Original Article

BX-795 inhibits HSV-1 and HSV-2 replication by blocking the JNK/p38 pathways without interfering with PDK1 activity in host cells

Ai-rong SU[1, #], Min QIU[1, #], Yan-lei LI[4, #], Wen-tao XU[1], Si-wei SONG[1], Xiao-hui WANG[1], Hong-yong SONG[1], Nan ZHENG[1, 2, 3, *], Zhi-wei WU[1, 2, 3, *]

[1]Center for Public Health Research, Medical School, [2]State Key Lab of Analytical Chemistry for Life Science, [3]Jiangsu Laboratory for Molecular Medicines, Nanjing University, Nanjing 210093, China; [4]Y-Clone Biomedical Technologies, Ltd, Suzhou 215028, China

Abstract

BX-795 is an inhibitor of 3-phosphoinositide-dependent kinase 1 (PDK1), but also a potent inhibitor of the IKK-related kinase, TANK-binding kinase 1 (TBK1) and IKKε. In this study we attempted to elucidate the molecular mechanism(s) underlying the inhibition of BX-795 on Herpes simplex virus (HSV) replication. HEC-1-A or Vero cells were treated with BX-795 and infected with HSV-1 or HSV-2 for different periods. BX-795 (3.125–25 µmol/L) dose-dependently suppressed HSV-2 replication, and displayed a low cytotoxicity to the host cells. BX-795 treatment dose-dependently suppressed the expression of two HSV immediate-early (IE) genes (ICP0 and ICP27) and the late gene (gD) at 12 h postinfection. HSV-2 infection resulted in the activation of PI3K and Akt in the host cells, and BX-795 treatment inhibited HSV-2-induced Akt phosphorylation and activation. However, the blockage of PI3K/Akt/mTOR with LY294002 and rapamycin did not affect HSV-2 replication. HSV-2 infection increased the phosphorylation of JNK and p38, and reduced ERK phosphorylation at 8 h postinfection in the host cells; BX-795 treatment inhibited HSV-2-induced activation of JNK and p38 MAP kinase as well as the phosphorylation of c-Jun and ATF-2, the downstream targets of JNK and p38 MAP kinase. Furthermore, SB203580 (a p38 inhibitor) or SP600125 (a JNK inhibitor) dose-dependently inhibited the viral replication in the host cells, whereas PD98059 (an ERK inhibitor) was not effective. Moreover, BX-795 blocked PMA-stimulated c-Jun activation as well as HSV-2-mediated c-Jun nuclear translocation. BX-795 dose-dependently inhibited HSV-2, PMA, TNF-α-stimulated AP-1 activation, but not HSV-induced NF-κB activation. Overexpression of p38/JNK attenuated the inhibitory effect of BX-795 on HSV replication. BX-795 completely blocked HSV-2-induced MKK4 phosphorylation, suggesting that BX-795 acting upstream of JNK and p38 MAP kinase. In conclusion, this study identifies the anti-HSV activity of BX-795 and its targeting of the JNK/p38 MAP kinase pathways in host cells.

Keywords: BX-795; PDK1 inhibitor; herpes simplex virus; infected cell polypeptide 0 (ICP0); antiviral agent; JNK; p38; MAP kinase; NF-kappa B

Introduction

Herpes simplex virus types 1 and 2 (HSV-1 and -2) are among the most prevalent human pathogens worldwide. Both HSV-1 and HSV-2 are neuroinvasive and become latent in neurons. After primary infection, some infected individuals experience sporadic viral reactivations. HSV-2 has been shown to increase the risk of human immunodeficiency virus type 1 (HIV-1) sexual acquisition approximately 3-fold in both men and women, and primary HSV-2 infection may have an even greater effect on HIV-1 susceptibility[1]. Currently, there is no cure or vaccine for HSV infection. Antiviral medications, such as acyclovir, penciclovir and valacyclovir, are proven to be an effective approach to shortening the length of herpes reactivation and reducing recurring outbreaks. Because these drugs specifically target HSV thymidine kinase (TK), drug-resistant mutants can arise in immunocompromised individuals[2, 3].

Eukaryotic cells respond to environmental stress and stimuli by recruiting or activating signal transduction pathways. The c-Jun NH$_2$-terminal kinase (JNK) and p38 MAP kinase are members of the mitogen-activated protein kinase (MAPK) family and are primarily responsible for the stress signal and proinflammatory cytokine stimulations. Activated JNK/p38 MAP kinase can transmit upstream signals to downstream fac-

tors, thus mediating cellular apoptosis, differentiation, growth or immune responses[4, 5]. JNK and p38 MAP kinase are also reported to be stimulated by many viruses or virus-associated proteins, including rotavirus[6], varicella-zoster virus (VZV)[7, 8], HIV-1[9], HSV-1[10, 11], coxsackievirus B3 (CVB3)[12], Epstein-Barr virus (EBV)[13], severe acute respiratory syndrome (SARS) coronavirus[14] and hepatitis B/C virus (HBV/HCV)[15, 16]. The activation of the JNK/p38 MAP kinase cascade induced by these viruses either facilitates the viral lytic cycle or serves as a defense mechanism of the host cells. Therefore, virus-induced activation of the JNK/p38 MAP kinase pathway is considered to be universally important for viral infection or cell survival[6, 7, 10, 12, 16].

The dynamic activation of JNK/p38 MAP kinase and the activator protein 1 (AP-1) caused by HSV-1 infection has been studied extensively[10, 11, 17]. Zachos *et al* found that HSV-1 infection activated JNK/p38 MAP kinase through upstream activators, mitogen-activated protein kinase kinase 4/stress-activated protein/ERK kinase 1 (MKK4/SEK1), which further stimulated AP-1[17]. McLean *et al.* reported that JNK/p38 activation by virus entry and immediate-early (IE) gene expression was correlated with c-Jun and ATF-2 *trans*-activation and that the activation of JNK was necessary for efficient HSV-1 replication[10]. Karaca *et al* indicated that inhibition of p38 MAP kinase activation would reduce HSV-1 viral propagation[11]. It was proposed that cellular JNK and p38 MAP kinase may serve as potential targets for preventing HSV-1 and HSV-2 viral infection.

BX-795, a pharmacological inhibitor, was synthesized and identified as a phosphoinositide-dependent kinase-1 (PDK1) inhibitor with potent activity in blocking PDK1/Akt/mTOR signaling[18, 19]. Further research demonstrated that BX-795 was also a potent and relatively specific inhibitor of TANK binding kinase 1 (TBK1) and IκB kinase ε (IKKε), which inhibited the phosphorylation, nuclear translocation, and transcriptional activity of interferon regulatory factor-3 (IRF-3)[19]. In the current study, we report that BX-795 exhibited inhibitory activity against HSV-1 and HSV-2 infection and demonstrated that the antiviral activity of BX-795 was independent of its anti-PDK1 and anti-TBK1/IKKε activities, and was instead mediated through disrupting the activation of the JNK/p38 MAP kinase pathway. To the best of our knowledge, this is the first report on the anti-viral activity of BX-795, and this study may facilitate the identification of new anti-HSV targets and the development of therapeutic agents.

Materials and methods
Reagents, cell lines, plasmids and viruses
LY294002, rapamycin, SB203580, SP600125, PD98059, BAY11-7082, phorbol-12-myristate-13-acetate (PMA), pyrrolidine dithiocarbamate (PDTC) and MG132 were obtained from Beyotime (Haimen, China). Glo Lysis buffer and luciferase assay kit were obtained from Promega Biotechnology (Promega Bio, Madison, USA). Cytoplasmic and nuclear protein extraction kit and BCA protein assay kit were purchased from Thermo Scientific Pierce (Rockford, USA). Lipofectamine 2000

transfection reagent, TRIzol reagent, DAPI and Alexa Fluor 488 goat anti-mouse IgG (H+L) were obtained from Life Technologies (Gaithersburg, USA). IRDye 680 goat-anti-rabbit, IRDye 800 goat-anti-mouse and LI-COR Blocking Buffer were obtained from LI-COR (Lincoln, USA). BX-795, prostaglandin A1 (PGA1), antibodies for gD-1/2, ICP0-1, ICP27-1, p65, p38, p-p38, ERK1/2, p-ERK1/2, JNK2, p-JNK1/2, c-Jun, p-c-Jun, GAPDH, β-catenin and β-actin and RIPA lysis buffer were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Antibodies for Akt, p-Akt and p-PI3K were obtained from SunshineBio (Nanjing, China). Antibodies for IκB-α, p-MKK4 and p-ATF-2 were obtained from Cell Signaling Technology (Beverly, USA). Recombinant human TNF-α was obtained from R&D Systems (Minneapolis, USA).

HEC-293T, HeLa, Vero and HEC-1-A cells were obtained from the American Type Culture Collection (ATCC, USA). Vero-ICP10-promoter was an HSV-2 infection indicator cell line, which was generated from Vero cells stably transfected with HSV-2 (G) ICP10 promoter-driven luciferase reporter plasmid.

NF-κB-luc and AP-1-luc reporter plasmids were from Clontech (California, USA). pcDNA3-ICP0-1-GFP and pcDNA3-ICP0-2-GFP were kindly provided by Dr Claus-Henning Nagel of Heinrich Pette Institute – Leibniz Institute for Experimental Virology. pCMV3-p38-C-GFPSpark and pCMV3-JNK1-N-GFPSpark were obtained from Sino Biological Inc (Beijing, China). HSV-1 (HF) and HSV-2 (G) were propagated and titrated on Vero cells as previously described[20]. HSV-2 titration was also performed on Vero-ICP10-promoter cells through detection of luciferase activity.

In vitro cytotoxicity assay
The *in vitro* cytotoxicity of BX-795 was measured using a commercial CCK-8 kit (Dojindo, Kumamoto, Japan) via the colorimetric method according to the manufacturer's instructions. Briefly, 2×10^4 cells per well were dispersed into 96-well plates and cultured for 24 h before serial concentrations of BX-795 were added in triplicate. After 24 h of culture, 10 μL of the CCK-8 reagent were dispensed into each well, and the plates were incubated at 37 °C for 2 h. Absorbance at 450 nm was measured using a TECAN Infinite M200 microplate reader (Männedorf, Switzerland). Cell viability was plotted as the percent viable cells of the mock-treated control cells.

Western blot and In-cell Western
Cells were lysed using RIPA lysis buffer in an ice bath for 30 min. The lysates were centrifuged and the supernatants containing total proteins were prepared. Nuclear and cytoplasmic total proteins were separated and extracted using a commercial kit. Western blot was performed as previously described[21].

In-cell Western assay was conducted according to our published protocols[21]. Briefly, the cell monolayers were fixed with 4% paraformaldehyde for 20 min and permeabilized by 5 washes with 0.1% Triton X-100 in PBS. The monolayers were then blocked with LI-COR Blocking Buffer and then incubated

with primary antibodies. After washing with PBS-T buffer, the cell monolayers were incubated with IRDye IgG. The plate was rinsed with PBS-T buffer and immediately scanned by the LI-COR Odyssey Infrared Imager.

Immunofluorescent staining and microscopy

Cells were seeded onto Φ10 mm glass coverslips in 24-well plates at a density of 2.5×10^5 cells per well. After antibody staining, the monolayers were rinsed with PBS and then fixed with 4% paraformaldehyde for 15 min at RT before permeabilized with 0.2% Triton X-100 for 15 min followed by PBS washes. PBS containing 1% BSA was added to each well to block non-specific binding. Cellular proteins were immunolabeled with the respective primary antibodies and then Alexa Fluor 488 IgG. Nuclei were visualized by staining with DAPI. Images were acquired using an Olympus FluoView FV10i confocal microscope (Tokyo, Japan).

Cell transfection and luciferase assay

HEC-1-A, Vero or 293T cells were seeded into 96-well plates. When reaching 90% confluence, the cells were transiently transfected with 100 ng AP-1 luciferase reporter plasmid alone or co-transfected with 50 ng NF-κB or AP-1 luciferase reporter plasmid and 150 ng ICP0-1/2-GFP expression plasmid. The cells were then cultured for 24 h and treated with inhibitors. The luminescence was determined using GloMax-96 Microplate Luminometer after 24 h treatment (Promega, Madison, USA).

HSV-2 was titrated as follows: HEC-1-A or Vero cells were treated in the absence or presence of serial concentrations of BX-795 and infected with HSV-2 (G) (moi=1) for 24 h. The cells were washed twice, exposed to the complete medium, and then frozen and thawed for three cycles to release infectious particles. The viral titration was determined using the Vero-ICP10-promoter luciferase reporter system.

RNA extraction and real-time PCR

Total RNA was extracted using TRIzol reagent following the manufacture's protocol. cDNA was synthesized using a ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan). Real-time PCR was performed in triplicate on an ABI Prism 7300 Sequence Detection System using the SYBR Green PCR Master Mix (TOYOBO) according to the manufacturer's protocol. Primer pairs used in this study were HSV-1 *ICP0*: Forward: 5'-TACGTGAACAAGACTATCACGGG-3', Reverse: 5'-TCCATGTCCAGGATGGGC-3'; HSV-1 *ICP27* Forward: 5'-CGCCAAGAAAATTTCATCGAG-3', Reverse: 5'-ACATCTTGCACCACGCCAG-3'; HSV-1 *gD* Forward: 5'-AGCAGGGGTTAGGGAGTTG-3', Reverse: 5'-CCATCTTGAGAGAGGCATC-3'; HSV-2 *ICP0* Forward: 5'-GTGCATGAAGACCTGGATTCC-3', Reverse: 5'-GGTCACGCCCACTATCAGGTA-3'; HSV-2 *ICP27* Forward: 5'-TGTCGGAGATCGACTACACG-3', Reverse: 5'-GGTGCGTGTCCAGTATTTCA-3'; HSV-2 *gD* Forward: 5'-CCAAATACGCCTTAGCAGACC-3', Reverse: 5'-CACAGTGATCGGGATGCTGG-3'; *GAPDH* Forward: 5'-TGCACCACCAACTGCTTAGC-3',

Reverse: 5'-GGCATGGACTGTGGTCATGAG-3'.

Statistical analysis

Statistical analysis was performed with a two-tailed Student *t*-test, using SPSS 18.0 software (SPSS for Windows Release 18.0, SPSS Inc). Statistical significance: $P<0.05$, $P<0.01$.

Results

BX-795 inhibited HSV gD expression and viral replication with low cytotoxicity

BX-795, initially developed as a PDK1/TBK1 inhibitor (molecular structure shown in Figure 1A)[18, 19, 22], was used to investigate the effect of TBK1/IRF-3 and downstream factors on HSV-2 replication. Surprisingly, we observed that BX-795 exhibited an inhibitory effect on the HSV-2-induced cytopathogenic effect (CPE) (data not shown). Using an In-cell Western assay, we analyzed HSV glycoprotein D (gD) expression in the presence of serial concentrations of BX-795 in HEC-1-A cells. gD protein is an HSV membrane protein representing late events in the viral life cycle. As shown in Figure 1B and C, BX-795 inhibited both HSV-1 (HF) (Figure 1B) and HSV-2 (G) (Figure 1C) replication in HEC-1-A cells in a dose-dependent manner. The same inhibitory activity of BX-795 was also demonstrated in Vero cells, as shown in Figure 1D and E. The inhibitory activity of BX-795 on HSV-1 and HSV-2 infectivity was also investigated using progeny virions released from infected HEC-1-A or Vero cells to infect Vero-ICP10-promoter cells. As shown in Figure 1F, BX-795 prevented the formation of HSV-2 infectious particles in both HEC-1-A and Vero cells (Figure 1F).

The cytotoxicity of BX-795 was investigated on two cell lines used in the inhibitory activity analyses above. As shown in Figure 1G, 80% of both cells remained viable at BX-795 concentrations up to 100 μmol/L, demonstrating appreciable Therapeutic Indexes (TI) and suggesting that the viral inhibitory activity was not due to cytotoxicity. In conclusion, BX-795 inhibited the replication of HSV-1 and HSV-2 in both HEC-1A and Vero cells.

The effect of BX-795 on HSV immediate-early (IE) gene expression

HSV lytic infection involves the temporally regulated expression of three classes of viral genes, the IE genes, early genes and late genes[23, 24]. The IE genes, expressed first following viral entry and capsid disassembly, regulate the expression of early and late viral genes[25]. We found that the expression of HSV-1 IE genes, ICP0 (Figure 2A), ICP4 (Figure 2B) and ICP27 (Figure 2C) was significantly inhibited by BX-795 in a dose-dependent manner, as measured by their protein levels 12 h postinfection. The results were consistent with a decreased gD expression, which is a late gene product that is regulated by IE genes and is critical for the formation of infectious virions.

To better understand the effect of BX-795 on IE gene transcription, we carried out real-time PCR analysis on the mRNA expression of ICP0 and ICP27, 6 and 12 h postinfection. As shown in Figure 2, when cells were pretreated with BX-795,
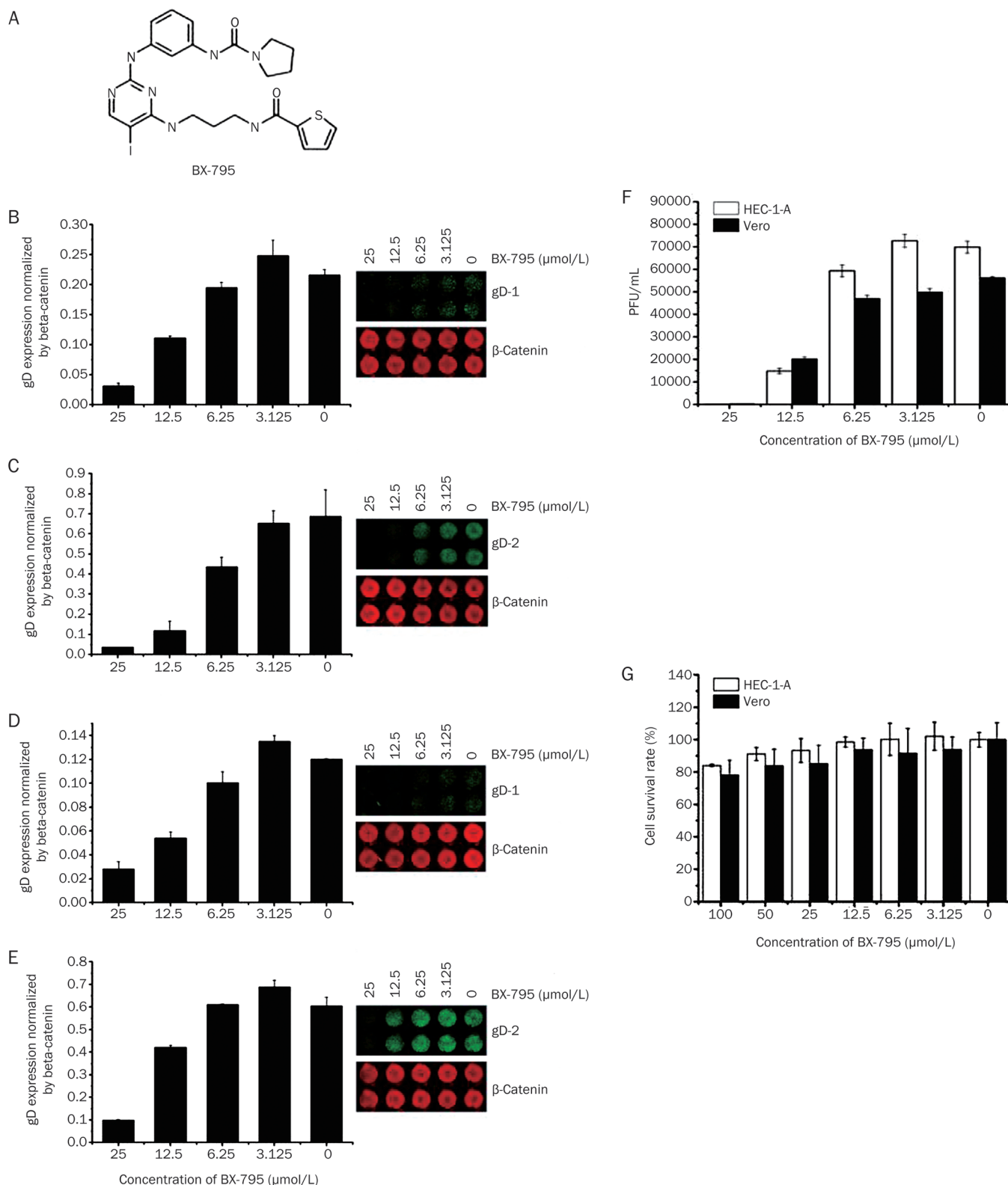
Figure 1. BX-795 inhibition of HSV replication and gD expression. (A) The molecular structure of BX-795. (B–E) BX-795 inhibition of HSV gD expression of HSV-1 (HF)/HEC-1-A (B), HSV-2 (G)/HEC-1-A (C), HSV-1 (HF)/Vero (D) or HSV-2 (G)/Vero (E); HSV was used at moi=1 and the gD-1/2 expression was determined by In-cell Western and normalized by β-catenin level 24 h postinfection. (F, G) BX-795 inhibition of HSV-2 replication in HEC-1-A and Vero cells (F). The cells were treated with BX-795 prior to HSV-2 infection (moi=1). The virus was released by three freeze-thaw cycles of the infected cells and the viral titers were determined on Vero-ICP10-promoter cells. The cytotoxicity of BX-795 on two cell lines used in the inhibitory activity analyses (G) All experiments were performed three times and the representative results are shown.
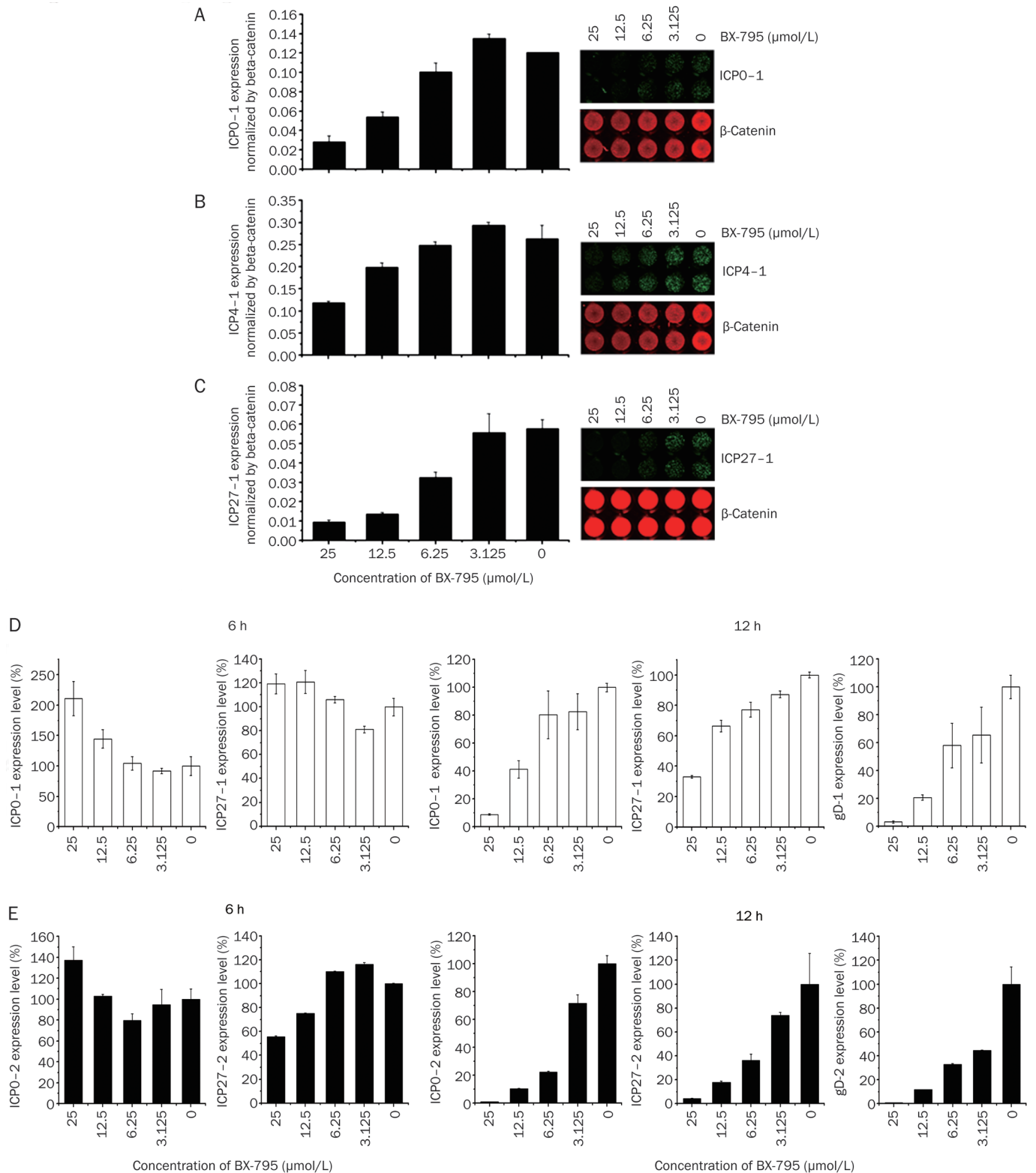
Figure 2. Effects of BX-795 on HSV IE gene expression at early stage of viral infection, and on the viral infection. (A–C) BX-795 inhibition of HSV IE expression. Confluent HEC-1-A cells were exposed to serial concentrations of BX-795 prior to infection with HSV-1 (moi=1). ICP0, ICP4 and ICP27 expression was determined by In-cell Western and normalized by β-catenin level 24 h postinfection. All experiments were performed three times and the representative results were shown. BX-795 did not block the expressions of HSV-1 (D) and HSV-2 (E) ICP0 and ICP27 6 h postinfection, but blocked their expressions 12 h postinfection. HEC-1-A cells were treated with serial concentrations of BX-795 and infected with HSV-1 and HSV-2 (moi=1), respectively. The levels of ICP0-1/2, ICP27-1/2 or gD-1/2 mRNA were quantified by qPCR analysis as described. All experiments were performed three times and the representative results are shown.

ICP0 and ICP27 mRNA transcription of HSV-1 (Figure 2D) and HSV-2 (Figure 2E) increased in a dose-dependent manner at an early stage of viral infection (6 h postinfection) (the effect of BX-795 is complex at 6 h pi, particularly the enhancing effect for ICP0 at 6 h pi). In contrast, at 12 h postinfection, BX-795 exhibited an inhibitory effect on the expression of not only these two IE genes but also on the late gene (gD) in a dose-dependent manner. Based on these observations, we speculate that BX-795 did not reduce HSV IE gene expression directly at the early period of the viral life cycle and that its apparent inhibitory activity on ICP0, ICP27 and gD mRNA level 12 or 24 h postinfection might be due to the decreasing level of infectious progeny virions in the presence of BX-795.

BX-795 inhibited HSV replication but not through its PDK1 inhibitory activity

BX-795 has been identified as a potent PDK1 inhibitor in the cell-free system *in vitro*[18, 22]. To investigate its antiviral mechanisms, we analyzed whether BX-795 acts by inhibiting PDK1 activity. We first investigated whether HSV infection would trigger intracellular PI3K/Akt pathway by Western blot analysis of phosphorylated-PI3K and phosphorylated-Akt (Thr308).

Thus, HSV-2 induced phosphorylation of PI3K and Akt 6 h postinfection, suggesting that viral replication and infection stimulate PI3K/Akt/mTOR pathway activation (Figure 3A), and BX-795 inhibited HSV-2-induced phosphorylation of Akt 12 h postinfection, as shown in Figure 3B. However, PI3K/Akt/mTOR pathway activation does not seem to be required for HSV-2 replication due to the fact that LY294002, a potent PI3K inhibitor, and rapamycin, an mTOR inhibitor, failed to exhibit any effect on HSV replication. As shown in Figure 3C, gD expression was minimally affected by even high concentrations (at 10 μg/mL and 100 ng/mL of LY294002 and rapamycin, respectively) of the inhibitors used, suggesting that the PI3K/Akt/mTOR pathway does not regulate HSV replication but rather the outcome of HSV infection. Therefore, BX-795 does not seem to inhibit HSV-1 and HSV-2 replication through its anti-PDK1 activity.

BX-795 inhibited HSV replication through blocking JNK/p38 pathway

We further investigated the effect of BX-795 treatment on JNK/p38 pathways. JNK/p38 pathways are two of the major stress-activated and inflammatory responsive pathways
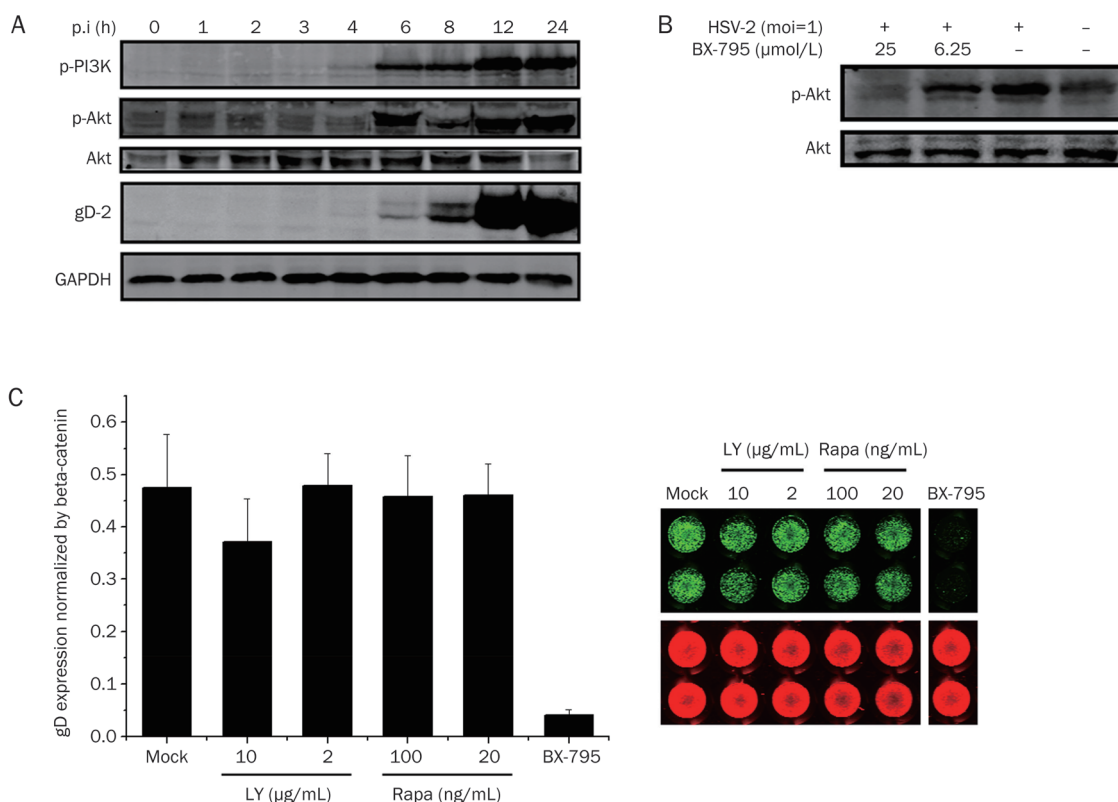

Figure 3. BX-795 inhibition of HSV replication was not mediated by its PDK1 inhibitory activity. (A) HSV-2 infection resulted in the activation of PI3K and Akt. HEC-1-A cells infected with HSV-2 (moi=1) were collected at the indicated time points and lysed. Akt, p-Akt, p-PI3K and gD were determined by Western blot. (B) BX-795 inhibited HSV-2-induced Akt phosphorylation and activation. HEC-1-A cells were either mock-infected or infected with HSV-2 (moi=1) in the presence or absence of BX-795. Cells were lysed 12 h postinfection. Akt and its phosphorylated form were determined by Western blot. (C) The blockage of PI3K/Akt/mTOR by their respective inhibitors did not affect HSV-2 replication. Confluent cells were treated with LY294002, rapamycin or BX-795 (25 μmol/L), and infected with HSV-2 (moi=1). Viral gD expression was determined by In-cell Western 24 h postinfection and normalized by β-catenin level. The experiment was carried out three times and the representative results are shown.
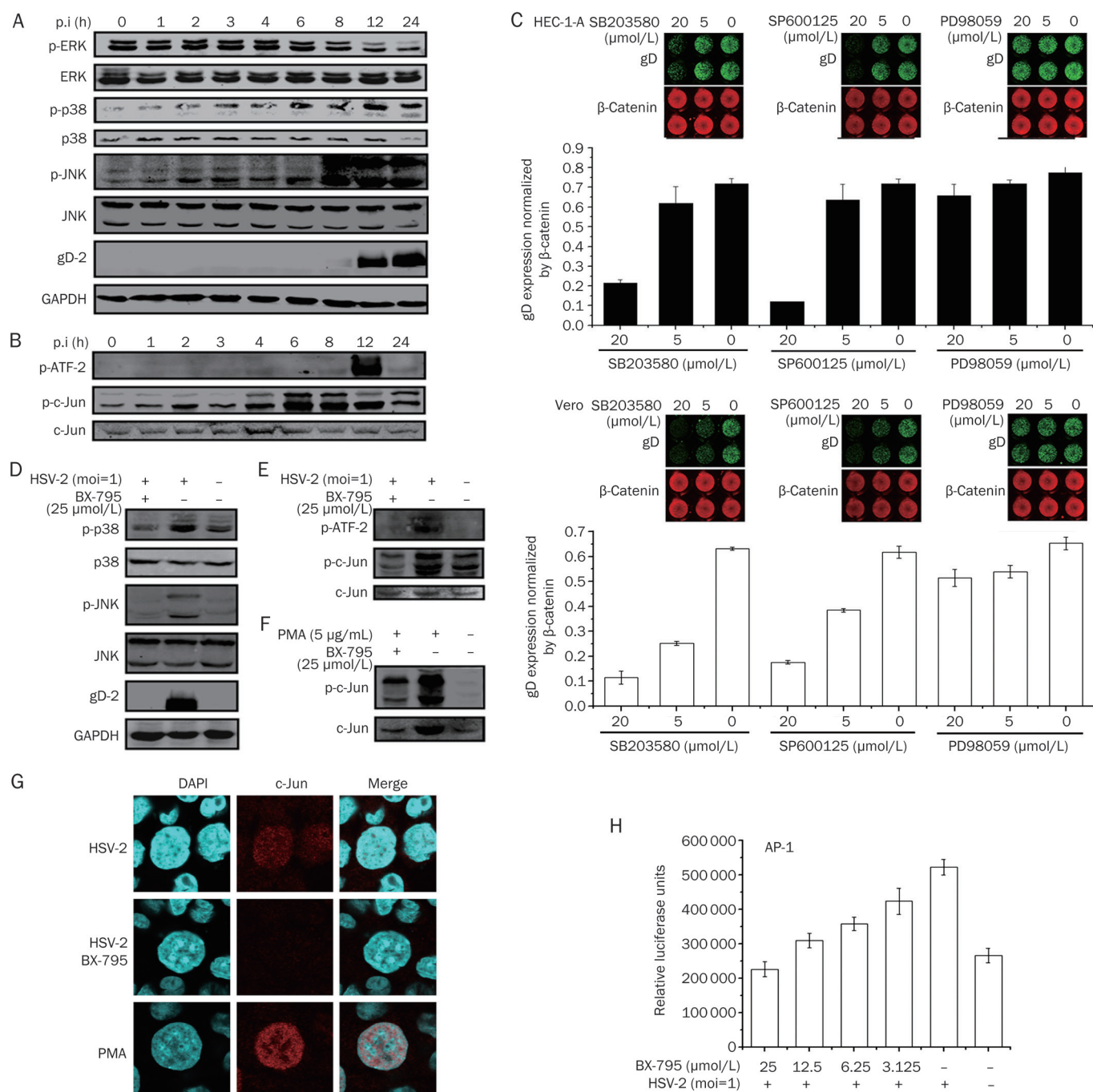
Figure 4A–4H. BX-795 inhibition of HSV replication by blocking JNK/p38 pathway. (A) HSV-2 infection stimulated JNK and p38 MAP kinase pathway, but not ERK pathway. (B) HSV-2 infection also activated the phosphorylation of c-Jun and ATF-2. Cells infected with HSV-2 (moi=1) were collected at the indicated time points. ERK, JNK, p38 MAP kinase, c-Jun and their phosphorylated forms and p-ATF-2 were determined by Western blot. (C) JNK and p38 MAP kinase inhibitors blocked HSV-2 replication. Confluent HEC-1-A cells or Vero cells were infected with HSV-2 (moi=1) in the presence of serial concentrations of SB203580, SP600125 or PD98059. gD expression was determined 24 h postinfection. (D) BX-795 inhibited HSV-2-induced activation of JNK and p38 MAP kinase pathways. (E) BX-795 inhibited further activation of c-Jun and ATF-2 caused by HSV-2 infection. HEC-1-A cells were either mock-infected or infected with HSV-2 (moi=1) in the presence or absence of BX-795. JNK, p38 MAP kinase, c-Jun and their phosphorylated forms and p-ATF-2 were determined 12 h postinfection. (F) BX-795 blocked PMA-stimulated c-Jun activation. HEC-1-A cells were either mock-treated or treated with PMA (5 µg/mL) in the presence or absence of BX-795. The cells were collected after 2 h treatment, and c-Jun and its phosphorylated form were determined. (G) BX-795 inhibited HSV-2-mediated c-Jun nuclear translocation. HEC-1-A cells were either mocked-treated or treated with PMA (5 µg/mL) or infected with HSV-2 (moi=1) in the presence or absence of BX-795. c-Jun nuclear translocation was determined by immunofluorescence staining 2 h post-treatment with PMA or 12 h post-infected with HSV-2. (H) BX-795 inhibited HSV-2-induced AP-1 activation in a dose-dependent manner. HEC-1-A cells were transfected with AP-1 *luc* reporter plasmid. After 24 h, the cells were either mock-infected or infected with HSV-2 (moi=1) in the presence of serial concentrations of BX-795. AP-1 activity was determined by luciferase assay.
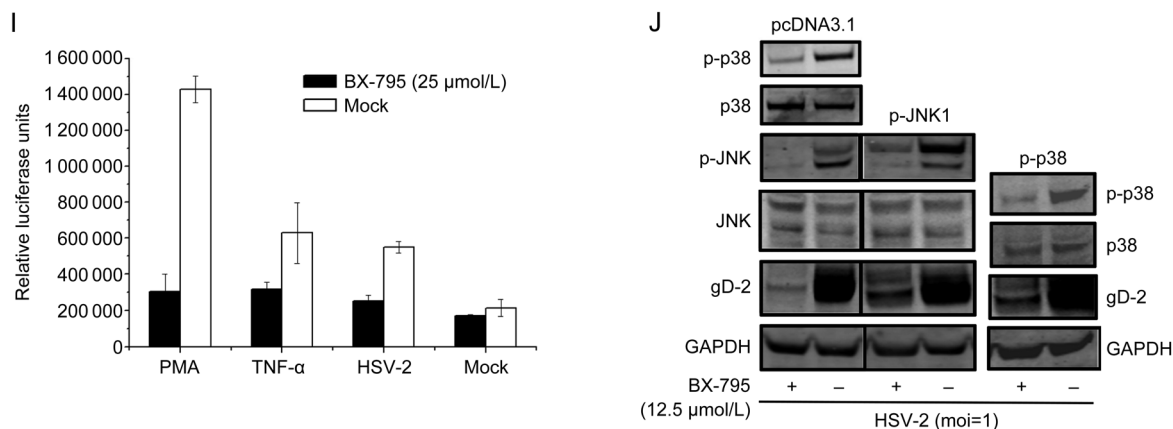
Figure 4I, 4J. BX-795 inhibition of HSV replication by blocking JNK/p38 pathway. (I) BX-795 also inhibited PMA and TNF-α stimulated AP-1 activation. HEC-1-A cells were transfected with AP-1 *luc* reporter plasmid. After 24 h, cells were mock-treated, treated with PMA (5 μg/mL) or rhTNF-α (100 ng/mL) or infected with HSV-2 (moi=1) in the presence of BX-795 (25 μmol/L). AP-1 activity was determined. (J) BX-795 inhibition on HSV replication was attenuated by overexpression of p38/JNK. HeLa cells with or without JNK1 or p38 overexpression were infected with HSV-2 and total cellular protein was prepared for WB analysis. gD-2, the phosphorylation level of p38 MAP kinase, JNK and their substrates were determined via Western blot.

stimulated by many viral infections[6–9, 12, 13, 15, 16, 26–31]. Evidence has shown that JNK and p38 MAP kinase pathways were also stimulated by HSV-1 infection and their activations play a central role in HSV replication[10, 11, 17]. As shown in Figure 4A, HSV-2 infection increased the phosphorylation of JNK and p38 but reduced ERK phosphorylation 8 h postinfection in human genital epithelial cells HEC-1A cells, suggesting that it only activated JNK and p38 MAPK pathways. Similar results were observed with HSV-1 (data not shown). We also investigated whether HSV-2 infection would influence the phosphorylation of c-Jun and ATF-2, which are downstream targets of JNK and p38 MAP kinase[32–34]. c-Jun was activated between 6–12 h postinfection, and ATF-2 was only activated and phosphorylated 12 h postinfection, as shown in Figure 4B.

To elucidate which pathways were crucial for HSV-2 replication, we used three specific MAPK inhibitors, SB203580 (p38 inhibitor), SP600125 (JNK inhibitor) and PD98059 (ERK inhibitor) in HSV-2-infected cells. The results in Figure 4C indicated that only SB203580 and SP600125, but not PD98059, inhibited viral replication in both HEC-1-A and Vero cells in a dose-dependent manner, suggesting that JNK and p38 pathways were the rate-limiting steps in HSV viral protein synthesis and replication, consistent with previous reports[10, 11, 17]. We found that BX-795 interfered with the phosphorylation and activation of JNK and p38 MAP kinase induced by HSV-2 infection of HEC-1-A cells 12 h postinfection (Figure 4D). Furthermore, BX-795 blocked HSV-2-induced activation of c-Jun and ATF-2 (Figure 4E), c-Jun phosphorylation and the activation stimulated by PMA (Figure 4F), suggesting that the BX-795 blockage of virus-induced JNK and p38 MAPK activation was not specific for the virus-associated protein, but rather, targeted certain cellular kinases associated with the viral infection. We also confirmed that BX-795 inhibited c-Jun nuclear translocation (Figure 4G) induced by both HSV-2 infection and PMA, consistent with the results shown in Figure 4D–4F.

It was also found that BX-795 inhibited HSV-2-mediated AP-1 activation in a dose-dependent manner as determined by a luciferase assay (Figure 4H) using an AP-1-luc reporter plasmid. Whether BX-795 inhibited AP-1 activation mediated by other stimuli was investigated. As shown in Figure 4I, PMA- and TNF-α-stimulated AP-1-binding site-driven luciferase expression was inhibited by BX-795. To further confirm that the effect of BX-795 on viral replication is JNK/p38 dependent, we investigated the effect of p38/JNK overexpression on HSV replication inhibited by BX-795. The results indicated that, when compared with the BX-795-pretreated cells, viral replication increased slightly when the cells overexpressed p38/JNK with BX-795 treatment (Figure 4J). The results indicated that inhibition of HSV replication by BX-795 can be at least partially rescued by overexpression of p38/JNK. In conclusion, BX-795 inhibition of HSV replication was mediated by the JNK and p38 MAP kinase pathways.

BX-795 acted upstream of JNK and p38 MAP kinase

SEK1/MKK4 acted upstream of JNK and p38, but not ERK, in response to various environmental stresses or mitogenic stimuli[35]. Evidence has shown that HSV-1 infection leads to SEK1/MKK4 activation[17]. In the current study, we investigated the effect of HSV-2 infection on MKK4 activation in HEC-1-A cells and showed that the viral infection stimulated MKK4 phosphorylation gradually, reaching its peak at 12 h postinfection (Figure 5A). BX-795 completely blocked HSV-2-induced MKK4 phosphorylation (Figure 5B), suggesting that BX-795 might inhibit JNK/p38 MAP kinase activation through interfering with MKK4 phosphorylation or its upstream activators.

BX-795 inhibited ICP0-mediated AP-1 activation but not virus-induced NF-κB activation

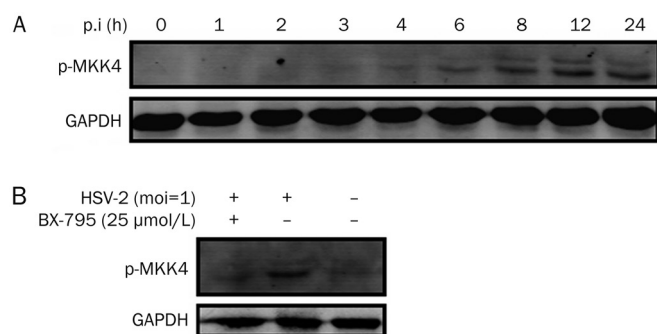ICP0, an important herpes IE protein, is a general potent

Figure 5. BX-795 acted at the upstream of JNK and p38 MAP kinase. (A) HSV-2 infection activated MKK4 in HEC-1-A cells. Cells infected with HSV-2 (moi=1) were collected at each time point. Cell lysates were prepared as described and p-MKK4 level were determined by Western blot. (B) BX-795 inhibited activation of MKK4 caused by HSV-2 infection. HEC-1-A cells were either mock-infected or infected with HSV-2 (moi=1) in the presence or absence of BX-795. p-MKK4 was analyzed 12 h postinfection.

activator of viral gene expression[36]. Expression of ICP0 has been reported to stimulate intracellular AP-1 and NF-κB activation[37, 38]. In the present study, we evaluated the effect of BX-795 on ICP0-induced AP-1 and NF-κB activation. As shown in Figure 6A, BX-795 impeded HSV-1 and HSV-2 ICP0-induced AP-1 activation in HEC-1-A, Vero and 293T cells. However, BX-795 did not inhibit ICP0-mediated NF-κB activation which was only blocked by MG132, confirming the observations by Diao *et al*[38]. Two other potent NF-κB inhibitors, PDTC and BAY11-7082 (Figure 6B), did not block ICP0-mediated NF-κB activation.

Although HSV-1 infection causes persistent activation of NF-κB, an essential part of HSV-1 viral replication[39, 40], and although we observed that HSV-2 infection led to IκB-α degradation (Figure 7A) in human genital tract epithelial cells (HEC-1-A), BX-795 failed to inhibit cytoplasmic IκB-α degradation 12 h postinfection (Figure 7B). In contrast, PGA1, a potent anti-inflammatory cyclopentenone prostaglandin and an NF-κB inhibitor, inhibited IκB-α degradation (Figure 7B).
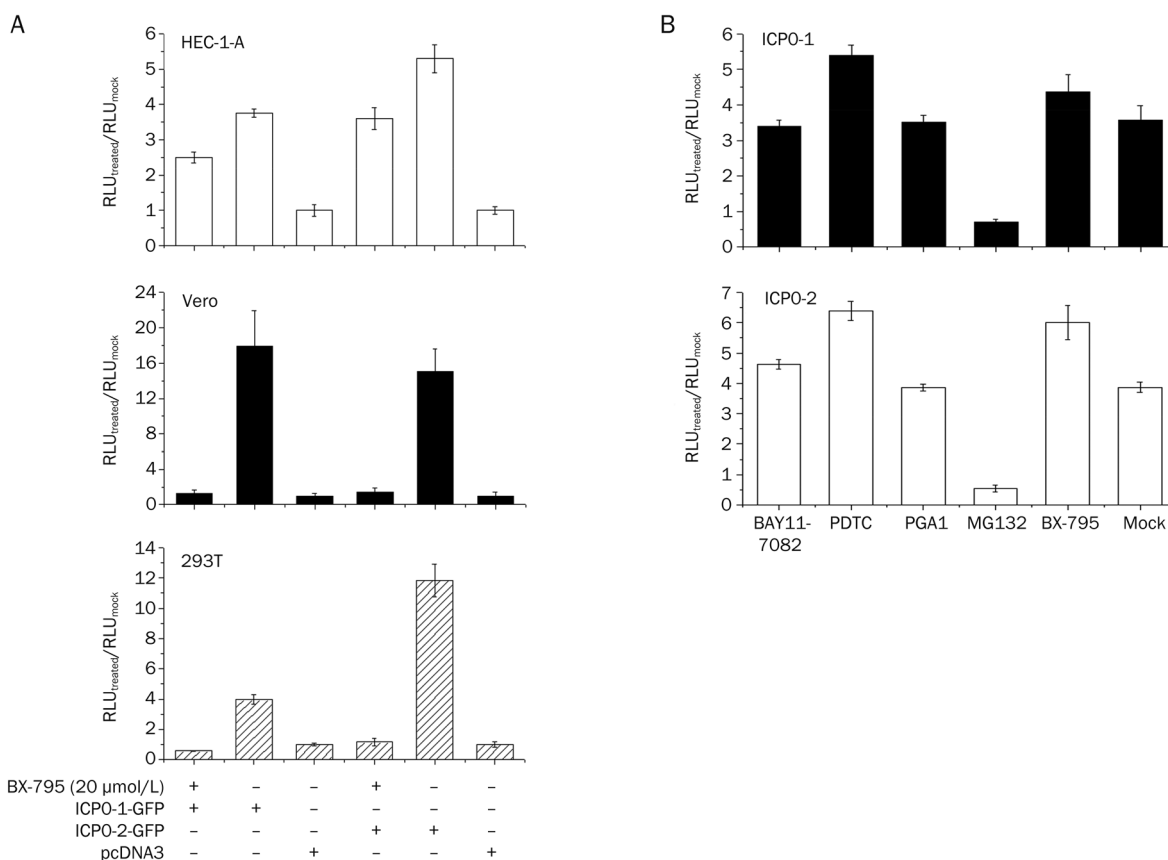

Figure 6. BX-795 inhibited ICP0-mediated AP-1 activation, but not NF-κB activation. (A) BX-795 inhibited ICP0-mediated AP-1 activation. HEC-1-A, Vero and 293T cells were co-transfected with pcDNA3-ICP0-1/2-GFP or pcDNA3 and AP-1 luc reporter plasmid. After 24 h, cells were either mock-treated or treated with BX-795 (25 μmol/L). The luciferase activities were measured 24 h post-treatment. (B) BX-795 did not impede ICP0-mediated NF-κB activation. HEC-1-A cells were co-transfected with pcDNA3-ICP0-1/2-GFP or pcDNA3 and NF-κB luc reporter plasmid. After 24 h culture, cells were either mock-treated or treated with BX-795 (25 μmol/L), BAY11-7082 (20 μmol/L), PDTC (10 μg/mL), PGA1 (10 μg/mL) or MG132 (20 μmol/L). The luciferase activities were measured 24 h post-treatment.
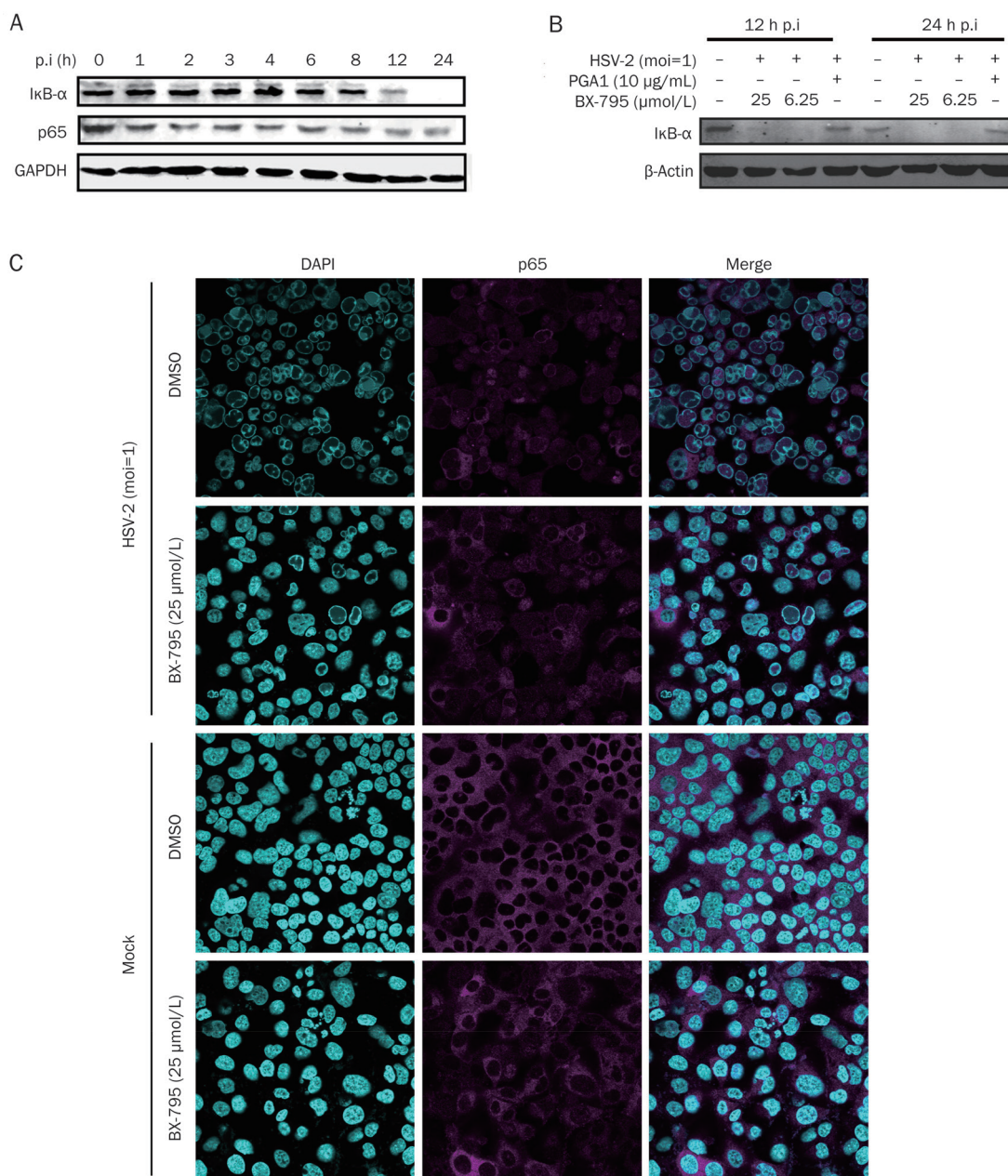
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Figure 7. BX-795 did not block HSV-induced NF-κB activation. (A) HSV-2 infection activated NF-κB in HEC-1-A cells. Cells infected with HSV-2 (moi=1) were collected at the indicated time points. Cell lysates were prepared as described in the context and p65 and IκB-α were determined by Western blot. (B) BX-795 did not inhibit HSV-2-induced NF-κB activation. HEC-1-A cells were either mock-infected or infected with HSV-2 (moi=1) in the presence or absence of BX-795 or PGA1. Cells were lysed and cytoplasmic proteins were extracted 12 or 24 h postinfection. Cytoplasmic IκB-α and β-actin were determined by Western blot. (C) BX-795 did not inhibit HSV-2-induced p65 nuclear translocation. HEC-1-A cells were either mock-infected or infected with HSV-2 (moi=1) in the presence of DMSO or BX-795, and p65 translocation was determined by immunofluorescence assay 24 h postinfection.

p65 translocation assay also confirmed that HSV-2 infection induced p65 nuclear translocation, which was blocked by PGA1 but not by BX-795 (Figure 7C).

Discussion

BX-795 was initially developed as an anti-PDK1 agent[22]. Recently, it was also reported as a potent and relatively specific inhibitor of TBK1, IKKε, Aurora B, ERK8 and MARK3[19].

In this report, we described novel bioactivity of BX-795 in the inhibition of HSV-1 and HSV-2 replication. To our knowledge, it was the report describing BX-795 as an antiviral agent.

PDK1 is an adaptor in the PI3K/Akt/mTOR pathway[41, 42]. Phosphatidylinositol-(3,4,5)-trisphosphate (PIP$_3$), the phosphorylated form of phosphatidylinositol-(3,4)-bisphosphate (PIP$_2$), which is catalyzed by PI3K, can trigger membrane colocalization of PDK1 and Akt and lead to downstream

mTOR activation[41, 42]. Hsu *et al* reported that HSV-1 infection induced activation of the Akt pathway in oral epithelial cells which was mediated by HSV IE gene expression[43]. BX-795 is a PDK1-inhibitory pyrimidine with an IC_{50} of 111 nmol/L in the cell-free system[22] and also active in inhibiting HSV-2-induced Akt phosphorylation and activation in a dose-dependent manner as shown in the current study (Figure 3B). However, the results with the specific PI3K inhibitor, LY294002, and an inhibitor of downstream mTOR, rapamycin, suggested that the PI3K/Akt/mTOR pathway did not affect viral late gene expression and replication because neither molecule inhibited the viral late gene expression (Figure 3C), although HSV-2 activated the PI3K/Akt pathway in human genital tract epithelial cells (Figure 3A). It was shown that the inhibition of PI3K attenuated HSV-1 ICP0 gene expression but did not affect thymidine kinase expression and viral replication. An alternative explanation could be that BX-795 can also act on a different target from those of LY294002 and rapamycin, such as PKC or downstream factors, leading to the activation of JNK/p38 mediated by MKK4, 7 and MAPK.

We showed in the current study that BX-795 did not inhibit either HSV-2-induced or ICP0-mediated NF-κB activation (Figure 6 and 7). It was reported that IKKα and IKKβ activation were not affected by BX-795[18, 19]. Clark *et al* also found that BX-795 did not affect phosphorylation of p65 or the degradation of IκB-α induced by LPS, IL-1α, TNF-α or poly (I:C)[19]. Altogether, the results suggested that the anti-HSV activity of BX-795 was independent of NF-κB activation.

JNK and p38 MAP kinase pathways are important for HSV gene expression and viral propagation[10, 11, 17]. McLean *et al* reported that activation of JNK was necessary for efficient HSV replication, and stable expression of JNK-interacting protein 1 (JIP-1, an inhibitor of JNK translocation to the nucleus) led to a 70% reduction in HSV-1 propagation[10]. Karaca *et al* found that the presence of SB203580 in HSV-1-infected cell culture attenuated 85%–90% virus yield[11]. This finding was consistent with our results on HSV-2 replication in HEC-1-A cells shown in Figure 4C. The activation of JNK/p38 MAP kinase may stimulate viral gene transcription via downstream AP-1 activation. JNK/p38 MAP kinase pathways can be induced not only by HSV-2 infection but also by stimuli, such as PMA and TNF-α (Figure 4). We demonstrated that HSV-2 infection induced c-Jun and ATF-2 phosphorylation and AP-1 binding activity (Figure 4B and 4H). c-Jun and ATF-2 are two components of the transcription factor AP-1, which regulates gene expression in response to a variety of stimuli, including cytokines, growth factors, stress and microbes[44]. These two proteins are substrates of JNK and p38 MAP kinase. The transcriptional activity of c-Jun is regulated by phosphorylation through JNK[45], and ATF-2 can be a target of the JNK or p38 MAP kinase signaling pathways[32–34]. The viral replication increased when cells were pretreated with BX-795 and overexpressed p38/JNK (Figure 4J), indicating that overexpression of p38/JNK partially rescued HSV replication inhibited by BX-795. We, therefore, postulate that BX-795 inhibited HSV infection by attenuating activation of the JNK/p38 MAP

kinase pathway and downstream AP-1 activation.

Some HSV proteins are known to induce JNK/p38 MAP kinase pathways. Zachos *et al* demonstrated that the virion transactivator protein VP16 was both necessary and sufficient for the activation of JNK/p38 pathways even in the absence of any other viral context[17]. However, Hargett *et al* employed HSV IE gene mutant viruses to investigate viral proteins necessary for JNK/p38 MAP kinase activation and showed that ICP27 alone was sufficient[46]. Although some controversies exist, it is apparent that viral immediate-early events are responsible for JNK/p38 MAP kinase activation. Evidence has suggested that activation of the JNK/p38 MAP kinase pathway was necessary for HSV early and late gene transcription. As shown in Figure 2, BX-795 exhibited no inhibitory effect on viral ICP0 and ICP27 transcription at 6 h postinfection but a significant inhibitory effect at 12 h postinfection. Therefore, we postulate that BX-795 exerted its anti-viral activity between IE and late gene transcription.

Notably, BX-795 inhibited HSV-2-induced MKK4 and downstream JNK/p38 MAP kinase activation (Figure 5B). However, using a protein kinase assay in a cell-free system, Clark *et al* reported that BX-795 did not influence MKK3/6 or MKK4/7 activation. The discrepancy between Clark's results and our results may be attributed to the different experimental systems used (cell-free in Clark's and cell-based assay in ours) or, although less likely, by the higher BX-795 concentrations used in our experiment. Another potential explanation is that the direct target of BX-795 in the JNK/p38 MAP kinase pathway may be upstream of JNK/p38 and MKK4. Reports have shown that BX-795 inhibited MLK1 (MAP3K9), MLK2 (MAP3K10) and MLK3 (MAP3K11), which could act as an upstream activator of MKK4 and JNK/p38 MAP kinase[19]. We postulate that BX-795 inhibited the JNK/p38 MAP kinase pathway by interfering with upstream MLK1-3. However, this hypothesis requires further investigation.

TBK1 is a kinase that activates IKK through direct phosphorylation of IKK[47]. TBK1 and IKKε play a vital role in coordinating the activation of IRF-3 and NF-κB in the innate immune response[48]. IRF-3 is a member of the interferon regulatory transcription factor family[49] and is pivotal for IFN production and the innate immune response to viral infection[49]. IFN is known to have an inhibitory effect on HSV IE and early protein expression[50]. Ma *et al*[51] reported that HSV infection inhibited TBK1 activity, which facilitated viral productive infection, indicating that TBK1 is a negative regulator of HSV propagation. BX-795 inhibited IKKε and TBK1 with IC_{50} at 41 and 6 nmol/L in a cell-free system, respectively. It was not clear whether its anti-TBK1/IKKε activity contributed to anti-HSV efficiency. Therefore, the effect of BX-795's anti-TBK1/IKKε activity on HSV infection, if any, would facilitate viral replication.

Many other viruses, such as rotavirus[6], VZV[7, 8], HIV-1[9], echovirus 1[26], CVB3[12], procine circovirus[27], dengue virus[28], EBV[13], SARS coronavirus[14], encephalomyocarditis virus[30], vaccinia virus[31] and HBV/HCV[15, 16], have also been reported to activate JNK/p38 MAP kinase pathways, and studies have

shown that these two pathways are important for viral replication[7, 27, 28]. The identification of the anti-HSV activity of BX-795 and its targeting of the JNK/p38 MAP kinase pathways raises the possibility that BX-795 may also possess biological activity against other viruses, which deserves further investigation.

Acknowledgements

Author contribution

Ai-rong SU participated in the design of the study, performed the statistical analysis and wrote the paper. Min QIU participated in the design of the study and helped to draft the manuscript. Yan-lei LI designed, performed and analyzed the experiments shown in Figures 1 and 2. Wen-tao XU designed, performed and analyzed the experiments shown in Figure 3. Si-wei SONG designed, performed and analyzed the experiments shown in Figure 4. Xiao-hui WANG and Hong-yong SONG designed, performed and analyzed the experiments shown in Figures 5, 6 and 7. Nan ZHENG helped to conceive and coordinate the study, provided technical assistance and contributed to the preparation of the Figures. Zhi-wei WU conceived, supervised the study and revised the paper. The first three authors contributed equally. All authors reviewed the results and approved the final version of the manuscript.

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