In Vitro and In Vivo Activity, Tolerability and Mechanism of Action of BX795 as an Antiviral against Herpes Simplex Virus-2 Genital Infection

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Abstract:

Herpes simplex virus type 2 (HSV-2) causes recurrent lesions in the ano-genital area that may be transmitted through sexual encounters. Nucleoside analogs such as acyclovir (ACV) are currently prescribed clinically to curb this infection. But in some cases, reduced efficacy has been observed due to the emergence of resistance against these drugs. In our previous study, we reported the discovery of a novel anti-HSV-1 small molecule, BX795 which was originally used as an inhibitor of TANK-binding kinase 1 (TBK1). In this study, we report the 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. Our investigations also revealed that the mechanism of action of BX795's antiviral activity stems from the reduction of viral protein translation via inhibition of protein kinase B phosphorylation. 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.

Keywords:

Herpes simplex virus-2, BX795, genital infection, protein kinase-R, antiviral drug.

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 (1-5). It is responsible for the majority of clinical cases of genital ulcers worldwide (6-8). 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 (9, 10). 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 (11-13). Extended infections can cause flu-like symptoms and eventually lymphadenopathy, cervicitis, and proctitis (13, 14).

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 (15-17) in the former case and acute retinal necrosis in the latter (18). The virus can also be passed from the mother to child during pregnancy, leading to skin lesions and poor prognoses (14). HSV-2 has also been shown to increase the risk of HIV acquisition, further elevating it as a public health concern (19-24).

The primary treatment for HSV-2 infections consists of nucleoside analogs, such as acyclovir, valacyclovir, and famciclovir (25). These antivirals inhibit viral DNA polymerase activity, preventing the virus from replicating successfully (26, 27). Dosages for herpes genitalis treatment are highly variable, depending on the progression of the disease and immune status of the patient (13). However, these nucleoside analogs suffer from multiple shortcomings. They do not directly obstruct viral protein synthesis (28), are prone to resistance and escape mutants (29-

31), and cause nephrotoxicity after extended usage (32, 33). In immunosuppressed patients, resistance to acyclovir and its analogs occurs in about 5% of cases (34). 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 (35). 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 (36, 37). 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 (38). 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 1A, 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 1D). Our results coincide with fluorescent microscopy (Figure 1E) and flow cytometry data (Figure 1H, 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 1F, G).

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 (36). 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 2A). 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 2B). 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 s1). 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 (36).

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 3A-top) at concentration ≥ 10 µM with no apparent cytopathic effect observed in brightfield (BF) images at concentrations as high as 80 µM (Figure 3A-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 3B,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 3D) 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 3E). 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 3E). These results were confirmed by plaque assays where a significant loss in viral load was observed both extracellular (Figure 3F) and intracellular (Figure 3G) viruses.

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 s2A). 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 s2B). However, plaque assay results showed a significant loss of infection at all time points except 2 hbi in both intracellular (Figure s2C) and extracellular (Figure s2D) viral loads.

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 4A, 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.

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 4C). 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 (39). 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

196 stimulus for the increased search for new and more effective antivirals against HSV-2. In our
197 recent article (36), we discovered that the off-target effect of a TBK-1 inhibitor, BX795 is
198 effective in controlling HSV-1 infection. We also provided preliminary data to support that
199 BX795 was effective against other herpesviruses including HSV-2. However, none of the studies
200 on HSV-2 were conducted on target cell lines or in murine models of genital infection. In this
201 study, we provide concise data on the antiviral efficacy and tolerability of BX795 using vaginal
202 epithelial cells in vitro and murine vaginal tissue in vivo.

203 Our initial results from this study show that BX795 is effective in controlling HSV-2 at a
204 previously reported concentration of 10 µM in vitro at the transcriptional and translational level.
205 These results are comparable to cells treated with ACV showing that BX795 is as effective as
206 currently used therapeutics at a much lower concentration. The mechanism of action of BX795
207 in controlling HSV-2 infection in vaginal epithelial cells was consistent with our previous reports
208 where we showed a significant loss in AKT phosphorylation in BX795 treated cells. While these
209 results indicate a potential mechanism that BX795 is a potent inhibitor of viral protein translation
210 and can be used to suppress HSV-2 infection in target cell type, the true mechanism of antiviral
211 action cannot be completely discerned in this study.

212 Another interesting result we observed in this study was the tolerability of BX795 in vaginal
213 epithelial cells. While our previous experiments, both in vitro and in vivo have shown good
214 tolerability of BX795, they were all performed at a much lower concentration. In this study, we
215 observed that even at a concentration as high as 80 µM, BX795 did not affect the viability of
216 vaginal epithelial cells, giving us the confidence that this drug can have a large therapeutic
217 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.

Materials and Methods:

The reagents used in this study are mentioned in Table 1

Unless specified otherwise, the concentration of BX795 and ACV used in this study are 10 µM and 50 µM respectively. DMSO was used to dissolve both the drugs and hence was used as the negative control for all experiments at the same volume as the drugs.

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 (40). 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 quantify through a real time PCR. The primers used in this study are tabulated in table 2.

Western Blotting:

Total protein from all the samples was isolated with RIPA (Radio immunoprecipitation assay) buffer using a protocol previously reported (41). Equal amounts of protein samples were loaded into 4 to 12% SDS–polyacrylamide gel and separated for three hours at the constant voltage of 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 a 1:1000 ratio and all the secondary antibodies were diluted at 1:10,000. The protein content was analyzed by the addition of the 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 minutes to fix the cells. The cells were then washed and permeabilized using 0.01% Triton-X (Fisher Scientific) for 10 minutes. The cells were then blocked for 1 hour in 1% Bovine Serum Albumin (BSA, Sigma-Aldrich) and incubated with the primary antibody in 1% BSA for 1 hour. Following washes, the cells were incubated with a secondary-conjugated FITC antibody in 1% BSA for 1 hour. DAPI was used to stain the nuclei as per the manufacturer's protocol. The cells were then washed multiple times before capturing images using an LSM 710 confocal microscope (Carl Zeiss) under 63x objectives. For image analysis, the MetaMorph or AxioVision (Carl Zeiss) software was 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 were 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 an MOI of 0.1. The requisite amount of virus was diluted in serum-free Opti-MEM media prior to its addition to cells. The 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 (42).

Murine model of HSV-2 infection:

The mice involved in this study were infected as per the protocol described previously (42). 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 × 106 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 (43). 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 minutes and then stained with Haematoxylin (Sigma-Aldrich) and washed thoroughly under running water. Slides were then dipped in 70% ethanol for 2 minutes, then in 100% ethanol for 1 minute, and incubated with eosin Y alcoholic, with phloxine (Sigma, HT110316) for 1 minute. Slides were then dipped in 70% ethanol for 1 minute, then in 100% ethanol for 1 minute, then xylene for 1 minute.

Coverslips 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.

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List of Tables

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	Selleckchem
ACV	Selleckchem
Anti-HSV-1 gD mouse monoclonal	Abcam
Anti-HSV-1 VP16 mouse monoclonal	Abcam
Anti-Akt rabbit monoclonal	Cell signaling technology
Anti-phospho-Akt- Ser473 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

473 **Table 1: List of reagents and their respective suppliers used for the study**

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’

474 **Table 2: A list of all the primer sequences used for q-RT-PCR amplification**

475 **Figure Legends**

476 **Figure 1. BX795 attenuates HSV-2 infection.** A. VK2 cells infected with HSV-2 333 at
477 0.1MOI then transcript levels of viral protein UL30 were measured 24 hpi with black
478 representing DMSO, red representing ACV (50μM), and grey representing BX795 (10μM)
479 treated cells. B. VK2 cells infected with HSV-2 333 at 0.1MOI then transcript levels of
480 immediate early viral protein ICP27 was measured 24 hpi. C. VK2 cells infected with HSV-2
481 333 at 0.1MOI then transcript levels of late viral protein gD were measured 24 hpi. D. VK2 cells
482 infected with HSV-2 333 at 0.1MOI then treated after infection with DMSO, ACV, or BX795.
483 Samples taken 0, 12, 24 and 36 hpi. Whole cell lysates were probed by immunoblotting with
484 antibodies against viral proteins VP16 and gD. E. VK2 cells were infected with HSV-2 333 GFP
485 at 0.1MOI then cells treated after infection with DMSO, ACV (50 μM), or BX795 (10 μM).
486 Fluorescent images were taken at 12, 24, and 36 hpi. F. VK2 cells infected with HSV-2 333 at
487 0.1MOI then media samples taken every 12 hpi till 48 hpi. Viral plaques of virus shed from cells
488 into media with black representing DMSO and grey representing BX795. G. VK2 cells infected
489 with HSV-2 333 at 0.1MOI then media samples taken every 12 hpi till 48 hpi. Viral plaques of
490 intra cellular virus with black representing DMSO and grey representing BX795(10 μM). H.
491 VK2 cells infected with HSV-2 333 GFP at 0.1MOI then treated with 10μM BX795 then cells
492 were collected and flow cytometry was performed measuring cell GFP florescence. I.
493 Quantification of H. *, $P < 0.05$; **, $P < 0.01$; ***,$P<0.001$; ****,$P<0.0001$.

494

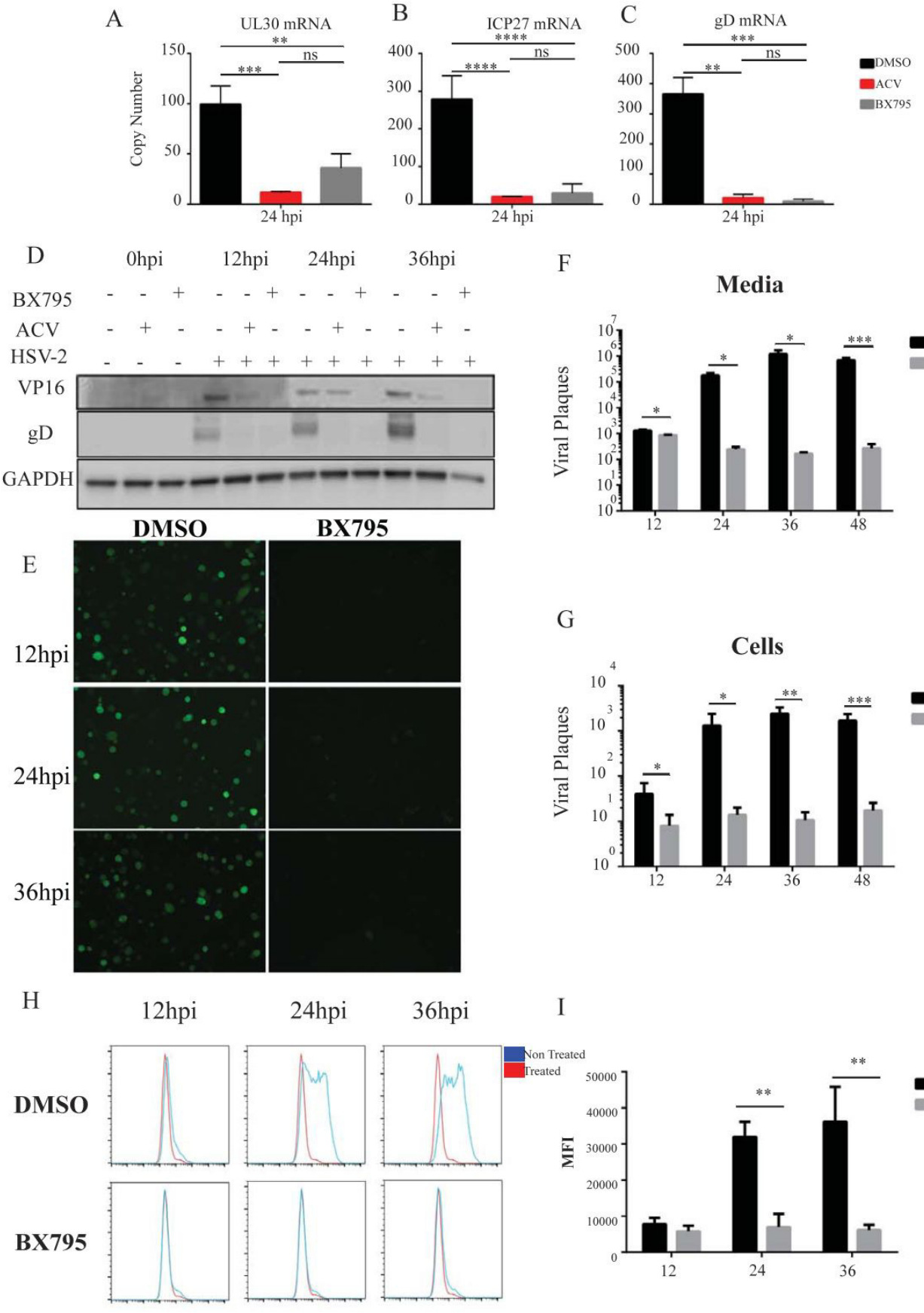
495 **Figure 2. Mechanism of antiviral action of BX795.** A. VK2 cells were infected with HSV-2
496 333 at 0.1MOI and then treated with either mock DMSO, BX795 (10 μM) or insulin (10 nM).
497 The cells were stained with Hoescht (nuclear blue stain) and an antibody against p-AKT. Green

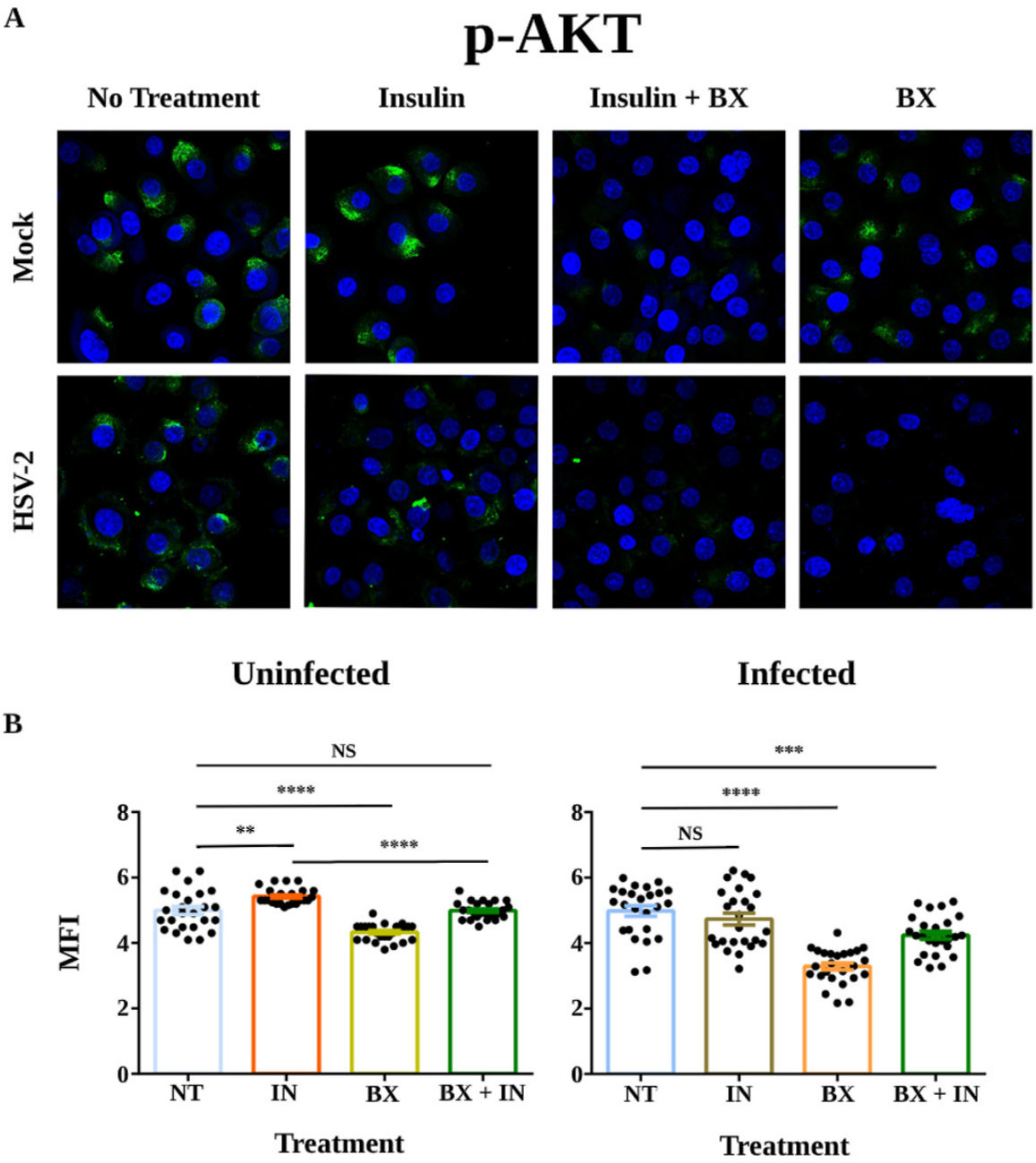
represents p-AKT expression. B. Quantification of A. *, $P < 0.05$; **, $P < 0.01$; ***,$P<0.001$; ****,$P<0.0001$.

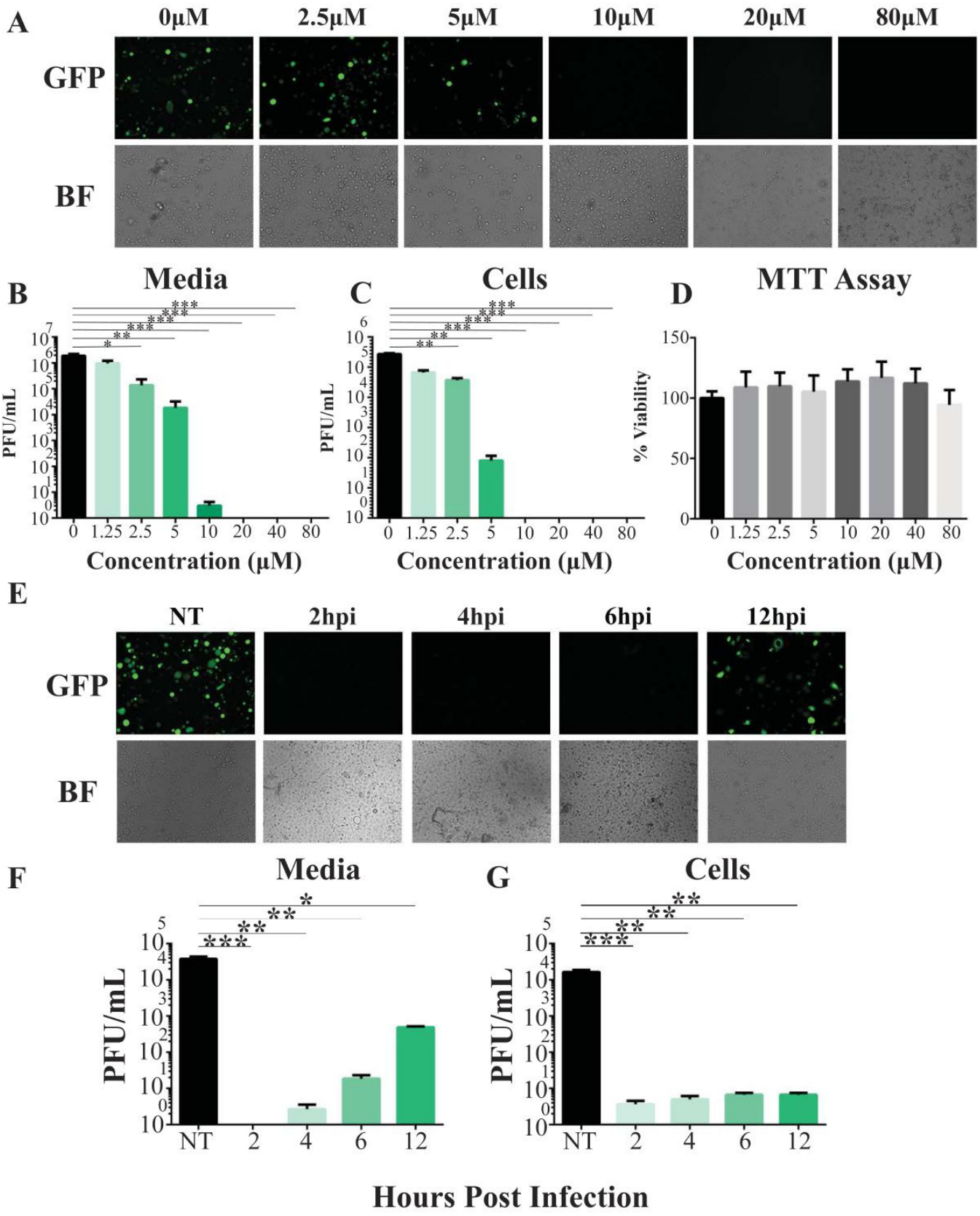
Figure 3 Efficacy of BX795 as a treatment for HSV-2 infection. A. Representative immunofluorescence microscopy images of VK2 cells where infected with HSV-2 333 GFP then treated with 0 μM to 80 μM BX795. Images were taken 24 hpi. B. Viral plaques of virus shed from cells into media after infection with 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.

Figure 4 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. *, $P < 0.05$; **, $P < 0.01$; ***,$P<0.001$; ****,$P<0.0001$.







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