Eludiacting the Interactions between Heparanase and Heparanase 2 During a Herpes Simplex 2 Infection

BY

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THESIS

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DEDICATION

This thesis is dedicated to my wife. Without her love and companionship I would have never been able to start or finish this program.

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List of Abbreviations

ACV	Acyclovir
BSA	Bovine Serum Albumin
Cath L	Cathepsin L
cDNA	Complementary Deoxyribonucleic Acid
CMV	Cytomegalovirus
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
dpi	Days Post Infection
EBV	Epstein-Barr virus
EGR1	Early growth response 1
ECM	Extra Cellular Matrix
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gB	Glycoprotein B
gC	Glycoprotein C
gD	Glycoprotein D
gE	Glycoprotein E
gH	Glycoprotein H
gI	Glycoprotein I
gK	Glycoprotein K
gL	Glycoprotein L
gM	Glycoprotein
HIV	Human Immunodeficiency Virus
hrs	Hours

HS	Heparan sulfate
HSPG	Heparan sulfate proteoglycans
HPSE	Heparanase
HPSE 2	Heparanase 2
HGF	Hepatocyte growth factor
HSV-1	Herpes Simplex Virus type-1
HSV-2	Herpes Simplex Virus type-2
HHV-1	Human Herpes Virus 1
HHV-2	Human Herpes Virus 2
HHV-3	Human Herpes Virus 3
HHV-4	Human Herpes Virus 4
HHV-5	Human Herpes Virus 5
HHV-6A	Human Herpes Virus 6A
HHV-6B	Human Herpes Virus 6B
HHV-7	Human Herpes Virus 7
HHV-8	Human Herpes Virus 8
KSV	Kaposi's sarcoma virus
MMP-9	Matrix metalloproteinase 9
MEM	Minimum Essential Medium
mins	Minutes
MOI	Multiplicity of Infection
mRNA	Messenger Ribonucleic acid
NT cell	Natural killer t cell
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
ONPG	O-nitrophenyl-β-D-galactopyranoside
P/S	Penicillin/Streptomycin
PBS	Phosphate Buffered Saline
PFU	Plaque Forming Units

PI	Propidium Iodide
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RIPA	Radioimmunoprecipitation Assay
RNA	Ribonucleic Acid
RT	Room Temperature
S.E.M.	Standard Error of the Mean
shRNA	Short hairpin Ribonucleic Acid
TLR4	toll-like receptor 4
VZV	Varicella-zoster virus
VEGF	Vascular endothelia growth factor
Vk2 cells	Vaginal epithelial HPV-16 E6/E7 transformed

Summary

Herpes simplex virus 2 (HSV-2) can productively infect many different cell types of human and nonhuman origin. Nucleoside analogs such as acyclovir (ACV) are currently prescribed clinically to curb this infection. Here we demonstrate interconnected roles for host enzymes, heparanase (HPSE), heparanase 2 (HPSE 2) and cathepsin L (Cath L), in HSV-2 release and inhibition in cells. In vaginal epithelial cells, HSV-2 causes heparan sulfate shedding and upregulation in HPSE, HPSE 2 and, Cath L levels during the productive phase of infection. Furthermore, inhibition of HPSE dramatically reduces HSV-2 release from vaginal epithelial cells, while HPSE 2 inhibition also show markedly less infection. Overexpression of HPSE leads to an increase in viral progeny, interestingly overexpression of HPSE 2 leads to less virus production. We propose that the HPSE increase after infection is mediated by an increased NF-_B nuclear localization and a resultant activation of HPSE transcription. Together these mechanisms contribute to the removal of heparan sulfate from the cell surface and thus facilitate virus release from cells. For HPSE 2 we propose that the increase after infection is a response to the clearing of HS that can lead to some inhibition of infection, though more work is needed on this. Finally, we also studied a small molecule inhibitor, BX795, as a new and alternative solution to reduce HSV burden in humans. We report evidence for strong antiviral efficacy of BX795 on HSV-2 infection in vaginal epithelial cells in vitro at 10 μM and in vivo at 50 μM. Additionally, through biochemical assays in vitro and histopathology in vivo, we show the tolerability of BX795 in vaginal epithelial cells at concentrations as high as 80 μM. Finally, using a murine model of vaginal infection, we show that topical therapy using 50 μM BX795 is well tolerated and efficacious in controlling HSV-2 replication.

CHAPTER 1: INTRODUCTION

Overview of Herpesviruses

One of the largest virus families is the family *Herpresviridae*, which Herpesviruses are members of. This virus family contains over 130 known well-characterized and morphologically similar viruses that can infect most vertebrates and at least one invertebrate. Of the 130 known herpesviruses, only nine infect humans: herpes simplex virus type-1 also known as human herpes virus 1 (HSV-1/HHV-1), herpes simplex virus type-2 (HSV-2/HHV-2), varicella-zoster virus (VZV/HHV-3), cytomegalovirus (CMV/HHV-4), Epstein-Barr virus (EBV/HHV-5), human herpesvirus 6a and 6b (HHV-6A and HHV-6B), human herpesvirus 7 (HHV-7), and Kaposi's sarcoma virus (KSV/HHV-8). The human Herpesviruses are divided into three subfamilies, α, β, and γ on the basis of tissue tropism and similarity of DNA sequence. The α herpesviruses are classified as neurotropic viruses and establish latency in neurons, while β and γ herpesviruses establish latency in lymphocytes and persist in lymphoid tissue. Herpesviruses are known to share six hallmark characteristics: ubiquity, latency, incurability, reactivation, unapparent infection, and opportunistic infection. Herpesviruses are extremely ubiquitous within the US population, about 50 percent of children and adults under the age of 50 are infected with HSV-1, and about one in eight are infected with HSV-2.

Alpha

Three herpes viruses are categorized as alpha herpes virus members, HSV-1, HSV-2 and VZV. HSV- 1 usually causes a labialis condition and is the most common cause for this condition worldwide. Recurrent reactivation episodes can be disfiguring while also being painful and very frequent. HSV-1 can also cause genital outbreaks with new genital cases being around 50 percent caused by HSV-1, ocular infections with it being the leading infection related cause of blindness in the world, oropharyngeal infections, and encephalitis in immune compromised populations(1-5). HSV-2 is the primary cause of genital herpes witch is characterized by painful lesions and

inflammation in the genital region. With males presenting with infection of the shaft of the penis or anal area, and women mainly in the vaginal region and can present in the cervix and perianal. HSV-2 genital infection is also associated with increased risk of HIV transmission and acquisition HSV-2 can also cause ocular infection and labialis infection albeit less so that HSV-1(3, 6-9). VZV is a highly ubiquitous neurotropic virus in which primary infection presents as a chickenpox infection. A primary varicella infection presents with inflamed ulcers on the skin, fever and fatigue (10, 11). Following initial infection VZV goes latent in neurons of the dorsal root ganglia, cranial nerve ganglia, and autonomic ganglia (11). As people age the experience a natural decline in T-cell-mediated immunity VZV (12). VZV frequently then reactivates to produce zoster or shingles, which is characterized with a rash and severe pain (13). Complications of zoster include postherpetic neuralgia, multiple neurologic disorders, and ocular disease.

Beta

The beta herpesvirus subfamily includes four human herpes viruses, CMV, HHV-6A, HHV-B and HHV-7. CMV is a very common virus in the United States with 33% of children under the age of 5 affected and over 50% of adults by age 40(14). CMV can present with fever, pharyngitis, and lymphadenopathy. Occasionally CMV can cause mononucleosis and hepatitis and with immune compromised individuals there can be more serious symptoms(15). Babies whom acquire congenital CMV can have symptoms at birth of jaundice, low birth weight, hepatosplenomegaly and seizers, with long term health complications of microcephaly, hearing loss, and vision loss(16). HHV-6A, 6B where discovered in 1986 and is present in 90% of the population and is generally acquired in childhood. HHV-6A and 6B share 90% sequence homology with each other and 50% with HHV-7. HHV-6A and 6B infect the central nervious system of humans and are able to integrate their genome into chromosomes which is rare in the

larger *Herpresviridae* family(17, 18). HSV-6 infections in childhood are generally mild or asymptomatic, there are cases of mesial temporal-lobe epilepsy that occur rarely in HHV-6B infections(19). HHV-7 is very similar to HHV-6 in that it is highly represented in the adult and adolescent population presenting with mild symptoms or asymptomatic(20). A difference occurs in solid organ transplant patients, where in up to 40% of patients there is reactivation that presents with fever and thrombocytopenia(21).

Gamma

The gamma herpes subfamily has two herpes virus which are EBV and KSV. More than 50 years ago EBV was discovered as the first human oncogenic virus. Around 90% of adults show evidence of past infection, with infection lifelong but asymptomatic(22). EBV has been found to be associated with a plethora of new and abnormal tissue growth diseases, such as Burkitt lymphoma, Hodgkin lymphoma, AIDs-related non-Hodgkin lymphoma, post-transplant lymphoproliferative disorders, diffuse large B cell lymphoma, NK/T cell lymphoma, nasopharyngeal carcinoma, and EBV-positive gastric cancer(23-26). KSV is less prevalent in world populations showing 2-50% of people infected, but it is heavily depended on the part of the world with 3-20% in south America and over 50% in sub-Saharan Africa(27). KSV commonly cause skin but, KSV also expresses as a highly metastatic tumor often involved with visceral organs. KSV tumors are characterized by vast proliferation of small vessels that lack a basement membrane, having leaky membranes, with micro hemorrhages and hemosiderin depositions(28, 29).

Herpes Simplex 2 Mediated Genital Infection

Herpes Simplex-2 is the leading cause of genital herpes which is one of the most persistent sexually mediated viral diseases. In the United States since 1999 the total seroprevalence of HSV-

2 has gone from around 19% to lower than 15% as of 2016(30, 31). In African Americans it has gone from around 43% to around 35%, this is alarming when you consider that the seroprevalence with the non-hispanic white population was around 14% in 1999 and has come down to below 10%(32). Also infection rates among women is higher than men in every subset of the US population. Most people who have genital herpes have mild symptoms or are asymptomatic and are not even aware that they have the disease, although they can transmit the virus readily (30).

Structure

The herpes simplex virus has a large double stranded DNA genome It very large where it has a diameter of ~200 nm and is comprised of 5 comprises four distinct components: an envelope, the envelope is covered in glycoproteins, the tegument which is a layer of proteins that lie in between the viral envelope and the nucleocapsid, an icosahedral capsid, and at the core is the double stranded DNA genome (Fig 1)(33). The glycoproteins are involved with attachment and subsequent entry of the virus into the cell. The capsid not only protects the viral genome from damage but also plays an important role in the release of viral genome into the nucleus of the host cell(34).

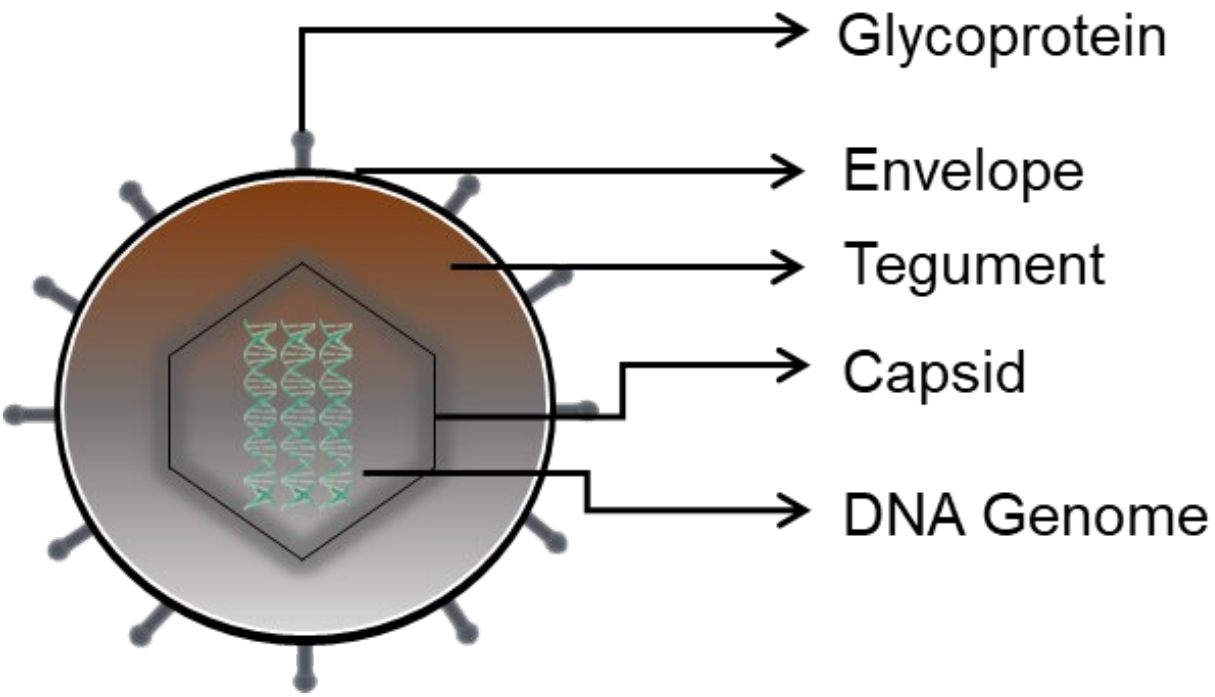

Figure 1. Schematic of HSV Structure

HEPARANASE

Human heparanase (HPSE) is an endoglycosidase (endo-β-Dglucuronidase) with the unique distinction of being the only mammalian enzyme capable of degrading heparan sulfate (HS)(35). HS is an evolutionarily conserved glycosaminoglycan that is present ubiquitously at the cell surface found covalently attached to a small set of extracellular matrix and plasma membrane proteoglycans of virtually all cells, HS can found covalently attached to a small set of extracellular matrix and plasma membrane proteoglycans (35). HPSE is initially translated as a pre-proenzyme, cleavage of a signal sequence by a signal peptidase, which leaves an inactive 65 kDa proHPSE, which must undergo further processing for activity14(35, 36). Proteolytic removal by Cathepsin L of a linker liberates an N-terminal 8 kDa subunit and a C-terminal 50 kDa subunit, which remain associated as a non-covalent heterodimer in mature active HPSE (Fig. 2)(37). Heparanase is preferentially expressed in human tumors and its over-expression in tumor cells causes the cleavage of HS leading to an invasive phenotype in experimental animals (36). HS has been shown an many studies to be a regulator of inflammatory responses at multiple levels, including but not restrained to sequestration of cytokines and chemokines in the extracellular space, modulation of ECM, and modulation of the innate immune response by sequestering or releasing at the cell surface, toll-like receptor 4 (TLR4)(38-42). This way, HS cleavage and ECM remodeling by heparanase can effect different levels of inflammatory reactions, including extravasation and migration towards inflammation sites, leukocyte recruitment, release of cytokines and chemokines anchored within HS(43-45). .It has recently been reported that host-encoded HPSE is upregulated and required for the release of viral progeny from parent cells after HSV-1 infection, HSV-2 infection, and in porcine reproductive and respiratory syndrome virus (PRRSV) infection (46-48).

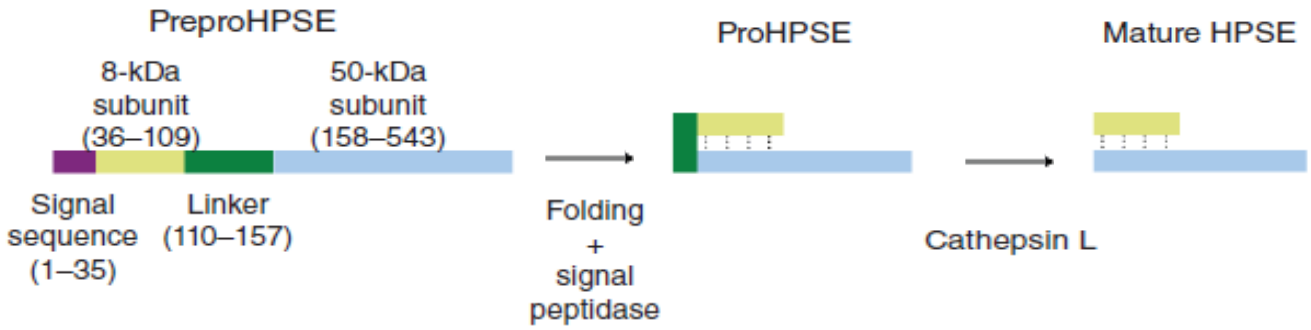
PreproHPSE
8-kDa subunit (36–109)
50-kDa subunit (158–543)
Signal sequence (1–35)
Linker (110–157)
Folding + signal peptidase
ProHPSE
Cathepsin L
Mature HPSE

Figure 2. Schematic representation of HPSE biogenesis

The signal sequence indicated in purple, the 8kDa subunit is in yellow, the linker segment that is cleaved by cathepsin L is in green, and the 50kDa subunit is indicated in blue.

Cancer

Under physiological conditions, in the majority of non-cancerous cells and tissues, HPSE promoter activity is constitutively inhibited, and posttranslational proteolytic processing is tightly regulated. HPSE expression is simulated by wild type P53, various mutant variants of P53, early growth response 1 (EGR1), reactive oxygen species, and inflammatory cytokines(35, 36). HPSE in cleaving cell surface HS, releases growth factors and cytokines, and HPSE is implicated in gene level regulation of vascular endothelia growth factor (VEGF), hepatocyte growth factor (HGF) and matrix metalloproteinase 9 (MMP-9) which promote aggressive tumor behavior(49, 50). In many cancers studies have shown elevated HPSE transcript and protein levels, and shown correlation with upregulated HPSE and increased tumor size, tumor progression, and poor patient prognosis(51). While studies involving tumor mouse models have shown treatment with heparanase inhibitors lead to a reduction in tumor size(52). This shows that looking into anti heparanase drug treatment for cancer is a viable worthy investigate.

Inflammation

There's been a link for decades between HS-degradation activity in neutrophils and inflammation, many studies have found a link between the two contributing to neutrophils ability of vascular escape and accumulation in a target tissue of the body(43, 45). This link is further strengthened by anti heparanase treatments demonstrating anti-inflammatory effects in many different studies(52, 53). Heparanase in epithelial cells can also have an inflammatory role in the

nucleus where it has been shown that it has a regulatory role in genes involving inflammation. Heparanase is also induced in the presence of inflammatory cytokines and in infection by ether bacteria or herpes viruses(49). Mounting evidence suggests that heparanase affects the activities of neutrophils, with neutrophils having a large role in acute inflammatory responses(54). It has also been reported that enzymatic degradation of endothelial cell surface HS and associated proteoglycans contribute to sepsis-associated lung injury(55). It was shown in a mouse model and in human samples taken from inflammatory lung disease patients that in sepsis-associated inflammatory lung disease, there is a large TNFα-dependent induction of heparanase activity(56-58). There are studies of heparanase in inflammatory hyperalgesia, neuroinflammation, modulation of macrophage responses can cause inflammatory bowel disease (IBD), ulcerative colitis and Crohn's disease(38, 49, 59, 60).

Infection

HSV-1 and HSV-2 use HS in a well-studied mechanism of attachment where herpes glycoproteins gB and gC bind to HS facilitating further entry into the cell(61, 62). Our lab has shown that upon infection there is a virus mediated increase in HPSE transcripts and in protein levels(46). It has also been shown that treatment with endogenous bacterial HPSE after infection reduce entry of HPSE into cells(36). Subsequent studies have shown the γ34.5 viral protein to be responsible for HPSE upregulation upon infection(63). Alongside an overall increase in protein levels there was also shown an increase in the 58kD heterodimer enzymatically active form of HPSE through cathepsin L activation, and large increase in the cell surface localization of HPSE(47). Our studies have shown in an HSV-1 mouse eye infection model that transfection with a constitutively active HPSE protein (GS3) leads to significantly worst infection in terms of clinical scores of disease and corneal ulceration and tissue damage(46). This damages was show

to be much less in HPSE shRNA treated mouse eyes that where then infected, there was less infection and much less damage the eye and disease to the mouse(46). HPSE is known to facilitate viral egress by cleaving cell surface HS during infection. While HS is important for attachment there is evidence that too much HS at the cell surface during egress can be detrimental to viral egress. In experiments where there was treatment with OGT 2115, which is a small molecule enzymatic inhibitor of HPSE, there was a large increase at the cell surface of HS, and during infection this lead to a large inhibition of virus(47, 63). This is important since during normal HSV infections there is a large loss of cell surface HS. While overexpression of HPSE lead to more virus released *in vivo* and *in vitro*. Other viruses also use HS as a surface receptor and/or use HS proteoglycans for entry, including KSHV, human papilloma virus, Dengue virus, respiratory syncytial virus, and porcine reproductive and respiratory syndrome virus(48, 64).

CHATHEPSIN L

There are eleven members of the cysteine cathepsin enzyme family, they have a wide range of expression and functional profile. They are Cathepsin L, B, C, , H, K, O, V, X, and S, they can involve in endocytosis, phagocytosis, autophagy and secretion, protein degradation, post-translational modifications of proteins, extracellular matrix (ECM) remodeling, and immune signaling(65). Cathepsin L (Cath L) is a papain like cysteine proteinase that is first synthesized as a preproenzyme. The linker peptide is removed in the endoplasmic reticulum and later Cath L is autoactivated in the acidic condition of the late endosome and lysosome. It resides in its mature form in the lysosome where it acts as an endopeptidase(65). Cathepsin L is the only known activator of proheparanase, it was shown in Cath L knockout cells that there was no HPSE activation at all and that overexpression of Cath L leads to augmentation of proheparanase

activation(37). In addition of Cath L‘s HPSE activity is widely implicated in a large variety of cancers(66).

Cancer

Cathepsin L is ubiquitously expressed and has been strongly associated in many models with malignancy. Cytokines that are tumor secreted and are involved in malignant tumor progression including: PDGF, EGF, NGF, INFγ, VEGF, FGF and IL-6, have been shown to lead to more activity of the CATH L promoter activity and a rise in Cath L protein levels(67). Showing concurrence with the tissue culture data, in a wide range of human cancers Cath L upregulation has been reported including: prostate, lung, gastric, breast, pancreatic, ovarian and colon cancers. Normally in tissues Cath L is highly regulated by Cath inhibitors for example cystatins and stefins(68, 69). These endogenous inhibitors have been shown significantly downregulated in chronic cancer patients leading to more Cath L expression(70).

Infection

Cath L through its HPSE activation and cell surface modulation has been shown to have a role in HSV infection. It’s been shown that upon infection that there’s an increase in Cath L transcripts and Cath L protein levels. Further that with treatment with Cath L enzymatic inhibitor during infection that there is a loss of active HPSE and a detrimental impact on viral spread (47). Shown in this thesis is that overexpression of Cath L is beneficial first to the amount of active HPSE increasing it, and is beneficial to virus replication. This viral activity through regulation of HPSE implicates roles to Cath L in many viral infections including KSHV, human papilloma virus, Dengue virus, respiratory syncytial virus, and porcine reproductive and respiratory syndrome virus(71, 72).

HEPEARANSE 2

McKenzie was the first to show the cloning of heparanase homolog named HPSE 2. HPSE 2 encodes three proteins generated by alternative splicing HPSE 2 a,b, and c, and shares an overall identity of 40 percent and sequence of 59 percent with heparanase. Full-length HPSE 2 gene consists of 2353 base pairs encoding proteins of 592 amino acids (HPSE 2c), 480 aa (HPSE 2a), and 534aa (HPSE 2b)(73). One of the main differences and cause of lack of enzymatic activity of HPSE 2 can be that of linker region of pro-heparanase is not conserved in HPSE 2. HPSE 2 unlike HPSE does not undergo Cath L mediated cleavage and hence lacks cell surface HS cleavage. Notably, only Hpa2c is secreted. HPSE 2c has the ability to bind HS but not cleave. Studies have shown that HPSE 2 has the ability to bind heparin and HS and shows a greater affinity towards heparin and HS than heparanase, thereby acting as a competitor for HS binding and inhibiting heparanase enzymatic activity there have also been studies that show the direct interaction of HPSE and HPSE 2 possibly showing a direct inhibition of HPSE by HPSE 2 but more studies in that area need to be done in that regard(74).

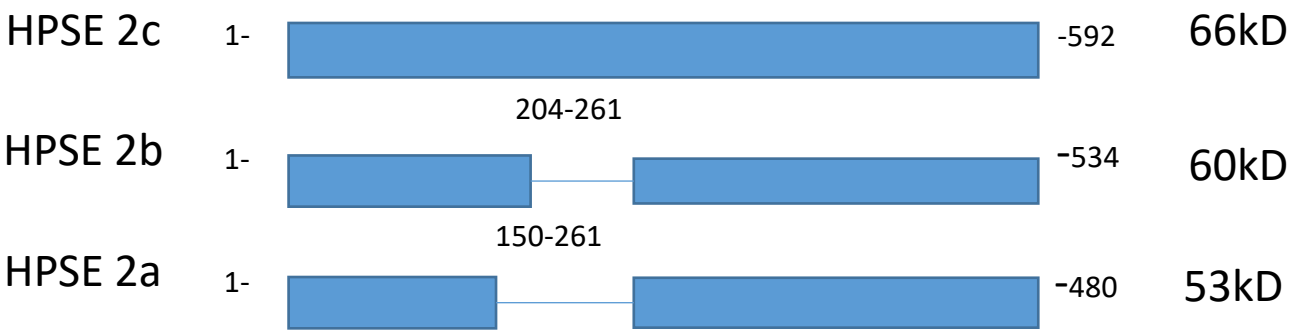

Figure 3. Splice variants of HPSE 2

HPSE 2 c is the longest at 66kD, then next is HPSE 2b at 60kD, and smallest is HPSE 2a at 53kD.

Cancer

HPSE 2 is associated with many human cancers in an expression pattern that inversely correlated with cell metastasis. In studies looking at head neck cancer it was shown that upon over expression of HPSE 2 that there was a decrease in tumor xenograft size compared to control cells. In bladder cancer where in HPSE 2 is normally expressed in the epithelium there is a large decrease in the expression the HPSE, which characteristic of a tumor suppressor(75). Tumors that exhibit high levels of HPSE 2 were diagnosed as low grade and stage. Also it was shown that tumors that received exogenous treatment with purified HPSE 2 exhibited attenuated migration and showed regression in size(76). In pancreatic cancer HPSE 2 has been show to attenuate cancer size and severity through ER stress stimulation leading to apoptotic cell death. In the exogenous treatment study they also showed that there was no change in HPSE activity upon treatment suggesting an alternative HPSE interaction independent method of action for tumor suppression (77).

Infection

HPSE 2 is the only known protein to sequester heparan sulfate (HS), a polysaccharide that is ubiquitously expressed at the cell surface, by inhibiting heparanase (HPSE). HPSE 2 inhibits HPSE competitively by targeting the same epitopes that HPSE targets with greater affinity (74). In this thesis I show that upon infection with HSV-2 there is a large increase to HPSE 2 transcripts and protein levels. After over expression of HPSE 2 and infection there is a large attenuation of viral replication and egress. Furthermore after knockdown of HPSE 2 there is also shown a detriment in viral replication. This is possibly through HS regulation, there is after infection an increase in cell surface localization and a sequestering of cell surface HS after overexpression of HPSE 2. This sequestering of surface HS could impact virus egress. HSPE 2 could also have some

HS and HPSE independent virus inhibitory roles do to all HPSE 2 variants having anti HSV properties though only HPSE 2c is excreted.

CHAPTER 2: Methods

Methods for Chapter 3

Cells and viruses.

Human vaginal epithelial (VK2/E6E7) cell line obtained from ATCC. VK2/E6E7 cells were passaged in Karatinocyte serum free medium (KSFM) (Gibco/BRL, Carlsbad, CA, USA) supplemented with epidermal growth factor (EGF), bovine pituitary extract (BPE) and 1% penicillin/streptomycin. For convenience this cell line is referred to as VK2 cells throughout. All infections were done with HSV-2 333 at MOI of 0.1 or 1 on VK2 cells unless mentioned otherwise. The Vero cell line cell line (African green monkey kidney) was generously given by Dr. Patricia G. Spear (Northwestern University, Chicago, IL) and cultured in DMEM (Gibco) with 10% FBS and 1% penicillin/streptomycin. Cathepsin L inhibitor IV (Santa Cruz Biotechnology) was used as a cathepsin L activity inhibitor (78). Cathepsin L inhibitor was used at a concentration of 10 μg/mL in DMSO unless otherwise specified. OGT 2115 (Tocris Biosciences) was used for heparanase activity inhibition and has been previously described as a HPSE inhibitor (61). OGT 2115 was used at 10 μM unless otherwise specified. Viruses used were wild type HSV-2 (333), and HSV-2 (333) GFP (79). Virus stocks were grown and tittered on Vero cells, and stored at −80°C.

Antibodies and plasmids.

HPSE antibody H-80 (Santa Cruz Biotechnology) was used for imaging (1:100) and flow cytometry studies (1:100). Cathepsin L antibody ab58991 (Abcam, Cambridge MA) was used for western blot analysis (1:2,000), imaging (1:100) and flow cytometry studies (1:200). Histone H3 antibody (Cell Signaling Technology, Danvers, MA) was used at a dilution of 1:500 for western blot analysis. Anti-human HS monoclonal antibody 10E4 (US Biological, Salem, MA) was used for flow cytometry (1:100) and cell imaging (1:100). NF-kB p65 antibody C-20 (Santa Cruz

Biotechnology) was used for imaging (1:500) and western blot analysis (1:2,000). GAPDH (Santa Cruz Biotechnology) was used for western blot analysis at dilution 1:2,000. The IkBa (S32/36A) plasmid was provided by Dr Michael Karin (University of California, San Diego, La Jolla, CA). All transfections were performed using Lipofectamine-2000 (Life Technologies).

Western blot analysis.

Proteins from samples in this study were collected using radio immunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, St Louis, MO) according to the manufacturer's protocol. After gel electrophoresis, membranes were blocked in 5% BSA for 1 h followed by incubation with primary antibody for 1 h, then incubation with respective secondary antibodies (anti-mouse 1:10,000, anti-rabbit 1:10,000) for 1 h. Protein bands were visualized using the SuperSignal West Femto maximum sensitivity substrate (Thermo Scientific, Waltham, MA) with Image-Quant LAS 4000 biomolecular imager (GE Healthcare Life Sciences, Pittsburgh, PA). The densities of the bands were quantified using ImageJ 1.52a image analysis software (NIH, USA).

PCR.

Trizol (Life Technologies) was used to obtain RNA following the protocol from the manufacturer then a cDNA Reverse Transcription Kit from (Applied Biosystems) was used to transcribe to DNA following the protocol from the manufacturer. Fast SYBR Green Master Mix (Applied Biosystems) was used to perform real-time quantitative PCR, using QuantStudio 7 Flex (Applied Biosystems). The primers used in this study are as follows: HPSE forward primer 5'-CTCGAAGAAAGACGGCTA-3' and reverse primer 5'-GTAGCAGTCCGTCCATTC-3'; NF-kB p65 forward primer 5'-TGGGGACTACGACCTGAATG-3' and reverse primer 5'-GGGGGCACGATTGTCAAAGA-3'; GAPDH forward primer 5'-

TCCACTGGCGTCTTCACC-3’ and reverse primer 5’-GGCAGAGATGATGACCCTTTT-3’; cathepsin L forward primer 5’- ATCTGGGCATGAACCACCTG-3’ and reverse primer 5’-CAGCAAGCACCACAAGAACC-3’.

Luciferase assay.

An empty vector, pGL3-Basic (Promega, Madison, WI), was used to control for transfection efficiency. The pHep1(0.7 kb)-luc plasmid that expresses firefly luciferase that is driven by the 0.7-kb upstream promoter for transcription of human HPSE was provided by Dr Xiulong Xu (Rush University). Luciferase activity was measured using Berthold detection systems Luminometer.

Flow cytometry.

Measurement of HS and HPSE cell surface expression was performed after HSV-2 333-WT infection. Monolayers of VK2 cells were infected at MOI 0.1 or 1 then harvested at different times post infection (12 hpi, 24 hpi, 36 hpi, and 48 hpi). Cells were harvested and fixed with 4% PFA for 10 min, then incubated with 5% BSA for 1 h followed by incubation with FITC-conjugated anti-HS diluted 1:100 in PBS with 1% BSA for 1 h. Cells were then suspended in FACS buffer (PBS, 5% FBS, 0.1% sodium azide). For detection of HPSE on the cell surface, cells were harvested then fixed with 4% PFA for 4 min, then incubated with 5% BSA for 1 h, then incubated with primary antibody diluted in PBS with 5% BSA for 1 h, followed by incubation with FITC-conjugated secondary antibody. Cells were then resuspended in FACS buffer. Cells stained with respective FITC-conjugated secondaries only were used as background controls. Entire cell populations were used for the mean fluorescence intensity calculations.

Immunofluorescence microscopy.

VK2 cells were cultured in glass bottom dishes (MatTek Corporation, Ashland, MA). Cells were fixed in 4% PFA for 10 min and permeabilized with 0.1% Triton-X for intracellular labelling, then incubated with 5% BSA for 1 hour then primary antibody diluted in PBS with 5% BSA for 1 hour then with FITC-conjugated secondary. For HPSE surface staining the protocol was 5% BSA for 1 hour then primary antibody diluted in PBS with 5% BSA for 1 hour then with FITC-conjugated secondary. Imaging was done with a Zeiss Confocal 710, Germany. Pinhole was set to 1 Airy Unit. Fluorescence intensity of images was calculated using Zen software.

Plaque assay.

Viral egress titers were measured using a plaque assay. Monolayers of VK2 cells were plated in twelve-well plates and infected with HSV-2 333 virus at MOI 0.1 or 1. Media were collected at different time points post infection and titered on Vero cells. Primary incubation of collected media was performed with DPBS+/+ (Life Technologies) with 1% glucose and 1% heat-inactivated serum for 2 h. Vero cells were then incubated with growth media containing 5% methylcellulose for 72 h followed by fixing with 100% methanol and staining with crystal violet solution.

Statistics.

Error bars of all figures represent standard error of three independent experiments, unless otherwise specified. Asterisks denote a significant difference as determined by Student's t-test; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, ns, not significant.

Methods for Chapter 4

Cells and viruses.

Human vaginal epithelial (VK2/E6E7) cell line obtained from ATCC. VK2/E6E7 cells were passaged in Karatinocyte serum free medium (KSFM) (Gibco/BRL, Carlsbad, CA, USA) supplemented with epidermal growth factor (EGF), bovine pituitary extract (BPE) and 1% penicillin/streptomycin. For convenience this cell line is referred to as VK2 cells throughout. All infections were done with HSV-2 333 at MOI of 0.1 or 1 on VK2 cells unless mentioned otherwise. OGT 2115 (Tocris Biosciences) was used for heparanase activity inhibition and has been previously described as a HPSE inhibitor (61). OGT 2115 was used at 10 μM unless otherwise specified. Viruses used were wild type HSV-2 (333), and HSV-2 (333) GFP (79). Virus stocks were grown and tittered on Vero cells, and stored at −80°C.

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Flow cytometry.

Measurement of HS and HPSE cell surface expression was performed after HSV-2 333-WT infection. Monolayers of VK2 cells were infected at MOI 0.1 or 1 then harvested at different times post infection (12 hpi, 24 hpi, 36 hpi, and 48 hpi). Cells were harvested and fixed with 4% PFA for 10 min, then incubated with 5% BSA for 1 h followed by incubation with FITC-conjugated anti-HS diluted 1:100 in PBS with 1% BSA for 1 h. Cells were then suspended in FACS buffer (PBS, 5% FBS, 0.1% sodium azide). For detection of HPSE on the cell surface, cells were harvested then fixed with 4% PFA for 4 min, then incubated with 5% BSA for 1 h, then incubated with primary antibody diluted in PBS with 5% BSA for 1 h, followed by incubation with FITC-conjugated secondary antibody. Cells were then resuspended in FACS buffer. Cells stained with respective FITC-conjugated secondaries only were used as background controls. Entire cell populations were used for the mean fluorescence intensity calculations.

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Viral egress titers were measured using a plaque assay. Monolayers of VK2 cells were plated in twelve-well plates and infected with HSV-2 333 virus at MOI 0.1 or 1. Media were collected at

different time points post infection and titered on Vero cells. Primary incubation of collected media was performed with DPBS+/+ (Life Technologies) with 1% glucose and 1% heat-inactivated serum for 2 h. Vero cells were then incubated with growth media containing 5% methylcellulose for 72 h followed by fixing with 100% methanol and staining with crystal violet solution.

Statistics.

Error bars of all figures represent standard error of three independent experiments, unless otherwise specified. Asterisks denote a significant difference as determined by Student's t-test; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, ns, not significant.

Methods for Chapter 5

The reagents used in this study are mentioned below

Table 1

REAGENTS	**SOURCE**
Vaginal epithelial cells (VK2)	P. G. Spear's laboratory at Northwestern University
African green monkey kidney (Vero) cells	P. G. Spear's laboratory at Northwestern University
HSV-2 (333)	P. G. Spear's laboratory at Northwestern University

HSV-2 (333 GFP)	P. G. Spear's laboratory at Northwestern University
Keratinocyte Media (KSFM)	Gibco
OPTI MEM	Gibco
Penicillin and streptomycin (P/S)	Sigma-Aldrich
Fetal bovine serum (FBS)	US origin Sigma-Aldrich
BX795	Selleckhem
Anti-HSV-1 gD mouse monoclonal	Abcam
Anti-HSV-1 VP16 mouse monoclonal	Abcam
Anti-EIF4E	Cell signaling technology
Anti-Akt rabbit monoclonal	Cell signaling technology
Anti-phospho-Akt- Ser473 rabbit monoclonal	Cell signaling technology
Anti-phospho-4E-BP1-Thr37/46 rabbit monoclonal	Cell signaling technology
Anti-GAPDH rabbit polyclonal	Proteintech
Anti-Mouse Secondary HRP antibody	Jackson Immunoresearch Lab
Anti-Rabbit Secondary HRP antibody	Jackson Immunoresearch Lab
Anti-Rabbit Secondary FITC antibody	Cell signaling technology

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Total RNA from samples was isolated from cells using TRIzol extraction method similar to previously reported study(80). Once the RNA was extracted, they were reverse transcribed using

a reverse transcription cDNA kit (Life Technologies) according to manufacturer's protocol. The cDNA was then mixed with Fast SYBR Green Master mix and pre-designed primers to run a real time PCR. The primers used in this study are tabulated below.

Table 2

Gene	Direction	Sequence
ICP27	Forward	5' TGT CGG AGA TCA ACT ACA CG 3'
ICP27	Reverse	5' GGT GCG TGT CCA GTA TTT CA 3'
UL30	Forward	5' GAC ACG GAC TCC ATT TTC GT 3'
UL30	Reverse	5' AGC AGC TTG GTG AAC GTT TT 3'
gD	Forward	5' TAC TAC GCA GTG CTG GAA CG 3'
gD	Reverse	5' CGA TGG TCA GGT TGT ACG TG 3'

Western Blotting:

Total protein from all the samples was isolated with RIPA (Radio immuno-precipitation assay) buffer using a protocol previously reported (81). Equal amounts of protein samples were loaded into 4 to 12% SDS–polyacrylamide gel, and run for three hours at the constant speed at 70V. The protein from gels was transferred to a nitrocellulose membrane prior to blocking the membrane with 5% skim milk (Difco). All the primary antibodies were diluted in 5% milk at 1:1000 ratio and all the secondary antibodies were diluted at 1:10,000. The protein content was analyzed by the addition of HRP substrate to the membranes and imaging under Image Quant LAS 4000 imager (GE Healthcare Life Sciences).

Immunofluorescence imaging:

All cell culture experiments requiring antibody staining for imaging purposes were performed on glass bottomed dishes (MatTek Corporation) using a protocol previously established . Cells were washed with PBS followed by the addition of 4% paraformaldehyde (PFA, Electron Microscopy Sciences, Hatfield, PA) for 15 mins to fix the cells. The cells were then washed and permeabilized using 0.01% Triton-X (Fisher Scientific) for 10 mins. The cells were then blocked for 1hr in 1% Bovine Serum Albumin (BSA, Sigma-Aldrich) and incubated with the primary antibody in 1% BSA for 1hr. Following washes, the cells were incubated with a secondary-conjugated FITC antibody in 1% BSA for 1hr. DAPI was used to stain the nuclei as per the manufacturer's protocol. The cells were then washed multiple times before capturing images using LSM 710 confocal microscope (Carl Zeiss) under 63x objectives. For image analysis, the MetaMorph or AxioVision (Carl Zeiss) software were used.

Fluorescence Cytometry:

The cells were washed with PBS and Hank's enzyme free dissociation buffer (Thermo Fisher Scientific) was added to dissociate the cells from the cell culture plates. Cells were collected, centrifuged and washed with PBS followed by the addition of 4% PFA to fix the cells. Cells were then washed and resuspended in PBS, ready for cytometry. Green fluorescence from HSV-2 infected cells was recorded under the FITC channel. Flow cytometry was performed on BD Accuri C6 Plus cytometer (BD), and the data was analyzed using FlowJo software. Unstained/non-infected cells were used as controls. The mean fluorescence intensity (MFI) was obtained for each treatment and normalized to the mock-treated cells.

HSV-2 Infection:

Unless specifically mentioned, all the infections mentioned in this study were performed at a MOI of 0.1. Requisite amount of virus was diluted in serum-free Opti-MEM media prior to its addition to cells. Virus was allowed to infect the cells for a period of 2 hours prior to the addition of fresh Keratinocyte media (KSFM with 10% FBS and 1% P/S).

BX795 treatment:

All the cell culture studies involving the use of BX795 were performed at a concentration of 10 μM, unless otherwise specifically mentioned. BX795 was received in a powder form from the supplier and dissolved in DMSO at a stock concentration of 10 mM. All the stocks were aliquoted into smaller volumes and stored at -80 °C until the day of use.

Cell viability (MTT) Assay:

Cell viability was determined using 3-(4, 5- Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide, MTT (Sigma-Aldrich) as per the protocol described previously.

Murine model of HSV-2 infection:

The mice involved in this study were infected as per the protocol described previously(82). Briefly, 8-week old C57BL6 female mice were injected subcutaneously (via scruff hold) with 2 mg of medroxyprogesterone (Depo-Provera). On day 5 after injection, mice were intravaginally infected with 1×10^6 PFU HSV-2 (333 strain). Starting 1 day post infection (1dpi), 10 μL of mock PBS or BX795 (dissolved in PBS) was administered intravaginally using a micropipette tip. The drug was administered 3 times every day for 4 days. Vaginal swabs were collected using calcium alginate tipped sterile applicators (Puritan: Calgiswab) on 2 and 4 dpi to assess the amount of viral replication in the vaginal epithelium via a plaque assay. Animals were monitored for any change in behavior and weight loss during this period. Animals showing signs of distress were euthanized

immediately for humane reasons. On 4 dpi, animals were euthanized and their genital tissue was collected and frozen in OCT (optimal cutting temperature) compound for histopathology analysis.

Plaque assay:

Plaque assay was performed to evaluate the number of infectious particles present in a given solution. Typically, Vero cells plated at a seating density of $5x10^4$ per well in a 24-well plate were used for a plaque assay. Upon confluency, the cell monolayers were washed with PBS, and virus samples diluted in OptiMEM were added in a log_{10} fold dilution series. After two hours of incubation with the infected samples, cells were washed twice with PBS, and DMEM mixed with 0.5% methylcellulose was overlaid on the cells. These plates were incubated for 72 hours at 37°C and 5% CO_2 before they were fixed with methanol and stained with crystal violet to determine the extent of plaque formation.

Histology staining:

Vaginal tissues collected from the animal groups were frozen, fixed and stained according to a previously described protocol(83). Briefly, vaginal tissue was embedded in OCT and frozen on a block of dry ice. Frozen sections were then affixed on a Cryostar NX-50 (Thermo Fisher Scientific) and 10 µm sections were cut and overlaid on glass slides. The tissue sections were fixed in pre-cooled acetone (Thermo Fisher Scientific) for 10 mins and then stained with Haematoxylin (Sigma-Alrdich) and washed thoroughly under running water. Slides were then dipped in 70% ethanol for 2 min, then in 100% ethanol for 1 min, and incubated with eosin Y alcoholic, with phloxine (Sigma, HT110316) for 1 min. Slides were then dipped in 70% ethanol for 1 min, then in 100% ethanol for 1 min, then xylene for 1 min. Cover slips with Permount mounting medium

(Thermo Fisher) were placed on the glass slides to cover them. Sections were visualized and photographed using a Zeiss Axioskop 2 plus microscope.

Statistical analysis:

GraphPad Prism software (version 4.0) was used for statistical analysis of each group. P values less than 0.05 were considered as the significant differences among mock-treated and treated groups.

CHAPTER 3: Heparanase

(Previously published as Hopkins J, Yadavalli T, Agelidis AM, Shukla D. Host Enzymes Heparanase and Cathepsin L Promote Herpes Simplex Virus 2 Release from Cells. J Virol. 2018;92(23):e01179-18. Published 2018 Nov 12. doi:10.1128/JVI.01179-18)

Importance

Genital infections by HSV-2 represent one of the most common sexually transmitted viral infections. The virus causes painful lesions, and sores around the genitals or rectum. Intermittent release of the virus (84)from infected tissues during sexual activities is the most common cause of transmission. At the molecular level, cell surface heparan sulfate (HS) is known to provide attachment sites for HSV-2. While the removal of HS during HSV-1 release has been shown, not much is known about the host factors and their regulators that contribute to HSV-2 release from natural target cell types. Here we suggest a role for the host enzyme heparanase in HSV-2 release. Our work reveals that in addition to the regulation of transcription by NF-kB, HPSE is also regulated post-translationally by cathepsin L and that inhibition of heparanase activity directly affects HSV-2 release. We provide unique insights into the host mechanisms controlling HSV-2 egress and spread.

Introduction

Genital herpes is one of the most common, persistent and highly infectious sexually transmitted disease caused by herpes simplex virus type-2 (HSV-2) and in many emerging first-time cases, by herpes simplex virus type-1 (HSV-1)(6, 85, 86). Primarily, the sites of infection include the vulva and the vagina, with some cases involving the cervix and perianal region in women and typically on the glans or the shaft of the penis in heterosexual men, whereas anal infection has also been reported with homosexual men (9, 87, 88). Primary and recurrent genital herpes infections result in lesions and inflammation around the genital area which are painful and cause distress (4). While there is no vaccination or cure against HSV-2, resistance against current therapies, such as Acyclovir, have been reported (89). Furthermore, these therapies are more than

a decade old and work on a single aspect of the viral life cycle, viral DNA replication. Novel therapeutic interventions that target different stages of viral infection including viral entry, viral protein translation and viral egress need to be addressed to successfully curb this distressing disease. One method to generate novel antiviral drugs that target these viral pathways is to understand host factors that help facilitate viral lifecycle. In this manuscript we focus on the host enzyme heparanase (HPSE) and its regulators that help facilitate egress of the HSV-2 virions.

Human HPSE is an endoglycosidase with the unique distinction of being the only enzyme capable of degrading heparan sulfate (HS)(35, 36), an evolutionarily conserved glycosaminoglycan that is present ubiquitously at the cell surface. HPSE is initially translated as a pre-proenzyme. Cleavage of a signal sequence by a signal peptidase leaves an inactive 65 kDa proHPSE, which undergoes further processing in the lysosomal compartment (35, 90). Proteolytic removal of an N-terminal 8 kDa linker by a lysosomal cysteine endopeptidase, cathepsin L, cleaves the C-terminal 50 kDa subunit, which remain associated as a non-covalent heterodimer in active HPSE(65, 91). Active HPSE is responsible for the degradation of cell surface HS, that is found covalently attached to a small set of extracellular matrix and plasma membrane proteins forming heparan sulfate proteoglycans (HSPG) (92, 93). Clearance of HS via HPSE modulates cell division and differentiation, tissue morphogenesis and architecture, and organismal physiology (93). HSV-2 encodes for two envelop glycoproteins, gB and gC, which bind HS at the cell surface and initiate viral entry (62). We first reported that host-encoded HPSE is upregulated and required for the release of viral progeny from parent cells after HSV-1 infection and subsequently similar findings were reported for porcine reproductive and respiratory syndrome virus (PRRSV) infection(46, 48).

The premise of this manuscript is to understand the role of HPSE in the egress of HSV-2 virus from its natural target cells. In this study, we show that HPSE is upregulated by the virus upon

infection and serves to aid in viral egress by preventing the newly released viral progeny from reattaching to cell surface HS. We also study transcriptional and post-translational regulators of HPSE and for the first time implicate cathepsin L in HSV release. We demonstrate that inhibition of HPSE and cathepsin L via commercially available inhibitors negatively impacts viral egress.

Results

Loss of cell surface HS during infection

To understand how HSV-2 infection modulates cell surface HS levels, we infected a natural target cell type, human vaginal epithelial cells (VK2), with HSV-2 with a multiplicity of infection (MOI) of 1 for a period of 48 hours. We observed that while mock infected cells consistently showed high amounts of cell surface HS, most HS was cleared in HSV-2 infected cells by 24 hours post infection (hpi) (Figure 4A, 1B). We also observed that there was a progressive loss of HS on the cell surface with time during infection using flow cytometric analysis (Figure 4C, 1D).

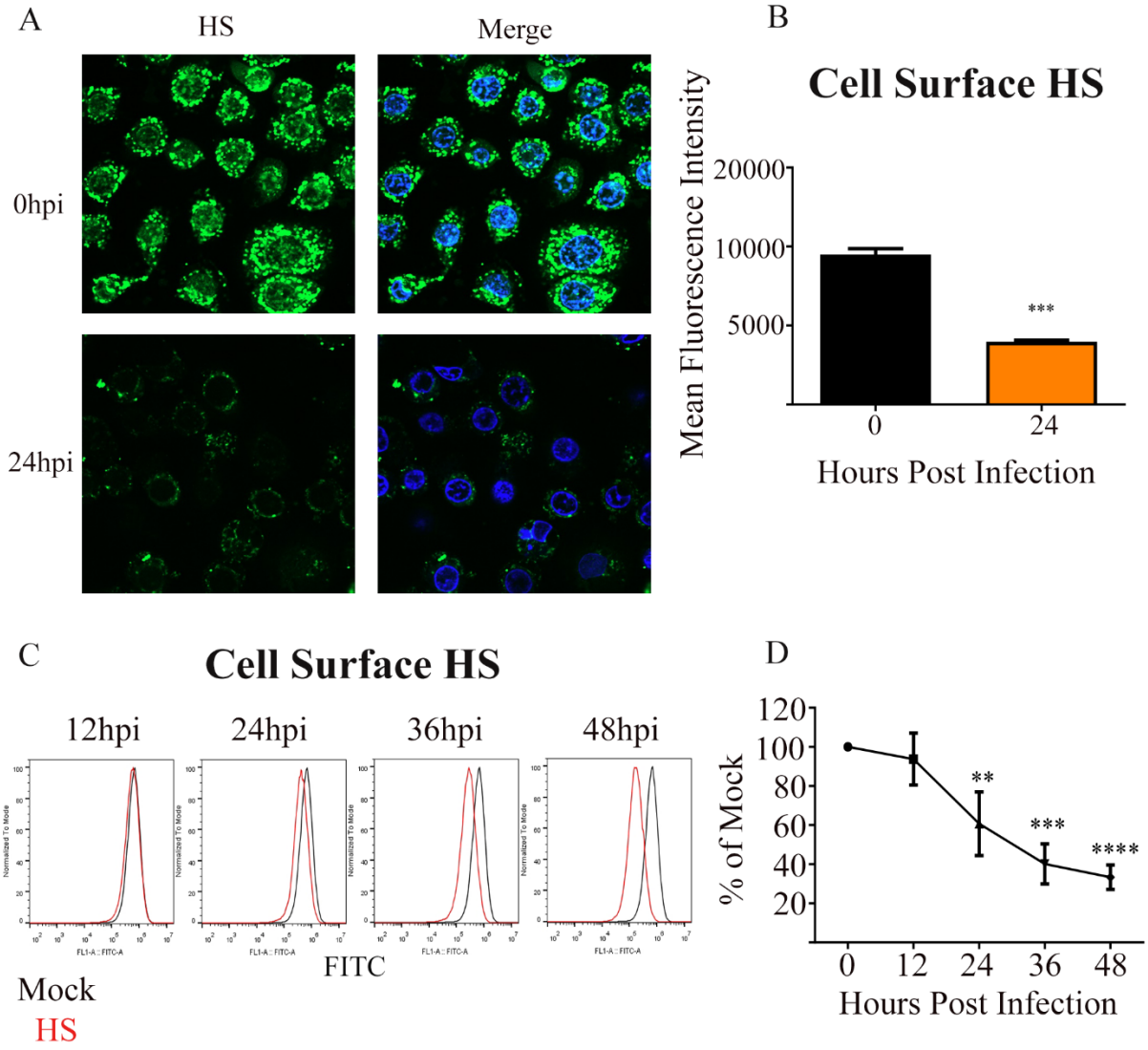
A
HS
Merge
0hpi
24hpi
B
Cell Surface HS
Mean Fluorescence Intensity
20000
10000
5000
***
0
24
Hours Post Infection
C
Cell Surface HS
12hpi
24hpi
36hpi
48hpi
FITC
Mock
HS
D
% of Mock
120
100
80
60
40
20
**
***
****
0
12
24
36
48
Hours Post Infection

Figure 4. Loss of cell surface HS during infection

A. Representative immunofluorescence images of HS stain. HSV-2 333 was used to infect cells at 1MOI for 24h. Upper left is HS stain only in uninfected sample, upper right is hoescht and HS stain merged for uninfected, lower left is HS stain only for infected sample at 24hpi, lower right is hoescht and HS stain merged for infected sample at 24hpi. B. Quantification of HS cell surface expression. C. Representative flow cytometry histogram showing change in cell surface HS expression with red representing infected samples and black showing uninfected control. D. Quantification of cell surface HS flow cytometry experiments. Asterisks denote a significant difference as determined by Student's *t*-test; *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$.

HPSE is upregulated after HSV-2 infection

Given that clearance of HS correlated with duration of HSV-2 infection, we hypothesized an upregulation of HPSE expression. HPSE is the only mammalian enzyme known to cleave HS (9-11). In order to test our hypothesis, we analyzed HPSE promoter activity at 12, 24, 36, and 48 hpi using a luciferase reporter assay. As expected, we observed a significant increase in the HPSE promoter activity during the duration of infection (Figure 5A). To understand this further we then looked at HPSE mRNA transcript levels after HSV-2 infection. We observed by quantitative real time-PCR (qRT-PCR) analysis that HPSE mRNA was significantly elevated at 12, 24 and 36 hpi (Figure 5B). Since HPSE expression was clearly upregulated inside the cells, we decided to look at HPSE translocation to the surface, which is the primary site for HS removal (93). Complementary to our HPSE mRNA results, after infection at an MOI of 1 there was a significant increase in cell surface HPSE protein levels that was observed first by immunofluorescence microscopy that showed a large and significant increase at 24 hpi (Figure 5C, 2D). This increase

in cell surface levels of HPSE was subsequently verified by flow cytometry (Fig. 5E, 5F). Taken together, our results confirmed an upregulation in HPSE levels upon infection.

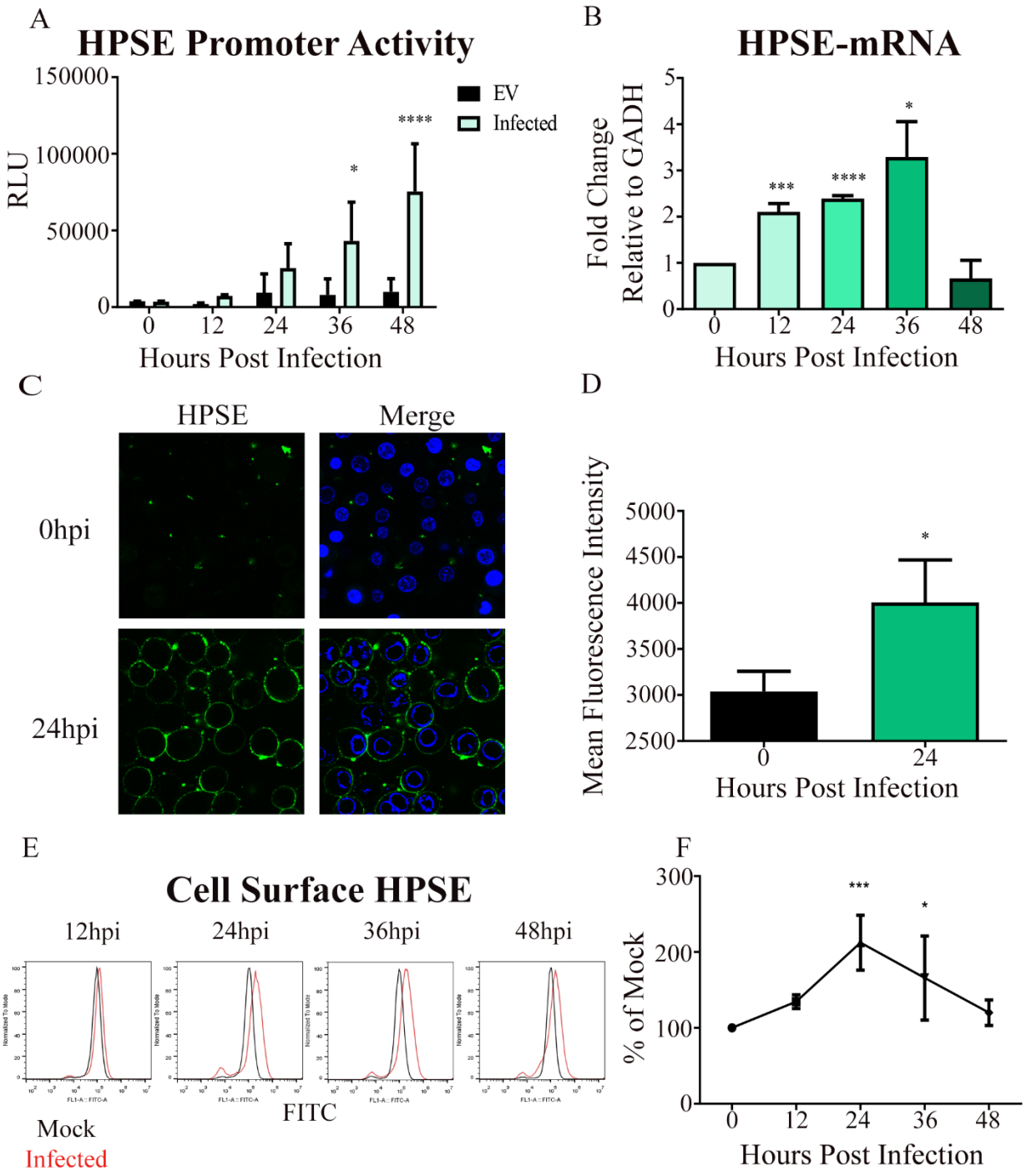
A
HPSE Promoter Activity
RLU
150000
100000
50000
0
EV
Infected
*
****
0
12
24
36
48
Hours Post Infection
B
HPSE-mRNA
Fold Change Relative to GADH
5
4
3
2
1
0
***
****
*
0
12
24
36
48
Hours Post Infection
C
HPSE
Merge
0hpi
24hpi
D
Mean Fluorescence Intensity
5000
4500
4000
3500
3000
2500
*
0
24
Hours Post Infection
E
Cell Surface HPSE
12hpi
24hpi
36hpi
48hpi
FITC
Mock
Infected
F
% of Mock
300
200
100
0
***
*
0
12
24
36
48
Hours Post Infection

Figure 5. HPSE is upregulated after HSV-2 infection

A. Increase in promoter activity of HPSE gene upon infection in HCE cells. HSV-2 333 was used to infect cells at a MOI of 1 for 12, 24, 36 and 48h. Shown is the average fold increase over uninfected control. Experimental values are normalized to those obtained with pGL3 as a control for transfection efficiency. B. Increase in HPSE mRNA levels, shown is the average fold increase over time point 0 hours post infection. C. Representative Immunofluorescence microscopy images of cell surface HPSE stain. HSV-2 333 was used to infect cells at a MOI of 1 for 24h. Upper left is HPSE stain only in uninfected sample, upper right is hoescht and HPSE stain merged for uninfected, lower left is HPSE stain only for infected sample at 24hpi, lower right is hoescht and HPSE stain merged for infected sample at 24hpi. D. Quantification of HPSE cell surface expression from multiple immunofluorescence images. E. Representative flow cytometry histogram showing change in cell surface HPSE expression with red representing infected samples and black showing uninfected control. F. Quantification of cell surface HPSE flow cytometry experiments compared to time point 0 hours post infection. Asterisks denote a significant difference as determined by Student's *t*-test; **P*<0.05, ***P*<0.01, ****P*<0.001, ****P<0.0001.

Mechanism of HPSE upregulation and activation upon infection

To understand the mechanism of HPSE regulation during HSV-2 infection, we studied transcriptional and post-translational regulators of HPSE. It is reported that during HSV-1 infection nuclear factor NF-kB (p65) activation and translocation to the nucleus could transcriptionally increase HPSE expression (94, 95). To understand if HSV-2 used a similar mode of action, we analyzed p65 activity during HSV-2 infection. As expected, there was a consistent increase in p65 mRNA expression in HSV-2 infected cells through 36 hpi (Figure 6A). We also

observed the nuclear translocation of p65 (Figure 6B) at 24 hpi using immunofluorescence microscopy. These results corroborated our western blot data, which showed significantly increased p65 in the nuclear fraction after HSV-2 infection (Figure 6D). Next we wanted to understand if the inhibition of p65 activation and nuclear localization would affect HPSE promoter activity. In this regard, we overexpressed a plasmid encoding mutant IkBa (S32A/S36A) in VK2 cells. This mutant IkBa is incapable of being phosphorylated and degraded and as a result, it acts as a dominant-negative protein that inhibits NF-kB activation and nuclear translocation (96). We did observe that expression of the IkBa mutant lead to a decrease in HPSE promoter activity (Figure 6C). Taken together our findings suggest that virus-induced activation of NF-kB regulates HPSE expression.

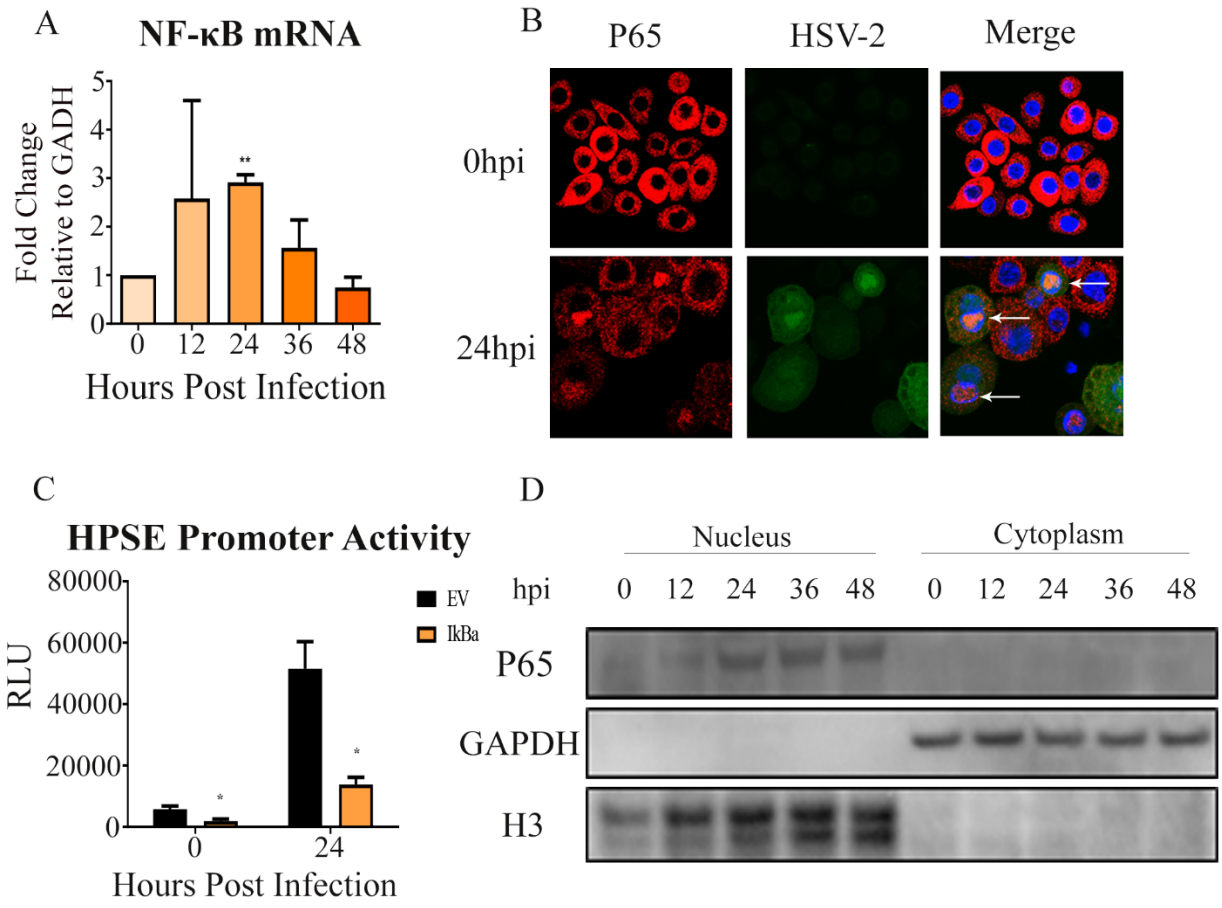
A
NF-κB mRNA
Fold Change Relative to GADH
0
1
2
3
4
5
**
0
12
24
36
48
Hours Post Infection
B
P65
HSV-2
Merge
0hpi
24hpi
C
HPSE Promoter Activity
RLU
80000
60000
40000
20000
0
EV
IkBa
*
*
0
24
Hours Post Infection
D
Nucleus
Cytoplasm
hpi
0
12
24
36
48
0
12
24
36
48
P65
GAPDH
H3

Figure 6. Nf-kB as mechanism for HPSE upregulation

A. Increase in Nf-kB P65 mRNA levels, shown is the average fold increase over uninfected control. B. Representative immunofluorescence microscopy images of nuclear translocation of P65 upon HSV-2 infection with HSV-2 333 GFP. With Nf-kB P65 labeled red, HSV-2 green, and the nucleus blue. C. Inhibition of NF-kB activation results in decreased HPSE promoter activity. VK2 cells were transfected with mutant IkBa incapable of degradation (S32A/S36A), thereby specifically inhibiting NF-kB activation and nuclear translocation. 24h after transfection cells were infected with HSV-2 333 for 24 hrs at a MOI of 1. Cell lysates were isolated, and luciferase assay was performed. Results shown are normalized to empty pGL3 vector (EV) as a control for transfection efficiency. D. Representative western blot of nuclear translocation of P65 upon HSV-2 infection with HSV-2 333. Asterisks denote a significant difference as determined by Student's *t*-test; *$P<0.05$, **$P<0.01$.

Cathepsin L as mechanism of HPSE activation

As described by others in the field, cathepsin L is the only known post-translational activator of HPSE in mammalian cells (65, 97). Given that HPSE is upregulated upon HSV-2 infection, we wanted to assess whether its lysosomal activator, cathepsin L, also increased with HSV-2 infection. As observed, cathepsin L mRNA levels were upregulated at 12 hpi when compared to 0 hpi and continued to increase at 24 and 36 hpi (Figure 7A). We observed a similar trend in mature cathepsin L which is known to be markedly stable (43), where protein levels were highest at 48 hpi by western blot analysis (Figure 7B). These important findings were confirmed using immunofluorescence microscopy (figure 7C, 7D) and flow cytometry analysis (Figure 7E,

7F). Collectively, our data suggests a connection between HSV-2 infection and HPSE/cathepsin L upregulation.

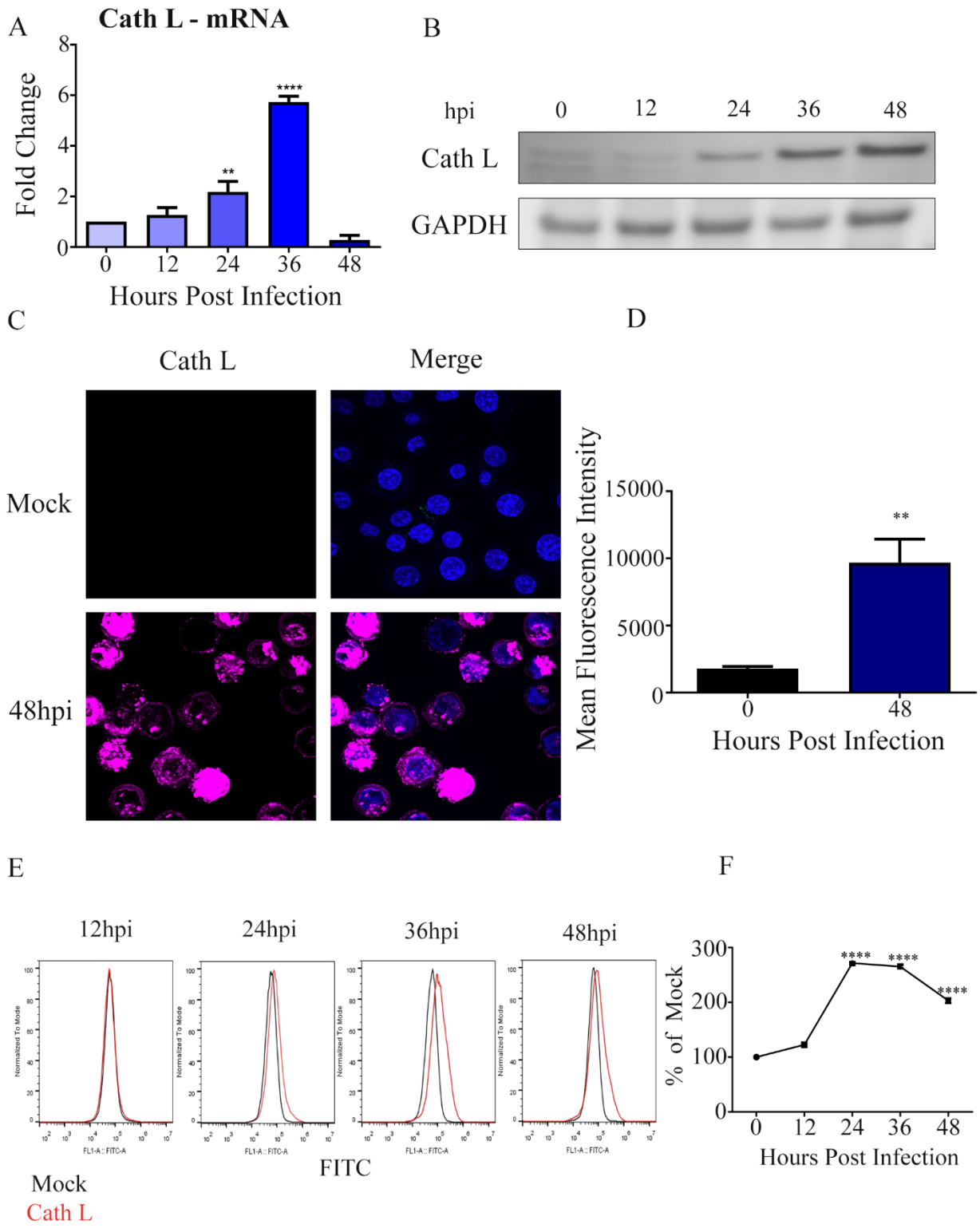
A
Cath L - mRNA
Fold Change
Hours Post Infection
B
hpi
Cath L
GAPDH
C
Cath L
Merge
Mock
48hpi
D
Mean Fluorescence Intensity
Hours Post Infection
E
12hpi
24hpi
36hpi
48hpi
FITC
Mock
Cath L
F
% of Mock
Hours Post Infection

Figure 7. Cathepsin L as mechanism of HPSE activation

A. Increase in Cathepsin L mRNA levels, shown is the average fold increase over time point 0 control. B. Representative western blot showing increase of Cathepsin L over time after infection with HSV-2 333 after a MOI of 1 infection. C. Representative immunofluorescence microscopy images of Cathepsin L stain. HSV-2 333 was used to infect cells at an MOI of 1 for 24h. Upper left is Cathepsin L stain only in uninfected sample, upper right is hoescht and Cathepsin L stain merged for uninfected, lower left is Cathepsin L stain only for infected sample at 24hpi, lower right is hoescht and Cathepsin L stain merged for infected sample at 48hpi. D. Quantification of total cathepsin L expression from multiple immunofluorescence images. E. Representative flow cytometry histogram showing change in Cathepsin L expression with red representing infected samples and black showing uninfected control. F. Quantification of Cathepsin L flow cytometry experiments. Asterisks denote a significant difference as determined by Student's *t*-test; *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$.

Effect of inhibition of cathepsin L and HPSE on infection

Having established a mechanism through which HPSE is upregulated and activated, we wanted to assess whether the inhibition of HPSE and cathepsin L would affect HSV-2 viral lifecycle. To ascertain the role of HPSE we used a well characterized and commercially available small molecule HPSE activity inhibitor, OGT 2115. This compound functionally blocks HPSE activity, and does not significantly affect its expression (98). To verify the inhibition of HPSE activity during infection we analyzed the cell surface HS expression. While cell surface HS expression during HSV-2 infection usually decreases, our results after pharmacological inhibition of HPSE showed a drastic increase in HS expression in OGT treated cells verses DMSO treatment (Figure 8A and 8B). Furthermore, we also observed a decrease in HSV-2 infection as measured

by GFP reporter activity in the presence of OGT 2115 at 10 □M concentration. (Figure 8C, 8D). Immunofluorescence microscopy data were in accordance with these results when we used the same HSV-2 GFP reporter virus and the same OGT 2115 concentration. (Figure 8E). We also observed a significant decrease in virus production and release using cell culture supernatant plaque assays through 48 hpi (Figure 8F). This data combined with observations showing higher levels of virus in cell lysates in OGT 2115 treated cells (Figure 9F) suggest that pharmacological inhibition of HPSE using OGT 2115 significantly increases HS expression on the cell surface while reducing the overall viral egress and spread.

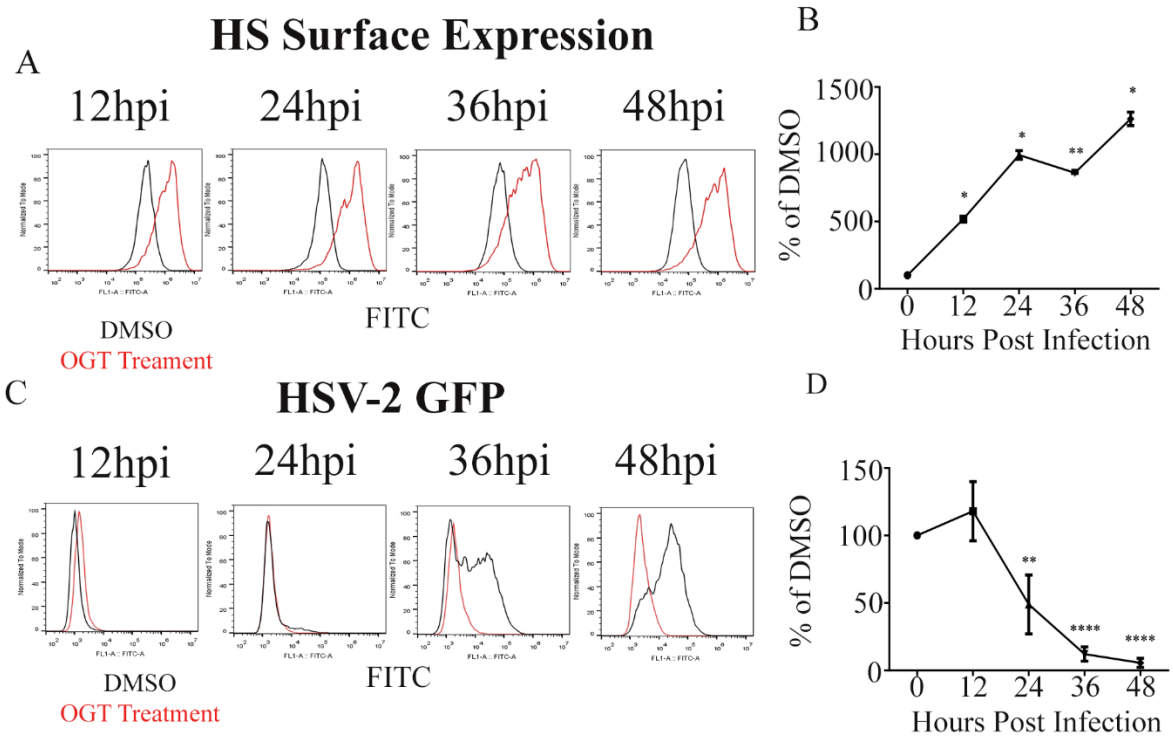
HS Surface Expression
A
12hpi
24hpi
36hpi
48hpi
DMSO
OGT Treament
FITC
B
% of DMSO
Hours Post Infection
C
HSV-2 GFP
12hpi
24hpi
36hpi
48hpi
DMSO
OGT Treatment
FITC
D
% of DMSO
Hours Post Infection
E
DMSO
OGT
F
HSV-2 in supernatant (PFU/mL)
DMSO
OGT
Hour Post Infection
G
HSV-2 in cell lysate (PFU/mL)
DMSO
OGT
Hour Post Infection

Figure 8. Inhibition of HPSE on HSV-2 infection

Representative flow cytometry histogram showing change in cell surface HS expression with red denoting OGT 2115 treatment and black representing DMSO treatment. Both samples were infected with HSV-2 333 at a MOI of 1. B. Quantification of cell surface HS OGT flow cytometry experiments. C. Representative flow cytometry histogram showing change in infection following OGT treatment, with red denoting OGT 2115 treatment and Black representing DMSO treatment. Both samples were infected with HSV-2 333 GFP. D. Quantification of infection after treatment with OGT flow cytometry experiments. E. Representative immunofluorescence microscopy images taken 24hpi after infection with HSV-2 333 GFP after treatment with OGT or DMSO with green representing infected cells. F. Average change in virus released from cells with and without OGT treatment every 12h up until 48h. G. Average change in virus from cell lysate with and without OGT treatment every 12h up until 48h Asterisks denote a significant difference as determined by Student's *t*-test; *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$.

Next we wanted to understand if the inhibition of cathepsin L, the lysosomal activator of HPSE, would affect HSV-2 viral egress and spread. We used a well characterized and commercially available small molecule inhibitor of cathepsin L, cathepsin L inhibitor IV (99). Given that active HPSE remains in the lysosomal compartment for a period of 48 hours before it is degraded (100), we hypothesized that cathepsin L would need to be inhibited for a period up to 48 hours prior to and during infection to make sure no active HPSE is present throughout. As hypothesized, we saw a loss of infection in cathepsin L inhibitor IV treated cells compared to mock DMSO control. These results were consistent when analyzed through immunofluorescence microscopy (Figure 9A) and flow cytometry (Figure 9C, 9D). We also observed through plaque

assay that the amount of egressed virus, found in the infected cell supernatant, also decreased in cathepsin L inhibitor IV treated cells compared to mock DMSO treated samples, reaching significance at 48 hpi (Figure 9B).

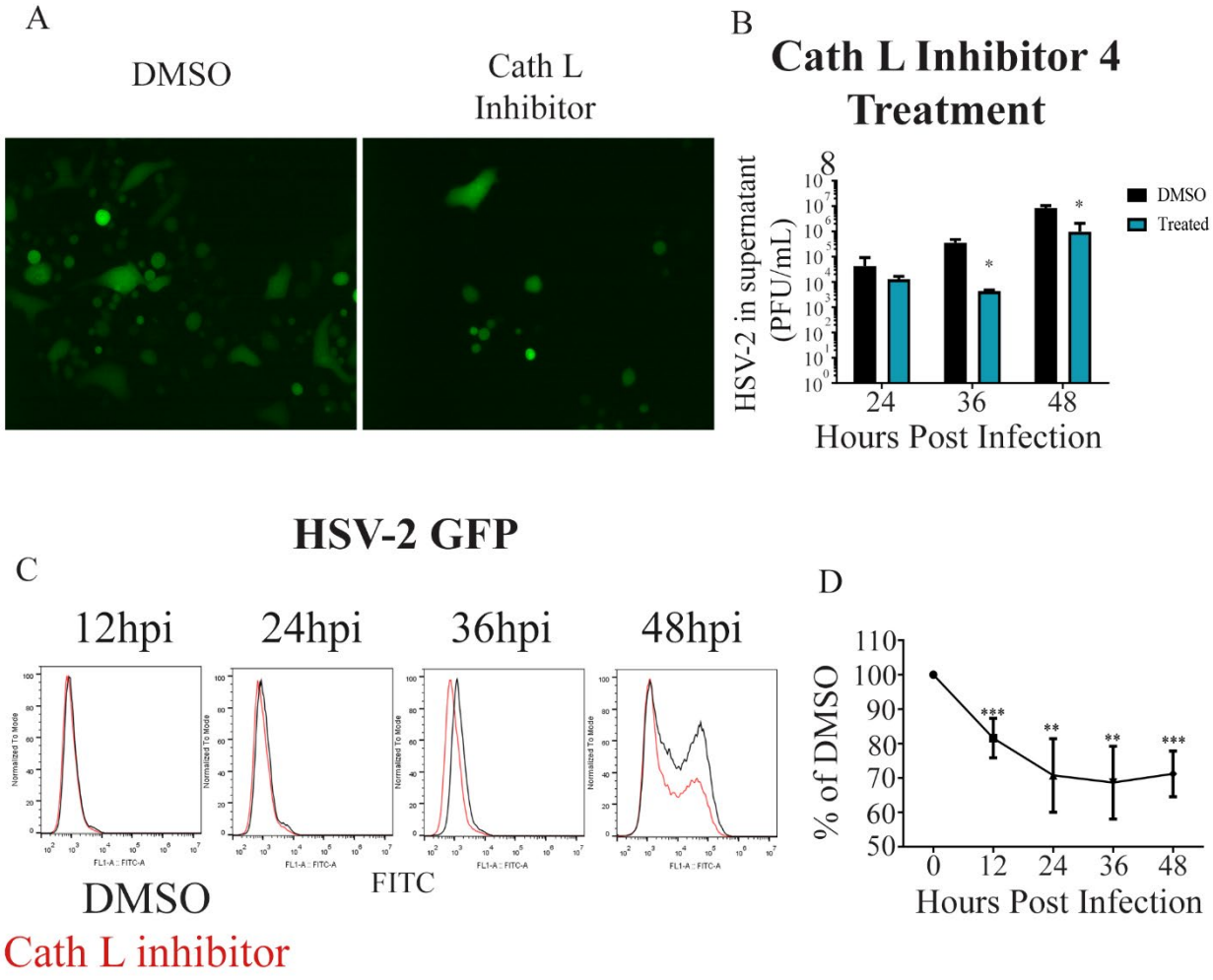
A
DMSO
Cath L Inhibitor
B
Cath L Inhibitor 4 Treatment
HSV-2 in supernatant (PFU/mL)
DMSO
Treated
24
36
48
Hours Post Infection
HSV-2 GFP
C
12hpi
24hpi
36hpi
48hpi
DMSO
Cath L inhibitor
FITC
D
% of DMSO
Hours Post Infection

Figure 9. Inhibition of Cathepsin L on HSV-2 Infection

A. Representative Immunofluorescence microscopy images taken 24hpi after infection with HSV-2 333 GFP after treatment with Cathepsin L inhibitor IV or DMSO with green representing infected cells. B. Average change in virus released from cells with and without Cathepsin L inhibitor IV treatment every 12h up till 48h. C. Representative flow cytometry histogram showing change in infection after Cathepsin L inhibitor IV treatment, with red denoting Cathepsin L inhibitor IV treatment and Black representing DMSO treatment. Both samples were infected with HSV-2 333 GFP. D. Quantification of infection after treatment with Cathepsin L inhibitor IV flow cytometry experiments. Asterisks denote a significant difference as determined by Student's t-test; *P<0.05, **P<0.01, ***P<0.001.

Effect of overexpression of HPSE during HSV-2 infection

Through the experiments in previous sections, we were able to establish that HPSE is important for HSV-2 release. Lastly, we wanted to understand if the overexpression of HPSE during a HSV-2 infection would be beneficial during viral proliferation. To study this, we overexpressed HPSE in VK2 cells for a period of 24 hours followed by infection with HSV-2 (333) GFP virus at a MOI of 1. The cells were plated without any methylcellulose to allow release and spread of the extracellular virions. We observed via immunofluorescence microscopy a clear increase in virally infected cell clusters in the HPSE transfected cells when compared to empty vector control (Figure 10A). It is important to note that although we saw less infection than expected, we normally find less infection in samples that have been previously transfected. Our results were in accordance with flow cytometry results (Figure 10C), which showed that overexpression of HPSE lead to a faster spread of infection, reaching significance at 36 and 48 hpi (Figure 10D). We also observed an increase in released virus after HPSE overexpression (Figure

10B) at 36 hpi and 48 hpi. Taken together our results suggest a direct connection between higher levels of HPSE and HSV-2 release in the culture supernatant.

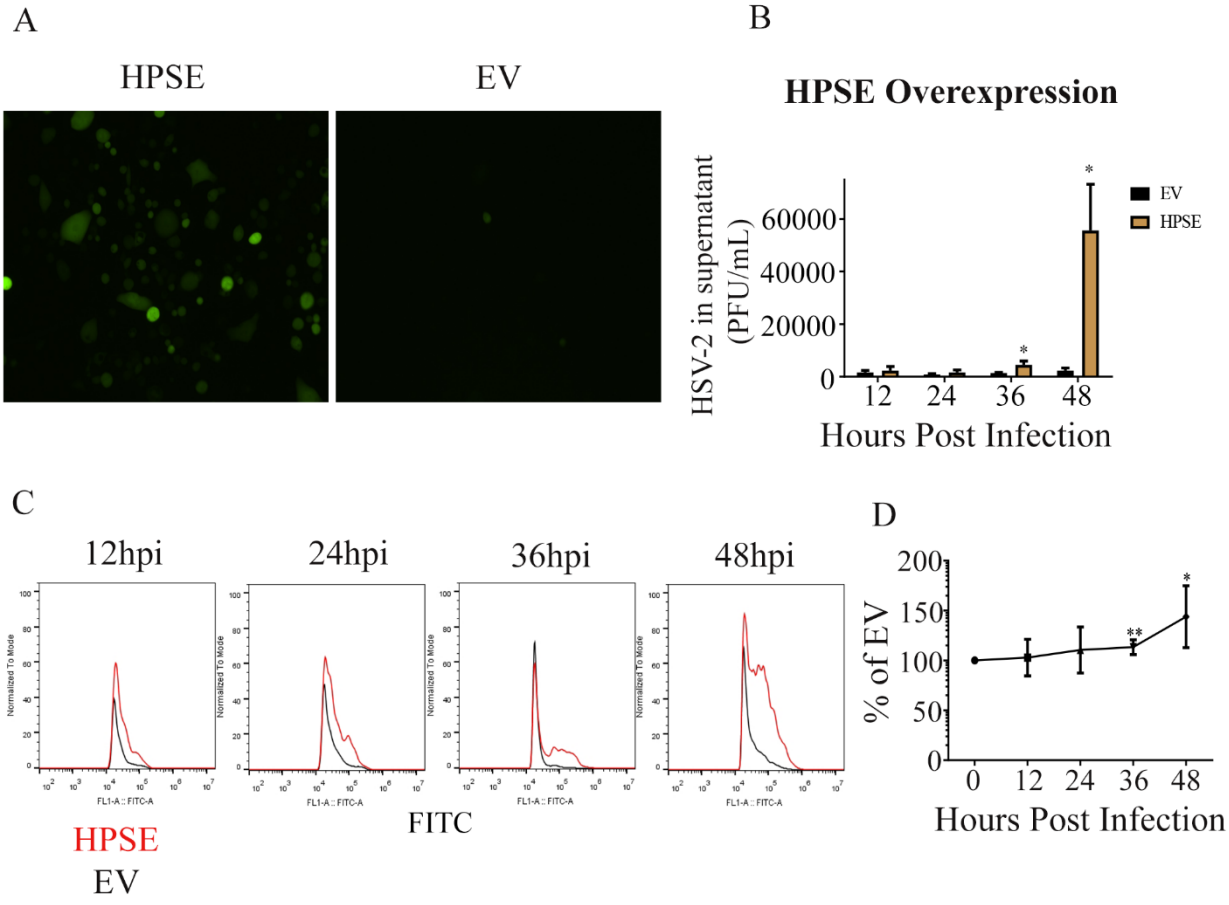
A
HPSE
EV
B
HPSE Overexpression
HSV-2 in supernatant (PFU/mL)
60000
40000
20000
0
12
24
36
48
Hours Post Infection
EV
HPSE
C
12hpi
24hpi
36hpi
48hpi
FITC
HPSE
EV
D
% of EV
200
150
100
50
0
0
12
24
36
48
Hours Post Infection

Figure 10. Effect of overexpression of HPSE during HSV-2 infection A. Representative Immunofluorescence microscopy images taken 24hpi after infection with HSV-2 333 GFP after overexpression of HPSE with green representing infected cells. B. Average change in virus released from cells overexpressing HPSE then infected with HSV-2 333 every 12h up till 48h. C. Representative flow cytometry histogram showing change in newly infected cells after overexpression of HPSE, with red denoting HPSE overexpression and black representing EV. Both samples were infected with HSV-2 333 GFP. D. Quantification of infection after overexpression of HPSE flow cytometry experiments. Asterisks denote a significant difference as determined by Student's *t*-test; **P*<0.05, ***P*<0.01.

Discussion

Active HPSE is responsible for the degradation of cell surface HS. HS is found covalently attached to a small set of extracellular matrix and plasma membrane proteins forming heparan sulfate proteoglycans (HSPG) (92, 93). Clearance of HS via HPSE modulates cell division and differentiation, tissue morphogenesis and architecture, and organismal physiology (93). HSV-2 encodes for two envelope glycoproteins, gB and gC, which bind HS at the cell surface and initiate viral entry (62). However, the new virions released can face a significant challenge if they bind with HS during viral egress. This phenomenon is commonly seen with other viruses including influenza where the viral hemagglutinin binds to host sialic acid chains for entry and if host sialic acid is not removed it can restrict viral release (101, 102). To eliminate the possibility of new virions binding upon release, the surface sialic acid residues are cleaved by a viral encoded sialidase, neuraminidase to prevent this problem for the influenza virus (103, 104). In this regard, HSV-2 does not have any known viral proteins that can cleave cell surface

HS. HSV-2 may have found the ability to cleave HS nevertheless from a host enzyme (46, 48). Given that HPSE is the only known mammalian enzyme capable of cleaving HS chains (36), we hypothesized and then demonstrated that its upregulation during infection may be beneficial for HSV-2 release from host cells.

Our evidence of HPSE upregulation during infection may suggest an additional role for HPSE in the exacerbation of the genital disease caused by HSV-2. During genital infection, distressing, painful lesions, and sores around the genitals or rectum can result from a productive HSV-2 infection**.** These lesions are accompanied by inflammation and localized loss to tissue architecture (9). Recent studies have implicated HPSE overexpression in multiple pathologic processes, including inflammation (40), angiogenesis (55), tumor metastasis (105), and atherosclerosis (106). It has been shown how heparanase contributes to HS cleavage and how this specifically results in rearrangement of the extracellular matrix as well as in controlling the release of many cell surface HS-linked molecules such as growth factors, cytokines and enzymes in larger tissue wide changes (36, 58).

To understand potential contributions of HPSE during HSV-2 infection, we first looked at HS chain expression on the cell surface during HSV-2 infection. It became very clear to us that HS expression decreases over time during infection. This information also strengthened our goal to see how HPSE is modulated during infection. As suspected, HPSE was upregulated both transcriptionally and translationally showing higher protein levels on the cell surface at 24, 36 and 48 hpi. Together, these results suggest that HSV-2 infected cells upregulate HPSE, which is then translocated to the cell surface, decreasing HS on the cell surface after infection.

Next we wanted to understand the mechanism behind the upregulation and activation of HPSE during infection. It was reported that nuclear factor NF-kB, is upregulated upon HSV infection and that NF-kB upregulation has been linked to the transcriptional regulation of HPSE (20). Also, it is well known that cathepsin L, a lysosomal endopeptidase, is the only known activator of HPSE in mammalian cells. Hence we looked at the transcriptional and protein levels of these two factors and how they change with infection. Through this study, we were able to show that HSV-2 infection of VK2 cells upregulated NF-kB and cathepsin L which in turn orchestrated the expression and activation of HPSE.

Finally, we asked whether the inhibition of HPSE and cathepsin L would downregulate viral egress and spread. To study this, we used two well-known inhibitors, OGT 2115 and cathepsin L inhibitor IV, on VK2 cells during HSV-2 infection. We saw a significant loss of infection using both these inhibitors compared to vehicle controls suggesting an alternate therapeutic modality against HSV-2 infections. Additionally, when we upregulated HPSE expression using a HPSE expression plasmid, we saw a significant increase in viral progeny and spread.

For our model we propose that during the productive phase of HSV-2 infection of VK2 cells HSV-2 causes an upregulation in HPSE levels that is mediated by an increased NF-kB p65 nuclear localization and a resultant activation of HPSE transcription. In parallel, increased levels of cathepsin L contribute to HPSE proenzyme cleavage and activation. Together they contribute to the removal of heparan sulfate from cell surface and thus, facilitate virus release from cells. Higher levels of HPSE may also be a trigger for the breakdown of extracellular matrix and eventually a trigger for local inflammation. While the latter part is yet to be demonstrated, our studies discussed here directly implicate HPSE and cathepsin L in HSV-2 release.

CHAPTER 4: HEPARANASE 2

Importance

HSV-2 is the most common cause of genital herpes which is a very transmissible sexually transmitted disease. The disease is hallmarked by lesions in the genital region that can be disfiguring and very painful. The burden of this disease comes at a cost of around 13 percent of the global population and an economic loss ranging from 2-13 billion a year in the United States. Heparan sulfate has been shown to be an attachment site for HSV-2 and has shown to be important in egress. How heparan sulfate is managed by the cell in response to viral mediated changes still needs more elucidation. Here we suggest that heparanase inhibitor heparanase 2 has a role in regulation of heparan sulfate during infection of vaginal epithelia cells. We show that modulation of heparanase 2 has direct negative impact on the HSV-2 life cycle. This study background and depth to the entry and egress stories of HSV-2 and gives new insight on possible pathways of controlling this disease.

Introduction

HSV-2 is the general cause of genital herpes which is characterized by painful lesions and inflammation in the genital region and anal region. With males presenting with infection of the shaft of the penis or anal area, and women mainly in the vaginal region and can present in the cervix and perianal(30). HSV-2 genital infection is also associated with increased risk of HIV

transmission and acquisition(107). HSV-2 can also cause ocular infection and labialis infection albeit less so that HSV-1(32). While there is no vaccination or cure against HSV-2, resistance against current therapies, such as Acyclovir, have been reported(108). Going farther, viral DNA replication is the only aspect of the viral life cycle that these therapies, which are more than a decade old, work on(109). New innovative therapeutic interventions that disrupt different stages, including viral entry, viral protein translation and viral egress, of viral infection need to be addressed to successfully slow down this terrible disease. One method would be to develop next drugs by finding new targets and new pathways to disrupt, and by understanding host factors that help facilitate viral lifecycle. In this manuscript we focus on the host enzyme heparanase 2 (HPSE 2) and how its management effects the HSV-2 lifecycle.

HPSE 2 is the only known protein to sequester heparan sulfate (HS), a polysaccharide that is ubiquitously expressed at the cell surface, by inhibiting heparanase (HPSE)(73, 74, 93). HPSE 2 inhibits HPSE competitively by targeting the same epitopes that HPSE targets with greater affinity. HPSE 2 has no enzymatic activity in contrast to HPSE, which cleaves HS(74). HPSE 2 inhibition of HPSE has been shown as a tumor suppressor in many studies, that it regulates selected genes that affect tumor vascularity, tumor fibrosis, cell differentiation, ER-stress and apoptosis(75, 76, 110-114, 114, 115). In recent studies by our lab we have investigated the pro-viral functions of HPSE in *in vitro* and *in vivo* models in HSV-1 infections, and in vitro models regarding HSV-2 infection(46, 47, 63). It is a natural progression that we should then investigate HPSE 2 which regulates HPSE. In this study we show that HPSE 2 negatively effects HSV-2 replication upon overexpression and knockdown. This shows HPSE 2 to be an important protein in defense upon HSV-2 infection.

Results

HPSE 2 Levels Increase During Infection

To understand how HSV-2 infection modulates HPSE 2 levels we infected an established cell type that is a normal target for the virus, human vaginal epithelia cells (VK2), with HSV-2 at a multiplicity of infection (MOI) of 0.1 for 48 hours. We observed a large and significant increase of HPSE 2 transcripts at 24hpi through 48hpi where there was near a 70 fold increase, at 12hpi there was an increase but it was not significant (Figure 11A). At the protein level by western blot we perceive an increase in HPSE 2 levels after a 1 and 0.1 infection from 12hpi though 48hpi. Note that there is discrimination of 3 separate bands denoting HPSE 2 splice variants a b and c, with a having the largest total increase (Figure 11B). Also by immunofluorescence microscopy after staining for HPSE 2 after infection with HSV-2 333 GFP, which produces GFP on a CMV promoter upon infection, we were able to see an increase in level with infection that agrees with our western blot data (Figure 11C).

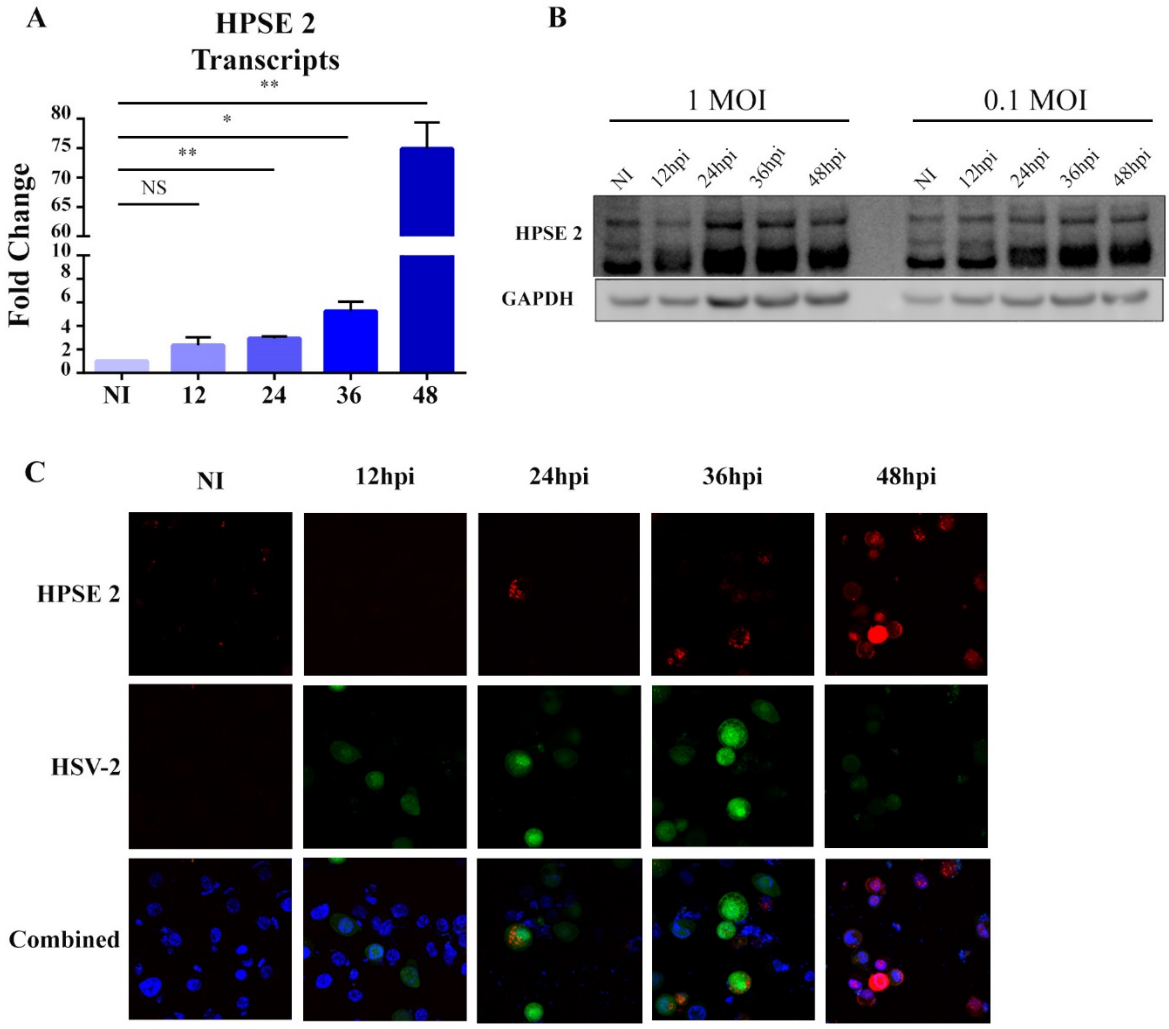
A
HPSE 2
Transcripts
Fold Change
NS
*
**
**
NI
12
24
36
48
B
1 MOI
0.1 MOI
NI
12hpi
24hpi
36hpi
48hpi
HPSE 2
GAPDH
C
NI
12hpi
24hpi
36hpi
48hpi
HPSE 2
HSV-2
Combined

Figure 11. After Infection HPSE 2 Transcript and Protein Levels Increase

A. Increase in HPSE 2 mRNA levels, vaginal epithelial cells were infected with HSV-2 333 and samples were collected at 0, 12, 24, 36, and 48 hours post infection. Shown is the average fold increase over uninfected control. B. Representative western blot of HPSE 2 expression after HSV-2 333 infection at 1 MOI and 0.1 MOI with samples taken at 0, 12, 24, 36, and 48 hours post infection. C. Representative immunofluorescence microscopy images of HPSE 2 stain. HSV-2 333 GFP was used to infect cells at a MOI of 0.1 then images where taken at 0, 12, 24, 36, and 48 hours post infection. The top row is a HPSE 2 in red, the middle row is HSV-2 in GFP and the last row is hoescht in blue, HPSE 2 stain in red, and HSV-2 in green merged. Asterisks denote a significant difference as determined by Student's *t*-test; ****P*<0.05, *****P*<0.01.

Effect of Overexpression of HPSE 2 on HSV-2 Infection

From the last section we found that expression of HPSE 2 changed during infection, we next wanted to realize if further modulation was beneficial for viral proliferation. To study this we overexpressed each of the splice variants of HPSE 2 in VK2 cells then infected with HSV-2 333 at an MOI of 1. We first looked at a viral late gene and glycoprotein gD that is very abundant, We found by western blot that there was a large decrease in gD across all of the time points and overexpressed splice variants verses the empty vector control (Figure 12A). This came as a surprise to us as the only variant with cell surface localization and HS interaction was HPSE 2c. We next looked at the cells were plated without any methylcellulose to allow release and spread of the extracellular virons then preformed a plaque assay of the media that would contain infectious virus that had egressed from cells. We found that there was significantly less virus in all three splice variant overexpressing cells verses the empty vector control, which goes along

with our results from the western blot data (Figure 12B). We next looked at the cell lysate from VK2 cells that were infected with HSV-2 at 1 MOI and performed a plaque assay after the cells were collected and sonicated to release any intracellular virus at 24hpi. We found that there was significantly less virus in all three splice variant overexpressing cells verses the empty vector control, which goes along with our results from plaque assay assessing extra cellular virus and the western blot data (Figure1 2C). Last we infected VK2 cells with 0.1 MOI with HSV-2 333 GFP after expressing the three splice variants in the cells then at 12, 24 and 36hpi we collected and performed flow cytometry on the cells. We found that there was less fluorescence from infected cells in the HPSE 2 overexpressing cells verses empty vector control (Figure 12D). All of the results are in accord with one another and suggest that overexpression of HPSE 2 negatively effects HSV-2 replication.

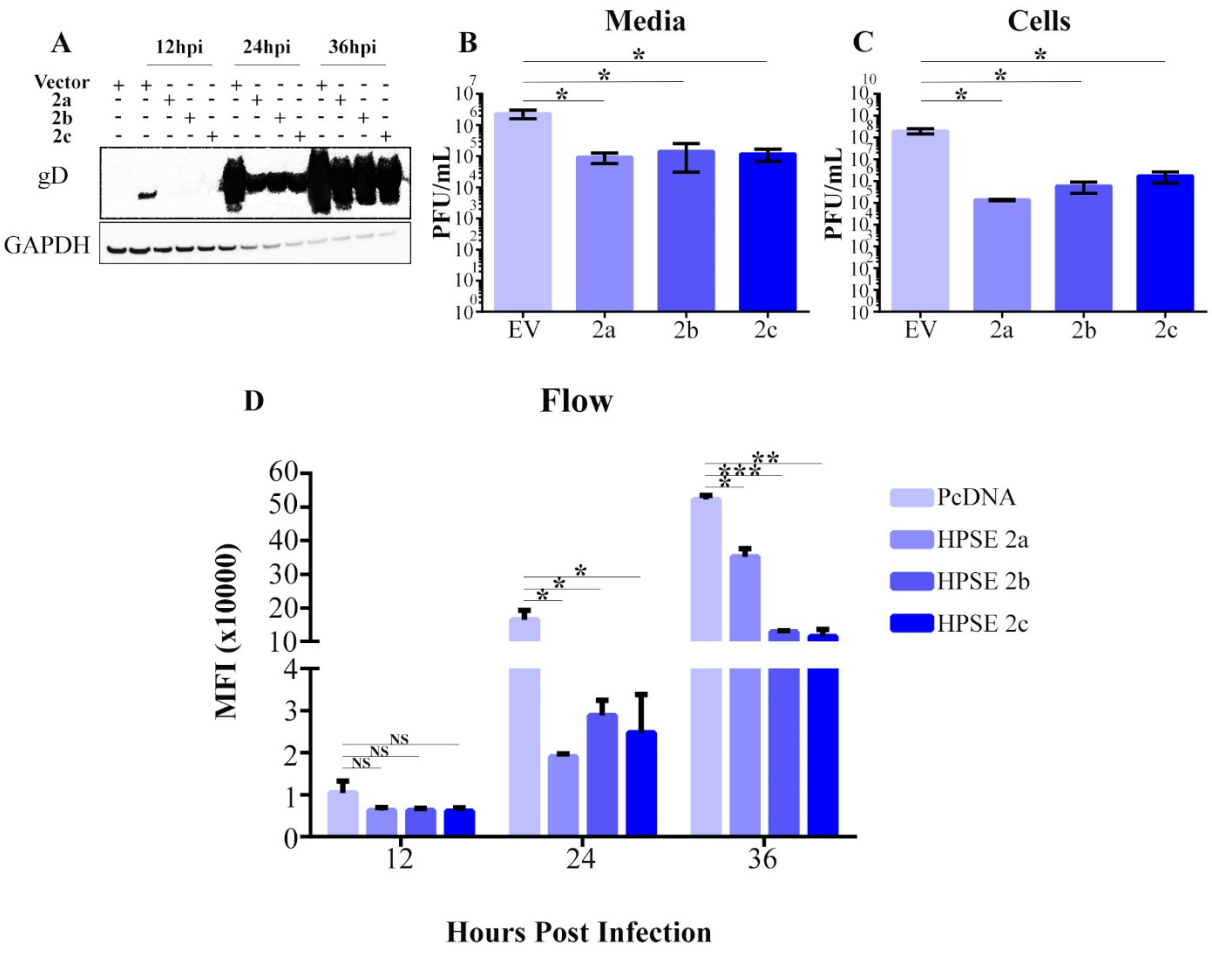
A
12hpi
24hpi
36hpi
Vector
2a
2b
2c
gD
GAPDH
B
Media
PFU/mL
EV
2a
2b
2c
C
Cells
PFU/mL
EV
2a
2b
2c
D
Flow
MFI (x10000)
NS
PcDNA
HPSE 2a
HPSE 2b
HPSE 2c
12
24
36
Hours Post Infection

Figure 12. Overexpression of HPSE 2 Leads to Lower Viral Replication

A. Representative Western blot of expression of viral protein gD after of overexpression of HPSE 2. VK2 cells were infected with HSV-2 333 24h after transfection with plasmids overexpressing HPSE 2 a, b, and c and a control. Samples were collected at 0 for the control and 12, 24, and 48 hours post infection for all for all categories. B. Quantification of plaque assay of media collected from cells that were overexpressing HPSE 2 a, b, and c and then were infected with HSV-2 at 1 MOI. Samples were collected at 24 hours post infection. C. Quantification of plaque assay of cell lysates collected from cells that were overexpressing HPSE 2 a, b, and c and then were infected with HSV-2 at 1 MOI. Samples were collected at 24 hours post infection. D. Quantification of infection after overexpressing HPSE 2 a, b, and c flow cytometry experiments, with samples collected 12, 24, and 36 hours post infection. Asterisks denote a significant difference as determined by Student's t-test; *P<0.05, **P<0.01, ***P<0.00.

Effect of Knockdown of HPSE 2 on HSV-2 Infection

So far we have seen that with infection of HSV-2 there is an overall HPSE 2 level rise and also that with overexpression of HPSE 2 that there is a detrimental effect on HSV-2 replication. Next we wanted to understand what the influence of lower levels of HPSE 2 would have on viral proliferation. We first designed an small interfering RNA mediated knockdown of HPSE 2 by making four different siRNA's targeting HPSE 2 named si1, si2, si3, and si4 with a scrambled si for control. We then picked the one that had the lowest HPSE 2 protein production by western blot, which was si4. We infected VK2 cells with HSV-2 333 at 0.1 MOI after verified knockdown of HPSE 2, also we treated scrambled control cells with small molecule HPSE inhibitor OGT 2115.We then looked for abundant viral protein gD. We found that there was a

large decrease in the production of this viral gene in the cells with HPSE 2 knocked down and in the OGT treated cells verses the cells treated with the scrambled si control (Figure 13A). Next we show a graph quantifying the western blot and in the graph we see a significant difference in gD expression between the scrambled si and the HPSE 2 and OGT 2115 treated cells (Figure 13B). This was a bit of a surprise to us considering in previous studies our lab has done increased HPSE lead to higher virus production. Next we looked at infectious virus produced in cells but not egressed and egressed virus by plaque assay. We found significantly lower amounts of virus in both experiments, which corresponds with the western blot data already shown (Figure 13C and 13D). Last we infected cells with HSV-2 333 GFP then measured the mean fluorescent intensity of cells as a measure of level of infection, and we found that over samples collected at 12, 24, 36, and 48hpi there was significantly lower level of infection in the cells with the knockdown of HPSE 2 and OGT 2115 treatment. This data agrees with the plaque data and western data shown that low levels of HPSE 2 is detrimental to viral replication (Figure 13E).

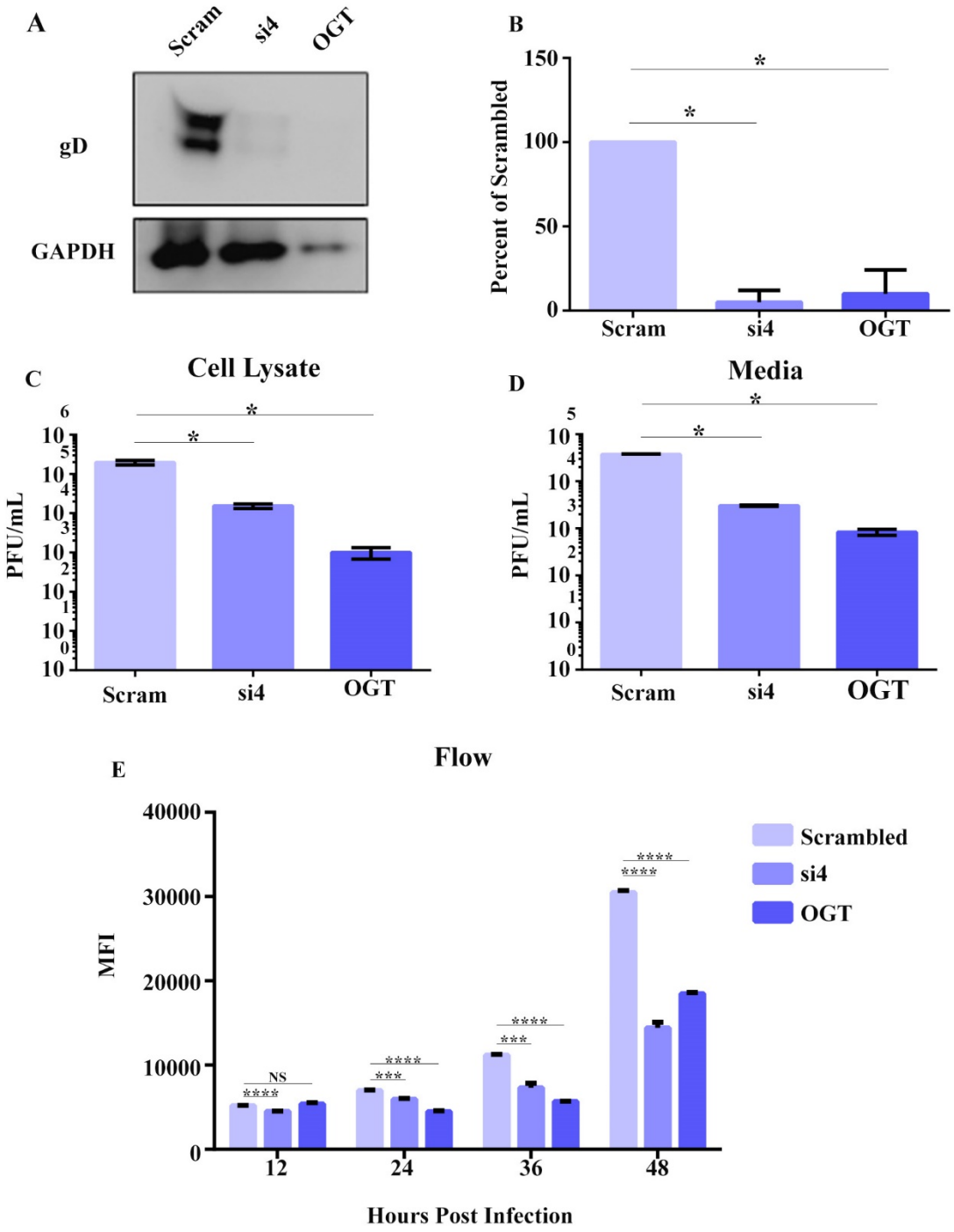
A
Scram
si4
OGT
gD
GAPDH
B
Percent of Scrambled
150
100
50
0
*
*
Scram
si4
OGT
C
Cell Lysate
PFU/mL
*
*
Scram
si4
OGT
D
Media
PFU/mL
*
*
Scram
si4
OGT
E
Flow
MFI
40000
30000
20000
10000
0
NS
****
***
****
****
***
****
****
****
Scrambled
si4
OGT
12
24
36
48
Hours Post Infection

Figure 13 Knockdown of HPSE Leads to Lower HSV-2 Replication

A. Representative Western blot of expression of viral protein gD after of knockdown of HPSE 2 and treatment with OGT 2115 after infection with HSV-2 333, samples collected 24 hours post infection. B. Quantification of Western blot of expression of viral protein gD after of knockdown of HPSE 2. C. Quantification of a plaque assay of cell lysate collected from cells that had a knockdown HPSE 2 or treated with OGT 2115 and then were infected with HSV-2 at 1 MOI. Samples were collected at 24 hours post infection. D. Quantification of a plaque assay of media collected from cells that had a knockdown HPSE 2 or treated with OGT 2115 and then were infected with HSV-2 at 1 MOI. Samples were collected at 24 hours post infection. E. Quantification of infection after treatment with OGT 2115 or knockdown HPSE 2 flow cytometry experiments. Asterisks denote a significant difference as determined by Student's t-test; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Effect of Modulation of HPSE 2 on Cell Surface HS

After discovering that overexpression of HPSE 2 negatively effects viral replication and that knockdown of HPSE 2 also negatively effects viral replication we wanted to know if the cause of these changes effects where through HPSE 2 regulating cell surface HS. First we look to discover if during infection there was a change in localization of HPSE 2 to the cell surface. We on a confocal microscope at 63x after infecting VK2 cells at 0.1 MOI stained for HPSE 2 at the cell surface (Figure 14A). We found a large increase of cell surface localization at 12hpi which then decrease by 24hpi and was only present at low levels through 48hpi. We then wanted to look at HPSE 2c since that was the only splice variant that has been reported on the cell surface. We overexpressed a HPSE 2c-myc construct where we then infected cells at 1 MOI, then stained for myc. We found that there was significant change in localization of myc and therefor HPSE 2c

upon infection, showing that HPSE 2c upon infection does localize to the cell surface (Figure 14B and 14C). We next show by immunofluorescence microscopy staining for cell surface HS that over expression of HPSE 2c can sequester HS at the surface through 24hpi (Figure 14D). We show in previous publications that by 24hpi that there is a large decrease in HS cell surface localization after infection. Last we show by staining for cell surface HS after overexpressing the HPSE 2 splice variants, over expressing GS3, treating with OGT 2115 and infection by HSV-2 333 at 24hpi that HPSE 2 across all variants can sequester HS at the cell surface (Figure 14E).

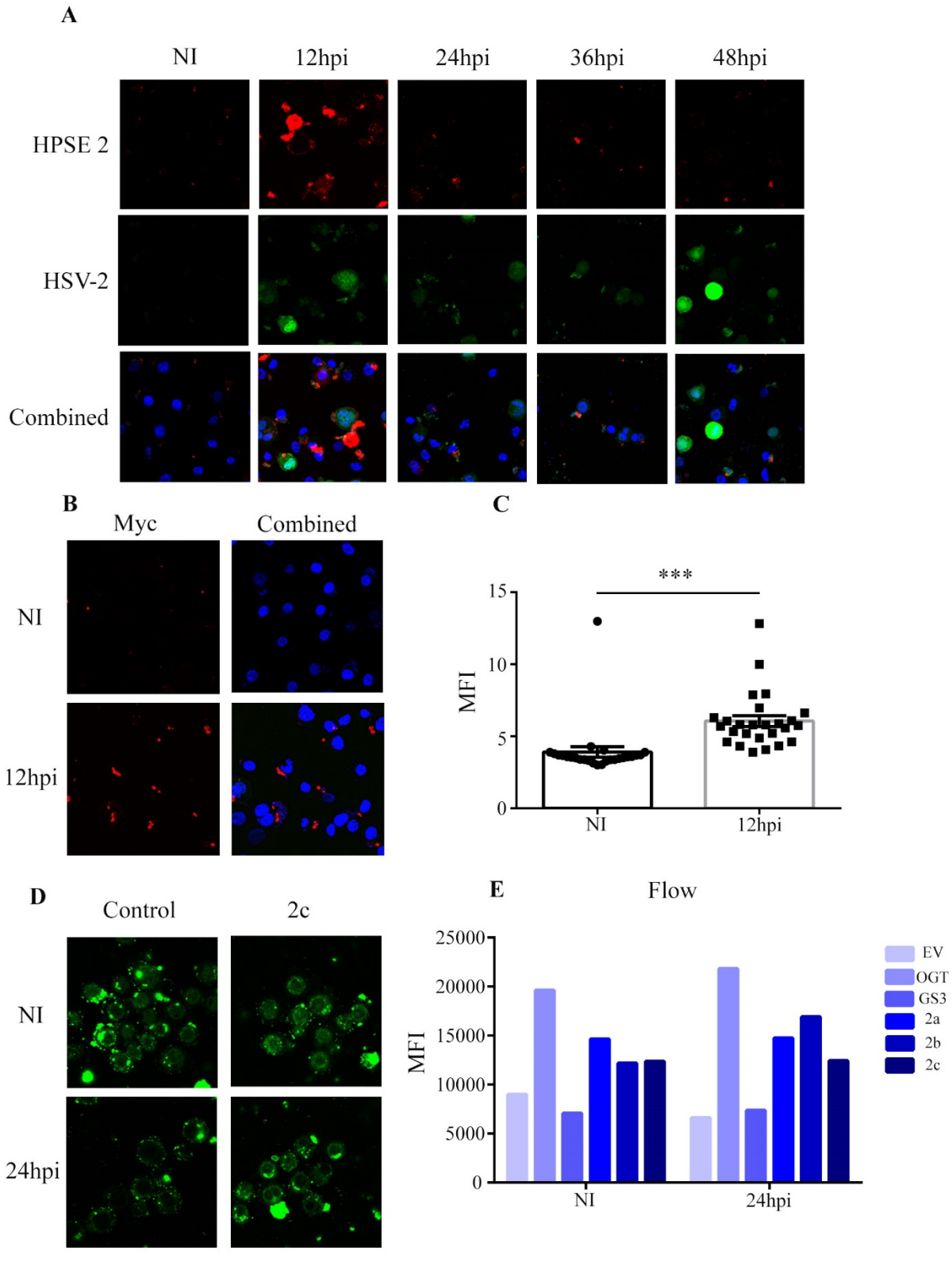
A
NI
12hpi
24hpi
36hpi
48hpi
HPSE 2
HSV-2
Combined
B
Myc
Combined
NI
12hpi
C
***
MFI
15
10
5
0
NI
12hpi
D
Control
2c
NI
24hpi
E
Flow
MFI
25000
20000
15000
10000
5000
0
NI
24hpi
EV
OGT
GS3
2a
2b
2c

Figure 14. Mechanism of HPSE 2 Inhibition of HSV-2 Infection

A. Representative Immunofluorescence microscopy images of HPSE 2 surface stain. HSV-2 333 GFP was used to infect cells at a MOI of 0.1 then images where taken at 0, 12, 24, 36, and 48 hours post infection. The top row is a HPSE 2 in red, the middle row is HSV-2 in GFP and the last row is hoescht in blue, HPSE 2 stain in red, and HSV-2 in green merged. B. Representative immunofluorescence images of surface myc stain. HSV-2 333 was used to infect cells at 1MOI for 12 hours. Upper left is myc stain only in uninfected sample, upper right is hoescht and myc stain merged for uninfected, lower left is myc stain only for infected sample at 12 hours post infection, lower right is hoescht and myc stain merged for infected sample at 24 hour post infection. C. Quantification of cell surface myc stain cell mean fluorescent intensity. D. Representative immunofluorescence images of HS stain. HSV-2 333 was used to infect cells at 1MOI for 24h after overexpression of HPSE 2c. E. Quantification of cell surface HS flow cytometry experiments. Asterisks denote a significant difference as determined by Student's t-test; *P<0.05, **P<0.01, ***P<0.001.

Discussion

In this study we have shown that upon infection with HSV-2 that there is an increase in total HPSE 2 protein levels. That modulation of HPSE leads to less virus replication whether through overexpression or through knockdown. We were surprised that during a knockdown of HPSE 2 you would see a decrease in viral replication. We have shown in other studies that an increase in HPSE results in more virus replication in HSV-1 and HSV-2. The thought process is that without an inhibitor HPSE would be free to cleave more HS at the cell surface which would allow more egress. Also we have shown that it could be through sequestering HS at the cell surface. HPSE 2

is a competitive inhibitor of HPSE by having a higher binding affinity to the same epitopes of HS. HPSE is the only known enzyme capable of cleaving cell surface HS. Through this sequestering of HS HPSE 2 can have a role in tumor suppression by segregating away HS bound growth and angiogenesis factors, leading to less cell migration and more cell differentiation.

These results have lead us to come up with a model for how HSPE 2 modulation is negatively affecting virus replication. Where if there is too little HPSE 2 before HSV-2 binding there will be not enough heparin sulfate at the cell surface level to facilitate viral binding and low levels of the heparin sulfate proteoglycans that are sequestered to the surface by heparin sulfate to facilitate entry. This lack of HPSE 2 Leads to HPSE at normal levels being able to cleave cell surface heparin sulfate leaving little to facilitate entry. On the opposite side, f there is higher HPSE 2 expression earlier in infection this leads to HPSE 2 shielding heparin sulfate from a virus mediated increase in HPSE which then we have shown to subsequently localize to the cell surface to cleave HS. This leads to more heparin sulfate at the cell surface and leads to a block of successful viral egress from cells (Figure 15).

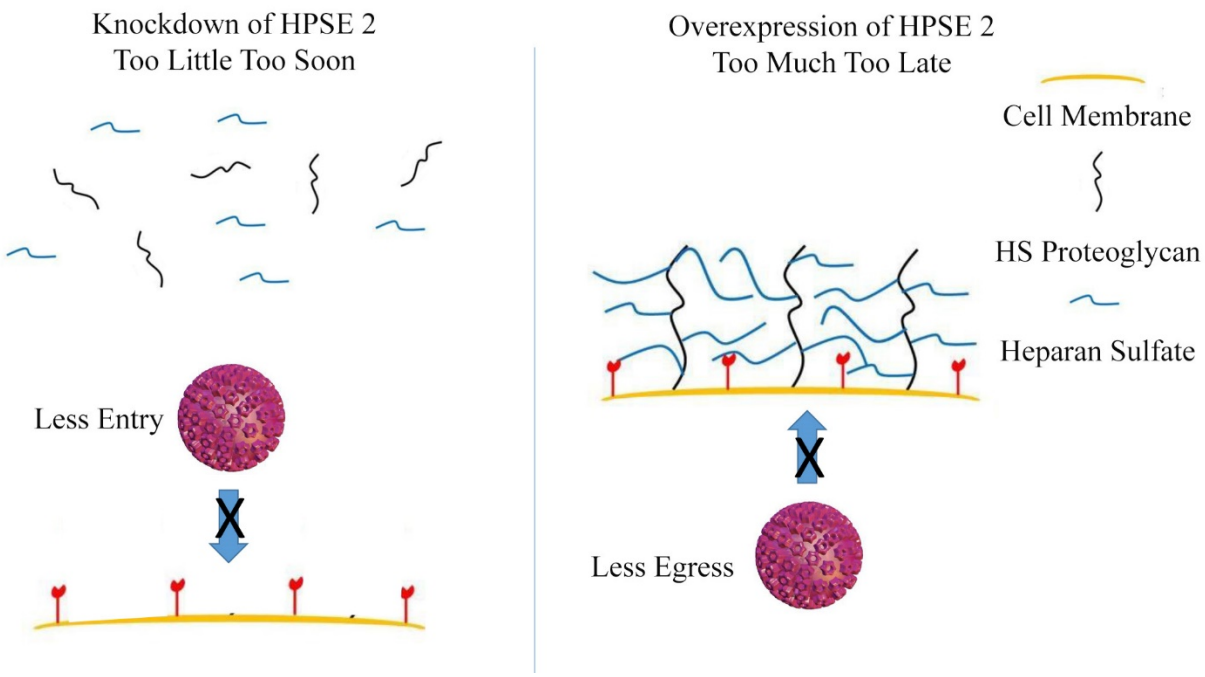
Knockdown of HPSE 2
Too Little Too Soon
Less Entry
X
Overexpression of HPSE 2
Too Much Too Late
Cell Membrane
HS Proteoglycan
Heparan Sulfate
X
Less Egress

Figure 15. Model of How of HPSE 2 Effects HSV-2 Infection

Starting on right. If there is too little HPSE 2 before HSV-2 binding there will be not enough heparin sulfate at the cell surface level to facilitate viral binding and low levels of the heparin sulfate proteoglycans that are sequestered to the surface by heparin sulfate to facilitate entry. This lack of HPSE 2 Leads to HPSE at normal levels being able to cleave cell surface heparin sulfate. On the right, if there is higher HPSE 2 expression earlier in infection this leads to HPSE 2 shielding heparin sulfate from a virus mediated increase in HPSE. This leads to more heparin sulfate at the cell surface and leads to a block of successful viral egress from cells.

To completely test this model we still need a lot of work. Entry and binding data in both overexpression and knockdown conditions will go far in confirming our model. Along with HS surface stains in the knockout condition with appropriate controls can confirm it. There are larger questions that rise from this study, namely how are spliced variants HPSE 2b and HPSE 2A are effecting virus replication without effect cell surface HS, and by what mechanism is HPSE binding and cleaving cell surface HS when there is rise in HPSE 2 at the cell surface also. While the again there are many questions our studies discussed here directly implicate HPSE 2 in sequestering cell surface HS that can protect cell from viral infection.

CHAPTER 5: BX795

(Previously published as Hopkins J, Yadavalli T, Suryawanshi R, Qatanani F, Volety I, Koganti R, Iqbal A, Shukla D. 2020. In vitro and In vivo activity, tolerability, and mechanism of action of BX795 as an antiviral against herpes simplex virus 2 genital infection. Antimicrob Agents Chemother 64:e00245-20. https://doi.org/10.1128/AAC.00245-20)

Introduction:

Herpes Simplex Virus Type-2 (HSV-2) is a ubiquitous infection, with prevalence rates ranging from 10% to more than 80% depending on the location, population age, behavior and gender (116-120). It is responsible for the majority of clinical cases of genital ulcers worldwide (121-123). HSV-2 is prone to reactivation as 60% of people experience recurring episodes and 20% of people experience more than 10 episodes of recurring genital ulcers during the first year of infection (124, 125). Even five years after the initial infection, HSV-2 reactivates twice a year on average. Initially, the infection presents as macules/papules, ulcers, pustules, and vesicles that can occur over time (126-128). Extended infections can cause flu-like symptoms and eventually lymphadenopathy, cervicitis, and proctitis (128, 129).

Unlike HSV-1, HSV-2 is communicated primarily through sexual contact. However, HSV-2 has a wide variety of bodily targets. Infections usually occur in the genitals, but HSV has been shown to infect the CNS and the eye, leading to Mollaret's meningitis and encephalitis (130-132) in the former case and acute retinal necrosis in the latter (133). The virus can also be passed from the mother to child during pregnancy, leading to skin lesions and poor prognoses (129). HSV-2 has also been shown to increase the risk of HIV acquisition, further elevating it as a public health concern (134-139).

The primary treatment for HSV-2 infections consists of nucleoside analogs, such as acyclovir, valacyclovir, and famciclovir (140). These antivirals inhibit viral DNA polymerase activity, preventing the virus from replicating successfully (141, 142). Dosages for herpes genitalis treatment are highly variable, depending on the progression of the disease and immune status of the patient (122). However, these nucleoside analogs suffer from multiple shortcomings. They do not directly obstruct viral protein synthesis (143), are prone to resistance and escape mutants (144-

146), and cause nephrotoxicity after extended usage (147, 148). In immunosuppressed patients, resistance to acyclovir and its analogs occurs in about 5% of cases (149). Patients with resistance are prescribed a pyrophosphate analog foscarnet, another viral DNA polymerase inhibitor, but its side effects include nephrotoxicity, anemia, and the onset of new genital ulcers (150). Better alternatives are needed for the treatment of genital HSV-2 infections.

BX795, a known inhibitor of TANK-binding kinase 1 (TBK1), has been shown to inhibit ocular HSV-1 infections in in vitro, in vivo, and ex vivo models (151, 152). It functions through an entirely separate mechanism from the nucleoside and pyrophosphate analogs that are used widely today. The mechanism of its action is not fully elucidated but involves the inhibition of protein kinase-B (AKT) phosphorylation and the subsequent hyperphosphorylation of 4EBP1. Through this mechanism, BX795 is able to impede viral translation, abrogating the production of virions as a result. Only one study has examined the effects of BX795 on HSV-2 infections, and it proposes that BX795 acts upstream of the JNK/p38 pathways (153). However, this study performed all of the experiments on a Vero cell line and in vivo efficacy was not tested. Hence, further research using physiologically relevant models such as natural target cells and murine models is needed to confirm the efficacy of BX795 on the treatment of HSV-2.

Here, we study the antiviral efficacy and drug tolerability of BX795 on HSV-2 infected vaginal epithelial cells and murine vaginal epithelium. We show that vaginal epithelial cells tolerate higher concentrations of BX795 than what has been previously reported on corneal epithelial cells. Through a murine model of vaginal HSV-2 infection, we show excellent antiviral efficacy of BX795 and no observable toxic effects during the drug course. These comprehensive results point to the applicability of BX795 in treating genital herpes infections through a topical mode of delivery.

Results:

BX795 attenuates HSV-2 infection:

To understand whether BX795 treatment inhibits HSV-2, we began by looking at viral transcript levels 24 hours post-infection (hpi) after a 0.1 MOI infection in VK2 cells followed by treatment with BX795 (10 μM). Across different classes of viral genes, ICP27 (immediate early), gD (late), and UL30 (a subunit of viral DNA polymerase), we found that BX795 treated cells showed significantly lower transcript levels (Figure 16A, B, C). These results were promising given that no significant differences between the ACV treatment and BX795 treatment group were seen. Similarly, viral protein (HSV-2 gD and VP16) levels at 24 hpi were significantly lower when measured through western blot analysis (Figure 16D). Our results coincide with fluorescent microscopy (Figure 16E) and flow cytometry data (Figure 16H, I) where a reporter HSV-2 (strain 333-GFP) that expresses GFP on a CMV promoter was used. We observed significantly lower GFP production in BX795 treated cells compared to DMSO control at 12, 24, and 36 hpi. BX795 also showed significant impairment of viral replication when measured through plaque assay (Figure 16F, G).

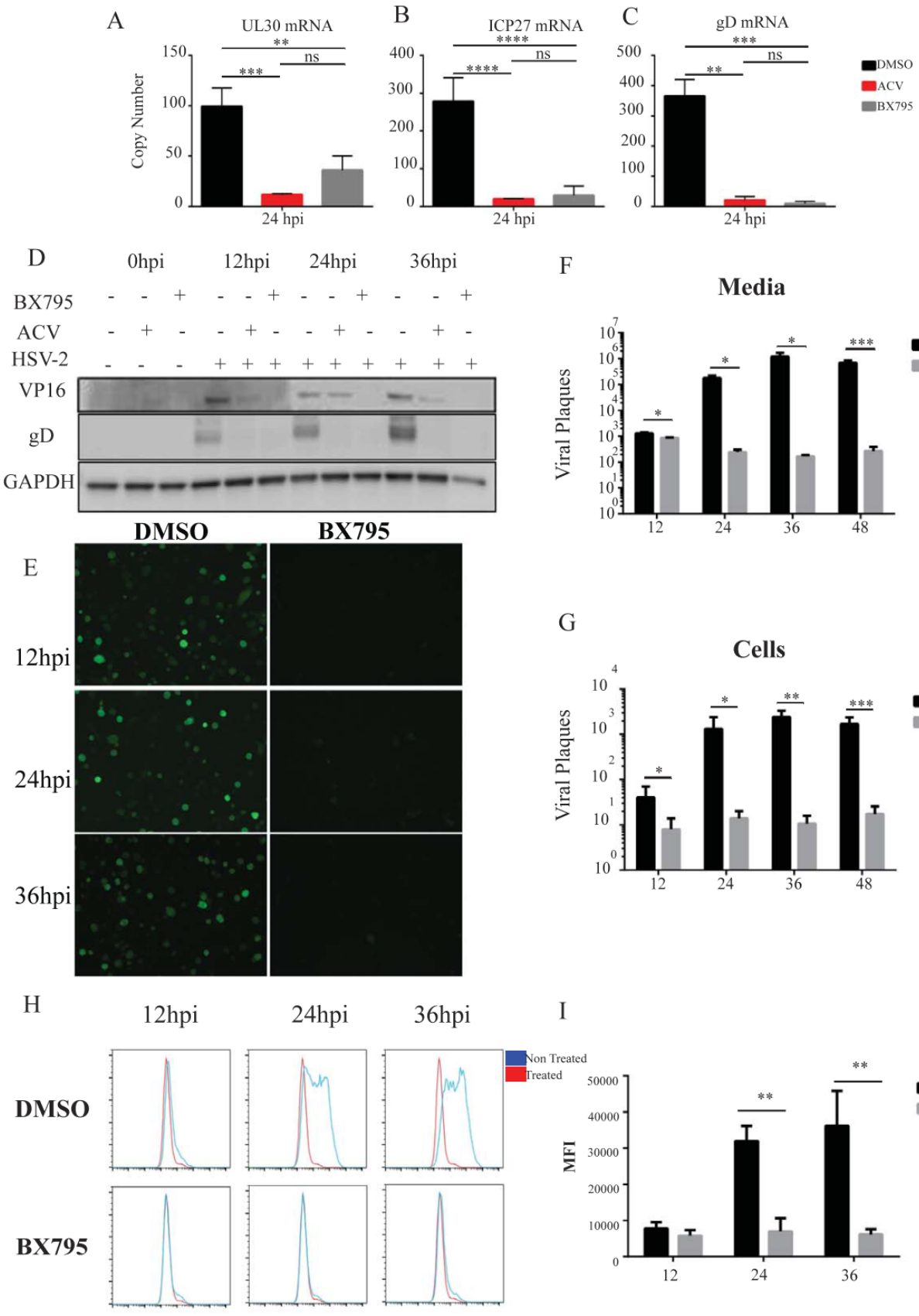
A
UL30 mRNA
Copy Number
24 hpi
B
ICP27 mRNA
24 hpi
C
gD mRNA
24 hpi
DMSO
ACV
BX795
D
0hpi
12hpi
24hpi
36hpi
BX795
ACV
HSV-2
VP16
gD
GAPDH
E
DMSO
BX795
12hpi
24hpi
36hpi
F
Media
Viral Plaques
DMSO
BX795
G
Cells
Viral Plaques
H
12hpi
24hpi
36hpi
DMSO
BX795
Non Treated
Treated
I
MFI
DMSO
BX795

Figure 16. BX795 attenuates HSV-2 infection.

A. VK2 cells infected with HSV-2 333 at 0.1MOI then transcript levels of viral protein UL30 were measured 24 hpi with black representing DMSO, red representing ACV (50μM), and grey representing BX795 (10μM) treated cells. B. VK2 cells infected with HSV-2 333 at 0.1MOI then transcript levels of immediate early viral protein ICP27 was measured 24 hpi. C. VK2 cells infected with HSV-2 333 at 0.1MOI then transcript levels of late viral protein gD were measured 24 hpi. D. VK2 cells infected with HSV-2 333 at 0.1MOI then treated after infection with DMSO, ACV, or BX795. Samples taken 0, 12, 24 and 36 hpi. Whole cell lysates were probed by immunoblotting with antibodies against viral proteins VP16 and gD. E. VK2 cells were infected with HSV-2 333 GFP at 0.1MOI then cells treated after infection with DMSO, ACV (50 μM), or BX795 (10 μM). Fluorescent images were taken at 12, 24, and 36 hpi. F. VK2 cells infected with HSV-2 333 at 0.1MOI then media samples taken every 12 hpi till 48 hpi. Viral plaques of virus shed from cells into media with black representing DMSO and grey representing BX795. G. VK2 cells infected with HSV-2 333 at 0.1MOI then media samples taken every 12 hpi till 48 hpi. Viral plaques of intra cellular virus with black representing DMSO and grey representing BX795(10 μM). H. VK2 cells infected with HSV-2 333 GFP at 0.1MOI then treated with 10μM BX795 then cells were collected and flow cytometry was performed measuring cell GFP florescence. I. Quantification of H. *, $P < 0.05$; **, $P < 0.01$; ***,$P<0.001$; ****,$P<0.0001$.

Mechanism of BX795 inhibition of HSV-2 infection is through prevention of AKT phosphorylation:

After showing its effectiveness as an HSV-2 inhibitor, we wanted to understand whether the mechanism of action of BX795 was similar to our previously reported study. We have previously

reported that BX795 inhibits viral protein translation via the inhibition of AKT (151). Using vaginal epithelial cells and HSV-2 infection, we performed immunofluorescence studies to estimate phosphorylation of AKT at ser-473 site in the presence and absence insulin (positive control for AKT phsophorylation), BX795 or both (Figure 17A). Quantification of mean fluorescent intensity (MFI) of individual cells over multiple images showed increased AKT phosphorylation in non-infected insulin treated samples which significantly decreased when the cells were treated with BX795 (Figure 17B). Interestingly, in HSV-2 infected cells, no significant increase in phosphorylation was observed when they were treated with insulin, however, BX795 treated cells both in the presence and absence of insulin showed decreased AKT phosphorylation. Immunoblotting studies using vaginal epithelial cells treated insulin revealed decreased phosphorylation of AKT at ser-473 site when treated with either AZD5363 (a known AKT inhibitor) or BX795 (Figure 18). Together, these results strongly correlate with our earlier reported findings which suggested that BX795 inhibits the viral recruitment of the cellular translational machinery thereby ensuring no viral protein synthesis and viral replication (151).

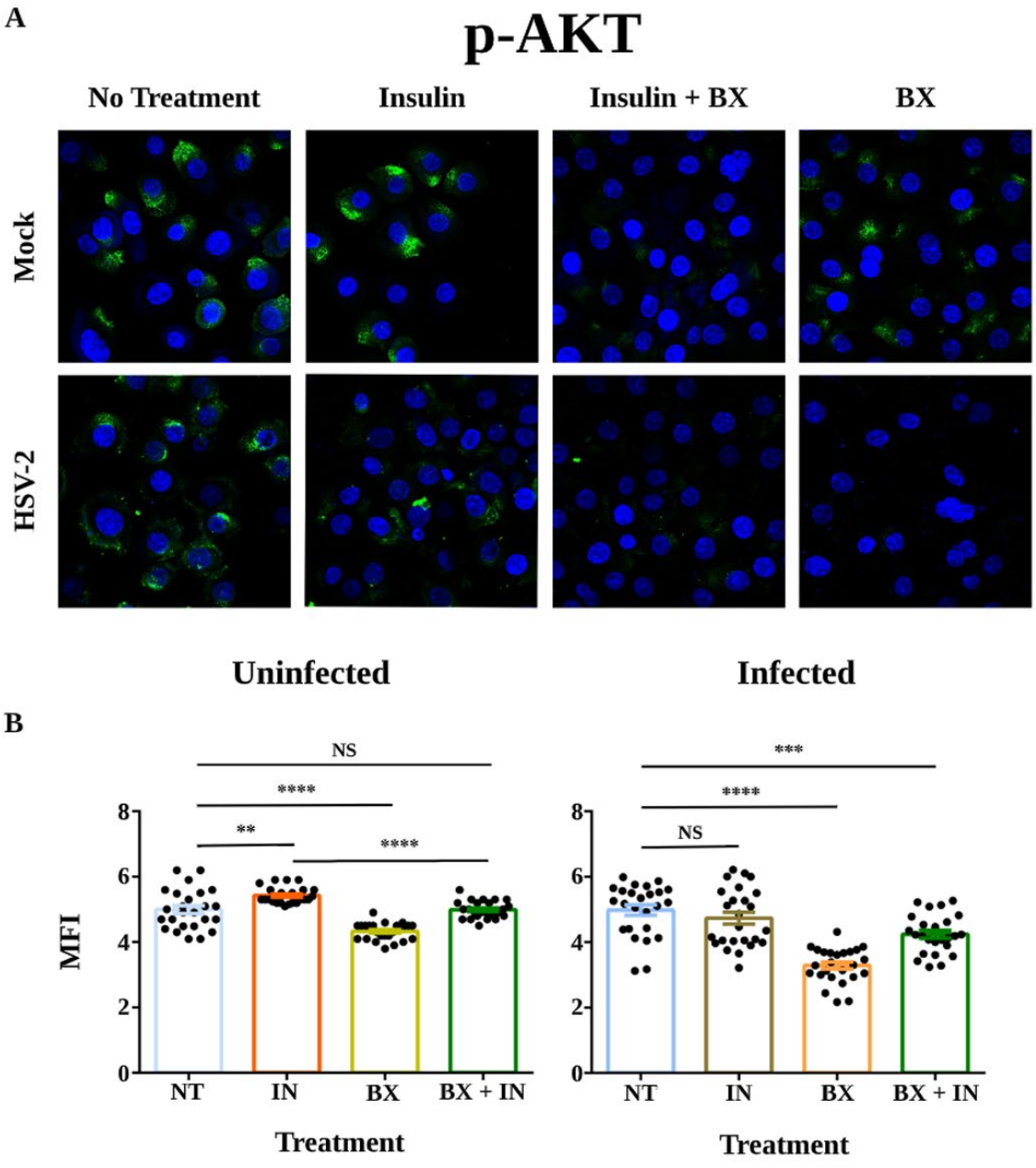
A
p-AKT
No Treatment
Insulin
Insulin + BX
BX
Mock
HSV-2
Uninfected
Infected
B
NS
****
**
****
***
****
NS
MFI
0
2
4
6
8
NT
IN
BX
BX + IN
Treatment

Figure 17. Mechanism of antiviral action of BX795.

A. VK2 cells were infected with HSV-2 333 at 0.1MOI and then treated with either mock, BX795 (10 μM) or insulin (10 nM). The cells were stained with an antibody against p-AKT. Green represents p-AKT expression. B. Quantification of A. *, P < 0.05; **, P < 0.01; ***,P<0.001; ****,P<0.0001.

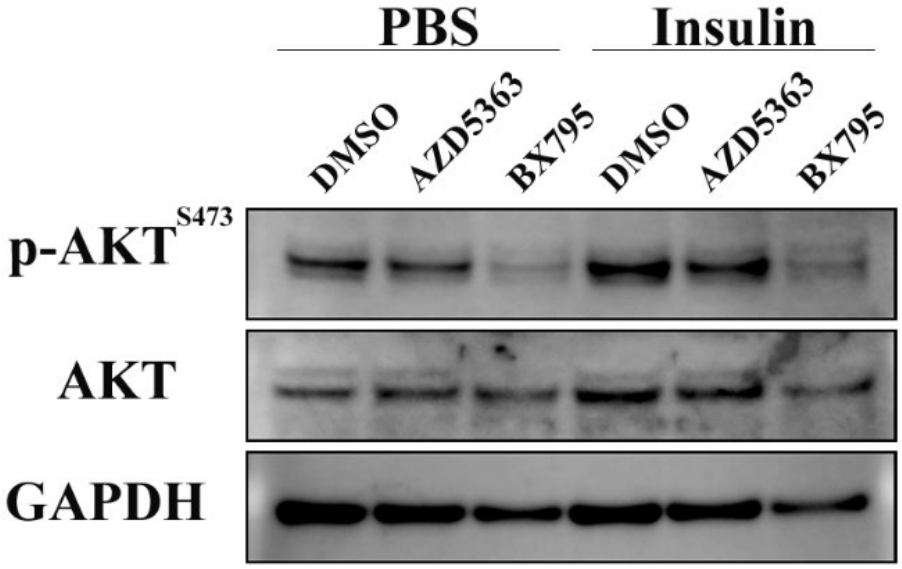

Figure 18: BX795 inhibits the phosphorylation of AKT at ser-473 site.

VK2 cells were treated with either DMSO, AZD5363 or BX795 at 10 µM concentration prior to the addition of mock PBS or Insulin at 100 nM for 10 minutes. The cells were lysed and immunoblotted for the shown antibodies. GAPDH was used as the loading control.

Therapeutic and prophylactic efficacy of BX795 against HSV-2 infection:

In our previous studies, we have described both therapeutic and prophylactic antiviral efficacy of BX795 against ocular HSV-1 infection. However, similar studies were not performed in vaginal epithelial cells to curb HSV-2 infection. Furthermore, most of our studies utilized BX795 at a therapeutic concentration of 10 μM. Hence in this study, we investigated the concentrations at which BX795 was antiviral against HSV-2 while nontoxic to vaginal epithelial cells. We observed loss of HSV-2 GFP production through fluorescent imaging (Figure 19A-top) at concentration ≥ 10 μM with no apparent cytopathic effect observed in brightfield (BF) images at concentrations as high as 80 μM (Figure 19A-bottom). Also, a significant reduction in viral load was seen in both extracellular supernatant and intracellular whole-cell lysates at concentrations as low as 2.5 μM, with complete inhibition seen at concentrations ≥ 10 μM (Figure 19B,C). On the other hand, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay to assess the viability of vaginal epithelial cells incubated with increasing concentrations of BX795 for a period of 48 hours (Figure 19D) showed no significant loss of viability in vaginal epithelial cells treated with BX795 at a concentration as high as 80 μM. This is very interesting given that in our previous study we reported a significant loss of viability at 100 μM BX795 in human corneal epithelial cells.

Once we confirmed the tolerability, we sought to understand the antiviral efficacy of BX795 in a delayed therapeutic treatment assay. Usually, in a cell culture experiment to test the therapeutic efficacy, we add a requisite concentration of the drug 2 hpi with HSV-2. However, in this study, using a time-course experiment, we tried to evaluate the extent of delay in drug administration before which BX795 is not effective anymore. To test this, vaginal epithelial cells were infected with 0.1 MOI HSV-2 GFP virus followed by the addition of BX795 at 2, 4, 6 and 12 hpi. All cells were imaged at 24 hpi to evaluate the extent of viral spread (Figure 19E). To our surprise, all

treatment groups including those that were treated 12 hpi showed little to no signs of viral infection at 24 hpi (Figure 19E). These results were confirmed by plaque assays where a significant loss in viral load was observed both extracellular (Figure 19F) and intracellular (Figure 19G) viruses.

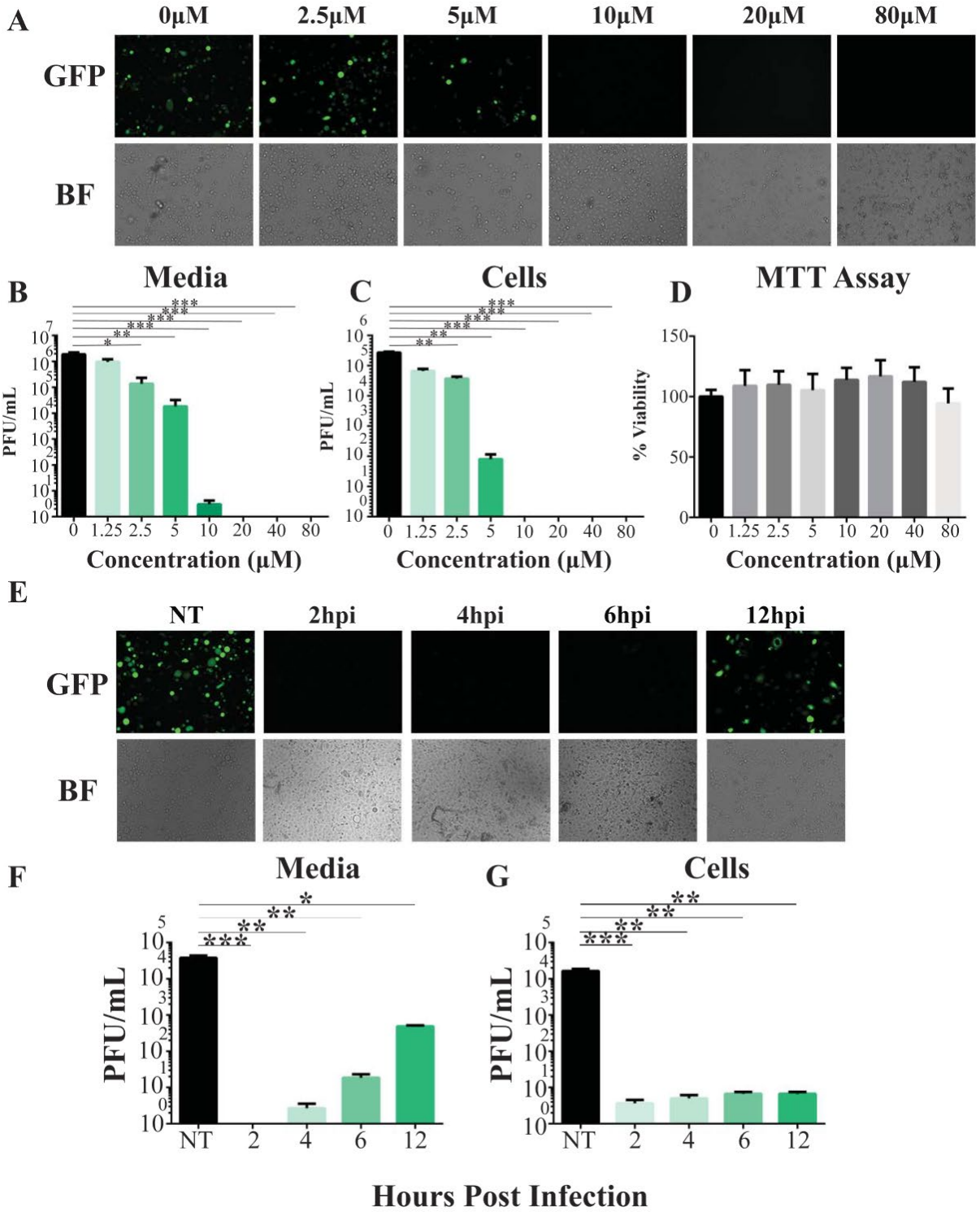
A
0μM
2.5μM
5μM
10μM
20μM
80μM
GFP
BF
B
Media
PFU/mL
Concentration (μM)
C
Cells
PFU/mL
Concentration (μM)
D
MTT Assay
% Viability
Concentration (μM)
E
NT
2hpi
4hpi
6hpi
12hpi
GFP
BF
F
Media
PFU/mL
G
Cells
PFU/mL
NT
2
4
6
12
Hours Post Infection

Figure 19. Efficacy of BX795 as a treatment for HSV-2 infection.

A. Representative immunofluorescence microscopy images of VK2 cells where infected with HSV-2 333 GFP then treated with 0 µM to 80 µM BX795. Images were taken 24 hpi. B. Viral plaques of virus shed from cells into media after infection with HSV-2 333 at 0.1MOI and subsequent treatment with increasing concentrations of BX795. With cells treated 2 hpi and samples collected 24 hpi C. Viral plaques of intracellular virus after infection with HSV-2 333 at 0.1MOI and subsequent treatment with increasing concentrations of BX795. With cells treated 2 hpi and samples collected 24 hpi. D. VK2 cells treated with 0 µM to 80 µM BX795 then collected 24 hours after treatment. Then a MTT assay was preformed to check viability. E. Representative immunofluorescence microscopy images of VK2 cells where infected with HSV-2 333 GFP at 0.1MOI then treated with 10 µM BX795 at 2, 4, 6, and 12 hpi. Images were taken 24 hpi. F. Viral plaques of virus shed from cells into media after infection with HSV-2 333 at 0.1MOI then treatment with 10 µM BX795 at 2, 4, 6, and 12 hpi. Samples were taken 24 hpi. G. Viral plaques of virus shed from cells after infection with HSV-2 333 at 0.1MOI then treatment with 10 µM BX795 at 2, 4, 6, and 12 hpi. Samples were taken 24 hpi.

Discerning the astonishing results from our delayed therapy experiment, we wanted to understand the prophylactic efficacy of BX795. Contrary to the experiments detailed above, we wanted to evaluate the extent of the prophylactic duration required to keep BX795 treated cells protected from HSV-2 infection (Figure 20A). It is pertinent to understand that in this experiment, once the cells were infected with the virus, the cells did not have access to BX795. Any antiviral efficacy being noticed must be a result of the BX795's prophylactic ability to protect cells. To test this, we treated vaginal epithelial cells with BX795 for 24, 12, 6, 4 and 2 hours infection (hbi) with HSV-

2 GFP in fresh media with no BX795. Fluorescent and BF images from this experiment indicated that treating VK2 cells 24 hbi showed loss of HSV-2 GFP, while treating them for 12 and 6 hbi showed partial protection from HSV-2 infection and no differences were found in treatment groups 4 and 2 hbi when compared to non-treated control groups (Figure 20B). However, plaque assay results showed a significant loss of infection at all time points except 2 hbi in both intracellular (Figure 20C) and extracellular (Figure 20D) viral loads.

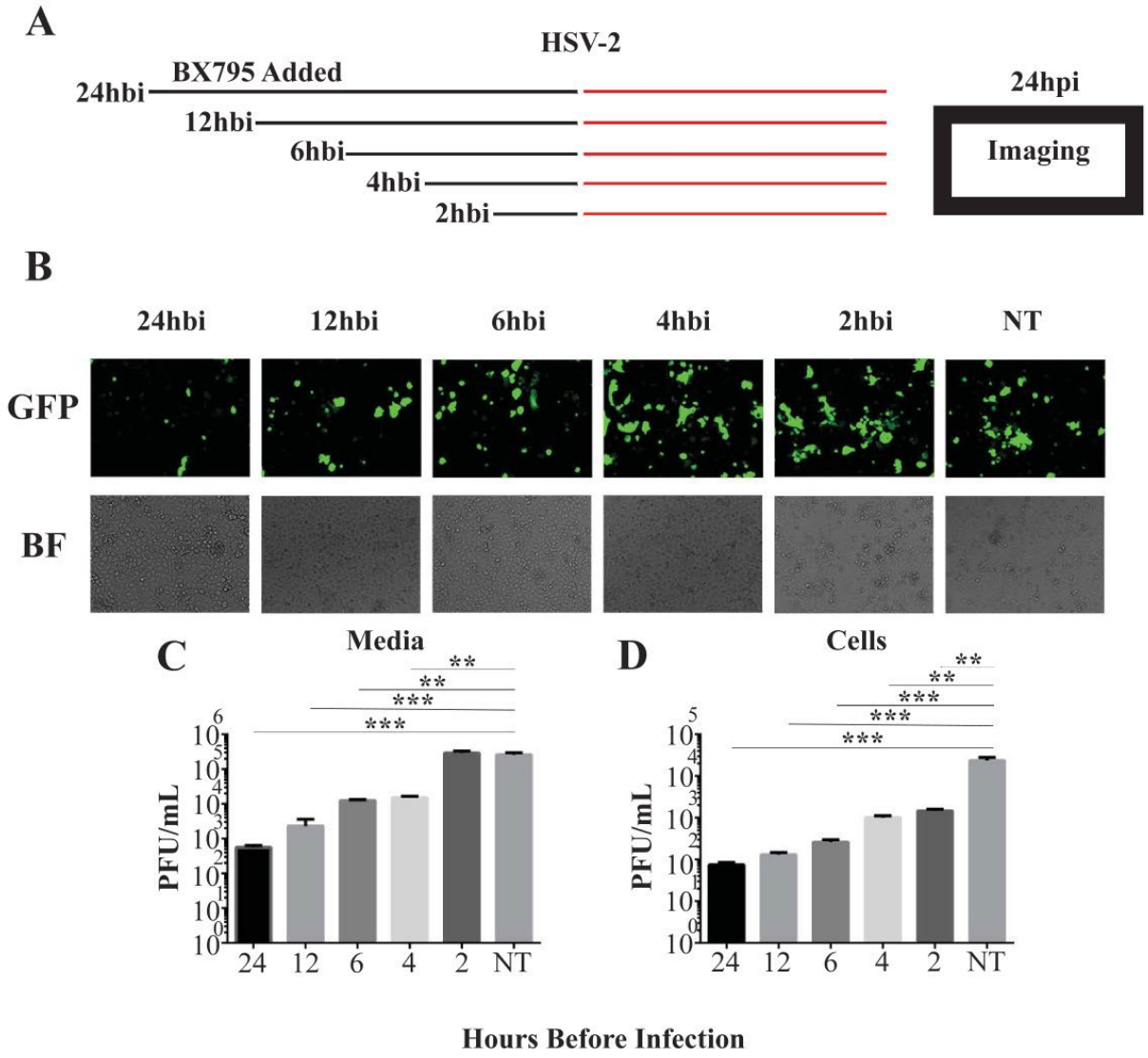
A
HSV-2
BX795 Added
24hbi
12hbi
6hbi
4hbi
2hbi
24hpi
Imaging
B
24hbi
12hbi
6hbi
4hbi
2hbi
NT
GFP
BF
C
Media
PFU/mL
**
**
***
***
24
12
6
4
2
NT
D
Cells
PFU/mL
**
**
***
***
***
24
12
6
4
2
NT
Hours Before Infection

Figure 20. Prophylactic antiviral efficacy of BX795.

A. Schematic of the experiment being conducted. VK2 cells were treated with BX795 (10 µM) for a period of either 24, 12, 6, 4, 2 or 0 hours before the media was changed to fresh media containing 0.1 MOI HSV-2 GFP virus and no BX795. B. The cells were incubated with the virus for an additional 24 hours before they were imaged using a fluorescent microscope to observe extent of viral GFP (green) and cytopathic effect (BF). C. The media supernatant containing the extracellular secreted virus and D. whole cell lysate containing total intracellular virus was collected and overlaid on Vero cells to perform a plaque assay to quantify viral load. One-way ANOVA was used to analyze the results for significant differences. *, P < 0.05; **, P < 0.01; ***,P<0.001.

In Vivo Efficacy of BX795 as a treatment for HSV-2 infection:

After confirming the tolerability and antiviral efficacy of BX795 in vitro, we wanted to assess whether topical BX795 treatment could be effective in protecting mice from a murine model of vaginal infection. The menstrual cycle of eight-week-old female mice (n=5 per group) was synchronized using subcutaneous medroxy-progesterone prior to intravaginal (1x106 PFU) HSV-2 infection. At 1 dpi, mice were treated topically via intravaginal route using DMSO, BX795 (10 µM) or BX795 (50 µM). Vaginal swabs were taken 2 and 4 days post-infection (dpi) from all the groups to assess the extent of viral spread through a plaque assay (Figure 21A, B). Interestingly, no evident protection was seen in animals treated with 10 µM BX795 when compared to DMSO control group mice. However, significant loss of infection was found in mice that were treated

with 50 μM BX795. This is interesting because ocular topical dosage using 10 μM BX795 had shown excellent therapeutic efficacy in our previous studies against HSV-1 infection.

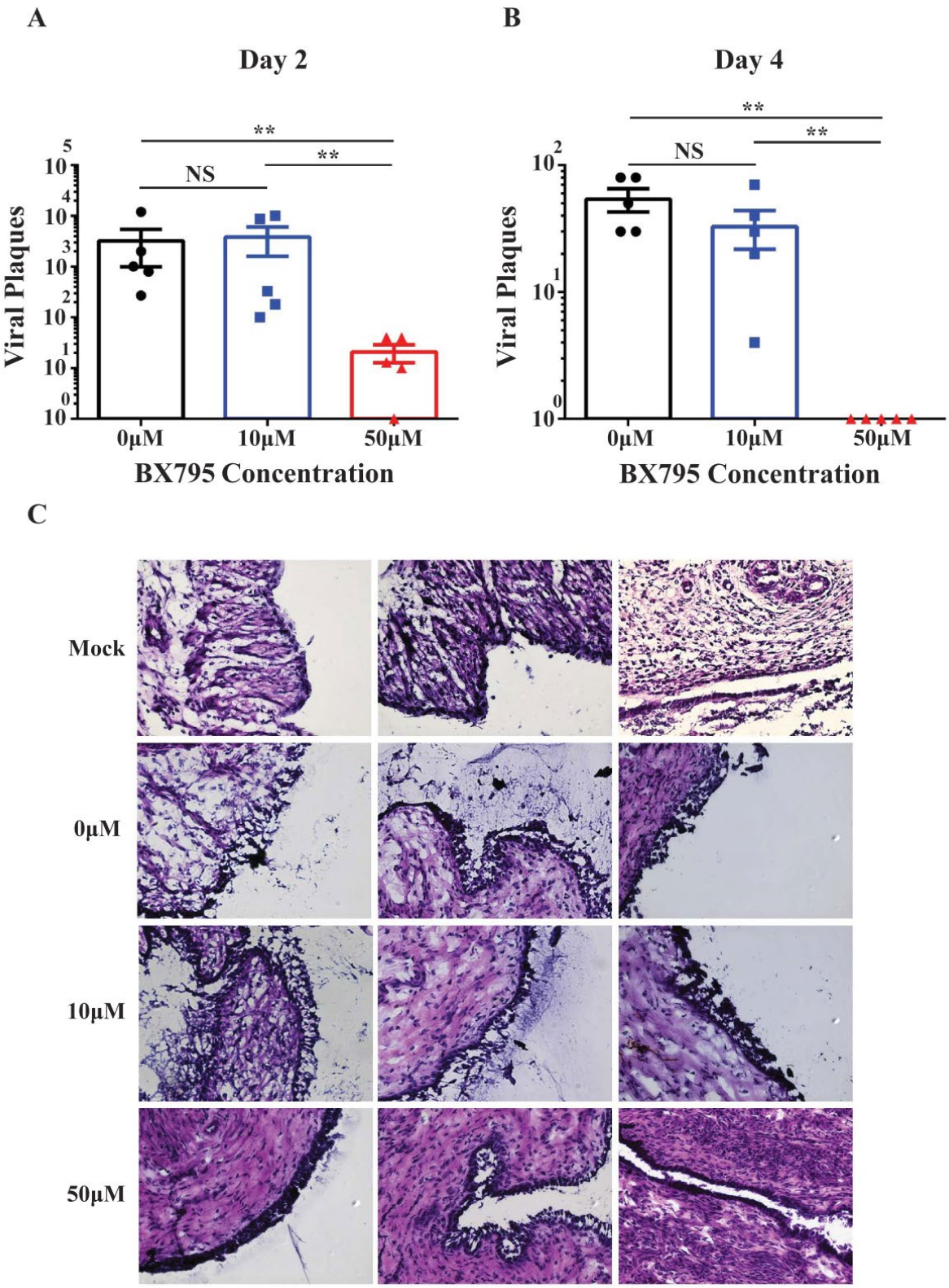
A
Day 2
**
**
NS
Viral Plaques
0μM
10μM
50μM
BX795 Concentration
B
Day 4
**
**
NS
Viral Plaques
0μM
10μM
50μM
BX795 Concentration
C
Mock
0μM
10μM
50μM

Figure 21. In Vivo Efficacy of BX795 as a treatment for HSV-2 infection.

A. Secreted virus titers assessed from the swabs of vaginas (n = 5 per treatment group) 2 days post infection. B. Secreted virus titers assessed from the swabs of vaginas (n = 5 per treatment group) 4 days post infection. C. Representative 10 micron sections of epithelium from mice, non-infected, infected non-treated, infected low dose, infected high dose. . Quantification of C. *, P < 0.05; **, P < 0.01; ***,P<0.001; ****,P<0.0001.

Lastly, we wanted to evaluate whether therapeutic treatment with BX795 at 50 μM concentration caused any significant morphological differences in the vaginal epithelium. To assess this, animals were sacrificed on 4dpi and their vaginal tissue was processed for histopathological study. Cryo-sectioned vaginal tissues were stained with hematoxylin and eosin (H&E) stain and 3 representative tissues from 3 different mice of the same group are shown (Figure 21). In DMSO and 10 μM BX795 treatment groups, we observed large disruptions of the vaginal epithelial surface however, no such disruptions were found in the 50 μM group. These results indicate that BX795 is well tolerated by vaginal epithelium both in vitro and in vivo and shown excellent antiviral efficacy at 50 μM concentration in vivo**.**

Discussion:

Herpes simplex virus type-2 (HSV-2) belongs to the neurotropic alphaherpesvirus subfamily of herpesviruses. The virus shares strong genetic homology with HSV-1 and both viruses result in very similar innate and adaptive immune responses from the human hosts. HSV-2 infects about 20% of the US population and anywhere from 10-50% worldwide. Primary infection of genital or anal mucosal epithelium is followed by spread to sacral ganglia where the virus establishes latency that lasts for the lifetime of the human host. This is further complicated by the fact that prior

infection with HSV-2 increases the chance for HIV/AIDS acquisition by 2-3 folds. According to the CDC Fact sheet on Incidence, Prevalence, and Cost of Sexually Transmitted Infections (STI) in the US, HSV-2 is the second most common STI after HPV. The US spends over $16 billion (in the year 2010) to treat STIs (154). The estimated number of known cases with HSV-2 includes over 24 million adults in the US alone. The actual seropositive numbers are suspected to be twice more.

While acyclovir (ACV) and related nucleoside analogs provide successful modalities for treatment and suppression, HSV remains highly prevalent worldwide. The emergence of ACV-resistant virus strains and the universal ability of HSV to establish latency coupled with adverse effects of the long-term systemic use of currently available anti-herpetic compounds provide a stimulus for the increased search for new and more effective antivirals against HSV-2. In our recent article (151), we discovered that the off-target effect of a TBK-1 inhibitor, BX795 is effective in controlling HSV-1 infection. We also provided preliminary data to support that BX795 was effective against other herpesviruses including HSV-2. However, none of the studies on HSV-2 were conducted on target cell lines or in murine models of genital infection. In this study, we provide concise data on the antiviral efficacy and tolerability of BX795 using vaginal epithelial cells in vitro and murine vaginal tissue in vivo.

Our initial results from this study show that BX795 is effective in controlling HSV-2 at a previously reported concentration of 10 μM in vitro at the transcriptional and translational level. These results are comparable to cells treated with ACV showing that BX795 is as effective as currently used therapeutics at a much lower concentration. The mechanism of action of BX795 in controlling HSV-2 infection in vaginal epithelial cells was consistent with our previous reports where we showed a significant loss in AKT phosphorylation in BX795 treated cells. While these

results indicate a potential mechanism that BX795 is a potent inhibitor of viral protein translation and can be used to suppress HSV-2 infection in target cell type, the true mechanism of antiviral action cannot be completely discerned in this study.

Another interesting result we observed in this study was the tolerability of BX795 in vaginal epithelial cells. While our previous experiments, both in vitro and in vivo have shown good tolerability of BX795, they were all performed at a much lower concentration. In this study, we observed that even at a concentration as high as 80 μM, BX795 did not affect the viability of vaginal epithelial cells, giving us the confidence that this drug can have a large therapeutic window for the treatment of HSV-2 infection in vivo. Furthermore, while the antiviral efficacy of BX795 is concentration-dependent, the goal of this study was to use it at a minimum inhibitory concentration of 10 μM, below which BX795 treated cells were visibly infected with the virus.

Through innovative time point studies, in this study, we showed that therapeutically treating vaginal epithelial cells even after 12 hpi is sufficient to control viral replication. Also, treating the cells for 24 or 12 hours before infection prophylactically can protect vaginal epithelial cells from HSV-2 infection. These results point to the opportunity that BX795 can confer protection for extended periods and the effect of its treatment lasts long after the drug is removed from culture media.

Finally, our in vivo results show excellent antiviral efficacy in controlling HSV-2 using a murine model of vaginal infection. However, it is interesting to note that BX795 did not function effectively when used at a concentration of 10 μM through intravaginal route. This is contrary to our previous findings where we reported that a topical 10 μM BX795 administered 3 times daily was sufficient to control ocular HSV-1 infection. In our study, we found that 50 μM BX795 was required to curb HSV-2 infection in the vaginal tissue. We hypothesize that the acidic pH of the

vaginal environment might be responsible for the fast degradation of the drug, which now requires an increased drug concentration to be effective against HSV-2 infection. While this is an excellent opportunity to utilize drug delivery systems to safeguard the drug until it reaches the desired tissue, it is out of the scope of this study and may be pursued in the future.

In conclusion, BX795 is an excellent alternative to current therapeutic options against HSV-1 and HSV-2 infections. This study has not only shown the therapeutic efficacy of BX795 against HSV-2 infection in target vaginal epithelial cells but has also demonstrated the safety and tolerability in the vaginal epithelium in vivo. Our results show a great promise for a novel antiviral that has a mechanism of action completely different from those clinically used.

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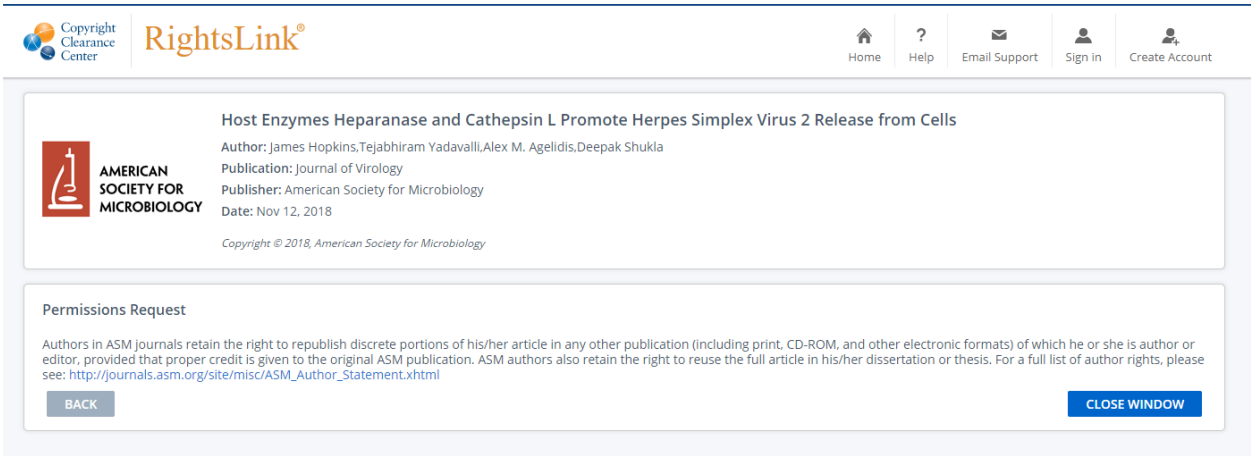
Appendices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Host Enzymes Heparanase and Cathepsin L Promote Herpes Simplex Virus 2 Release from Cells
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In Vitro and *In Vivo* Activity, Tolerability, and Mechanism of Action of BX795 as an Antiviral against Herpes Simplex Virus 2 Genital Infection

Author: James Hopkins,TeJabhiram Yadavalli,Rahul Suryawanshi,Farreh Qatanani,Ipsita Volety,Ram Koganti,Aqsa Iqbal,Deepak Shukla

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VITA

James J. Hopkins

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Education

Northeastern Illinois University: Chicago, IL **May 2015**
Bachelor of Science
Major: **Biology**

University of Illinois at Chicago **Expected August 2020**
PhD Microbiology Immunology

Research Experience

Shukla Laboratory Chicago, IL **May 2016-Present**

- Characterization of life the cycles of Herpes Simplex 1 and 2.
- Development of novel research direction to characterize the function Heparanase during a HSV 2 infection
- Characterization of novel HSV-2 inhibitor
- Extensive familiarity with the different Microbiological techniques used in the Shukla lab.
- Mentoring of new members to the lab.

Stojković Laboratory Chicago, IL **May 2013-2015**

- Characterization of the photochemistry of a bacteriophytochrome (BphP), bacterial red-light photoreceptor, through extensive UV-vis spectroscopy
- Development of novel research direction to characterize the function of BphPs in non-photosynthetic bacteria through literature review and protocol formation.

- Extensive familiarity with protein purification using metal affinity chromatography, plasmid extraction, PCR, bacterial transformation, SDS-PAGE electrophoresis, DNA gel electrophoresis, and general bacterial handling techniques.
- Mentoring of new students to the lab.
- Competence in presenting my research, as well as other projects in the lab, at national conferences

Publications and Presentations

Hopkins, J, Yadavalli T, Suryawanshi R, Qatanani F, Volety I, Shukla D. **Heparanase 2 plays a key role in regulating virus herpes simplex virus entry and egress through surface heparan sulfate regulation**. In preparation. 2020

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Yadavalli T, Ames J, Agelidis A, Suryawanshi R, Jaishankar D, **Hopkins J**, Thakkar N, Koujah L, Shukla D. **Drug-encapsulated carbon (DECON): A novel platform for enhanced drug delivery.** Sci Adv. 2019

Hopkins J, Yadavalli T, Agelidis AM, Shukla D. **Host Enzymes Heparanase and Cathepsin L Promote Herpes Simplex Virus 2 Release from Cells.** J Virol. 2018

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James J Hopkins, Alex M. Agelidis, Dinesh Jaishankar, Deepak Shukla. **Preliminary Investigation into OGT 2115 Antiviral Activity.** Graduate Education in the Medical Sciences (GEMS) Symposium, Chicago, IL, 2016

James J Hopkins, Patricia Waltz, Hardik Patel and Nima Missaghian, Aaron Schirmer and Emina A. Stojković. **Crystallization and preliminary X-ray crystallographic studies of unusual red-light photoreceptors from photosynthetic and non-photosynthetic bacteria.** Society for Advancement of Chicanos and Native Americans in Science (SACNAS), San Antonio, TX. 2013.

James J Hopkins, Patricia Waltz, Hardik Patel and Nima Missaghian, Aaron Schirmer and Emina A. Stojković. **Crystallization and preliminary X-ray crystallographic studies of unusual red-light photoreceptors from photosynthetic and non-photosynthetic bacteria.** 5th Annual NEIU-SCSE Symposium, Chicago, IL. 2013.

James J Hopkins, Patricia Waltz, Andrei Halavaty and Emina A. Stojković**. X-ray Crystallographic Studies of a Bacteriophytochrome from Myxobacterium Stigmatella aurantiaca.** Annual Biomedical Research Conference for Minority Students (ABRCMS), San Antonio, TX. 2014.

James J Hopkins, Patricia Waltz, Andrei Halavaty and Emina A. Stojković**. X-ray Crystallographic Studies of a Bacteriophytochrome from Myxobacterium Stigmatella aurantiaca.** NEIU 22nd Annual Student Research & Creative Activities Symposium, Chicago, IL. 2014.

Awards and Honors

January 2012	McNair Scholar
October 2013	Travel Scholarship Annual SACNAS Conference, October 2013, San Antonio, TX
November 2014	Travel Scholarship Annual ABRCMS Conference, November 2014, San Antonio, TX
	Protein Society Outstanding Student Poster Award Annual ABRCMS Conference, November 2014, San Antonio, TX
July 2015	NSF LSAMP Fellow

January 2018	Illinois Society for the Prevention of Blindness (ISPB) Fellow

Professional Activity

2018-Present	Volunteer with the Illinois Science Council
2018-Present	Volunteer with the Chicago Council on Science and Technology
2017- Present	Member of Graduate Education in the Medical Sciences Student Association-Outreach Coordinator
2016- Present	Member of Graduate Education in the Medical Sciences Symposium planning committee
2018-Present	Member of the Intellectual Property Law Association of Chicago
2014-2019	Member of Protein Society
2014-2016	Member of Society for Advancement of Chicanos/Hispanics and Native Americans in Science