A 3-O Sulfated Heparan Sulfate Binding Peptide Preferentially Targets Herpes Simplex Virus Type-2 Infected Cells.

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Abstract

Herpes simplex virus type-2 (HSV-2) is the primary cause of genital herpes, which is one of the most common sexually transmitted viral infections worldwide, and a major cofactor for human immunodeficiency virus infection. The lack of an effective vaccine or treatment and the emergence of drug-resistant strains highlight the need for developing new antivirals for HSV-2. Here, we demonstrate that a low molecular weight peptide isolated against 3-O-sulfated heparan sulfate (3-OS HS) can efficiently block HSV-2 infection. Treatment with the peptide inhibited viral entry and cell-to-cell spread both in vitro and in vivo using a mouse model of genital HSV-2 infection. Quite interestingly, the peptide showed a preferential binding to HSV-2 infected cells with more than 200% increased binding compared to uninfected cells. Our additional results show that heparan sulfate expression is up-regulated by 25% upon HSV-2 infection, which is a significant new finding that could be exploited for designing new diagnostic and treatment strategies against HSV-2 infected cells. In addition, our results also raise the possibility that 3-OS HS modifications within HS may be up-regulated even more to accommodate for a significantly higher increase in the peptide binding to the infected cells.
Introduction

Herpes Simplex Virus type 2 (HSV-2) is responsible for approximately two thirds of the sexually transmitted mucocutaneous lesions commonly known as genital herpes in the United States, with a gender, age, and racial disparity in its seropositivity prevalence that ranges from 3.4% in Caucasian males and 18.2% in Black males around their early twenties to 31.4% in Caucasian women and 66.5% in black women around their late forties (40, 52). HSV-2 enters into susceptible cells by first attaching to heparan sulfate (HS) linear polysaccharide side chains of cell surface heparan sulfate proteoglycans (HSPG) (48). Attachment is mediated by the viral envelope glycoproteins, gB and gC (19,48). The interaction between HSV attachment glycoproteins and HS most probably relies on the non-covalent associations between the positively charged amino acid residues of the HS-binding sites of viral glycoproteins and the negatively charged sulfate and carboxylate groups of HS chains (11). The absence of this interaction does not prevent entry, but simply slows the process (18). This is followed by a post attachment receptor mediated docking of HSV virions to one of gD receptors: herpes virus entry mediator (HVEM), nectin-1, or nectin-2 (27, 17, 45, 23). The binding of gD to its receptor initiates the penetration process involving gB, gH and gL and possibly gB and gH receptor(s) (5,33, 35,36).

Recently, the role of HS in HSV infection has been shown to be more than just providing attachment sites for the virus. Some rare modifications in HS can create a HSV-1 gD receptor (31). It is also involved in negatively regulating virus induced cell-to-cell fusion and mediating HSV movement along plasma membrane protrusions, a process that is termed surfing (30, 32). Moreover, HS has been shown to serve as co-receptor for many viruses including human
immunodeficiency virus (HIV), human cytomegalovirus (HCMV), and Varicella-zoster virus (VZV) (44, 14, 20).

The biosynthesis of HS and subsequent modifications are performed by multiple enzymes. Combinatorial expression of these enzymes gives rise to the structural diversity of HS chains to modulate a wide variety of tissue-specific functions (16). One of the most critical modifications in HS structure, 3-O sulfation, is catalyzed by a family of enzymes called 3-O-sulfotransferases (3-OSTs) (24). Seven members have been identified in this family where each isoform has the ability to recognize a distinct saccharide sequence around the modification site, and thus generate their own unique 3-O-sulfated motifs. Some of the unique motifs have been shown to have strong regulatory functions while others remain poorly understood (39, 25). 3-O-sulfated HS (3-OS HS) modified by the 3-OSTs isoforms except for the isoform 3-OST-1 has been shown to function as a gD receptor for HSV-1. However, 3-OS HS fails to provide gD receptor activity for HSV-2 entry into HSV resistant cells that lack a gD receptor (49, 50, 37, 41, 31). It probably only mediates HSV-2 attachment to cells (38). In this regard, it is not very clear whether blocking 3-OS HS can block HSV-2 infection in vivo and likewise, very limited information is available on the expression of 3-OS HS in vitro and in vivo.

Cationic peptides targeting HS on the surface of the host cell provide an attractive approach for the development of anti-herpetic agents. Some recently identified peptides include human apolipoprotein E derived peptide (apoEdp), rabbit neutrophil peptide-1 (NP-1), Lactoferrin (LF), indolicidin (a tryptophan-rich peptide from bovine neutrophils) and brevinin-1 (a peptide found in frog skin) (8, 34, 3, 51, 2). We recently used a phage display random peptide library screening...
to isolate a 12-mer cationic peptide (G2 peptide) that binds 3-OS HS, and blocks HSV-1 infection (43). Very interestingly, we show here that despite the inability of 3-OS HS to allow HSV-2 entry, G2 peptide blocks HSV-2 infection and cell-to-cell fusion mediated by the virus. We also provide new details on the mechanism of antiviral activity of G2 peptide in vitro and demonstrate its efficacy in vivo using a mouse model of genital herpes infection. Additionally, our study demonstrates that HSV-2 infected cells show a significantly higher binding of G2 peptide compared to un-infected cells. We also observed an increase in HS expression and propose an even higher increase in 3-OS HS modifications, which may explain the 200% increase in the peptide binding to infected cells. The enhanced HS expression and preferential binding of G2 are novel pieces of information with significant long-term implications for anti-HSV-2 prognosis and future drug designs for therapy.

Materials and Methods

Cell culture and viruses. African green monkey kidney cells (Vero) were provided by P. G. Spear (Northwestern University, Chicago, IL, USA). Human cervical (HELA) cells were provided by B. S. Prabhakar (University of Illinois at Chicago, Chicago, IL, USA). The human corneal epithelial (HCE) cell line (RCB1834 HCE-T) was provided by Kozaburo Hayashi (National Eye Institute, Bethesda, MD) (6). Vero and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 ug/ml streptomycin sulfate at 37°C in an atmosphere of 5% CO₂. HCE cells were maintained in Minimum Essential medium (MEM; Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 U/ml...
penicillin, and 100 μg/ml streptomycin sulfate at 37°C in an atmosphere of 5% CO₂.

Three strains of HSV-2 were used; HSV-2 strain 333, the galactosidase enzyme inducing (gJ-) HSV-2 strain 333, (both Provided by Dr. P. G. Spear, Northwestern University) and GFP tagged HSV-2 derived from HSV-2 strain 333 with an EF-1alpha promoter expressing GFP inserted between UL26 and UL27 (Provided by Dr. J. Vieira, University of Washington, Seattle, WA, USA). High titer stocks of all of the used strains were prepared by infecting Vero cells at a low multiplicity of infection (MOI = 0.01) and harvesting the cells when the cytopathic effect was 90 – 100%. Cell bound virus particles were released by sonication. Purified stocks of all HSV-2 strains were prepared by purification on a sucrose gradient.

**Isolation and synthesis of HS antagonist peptide.** Phage-displayed combinatorial 12-mer peptide libraries were screened for phage populations exposing peptides that are able to bind purified Heparan Sulfate (HS) and 3-O sulfated HS (3-OS HS) that has been modified by 3-O sulfotransferase isoform 3 (3-OST - 3) as described previously (43). G2 and a control peptide Cp (RVCGSIGKEVLG) were synthesized using the Fmoc synthesis method at university of Illinois research resources center proteomics laboratory with the purity of the synthesized peptide measured by MS / HPLC to be more than 90% (29). Working stock of the peptides were prepared by dissolving in phosphate buffered saline 1× PBS and filter sterilization through a 0.2μm low protein binding syringe filter (Millipore Corp., Billerica, MA) followed by protein estimation.
Heparanase assay. Vero cell monolayers were pretreated with 10 mIU of heparinase III (Provided by Dr. J. Liu, University of North Carolina, Chapel Hill, NC, USA) in reduced serum medium Opti-MEM (Invitrogen, Carlsbad, CA) or with medium alone for 2 h at 37°C. Cells were then washed three times with warm 1× PBS then incubated with FITC conjugated G2 peptide at room temperature for 30 min. Detection of bound fluorescent G2 peptide was performed by fluorescence microscopy and flow cytometry. Images were captured using Nikon Eclipse T2000 equipped with a low light sensitive CCD camera (Photometrics Cascade II) under control of imaging software Metamorph (Molecular Devices, Sunnyvale, CA). For flow cytometry, cells were detached from culture plates using enzyme free cell dissociation buffer (Life Technologies, Grand Island, NY) and cells treated with Heparanase III only were used as background control.

Viral entry assays. The effect of G2 peptide on HSV-2 entry was assessed using standard entry assay as described previously (37). Briefly, confluent cultures of HeLa cells in 96 well plate were incubated for 1 h with a serial dilution of G2 peptide. Cells were then infected with the β-galactosidase expressing recombinant HSV-2(333) gJ- at a high multiplicity of infection (MOI) of 20 PFU/cell for 6 h. Enzymatic activity was then assayed using the galactosidase enzyme substrate O-nitrophenyl-β-D-galactopyranoside (ONPG; Pierce, Rockford, IL), and the optical density (OD) for the reaction was measured at 410 nm using a micro-plate reader (Spectra Max 190 Molecular Devices, Sunnyvale, CA). Fluorescence microscopy was also utilized to assess HSV-2 entry after G2 peptide treatment. Confluent cultures of Vero cells were not treated, treated with control nonspecific peptide, or treated with G2 peptide at 1 mg/ml for 1 h at 37°C. Cells were then infected with
GFP-expressing HSV-2 (GFP-HSV-2) at MOI of 10 for 2 h at 37°C, washed, and incubated for another 12 h at 37°C. Imaging was performed using 10× objective on a Zeiss Axiovert 200 fluorescence microscope.

**Western blotting for viral protein VP16.** Confluent cultures of HeLa cells were either untreated or treated with 2mg/ml G2 peptide for 30 min at 37°C. Cell were then infected with HSV-2(333) at an MOI = 10 for 4 h at 37°C. After 4 h, cells were washed 3 times with 1× PBS and once with citrate buffer (pH=3) to remove any bound virus particles. The Western blot assay was performed according to protocols described previously (21). Briefly, Whole cell lysates were denatured in NuPAGE LDS Sample Buffer (NP0007; Invitrogen, Carlsbad, CA) and heated to 86 °C for 8 min before gel loading. Equal amounts of protein were subjected to 4-12% SDS-PAGE and electroblotted onto a nitrocellulose membrane. Nonspecific binding was blocked using 5% nonfat milk in tris buffered saline (TBS) for 2 hours at 37°C. The membranes were then incubated with primary mouse monoclonal antibodies to VP16 (sc-7545; Santa Cruz Biotech, Santa Cruz, CA; 1:500) overnight at 4°C. The blots were rinsed 5 times with 0.1% TTBS (0.1% Tween 20 in TBS) for 5 min followed by incubation for 1 h at room temperature with horseradish peroxidase-conjugated anti-mouse IgG (115-035-062, Jackson ImmunoResearch Laboratories, West Grove, PA; 1:25000). Protein bands were detected using SuperSignal West Femto maximum sensitivity substrate (34096; Pierce, Rockford, IL), and visualized using ImageQuant LAS 4000 imager (GE Healthcare Life Sciences; Piscataway, NJ). Protein bands were quantified using ImageQuant TL image analysis software (version: 7).

**Plaque reduction assay.** Plaque reduction assays were performed in a pretreatment...
approach to test for the antiviral properties of the G2 peptide. Confluent monolayers of Vero cells in 24 well plates were pretreated with serial dilutions of the G2 peptide or with PBS for 1 h at 37°C. Cells were then infected with HSV-2 (333) virus or green fluorescent protein (GFP)-tagged HSV-2 virus to yield 20-30 plaques per well for 2 h to allow virus adsorption. After 2 h, cells were washed three times with 1× PBS to remove unbound viral particles then overlaid with 1% methylcellulose in DMEM medium supplemented with 0.05% human pooled immunoglobulin G (IgG) (SIGMA, St. Louis, MO) to neutralize any unbound virions. 48 to 72 h later, cells infected with HSV-2(333) were fixed with 100% methanol and stained with 0.1% crystal violet and the plaques were enumerated using a microscope or alternatively by direct visualization. In the case of plaques formed by GFP-tagged HSV-2, plaques were visualized using fluorescent microscopy (Zeiss Axioscope microscope equipped with a digital low light CCD camera under control of the imaging software Axiovision) at a 100× magnification and plaque size was measured using Axiovision software (version: 4).

Viability and cytotoxicity assays. 50% confluent Vero and HCE cells in 24 well plates were either untreated or treated with a serial dilution of G2 peptide in complete medium for 24 h at 37°C. Cells were then either visualized under the bright field directly for HCE cells, or stained with the Hoechst 33342 live-cell nuclear stain in case of Vero cells to count viable cells’ nuclei using a direct cell counting method.

Cell-to-Cell Fusion Assay. Standard cell-to-cell fusion assay was used as previously described (43). Cells were split into two populations. “Target” cells were transfected with plasmid expressing Nectin-1 as a gD receptor (1.0 µg) and the luciferase gene (0.5 µg).
“Effector” cells were transfected with plasmids expressing HSV-2 glycoproteins gD, gB, gH, and gL and T7 RNA polymerase (0.5 µg each). 24 h post-transfection, target cells were either treated with serial dilution of G2 peptide for 1 h at 37°C or left untreated. Target cells were then washed 3 times with 1× PBS, and were mixed with effector cells in a 1:1 ratio and replated in 24-well dishes. Luciferase activity was measured after 18 h. As a negative control, target cells were mixed with effector cells that lack HSV-2 gB.

**Flow cytometry.** HS cell surface expression was detected after HSV-2(333) infection. Vero cells were either uninfected or infected with HSV-2 (333) (MOI of 10) for 2 h. Cells were then washed with PBS, harvested and incubated with mouse anti-human HS mAb 10E4 (US Biological, Swampscott, MA; 1:50) in 1% BSA in 1× PBS for 30 min at 4°C. After that cells were washed and incubated for 30 min at 4°C with FITC-conjugated anti-mouse IgM (F9259 Sigma; 1:100). Cells stained only with FITC-conjugated anti-mouse IgM were used as background controls.

**G2 peptide binding assay.** Vero cells were either uninfected or infected with HSV-2(333) at MOI=10 for 2h at 37°C. Cells were then washed and treated with 1mg/ml FITC conjugated G2 peptide (G2-FITC) for 30 min at 37°C. G2-FITC binding to Vero cells was examined by fluorescence microscopy at 100X magnification. Images were analyzed using NIH imageJ v1.41 histogram analysis function. Also G2-FITC binding to uninfected or HSV-2 infected cells was analyzed by flow cytometry where untreated cells were used as background control.
**In Vivo** study to examine the G2 peptide inhibitory effect in a mouse model of herpesvirus genital infection. 4 Balb/c female mice were pretreated for 1h with either PBS (N=2) or 25ul G2-peptide (2mg/ml) (N=2). Mice were then infected with $1 \times 10^3$ pfu β-galactosidase expressing HSV-2 (gJ-). 24h after infection, mice were sacrificed by euthanasia. Genital organs were harvested, and vaginal tubes were longitudinally opened and fixed in 2% paraformaldehyde plus 0.02 % Nonident for 12 h, then tissues were incubated for 24 h in X-gal in ferricyanide buffer to stain infected cell colonies with dark blue color. Tissue samples were compressed to a glass slide and imaged using Zeiss AxioScope microscope equipped with a digital camera at 100× magnification. Mice were housed in the animal facility at the University of Illinois at Chicago Medical School. Experimental protocols involving animals were performed in accordance with the guideline of the University of Illinois at Chicago.

**Results**

Hydrophilic properties of G2 peptide and distribution of negative charges on 3-O sulfated HS. G2 peptide contains mostly positively charged amino acids in the sequence "MPRRRRISTRQK" with a MW of 1.71 kDa. Our analysis shows that its estimated charge at pH 7.00 is approximately +8 and the ratio of its hydrophilic residues to the total number of residues is 75% (Fig. 1A). We also found that G2 peptide is soluble in PBS and serum reduced Opti-MEM media without any significant pH shift for up to 20 mg/ml (11.6 mM). The putative cellular target of G2 peptide is 3-O sulfated HS (3-OS HS), which is a polysaccharide with repeating units of a disaccharide (Fig. 1B) (43). A complex distribution of negative charges originates from multiple modifications including sulfations during the biosynthesis of 3-OS HS.
G2 peptide binds to cell surface HS. In order to examine the affinity of G2 peptide to cell surface HS, it was conjugated with FITC to generate a fluorescent peptide (G2-FITC). Cells were pretreated with heparinase III (10 IU) that selectively cleaves HS chains from cellular surfaces and from extracellular matrices (22). G2-FITC binding to Heparinase-treated cells was determined by fluorescence microscopy (Fig. 2A) and by flow cytometry (Fig. 2B) and compared with G2-FITC binding observed with untreated cells. Removal of HS from the cell surface resulted in significant loss of G2-FITC binding to Vero cells and thus, less fluorescence signals were detected compared to untreated cells (Fig. 2). Vero cells treated with heparinase III only were used as background control. Similar results were obtained with HeLa cells (data not shown).

G2 peptide inhibits HSV-2 entry into host cells. After verifying that G2 peptide binds cell surface HS, the effect of the peptide on HSV-2 entry into HeLa cells was examined. A standard HSV entry assay, that utilizes a recombinant HSV-2 (333 gJ-) virus, which expresses a reporter β-galactosidase upon viral entry, was used (1). G2 peptide treatment resulted in an inhibition of HSV-2 entry into HeLa cells compared to untreated cells. The concentration of G2 peptide that showed its maximum inhibitory effect was determined to be around 2.5 mg/ml (Fig. 3A). It was interesting that the peptide concentrations higher than 2.5 mg/ml were little less effective. To demonstrate that the antiviral effect of the G2 peptide on HSV-2 entry was not cell type specific, HSV-2 entry was analyzed by fluorescence microscopy using Vero cells. Vero cells treated with 1mg/ml G2-FITC peptide showed less fluorescence signal compared to untreated or control peptide treated cells (Fig. 3B). For further confirmation, the effect of G2
peptide treatment on HSV-2 entry was analyzed by examining viral protein expression by Western blot analysis. HeLa cells were either untreated or pretreated with 2mg/ml G2 peptide, followed by HSV-2 infection. G2 peptide treatment resulted in around 80% reduction in VP16 levels indicating that less virus was able to enter these cells compared to untreated HeLa cells (Fig.4). The positive control represents a control sample for Western blot. Together, these results suggest that G2 peptide inhibits HSV-2 entry into host cells.

In order to determine the concentration of G2 peptide that produces 50% of its maximum inhibitory effect or IC$_{50}$ value, plaque reduction assays were performed using two different HSV-2 strains: HSV-2 strain 333 (gJ-) and the GFP tagged HSV-2 (GFP-HSV-2). G2 peptide pretreatment of Vero cells resulted in smaller size plaques, as well as, fewer plaques compared to untreated cells (Fig. 5A). Based on the inhibition levels of both plaque size and number after G2 peptide treatment, IC$_{50}$ value for G2 peptide is estimated to be approximately 1mg/ml. It was also interesting to note that the best inhibition was observed at 2.5mg/ml and increasing G2 peptide concentration further did not produce better results. Representative images of plaques formed after HSV-2 (333) gJ- and GFP-HSV-2 infection are shown in Fig. 5B.

**G2 peptide is non-toxic at 2.5 mg/ml or lower concentrations.** In order to examine the effect of G2 peptide on cell viability, direct cell counting methods to estimate the fraction of viable cells in treated cells were utilized. The morphology of HCE cells treated with serial dilution of G2 peptide showed that G2 peptide has cytotoxic effects at concentrations above 2.5 mg/ml. Brightfield microscopy images of HCE cells treated with serial dilution of G2 peptide are shown in Fig. 6A. Additionally, Cell viability of G2 peptide treated Vero cells was evaluated by counting fluorescent nuclei stained with live-cell nuclear stain Hoechst 33342. The number of
live cells fluorescent nuclei of G2 peptide treated Vero cells indicates that G2 cytotoxic properties are exhibited at concentrations above 2.5 mg/ml (Fig. 6B). Taken together, G2 peptide does not affect cell viability at concentrations below 2.5 mg/ml. Therefore, the determined value of G2 peptide IC₅₀ (1mg/ml) has no effect on cell viability.

**Pretreatment with G2 peptide affects cell-to-cell fusion.** G2 peptide was isolated based on its ability to bind 3-OS HS (43). Although 3-OS HS is identified as an HSV-1 entry receptor, studies have shown that it does not function as a receptor for HSV-2 (37). However, since G2 peptide resulted in significant inhibition of HSV-2 entry and plaque formation in various HSV-2 susceptible cell lines, we sought to investigate whether G2 peptide is capable of blocking HSV-2 induced cell-to-cell fusion. Membrane fusion represents a critical step during HSV entry, and an important event during virus spread (28, 42).

HSV-2 induced cell-to-cell fusion can be studied by co-cultivating two cell populations: “Target” and “Effector” cell populations. Target cells express gD receptor and the luciferase reporter gene under the control of T7 promoter. Effector cells express HSV-2 glycoproteins that are absolutely required for virus fusion (gB, gD, gH, and gL) plus T7 polymerase (33). In order to quantify cell-to-cell fusion, luciferase reporter gene activity is determined 18 h post mixing the two populations. As a negative control, target cells are mixed with effector cells that lack HSV-2 gB, where cell-to-cell fusion is expected to be reduced dramatically (28). The Chinese hamster ovary (CHO-K1) cells were used in cell-cell fusion assay, and were transfected with nectin-1 plasmid as a gD receptor. Target CHO-K1 cells were treated with a serial dilution of G2 peptide for 1 h at 37°C, then washed and mixed with effector cell population. Interestingly, G2 peptide treatment inhibited HSV-2 induced cell-to-cell fusion in a dose dependent manner (Fig. 7). This result indicates that G2 peptide acts on HSV-2 entry by blocking membrane fusion.
G2 peptide preferentially binds to infected cells. In order to investigate the binding of G2 peptide to HSV-2 infected cells, G2-FITC attachment to Vero cells that were pre-infected with HSV-2 was compared to the attachment of G2-FITC to uninfected Vero cells. According to the results, G2-FITC peptide showed more binding to HSV-2 infected cells in comparison to uninfected cells. G2-FITC attachment was evaluated by fluorescence microscopy, which demonstrates about 400 times increase, as well as, flow cytometric analysis, which shows about 214 times increase (Fig. 8). This result raises the possibility that HS expression is enhanced upon HSV-2 infection. It also suggests that G2 peptide has the capability of being used in targeted therapy, as it preferentially binds to HSV-2 infected cells.

HSV-2 infection results in an increase in HS expression on the surface of infected cells. Next, we sought to examine the possibility that HSV-2 infection affects HS expression on the cell surface. Vero cells were infected with HSV-2 (333) at an MOI of 10 for 2 h, HS expression on the cell surface was then examined using flow cytometry and compared to the levels of HS on uninfected cells. HSV-2 infection resulted in 25% increase in HS cell surface levels compared to HS levels on uninfected cells (Fig. 9). The increase in HS cell surface expression after HSV-2 infection may explain, at least partially, the higher affinity of G2 peptide to HSV-2 infected cells compared to uninfected cells as was shown in Fig. 8.

Intravaginal G2 peptide pretreatment reduces the number of HSV-2 infected cell colonies. A mouse genital herpes model was utilized to investigate whether G2 peptide’s antiviral action can lead to protection in vivo. Mice were pretreated with either PBS or 25μl G2
peptide (2mg/ml) for 1h. Mice were then inoculated with β-galactosidase expressing HSV-2 (gJ-) and sacrificed 24 h post infection. Vaginal tissues were dissected and stained with X-gal, and the number of genital lesions was counted. G2 peptide treated mice showed significantly lower number of genital lesions compared to PBS treated mouse (Fig. 10). Results suggest that G2 peptide is effective as a prophylactic drug against HSV-2. Importantly, this is the first study to show the importance of a 3-OS HS binding peptide in protection against HSV-2 genital infection in vivo.

Discussion

Our study has shown that G2 peptide, a 12-mer cationic peptide, exhibits antiviral activity against HSV-2. It inhibits HSV-2 entry, cell-to-cell fusion, and virus-induced plaque formation. Even more interestingly, G2 peptide demonstrates a significantly enhanced targeting of HSV-2 infected cells. As a possible explanation for that, we also demonstrate that HSV-2 infected cells show an increase in HS expression. This and virtually all other results shown here are novel and highly significant. They strongly raise the possibility that anti-3-OS HS agents can inhibit HSV-2 infection in vivo and therefore, provide significant protection from the development of disease symptoms. In addition, our data suggests that G2 peptide can be used to perform two independent roles in anti-HSV-2 therapy. It could be used to as a microbicide against the infection and in parallel; it can also be exploited for preferential delivery of other anti-viral drugs to infected cells.

The effect of G2 peptide is not only at the level of virus attachment; in addition, it also affects membrane fusion during entry. The evidence comes from our observation that G2 peptide blocks
cell-to-cell membrane fusion induced by HSV-2 glycoproteins (Fig. 7). In addition, G2 peptide treatment significantly reduced the number of plaques formed by HSV-2 and it also resulted in smaller plaques compared to untreated cells (Fig. 5). Plaque assays were performed in the presence of neutralizing antibodies, where plaque sizes depended exclusively on cell-to-cell spread of the virus, suggesting that G2 peptide reduces the efficiency of virus spread, most likely by blocking the membrane fusion required for spread. Interestingly, the concentration that most effectively blocks HSV-2 entry and plaque formation is at 2.5mg/ml (Fig. 3 and Fig. 5), increasing the concentration further shows less inhibitory effects. The rebound in percent reduction in plaque sizes and numbers noticed at 5mg/ml and 10mg/ml might be attributable to transmembrane channel opening, since G2 peptide has structural similarity to cell penetrating arginine rich peptides (44). The higher concentrations might result in the disruption of the host cell membrane integrity, and a receptor-independent entry and similarly, spread of the virions may be less inhibited. Studies will be performed in the future to further investigate this possibility.

Since G2 peptide bind was isolated against 3-OS HS, the suggested mechanism of activity is that G2 peptide binds to modified 3-OS HS on the cell surface preventing virus attachment and eventually the fusion of the virions with cells, and thus reducing virus entry. Consistent with that, Heparanase treatment to cells reduced the ability of G2 peptide to bind cultured cells (Fig. 2). The effect on membrane fusion was indicated by the observation that G2 peptide treatment to target cells inhibited HSV-2 induced cell-to-cell fusion (Fig. 7). So, the intriguing question is that if 3-OS HS is not a receptor for HSV-2 then what is the mechanism by which G2 peptide blocks entry and fusion? One possible explanation is that HSV-2 gD may contain a partial 3-OS HS
binding domain and blocking of which may block the membrane fusion altogether. The putative 3-OS HS binding domain of HSV-1 gD overlaps with HVEM and/or nectin-1 binding domains and that is why blocking 3-OS HS binding domain blocks entry via all the receptors (12,43). Therefore, the suggested mechanism of activity of the G2 peptide is a twofold effect by inhibiting virus attachment as well as membrane fusion during virus entry.

A related interesting finding is that G2 peptide binding by HSV-2 infected cells was significantly higher compared to uninfected cells. The dramatic, 200% increase in G2 peptide binding to HSV-2 infected cells (Fig. 8) cannot be explained solely in light of the increased HS availability on the surface of HSV-2 infected cells. Our interesting results indicate that HS cell surface expression also increased but only by 25% (Fig. 9). We propose that the infection-dependent increase in HS is accompanied by hyper 3-OS HS modifications and that may be a reason why the 3-OS HS binding G2 peptide shows a 200% increase in binding to the infected cells while the total HS is only increased by 25%. It has been reported that modifications within HS are altered in response to certain inflammatory and stress signals mediated by inflammatory cytokines (13). HSV infection is considered a viral inflammatory condition with proinflammatory cytokines and chemokines released from HSV infected cells during different phases of the infection. It is possible that the cytokines enhance the enzymatic activities of 6 or more 3-O sulfotransferases (25,50), which in turn, can induce hyper 3-O sulfation in newly synthesized HS moieties during HSV-2 infection. Currently, the precise functions of 3-O sulfotransferases are not very clear and it is possible that they respond to stress by increasing the 3-O sulfation in HS. Further studies must be done to identify the structural changes induced in HS upon HSV-2 infection.
The therapeutic margin of the G2 peptide is relatively narrow as its IC50 equals 1mg/ml while its CC50 (50% cytotoxic concentration) is around 10 mg/ml. Nevertheless, the enhanced binding of G2 peptide by infected cells is more than 200% higher than that of non-infected cells. This opens the door for targeted therapeutic potential and considerably widens the therapeutic margin of the G2 peptide. In addition, the high affinity of G2 peptide to HSV-2 infected cells versus uninfected cells advances the therapeutic applications of G2 peptide to be used as a delivery molecule in addition to its antiviral effect. It also paves the way for the discovery of new HS binding small molecules that can be used for preferential targeting of infected cells, which will avoid healthy cells and thereby reduce any associated cytotoxicity to healthy cells.

To our understanding, our animal study provides a first time proof on the importance of blocking 3-OS HS during HSV-2 infection in vivo. In this regard, administering G2 peptide to Balb/c female mice before HSV-2 challenge significantly reduced the number of vaginal herpetic lesions compared to untreated mice (Fig. 10). The peptide was well tolerated and the animals did not exhibit any adverse reaction to its application before or after the infection. However, extensive toxicology studies and more work needs to be done to determine whether a preventive application of the peptide can block the virus from establishing latency and likewise, the efficacy of the peptide needs to be examined under therapeutic conditions for treating an existing infection.

In summary, our results demonstrate that the cationic G2 peptide inhibits HSV-2 infection in cell culture as well as in vivo using the mouse model of genital herpes. G2 peptide binds to 3-OS HS disrupting virus attachment and entry into cells. G2 peptide protects mice from HSV infection.
and resulted in less herpetic genital lesions compared to untreated animals. These studies show the promise that G2 peptide or similar 3-OS HS binding agents can be developed into effective anti-HSV agents. Similarly, the 200% increase in G2 peptide binding to HSV-2 infected cells can be exploited in the future for diagnosis and targeted delivery of antiviral agents to the infected cells. The possibility that 3-O sulfation of HS itself may increase upon HSV-2 infection will almost certainly spark new studies on HS modifications and how they may be regulated in response to infection and stress.

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FIGURE LEGENDS

Fig. 1. G2 peptide structure. (A) The arrangement of the hydrophilic (grey) and hydrophobic (black) amino acid residues of the G2 peptide as calculated by Innovagen peptide property calculator (Hopp & Woods) accessible from http://www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp (as of August 2011). (B) 3-O sulfated HS. The disaccharide structure representative of 3-O sulfated HS is shown. The solid arrow shows the 3-O position of the glucosamine residue where sulfation is essential for HSV-1 gD binding. The broken arrows show the additional modifications, one or more of which may also be present as part of the gD-binding 3-OS HS.

Fig. 2. G2 peptide binds specifically to HS. (A) FITC conjugated G2 peptide (FITC-G2) binding to Vero cells with or without heparanase III treatment imaged by fluorescence microscopy. Vero cells were grown in chamber slides and either treated or not treated with heparanase III (10mlU) for 2h at 37°C. This was followed by incubation at 37°C for 30 min with FITC-G2 peptide. (B) FITC-G2 peptide binding to Vero cells with or without heparanase III treatment quantified by flow cytometry. Vero cells treated as described in A, and processed for flow cytometry. Vero cells treated with heparanase III only were used as background control. Results are representative of three independent experiments.

Fig. 3. G2 peptide inhibits HSV-2 entry into HeLa cells. (A) Confluent cultures of HeLa cells in 96 well plate were either left untreated or treated with serial dilutions of G2 peptide for 1h at 37°C. Cells were then infected with the recombinant β-galactosidase expressing HSV-2 strain (gJ-) at an MOI of 20. After 6h, the soluble substrate ONPG was added and enzymic activity...
was measured. Results are representative of three independent experiments. (B) Effect of entry blocking activity of G2 peptide was examined by fluorescence microscopy. Vero cells were not treated (No G2), treated with control nonspecific peptide (Cont. peptide), or treated with G2 peptide (G2) at 1mg/ml for 1h. Cells were then infected with GFP-expressing HSV-2 (GFP-HSV-2) at MOI = 10 for 2h, washed, and incubated for another 12h. Imaging was performed using 10× objective on a Zeiss Axiovert 200 fluorescence microscope. Representative images from one experiment performed in triplicates are shown. Images of G2 peptide treated cells are shown under both the fluorescent light and the bright field.

**Fig. 4. G2 peptide pretreatment reduces VP16 protein translocation into cells.** As a marker for HSV-2 entry, VP16 level was measured by Western blotting using lysates from HeLa cells untreated or treated with G2 peptide (2mg/ml) for 30 min, followed by HSV-2 infection for 4 h. Representative Western blot is shown. The positive control represents lysates from cells transiently-expressing VP16. The results are expressed as means ± 1SD values from two independent experiments.

**Fig. 5. IC_{50} of G2 peptide is approximately 1 mg/ml by plaque reduction assay.** Vero cells were either untreated or treated with serial dilution of G2 peptide for 1h at 37°C. Cells were then infected at 20-30 PFU/well with either HSV-2 (333) gJ- or with GFP tagged HSV-2 (GFP-HSV-2) for 2h at 37°C. After 2 h, cells were washed and overlaid with 1% methylcellulose in DMEM medium supplemented with 0.05% human pooled immunoglobulin G (IgG) for 48 to 72 h. (A) HSV-2 plaque number and size. Plaque number was counted under the microscope. Plaque size was measured using Axiovision software version 4 program. Results are representative of three
independent experiments. (B) Representative images of HSV-2 (333) gJ- plaques after crystal violet staining (upper panel), and GFP-HSV-2 plaques under fluorescent excitation light. Imaging was performed using Zeiss Axioscope microscope equipped with a digital low light CCD camera under control of the imaging software Axiovision at a 100x magnification.

Fig. 6. **G2 peptide affects cell viability at concentration above 2.5 mg/ml in a dose dependent manner.** (A) HCE cells were either untreated or treated with serial dilutions of G2 peptide in MEM complete medium for 24 hours at 37°C / 5% CO2. Morphological appearance of treated HCE cells was observed and compared to untreated cells at 100X magnification. (B) Cell viability of Vero cells, that were either untreated or treated with a serial dilutions of G2 peptide in DMEM complete medium for 24 hours at 37°C / 5% CO2, was examined by counting fluorescent nuclei of viable cells after staining the cells with Hoechst 33342 live-cell nuclear stain. Representative images of each condition are shown, as well as, the relative number of viable cells. Results are representative of three independent experiments.

Fig. 7. **Pretreatment of G2 peptide to Target cells affects cell-to-cell fusion in a dose dependent manner.** Target CHO-K1 cells expressing HSV-2 gD receptor nectin-1 and luciferase gene under the control of T7 promoter were preincubated with a serial dilution of G2 peptide or left untreated for 1 h at 37°C. Target cells were then mixed with effector cell population that expresses HSV-1 fusion glycoproteins plus T7 polymerase. As a negative control for the cell fusion assay, untreated target cells were mixed with effector cells that lack HSV-2
Luciferase reporter gene activity was determined to quantify cell-to-cell fusion. Results are presented as mean ± 1 SD of 3 independent experiments.

Fig. 8. G2 peptide is preferentially bound by infected cells population. Vero cells were either uninfected or infected with HSV-2 at MOI=10 for 2h at 37°C. Cells were then treated with 1mg/ml FITC conjugated G2 peptide (G2-FITC) for 30 min at 37°C. (A) G2-FITC binding was examined by fluorescence microscopy at 100X magnification. Images were analyzed using imageJ histogram analysis function. (B) G2-FITC binding by uninfected or HSV-2 infected cells was further quantified using flow cytometry analysis. Results are representative of six independent experiments.

Fig. 9. HSV-2 infection results in an increase in HS expression on the surface of infected Vero cells. The effect of HSV-2 infection on HS expression was analyzed using flow cytometric analysis of HS expression probed by FITC conjugated anti-HS Ab specific to epitope 10E4. Vero cells were infected with HSV-2 strain 333 at MOI=10 for 2 hours. Vero cells incubated with a FITC conjugated secondary Abs (isotypic control) were used as background control. Results are representative of three independent experiments.

Fig. 10. Intravaginal G2-peptide pretreatment reduces the number of HSV-2 infected cell colonies. Balb/c female mice were pretreated for 1h with either PBS or 25ul G2-peptide (2mg/ml). Mice were then infected with 1×103 pfu β-galactosidase expressing HSV-2 (gJ-). 24 h
after infection, mice were sacrificed and the vaginal tissue were harvested, fixed, and incubated in X-gal in ferricyanide buffer to stain infected cell colonies. Tissue samples were compressed to a glass slide and imaged using Zeiss Axioscope microscope equipped with a digital camera at 100x magnification. (A) Pictures of vaginal tissue from mice of the indicated treatments. (B) Pictures of HSV-2 (gJ-) vaginal lesions found in the vaginas from mice of the treatments noted (C) Quantitation of the vaginal lesions.
A

![Graph showing data on G2 peptide](image)

B

No G2  Cont. peptide  G2  G2 Bright Field