	L784P	-	-	-	-
C3	4.3×10^3	+	0.9	93.1 (4.067)	N13K A516T L784P	-	-	-	-
C5	5.6×10^3	+	1.5	49.9 (0.685)	A327V	-	-	-	-
C6	2.6×10^4	+	1.5	NT	L784P	-	-	-	-

The most PI-88 resistant variants were identified in the AC group both for HSV-1 and HSV-2. The PI-88 resistant variants of HSV-1 will be discussed first. The gC positive AC1-4, but not the gC negative AC5-6, were 6-12 times more resistant to PI-88 than parental KOSc strain in the plaque number reduction assay. In the plaque size reduction assay all AC and C variants were more resistant to PI-88 than KOSc. In contrast, all variants A were sensitive to PI-88 in both assays.

Nucleotide sequence analysis revealed that most of the mutations were in gC, the major attachment protein for HSV-1. The most resistant variants AC1-4 all had an identical deletion of a.a. 33-116 of gC. Variant AC2 also had two point mutations in gB ectodomain while AC3 one point mutation in the gB endodomain. Alteration in the gB endodomain (R770W) was also observed in MP R15 variant. Variants AC3 and AC6 carried the same Q27P a.a. substitution in gD. Similar alteration (Q27R) was found in variant R6 of gC^{-}39 strain. Mutations in the group A occurred in gC and gK genes although as mentioned above these variants were not found to be resistant to PI-88.

The HSV-2 variants generated by selective pressure from PI-88 had the low infectious titers and produced syncytia in cultured cells which suggests that the drug adaptative alterations may greatly impair HSV-2 infectivity. All variants AC (except AC10) and variant A9 were resistant to PI-88 presence during initial infection of cells (plaque number reduction assay) as they all had significantly higher IC50 values than parental 333 and 333p10 strains. All variants AC (except AC1 and AC4), C3 and C5 were partly resistant to PI-88 presence during the cell-to-cell spread of the virus (plaque size reduction assay).

The major alterations detected in all HSV-2 variants resistant to PI-88 presence during initial infection of cells (plaque number reduction assay) were the frameshift mutations in the gG gene (AC1, AC8 and AC9: ▲c1655; AC2: ▲c1341, c1655; AC3: ▼ c624; AC4: ▼ g268, ▲ c1593; AC6: ▲ c1857; A9: ▲ c783) that resulted in the premature termination codon (see Table 5) and lack of expression of viral glycoprotein G, an observation confirmed by plaque immunostaining with anti-gG2 monoclonal antibody. Many of PI-88-selected variants of HSV-2 had alterations in the gB ecto- or endodomain (the L784P change occurred in 10 out of 16 variants). However, these mutations in gB did not match the pattern of variant sensitivity to PI-88 presence either during initial infection of cells or during the cell-to-cell spread of the virus. Instead, occurrence of these mutations in gB positively correlated with the syncytium-forming activity of particular variant. Two variants also carried the Q27R alteration in gD, i.e., an identical change to those found in HSV-1 gD. Interestingly, the PI-88 resistant variants of HSV-2 exhibited no alterations in gC.

The major alteration found in the PI-88 resistant variants of HSV-1 was the deletion of 83 a.a. segment forming the mucin like region in the N-terminal part of gC (Fig. 4). The N-terminal part of gC1 has a net negative charge, due to negatively charged a.a. residues in the protein backbone, while the C-terminal part of the mucin like region and adjacent region are positively charged. The mucin like region of HSV-1 gC is known to be important for binding to cell surface HS (Tal-Singer et al., 1995) either by direct interaction with HS or by modulating the gC conformation. The positively charged region adjacent to or partly overlapping with the mucin-like segment is also involved in HSV-1 attachment to cell surface HS (Mårdberg et al., 2001; 2002; Trybala et al., 1994). It is interesting that HSV-1 gC with this specific deletion but not the gC negative viral variants appeared to be resistant to PI-88. In contrast to PI-88 data, the gC-negative mutants of HSV-1 were found to be resistant to heparin (Pertel and Spear, 1996). It is likely that absence of whole gC may uncover the PI-88 sensitive site present on another viral protein involved in interaction with sulfated oligosaccharides, however, other possibilities cannot be excluded. The mucin like region is not found in HSV-2 gC2, and as mentioned above no alteration was found in the gC2 of PI-88 resistant variants of this virus. HSV-2 gC2 is not considered as an important attachment protein for HSV-2 (Gerber et al., 1995; Herold et al., 1996). Instead we found alterations in HSV-2 glycoprotein gG, that like HSV-1 gC contains a mucin like region (Fig. 4). All the HSV-2 variants that were resistant to the presence of PI-88 during initial infection of cells

were gG negative due to frameshift mutations. This indicates that PI-88 targets the viral proteins comprising the mucin like regions, and that gC1 and gG2 may have similar functions in virus attachment to cells, at least to GMK cells. The HSV-2 gB was reported to mediate the virus attachment to cells (Cheshenko and Herold, 2002; Herold et al., 1996). We detected several mutations in gB, both for HSV-1 and HSV-2, but neither of them was clearly coupled to the resistance to PI-88 during initial infection of cells. Instead, mutations in the endodomain of gB were found to be responsible for syncytial phenotype of HSV-1 resistant to heparin (Goodman and Engel, 1991).

The Q27P/R a.a. substitution in gD were found in several PI-88 resistant variants of both HSV-1 and HSV-2. This mutation is known to abolish binding of gD1 to HVEM and 3-O-sulfated HS (Montgomery et al., 1996; Whitbeck et al., 1997; Yoon and Spear 2004), and to confer the binding of gD1 to nectin-2 (Lopez et al., 2000; Warner et al., 1998; Yoon et al., 2003), which normally is a receptor for gD2 only. Hence, one can speculate that this alteration in gD may confer HSV-1 resistance to PI-88 by binding of gD to nectin-2 instead of 3-O-sulfated HS or by abolishing the PI88-gD interaction, however, other possibilities cannot be excluded.

It is possible that some mutations found in our study were randomly selected for. To confirm that mutations detected in our investigation were actually responsible for the PI-88 resistant phenotype, we purified (i) the viral DNA from parental viruses KOSc, MP or gC$^-$39, and (ii) the DNA fragments comprising the mutated genes of interest from PI-88 variants (see table 6) and then co-transfected the mixture into GMK AH1 cells. The resulting recombinant viruses were sequenced to confirm the transfer of altered DNA (marker) to the background of parental virus DNA, and then tested in plaque number- and plaque size- reduction assays. The results are shown in table 6.

Table 6. Features of recombinant HSV-1 variants prepared in the marker transfer experiment

Virus variant	Phenotype		PI-88 sensitivity	
	gC	Syn	Plaque nr-reduction assay IC_{50} (μg/ml)	Plaque size-red. assay % of control
KOSc	+	-	8	28
gC-39	-	-	1.2	17
MP	-	+	16	14
gC-39+AC3gC (Δ33-116)	+	-	27	21
KOSc+AC3gD (Q27P)	+	-	9	38
gC$^-$39+R6gD (Q27R)	-	-	1.6	63
MP+R15gB (R770W)	-	+	7	55
KOSc+A1gK (L325I)	+	+	NT	NT

The AC3gC gene with the deletion of a.a. 33-116 was cotransfected with gC$^-$39 DNA into cells. The resulting progeny virus was resistant to PI-88 in the plaque number reduction assay, but not in the plaque size reduction assay, thus confirming that this alteration in gC is responsible for HSV-1 resistance to PI-88 during initial infection of cells. In contrast, the

recombinant viruses with “transferred” alterations in gB (R770W) or gD (Q27P or Q27R) were partly resistant to PI-88 in the plaque size but not the plaque number reduction assay, thus confirming that these mutations confer HSV-1 resistance to PI-88 presence during the cell-to-cell spread of the virus. The marker transfer experiment also revealed that the L325I alteration in gK was responsible for the syncytial phenotype of HSV-1 variants.

To investigate of how specific mutations in gC (Δ33-116) or in gB (point alterations in the ecto- or endodomain), can confer resistance to PI-88, we tested the effect of PI-88 on binding to cells of purified virions or isolated gB or gC (Fig. 10). The binding to cells of variant AC1 (Δ33-116) was more resistant to PI-88 than that of parental KOSc strain or variant AC5 (gC negative) indicating that this alteration in gC enabled efficient HSV-1 attachment to cells in the presence of PI-88. Also the binding to cells of gC (Fig. 10B) or gB (Fig. 10C), purified from variants AC2 (Δ33-116 in gC, and the T23N and E244K changes in gB) or AC3 (Δ33-116 in gC, and the R828H change in gB) were somehow less efficiently inhibited by PI-88.

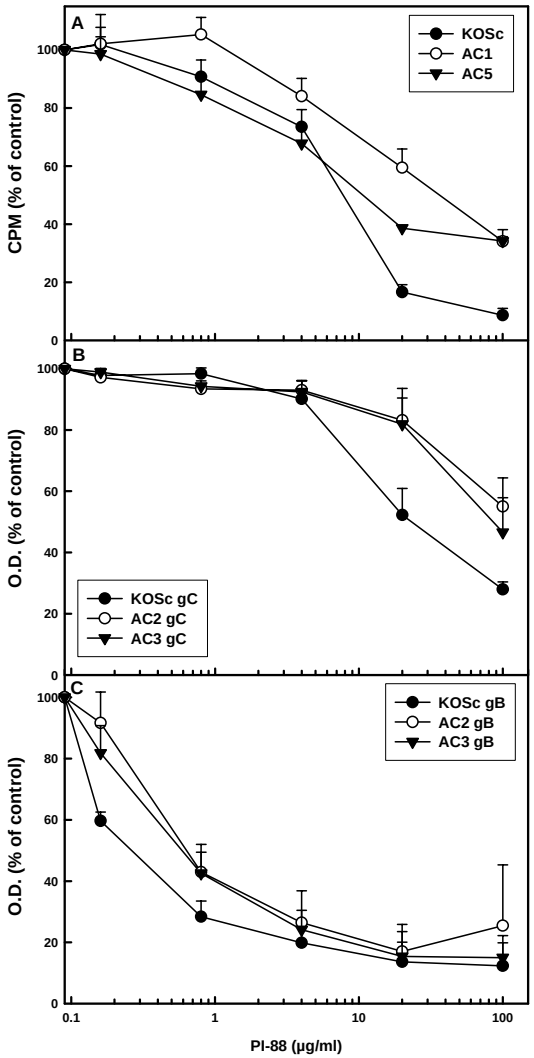

Fig. 10. The effect of PI-88 on binding to cells of purified HSV-1 virions or HSV-1 glycoproteins. PI-88 was incubated with purified virions of HSV-1 KOSc, AC1 or AC5 variant (A) or isolated glycoproteins gC (B) or gB (C) of KOSc, AC2 or AC3 variant. The results are shown as a percentage of attached viral cpm, or absorbance of cell-bound viral glycoprotein detected with PI-88 treated samples relative to mock-treated controls.

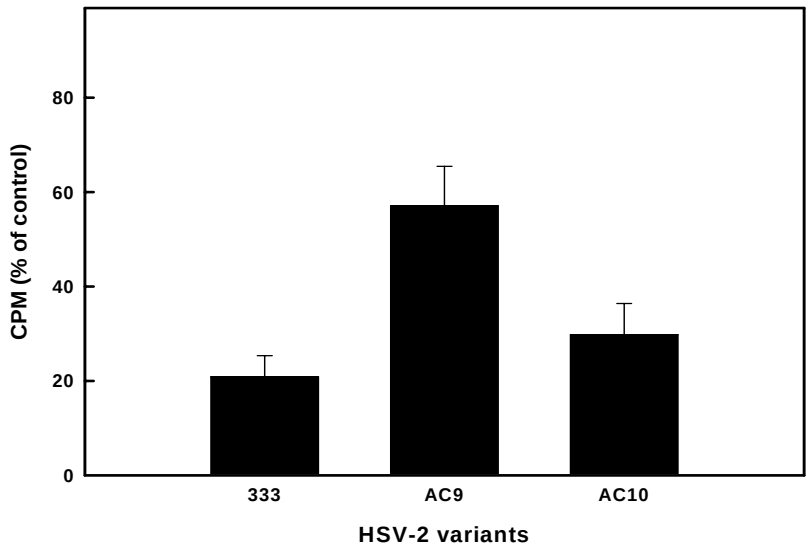

Fig. 11. The effect of PI-88 on binding to cells of purified HSV-2 virions. PI-88 at 100 µg/ml was incubated with purified virions of HSV-2 333, AC9 (gG-negative) or AC10 (gG-proficient) variant for 15 min prior to and during 1 h period of virus adsorption to GMK AH1 cells. The results are shown as a percentage of attached viral cpm detected with PI-88 treated samples relative to mock-treated controls.

We also tested the effect of PI-88 (100 µg/ml) on binding to cells of HSV-2 variants AC9 (gG negative) that was resistant to PI-88 presence during initial infection of cells (Table 5). This variant but not parental 333 strain or variant AC10 (gG-proficient) exhibited relative resistance to PI-88, indicating that the deletion of entire gG mediate the PI-88 resistant attachment of HSV-2 to cells (Fig. 11). AC9 virions were considerably more resistant to PI-88 than 333 strain during the attachment fase of infection.
In conclusion, our results indicate that PI-88 primarily target HSV proteins that comprise the mucin-like regions, i.e, HSV-1 gC1 and HSV-2 gG2, and that this block occurs during initial infection of cells. An issue of why and how PI-88 targets mucin-like regions of proteins is of great interest and requires further studies. As mentioned above, the mucin-like regions of a protein may interact with HS, cover the receptor-binding sites, or modulate protein conformation. However other possibilities cannot be excluded.

The low molecular weight disulfated cyclitols and their analogues, as well as an analogue of PI-88, exhibit virucidal properties (papers IV and V)

We screened for anti-HSV activity a mini-library of 125 small sulfated compounds. These compounds were synthesized as potential inhibitors of the HS-protein interactions, but in contrast to conventional sulfated polysaccharides, they were of low molecular weight and of decreased hydrophilicity. Two compounds, i.e., disulfated cyclitol 1 (DSC1) and DSC3 (Fig. 5), which inhibited HSV-1 and HSV-2 infectivity in the screening assay, were further analysed in plaque number- and plaque size reduction assays (Table 7), and in the virus-inactivation (virucidal) assay.

In the paper V, several analogues of PI-88 prepared by attachment of different hydrophobic/aromatic groups to the reducing end of oligosaccharide chain, and analogues of DSC3 prepared by naphthalene-for-indole substitution and by attachment of hydrophobic/aromatic groups to pyrrole or benzene rings, were analyzed (Table 7).

Table 7. Functional features of disulfated cyclitols and PI-88 and analogues

	Plaque number reduction assay IC50 (μM) or (μg/ml) (Selective index CC_{50}/IC_{50})		Plaque size reduction assay IC50 (μM)	
	HSV-1	HSV-2	HSV-1	HSV-2
DSC1	4.2	3.7	~5	~3
DSC2	30	42.7	NT	NT
DSC3	1.4	0.46	~10	~3
			Plaque size reduction at 10 μM	
DSC3			30 %	85 %
3057	21 (>19)	11 (>36)	NT	NT
3058	11 (18)	11 (18)	55 %	65 %
3059	1.6 (39)	1.2 (52)	100 %	85 %
3060	5 (14)	4 (18)	100 %	85 %
			Plaque size reduction at 10 μg/ml	
PI-88	7 (>143)	1.2 (>833)	65 %	70 %
519	2.1 (>190)	0.8 (>500)	65 %	100 %
521	1.8 (>222)	1.0 (>400)	NT	NT
536	2.1 (52)	1.6 (69)	100 %	100 %

The virus-inactivating (virucidal) activity of a compound is a functional feature of importance for its in vivo antiviral activity. Thus, certain non-virucidal sulfated polysaccharides such as dextran sulfate or cellulose sulfate which, while efficient at inhibiting HSV-2 or HIV infectivity in cultured cells, poorly protected mice or humans against respective viruses (Neyts and De Clercq, 1995). However, sulfated polysaccharide lambda carrageenan which is known to possess virucidal properties exhibited both potent anti-HSV-2 activity in cultured cells and in laboratory mice (Carlucci et al., 2004). DSC1 and DSC3 as well as their analogues 3058 and 3059 did, to some extent, inactivate viral particles (Table 8). As expected, PI-88 did not inactivate HSV and RSV virions, however its analogue 536 (Fig. 12) completely inactivated HSV particles. Compound 536 is composed of PI-88 oligosaccharide and lipophilic cholestanol group attached to the reducing end of oligosaccharide chain. It is likely that while sulfated oligosaccharide chain can bind to and block the virus attachment proteins, the cholestanol group may insert to and destabilize the viral lipid envelope thus irreversibly inactivating the virus particle.

Table 8. Inactivation of HSV and RSV by DSC, PI-88, and their derivatives

Virus	Compound concentration	Compound						
		PI-88	519	536	DSC1	DSC3	3058	3059
HSV-1	100	97.6	79.0	0	0.8	0	0.4	0
	10	100.0	91.1	0	NT	46.4	61.8	17.3
	1	94.9	87.7	76.7	NT	78.5	64.9	73.7
HSV-2	100	100	85	0	36	10	0	0
RSV	100	94.0	47.3	NT	NT	27.0	62.2	9.1

We also tested the effect of PI-88 analogue 519, DSC1, and DSC3 and its analogue 3059 on binding of purified radiolabeled HSV-1 and HSV-2 to cells. Both PI-88 and to some extent DSC3 analogues inhibited virus attachment to cells (see Fig. 4 in paper IV, and Fig. 3 in paper V). This suggests that apart from virucidal properties, these compounds may act, at least in part, through interference with the virus attachment to cells.

In conclusion, chemical modifications based on the introduction of specific hydrophobic/aromatic group(s) at the reducing end of PI-88 oligosaccharide chain, enhanced its capability to inhibit infection of cells and the cell-to-cell transmission of HSV and/or RSV. In addition modification of PI-88 with cholestanol group provided the analogue with the HSV-inactivating activity. This analogue is therefore a promising candidate for development as virucide for topical application in humans.

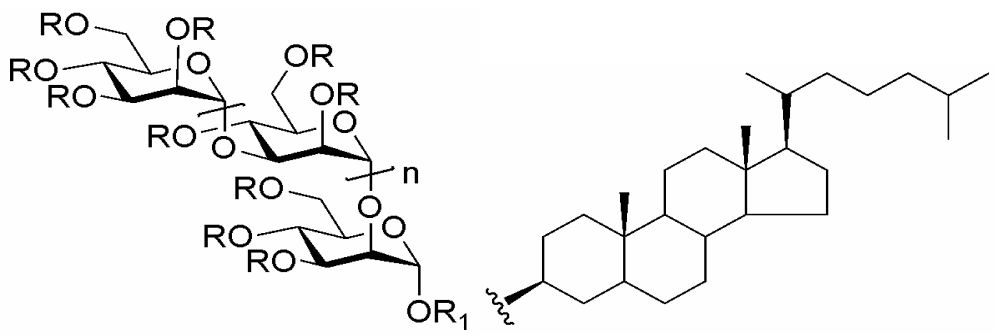

Fig. 12. Structure of PI-88 analogue 536

Summary

The novel findings presented in this thesis were as follows.

(i) The low molecular weight, HS-mimetic PI-88, in contrast to conventional sulfated polysaccharides, efficiently reduced the cell-to-cell spread of HSV.
(ii) Analysis of the PI-88 resistant variants of HSV revealed that the viral envelope glycoproteins comprising the mucin-like regions, i.e., HSV-1 gC or HSV-2 gG were primarily targeted by sulfated oligosaccharide PI-88.
(iii) Two disulfated cyclitols, i.e., DSC1 and DSC3 exhibited an HSV-inactivating activity.
(iv) Modification of PI-88 oligosaccharide with cholestanol provided the compound with the ability to inactivate HSV particles. This PI-88 analogue as well as DSC1 and DSC3 compounds are therefore promising candidates for the development as topical virucides for the prevention of the host-to-host transmission of HSV infections in humans.

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