Novel Hsp90 inhibitor JD-02 inhibits HSV-1 infection via the Raf/MEK/ERK signaling pathway

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Abstract. Herpes simplex virus type 1 (HSV-1) is a neurotropic pathogen with an extremely high infection rate. The excessive use of acyclovir (ACV) and nucleoside analogs has resulted in the emergence of drug-resistant HSV-1 strains, thereby underscoring the need for the development of novel therapeutic agents against HSV-1. The present study sought to evaluate the efficacy and elucidate the mechanism of action of the novel Hsp90 inhibitor, JD-02, in the context of HSV-1 infection, as well as to assess its potential as an anti-HSV-1 therapeutic agent. The results of the present study demonstrated that JD-02 exhibits lower cytotoxicity relative to the conventional Hsp90 inhibitor, AT533, and effectively inhibits infection by both standard and ACV-resistant HSV-1 strains *in vitro*. Additionally, JD-02 markedly suppresses the expression of viral-associated genes and proteins. The present investigation further revealed that the Raf/MEK/ERK signaling pathway is activated during HSV-1 infection, and that JD-02 exerts its antiviral effects through the inhibition of this pathway. Moreover, the *in vivo* administration of JD-02 mitigated the symptoms of Herpes simplex encephalitis (HSE), extended the lifespan of mice with HSE, and decreased both the viral gene copy number and the expression of inflammatory factors. In contrast to targeting viral DNA polymerases, Hsp90 inhibitors, which target host proteins, exhibit a significantly lower likelihood of inducing drug resistance. These findings indicate that JD-02, a novel HSP90 inhibitor, holds promise for development as a therapeutic agent for the treatment of HSV-1 infection and associated diseases.

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Abbreviations: ACV, acyclovir; CPE, cytopathic effect; MOI, multiplicity of infection; HSV-1, Herpes simplex virus 1; HSE, Herpes simplex virus encephalitis; IFN, interferon; RT-qPCR, reverse transcription-quantitative PCR; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PFU, plaque-forming units; PVDF, polyvinylidene difluoride

Key words: HSV-1, JD-02, antiviral, UL30, Raf/MEK/ERK signal pathway

Introduction

Herpes simplex virus type 1 (HSV-1) is a neurotropic human pathogen that has infected over 67% of the global population (1,2). When it infects the central nervous system (CNS), it can lead to Herpes simplex encephalitis (HSE), posing significant risks to the health and survival of neonates and immunocompromised individuals (3). Furthermore, HSV-1 infection has been associated with an elevated risk of HIV-1 infection and transmission (4,5) and is closely linked to the pathogenesis and progression of neurodegenerative disorders, including Alzheimer's disease (6-8). Notably, there is currently no clinically approved vaccine available to prevent HSV-1 infection (9). Presently, nucleoside analogs such as acyclovir (ACV) and valacyclovir are primarily employed to mitigate the symptoms associated with HSV-1 infection and to suppress viral reactivation. However, the prolonged use of these antiviral agents has led to the emergence of drug-resistant strains (10), which pose a significant threat to the

health of immunocompromised patients (11,12). Consequently, the development of novel therapeutic agents effective against HSV-1 infection is of paramount importance.

Viruses, as parasitic entities, typically depend on the host's cellular synthesis and metabolic pathways to complete their lifecycle (13). Among these pathways, heat shock protein 90 (HSP90), a highly conserved molecular chaperone, emerges as a critical host factor essential for the lifecycle of numerous viruses (14). This includes processes such as viral entry, transport, assembly, and the release of viral particles, all of which necessitate host molecular chaperones. The RAF/MEK/ERK signaling pathway, a component of the classical MAPK cascade, plays a pivotal role in cell proliferation and development (15). RAF family proteins, which serve as client proteins of HSP90, are exploited by viruses such as the influenza virus, SARS-CoV-2 and HSV-1 to co-opt the host's Raf/MEK/ERK pathway, thereby enhancing their replication and proliferation (13,16). The interaction between host and pathogen results in substantial reorganization of the host protein network, leading to dysregulation of signaling pathways and the establishment of a pathological environment (17). Consequently, the RAF/MEK/ERK pathway represents a promising target for antiviral intervention (18). A comprehensive understanding of the intricate dynamics of host-pathogen interactions is imperative for the development of strategies aimed at inhibiting viral replication. In contrast to antiviral agents targeting viral components, those designed to interact with host factors demonstrate a reduced susceptibility to the emergence of drug-resistant viral strains (19,20). While HSP90 inhibitors have shown broad-spectrum antiviral efficacy, their approval by the FDA remains pending due to concerns regarding toxicity and limited efficacy (21). Consequently, advancing the development of safe and effective HSP90 inhibitors represents a critical focus for current antiviral drug research and development.

The present study assessed JD-02's potential as an anti-HSV-1 treatment. Among six benzamide-based compounds, JD-02 showed anti-HSV-1 activity in Vero cell assays. As a novel HSP90 inhibitor, it blocks HSV-1 infection by targeting the Raf/MEK/ERK pathway. *In vivo* mouse model studies confirmed JD-02's effectiveness in reducing HSV-1-related disease symptoms.

Materials and methods

Compounds, antibodies and reagents: Six benzamide derivatives were synthesized by Professor Daohua Xu from the School of Pharmacy, Guangdong Medical University (22). The compounds were purified by silica gel column, and the purity was tested by High Performance Liquid Chromatography analysis [Agilent 1260 Infinity II HPLC system (Agilent Technologies, Inc.) with a Zorbax Eclipse Plus C18 column (4.6x150 mm, 5 μm; Agilent Technologies, Inc.). Conditions included a 30˚C column temperature, 20 μl injection volume, mobile phase consisting of solvent A (water/0.1% formic acid) and solvent B (acetonitrile/0.1% formic acid) at 1.0 ml/min flow rate]. All compounds were dissolved in dimethyl sulfoxide at a concentration of 50 mM.

Antibodies against ICP0 (cat. no. ab6513), gD (ab6507), ICP27 (cat. no. ab53480) and Anti-HSV-1 (cat. no. ab9533) were obtained from Abcam. Antibodies against GAPDH (cat. no. GTX100118) were purchased from GeneTex, Inc. Antibodies against phospho-ERK1/2 (cat. no. 4370S), ERK1/2 (cat. no. 4695S), phospho-MEK1/2 (cat. no. 9154T), phospho-B-Raf (cat. no. 2696T), HA (cat. no. 3724S) and Flag (cat. no. 14793S) were obtained from Cell Signaling Technology, Inc. Antibodies against VP5 (cat. no. sc-13525), gB (cat. no. sc-56987) and Raf-B (C-19) (cat. no. sc-166) were obtained from Santa Cruz Biotechnology, Inc. Antibodies against MEK1/2 (cat. no. AF6385) were obtained from Affinity Biosciences.

ACV was purchased from MilliporeSigma, which was dissolved in dimethyl sulfoxide (DMSO) with a concentration of 20 mM. The MEK1/2-inhibitor U0126 was purchased from Cell Signaling Technology, Inc. and was dissolved in DMSO with a concentration of 10 mM.

Cell lines and virus. SH-SY5Y [cat. no. CRL226; American Type Culture Collection (ATCC)] and HaCaT (cat. no. 300493; CLS Cell Lines Service GmbH) and were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.). Vero cells (cat. no. CCL81; ATCC) were cultured in DMEM with 10% FBS (Hangzhou Biology Engineering Materials Co., Ltd.). All the cells were cultured at 37˚C in a humid atmosphere with 5% CO_2.

HSV-1 strain F was acquired from the Hong Kong University and propagated in Vero cells. The reporter virus EGFP-HSV-1, which expresses EGFP-tagged viral protein Us11, was acquired from the Anti-Stress and Health Research Center, College of Pharmacy, Jinan University. ACV resistant HSV-1 strains, HSV-1/Blue and HSV-1/153, were kind gifts from Prof Tao Peng (Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences) and prepared as previously methods (23).

Cytotoxicity assay. The cytotoxic effects of JD-02 were assessed utilizing the Cell Counting Kit (CCK-8) assay kit (cat. no. 96992; MilliporeSigma). Initially, Vero, HaCaT, SH-SY5Y and BV2 were seeded in triplicate into 96-well plates at a density of 10,000 cells per well and incubated for 24 h. Following this incubation period, the cells were exposed to varying concentrations of JD-02 (0.125, 0.25, 0.5, 1, 2, 4, 8 and 16 μM). Following a 24-h incubation at 37˚C, 10 μl of CCK-8 reagent was added to each well, and the optical density at 490 nm (OD490) was measured using a microplate reader (24). The experiment utilized non-cytotoxic compound concentrations.

Viral plaque assay. Viral plaque assay was performed in Vero cells (25). Briefly, once the Vero cells formed a confluent monolayer, they were infected with HSV-1 [multiplicity of infection (MOI)=1] and treated with drugs or ACV at 37˚C to facilitate optimal viral particle absorption. Following this, the medium was replaced with a maintenance medium containing 1% methylcellulose (cat. no. M0512; MilliporeSigma) with or without JD-02 and ACV in each well. The cells were incubated for 72 h before the cells were fixed with 4% polyoxymethylene for 20 min at room temperature and stained with 1% crystal violet (cat. no. C0121; Beyotime

Institute of Biotechnology) for 30 min at room temperature. The total number of plaques was counted, and the inhibition rate was determined.

Virus titration assay. Virus titers were quantified by assessing the cytopathic effects (CPEs) induced by HSV-1. Viral titration was conducted by assessing the CPEs in Vero cells infected with HSV-1. The cells were cultured in 96-well plates at a density of $1.5x10^5$ cells per well and were exposed to a culture medium containing serial 10-fold dilutions of HSV-1 viral particles (26). Following a 72-h incubation period, the CPEs were evaluated to determine the 50% tissue culture infectious dose ($TCID_{50}$), which was subsequently converted to plaque-forming units (PFU) per ml using the formula: PFU/ml=$TCID_{50}$/ml x 0.7. CPE grade: '-' for no CPE; '+' for 1-25% CPE; '++' for 26-50% CPE; '++' for 51-75% cytopathic as '++++'; 76-100% cytopathic as '++++'.

Viral inactivation, attachment, penetration assay. For virus inactivation analysis, HSV-1 was incubated with JD-02 (0.5 μM) at 37˚C for 2 h, then diluted and used to infect Vero cells at 37˚C for another 2 h. After replacing the solution, incubation continued for 72 h (27). Empty spot reduction assays were conducted. For the virus-attached vacuole assay, HaCaT cells were chilled at 4˚C for 1 h, exposed to HSV-1 (30 PFUs/well) with or without JD-02 (0.5 μM) at 4˚C for 2 h, then washed with cold PBS and incubated for 48 h with a complete solution for empty plaque experiments. For the viral DNA copy number assay, HSV-1 (MOI=5) and JD-02 (0.5 μM) were applied to HaCaT cells at 4˚C for 2 h, washed with cold PBS, subjected to three freeze-thaw cycles. For the virus-penetration empty plaque assay, HaCaT cells were infected with HSV-1 at 4˚C for 2 h, treated with JD-02 at 37˚C for 10 min, then exposed to acidic and alkaline PBS for 1 min each, and incubated with an overlay solution for 72 h. For the viral DNA copy number assay, HaCaT cells were treated with HSV-1 and JD-02 at 4˚C for 2 h, followed by JD-02 at 37˚C for 10 min, and exposure to acidic and alkaline PBS for 1 min each (28).

Immunofluorescence assay. The cells to be treated were fixed with 4% paraformaldehyde (PFA), penetrated with 0.1% NP-40 for 4 min, and then blocked with 5% bovine serum albumin (BSA) for 90 min before staining with VP5 (1:100; cat. no. sc-13525; Santa Cruz Biotechnology, Inc.) overnight at 4˚C. The cells were then incubated with Alexa Fluor-conjugated secondary antibody (1:1,000; cat. no. A32723; Thermo Fisher Scientific, Inc.) at room temperature. Nuclei were next labeled with DAPI (cat. no. C1006; Beyotime Institute of Biotechnology) for 15 min at room temperature. It should be noted that each step of these processes was washed with PBS for 3 min. Finally, fluorescence images were acquired using a Zeiss LSM510 Meta confocal system (Zeiss GmbH).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from the indicated treated cultured cells using TRIzol Reagent (TRIzol; cat. no. DP424; Tiangen Biotech Co., Ltd.), and a PrimeScript RT kit (cat. no. RR036A-1; Takara Bio, Inc.) was used for cDNA synthesis according to the manufacturer's instructions. The RT-qPCR assay was performed in a CFX96 Touch-Real-Time PCR detection system (Bio-Rad Laboratories, Inc.) using the TB Green Premix Ex Taq II kit (cat. no. RR820; Takara Bio, Inc.) according to the manufacturer's instructions (28). Gene expression levels were normalized to the internal regulatory gene GAPDH. To determine the viral genomic DNA, HaCaT cells were infected with HSV-1 (MOI=0.1) while incubated with the addition of JD-02. After 24 h, samples were placed in -80˚C refrigerator and repeatedly frozen and thawed three times, after which the supernatant and cell pellet were collected. The viral genomic DNA was obtained using EasyPure® Viral DNA/RNA Kit (TransGen Biotech Co., Ltd.), which were subjected to RT-qPCR for quantification. The thermal cycling conditions were set as follows: Initial denaturation at 95˚C for 30 sec; 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. The $2^{-\Delta\Delta Cq}$ method was applied for mRNA analysis and GAPDH was used as the reference gene (29). All primer sequences are shown in Table SI.

Transfection of plasmids or small interfering RNA (siRNA). The HA-UL42 plasmids and Flag-UL30 plasmids were synthesized and constructed by Shanghai GenePharma Co., Ltd. All siRNAs were synthesized and constructed by Integrated Biotech Solutions. Cells were transfected with plasmid (2 μg) or siRNA (100 nM) using jetPRIME®transfection reagent (cat. no. PT114-15; Polyplus-transfection). After 24 or 48 h, the cells were infected with HSV-1 with or without chemicals. All primer sequences of siRNA are displayed in Table SII.

Western blot assay. Samples were extracted from HSV-1-infected cells using RIPA buffer (cat. no. P0013B; Beyotime Institute of Biotechnology) supplemented with 1% PMSF (cat. no. ST506; Beyotime Institute of Biotechnology). The lysates were clarified by centrifugation at 12,000 x g for 10 min at 4˚C. Protein concentrations were normalized using the bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology). Proteins (30 μg total per lane) were separated on an 8 to 12% gradient SDS-PAGE gel and transferred onto polyvinylidene fluoride membranes (MilliporeSigma). Membranes were blocked with 5% BSA for 1 h at room temperature and incubated with primary antibodies at 4˚C overnight, followed by incubation with secondary antibodies (1:5,000; cat. nos. 31430 and 31460; Invitrogen; Thermo Fisher Scientific, Inc.,) for 60 to 90 min at room temperature. These primary antibodies included ICP0 (1:1,000; cat. no. ab6513; Abcam), ICP27 (1:1,000; cat. no. ab53480; Abcam), gD (1:1,000; cat. no. ab6507; Abcam), gB (1:500; cat. no. sc-56987; Santa Cruz Biotechnology, Inc.), Raf-B (1:500; cat. no. sc-166; Santa Cruz Biotechnology, Inc.), p-BRAF (1:1,000; cat. no. 2696T; Cell Signaling Technology, Inc.), ERK (1:1,000; cat. no. 4695S; Cell Signaling Technology, Inc.), p-ERK (1:1,000; cat. no. 4370S; Cell Signaling Technology, Inc.), MEK1/2 (1:1,000; cat. no. AF6385; Affinity Biosciences), p-MEK1/2 (1:1,000; cat. no. 9154T; Cell Signaling Technology, Inc.), Flag (1:1,000; cat. no. 14793S; Cell Signaling Technology, Inc.), HA (1:1,000; cat. no. 3724S; Cell Signaling Technology, Inc.). Target proteins were visualized using enhanced chemiluminescence (ECL) solution (cat. no. 36208ES60; Shanghai Yeasen Biotechnology Co., Ltd.) and images were captured

with a Tanon 5200 Image Analysis System (Tanon Science and Technology Co., Ltd.). ImageJ software (version 1.54f; National Institutes of Health) was used for densitometric analysis.

HSE mice model. All animal experiments were approved (approval no. 20200402-07) by the Animal Care and Use Committee of Jinan University (Guangzhou, China). A total 50 male BALB/c mice, aged 5 weeks and weighing between 20-22 g, were procured from the Guangdong Medical Laboratory Animal Center. The mice were maintained under a controlled environmental conditions, with a standardize temperature range of 20-26˚C. They were housed to a regulated 12/12-h reverse light/dark cycle and were provided with *ad libitum* access to food and water. Following an acclimatization period, the mice were randomly allocated into different experimental groups and subsequently inoculated intranasally with HSV-1 at a concentration of $2x10^6$ PFU per mouse. Infected mice were injected intraperitoneally with JD-02 (10 or 20 mg/kg/day) or ACV (20 mg/kg/day) for 5 consecutive days. Correspondingly, mice in the mock and HSV-1 control groups were treated with saline (0.9%). Body weight and HSE symptoms were recorded daily for all mice. The scoring rules for HSE symptoms were based on previous studies: Eye swelling/lesions (0: no symptoms, 1: mild eyelid swelling, 2: moderate eyelid swelling with crusting > 50% 3: severe eyelid swelling with crusting), hair loss (0: none hair loss, 1: minimal periocular hair loss, 2: moderate periocular hair loss, 3: severe hair loss limited to the periocular region); hydrocephalus (0: none, 1: minor bump, 2: moderate bump, 3: large bump). Mice were sacrificed for histological analysis and viral quantification as soon as they lost ~20% of their body weight (the 6th day after infection). The mice were euthanized using isoflurane (5% induction), followed by cervical dislocation. Death was confirmed by the absence of respiration and heartbeat. Whole brains were primarily dissected and promptly stored at -80˚C for subsequent viral titer and gene expression analysis. Parts of the heart, liver, spleen, lung, kidney and intestine were fixed in 4% PFA solution, at room temperature for 24 h, embedded in paraffin, sectioned, stained with hematoxylin-eosin (H&E) solution or anti-HSV-1 antibody, and examined under the light microscope. In addition, total RNA was extracted from brain tissues, and the samples were subjected to RT-qPCR.

Histology assay. The primary organs from mice infected with HSV-1 and subsequently treated with JD-02 at six days post-infection (n=4) were preserved in 4% PFA, embedded in paraffin, deparaffinized, stained with H&E (cat. no. G1120; Beijing Solarbio Science & Technology Co., Ltd.) The specimen was stained with hematoxylin solution for 15 min and eosin for 3 min, both at room temperature and subsequently examined using light microscopy.

Statistical analysis. Data are presented as the mean ± SD of the results from at least 2 independent experiments. The unpaired Student's t-test analysis was executed to compare the means of two groups. $^*P<0.05$ was considered to indicate a statistically significant difference. Statistical analysis was carried out using GraphPad Prism 8.0 software (Dotmatics).

Results

Anti-HSV-1 activity and cytotoxicity of JD-02. The structures of the six novel benzamide derivatives are detailed in Table I. Initially, the antiviral efficacy of these derivatives against HSV-1 was assessed in Vero cells using a CPE. Observations via light microscopy revealed that JD-02 significantly inhibited HSV-1-induced CPE in Vero cells more effectively than the other compounds tested (Table II). Consequently, JD-02 was selected for further experimentation.

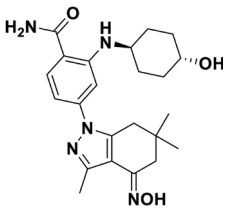
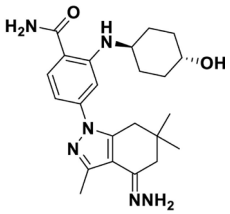
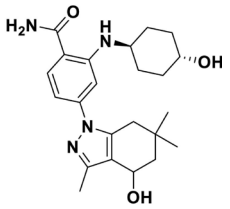
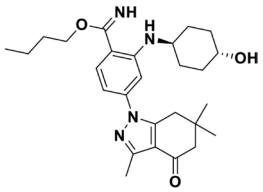
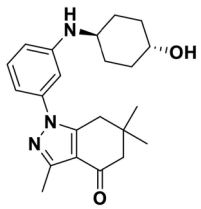
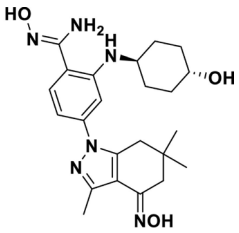
The cytotoxicity of JD-02 was compared with that of the commercial benzamide Hsp90 inhibitor AT533 across various cell lines, including Vero, HaCaT, SH-SY5Y and BV2, utilizing the CCK-8 assay (Fig. S1). The findings indicated that JD-02 as Hsp90 inhibitor exhibited relatively low toxicity in Vero, HaCaT and BV2 cells compared with AT533. The 50% cytotoxic concentration (CC_{50}) values for JD-02 exceeded 32 μM in Vero cells, 5.503 μM in HaCaT cells, 10.75 μM in BV2 cells and 3.374 μM in SH-SY5Y cells. Therefore, it was chosen to use a concentration below its CC_{50} for subsequent studies.

JD-02 suppresses HSV-1 replication. Among the compounds evaluated, JD-02 (Fig. 1A) demonstrated significant inhibitory activity against HSV-1 infection across all tested concentrations. The anti-HSV-1 efficacy of JD-02 was assessed using viral plaque assays, which revealed a dose-dependent decrease in the number of viral plaques formed in Vero cells infected with HSV-1 and the half-maximal effective concentration was determined to be 0.1396 μM (Fig. 1B). JD-02 exhibited inhibition rates against HSV-1 infection that were comparable to those of ACV at equivalent concentrations. Additionally, RT-qPCR analysis indicated that JD-02 significantly reduced the DNA copy number of several viral genes, including UL54, ICP0, UL52 and UL27, at concentrations of 0.5 and 1 μM (Fig. 1C). Besides, western blot analysis further confirmed a dose-dependent inhibitory effect of JD-02 on the expression of the viral immediate-early protein ICP0, early proteins ICP27 and gD, as well as the late protein gB (Fig. 1D). Collectively, these findings suggest that JD-02 possesses a potent capacity to inhibit HSV-1 infection.

To further investigate the antiviral efficacy of JD-02, an EGFP-tagged HSV-1 was employed. Fluorescence analysis revealed a dose-dependent reduction in fluorescence intensity in Vero cells treated with JD-02. Moreover, JD-02 demonstrated greater effectiveness in inhibiting fluorescence intensity compared with ACV (Fig. 2A). Considering that HSV-1 is a neurotropic virus capable of accessing the CNS via epithelial cells and potentially inducing severe HSE (3), and acknowledging that microglia function as the primary immune cells within the brain, the effects of JD-02 on HSV-1 infection in microglia were investigated. Fluorescence data revealed a concentration-dependent decrease in fluorescence intensity with the treatment of JD-02 (Fig. 2B). Additionally, RT-qPCR analysis showed a significant, concentration-dependent reduction in the copy number of the viral gene ICP0 following JD-02 administration in Vero, BV2 and HaCaT cells (Fig. 2C-E). These findings substantiate the efficacy of JD-02 in inhibiting HSV-1 infection across various cell types.

JD-02 inhibits normal and ACV-resistant HSV-1 strains infection. The emergence of ACV-resistant strains poses a

Table I. Structures of 6 novel benzamide derivatives.

Compound	Structure	Molecular formula	Molecular weight (Da)
JD-01		$C_{23}H_{31}N_5O_3$	425.53
JD-02		$C_{23}H_{32}N_6O_2$	424.54
JD-03		$C_{23}H_{32}N_4O_3$	412.31
JD-04		$C_{27}H_{38}N_4O_3$	466.35
JD-05		$C_{22}H_{29}N_3O_2$	367.49
JD-06		$C_{23}H_{32}N_6O_3$	440.54

considerable challenge in the clinical management of diseases associated with HSV-1 (11,30). In the present study, the antiviral efficacy of JD-02 was evaluated against two ACV-resistant strains, HSV-1/153 and HSV-1/Blue, in HaCaT cells. A viral plaque assay was performed to determine the antiviral efficacy of JD-2 against these ACV-resistant strains. The results indicated that ACV was ineffective in inhibiting the infection efficiency of these two viral strains (Fig. 3A). Conversely, JD-02 significantly reduced the infection efficiency of HSV-1/153 and HSV-1/Blue (Fig. 3B) in a dose-dependent manner, with half-maximal inhibitory concentration (IC_{50}) of 1.52 μM (HSV-1/153) and 0.72 μM (HSV-1/Blue). Additionally, AT533 also inhibited the infection of HSV-1/153 and HSV-1/Blue (Fig. 3C), with IC_{50} values of 1.74 μM (HSV-1/153) and 1.39 μM (HSV-1/Blue), although its efficacy was inferior to that of JD-02. Similarly, JD-02 (IC_{50}=0.11 μM) exhibited

Table II. Antiviral screening based on the CPE assay.

Compound	1 μM	2 μM	4 μM
JD-01	++++	+++	+++
JD-02	+	+	+
JD-03	++++	++++	++++
JD-04	+++	++	++
JD-05	++++	+++	+++
JD-06	++++	+++	+++
Cell	-	-	-
HSV-1	++++	++++	++++

CPE grade: '-' for no CPE; '+' for 1-25% CPE; '++' for 26-50% CPE; '++' for 51-75% cytopathic as '++++'; 76-100% cytopathic as '++++'. CPE, cytopathic effect.

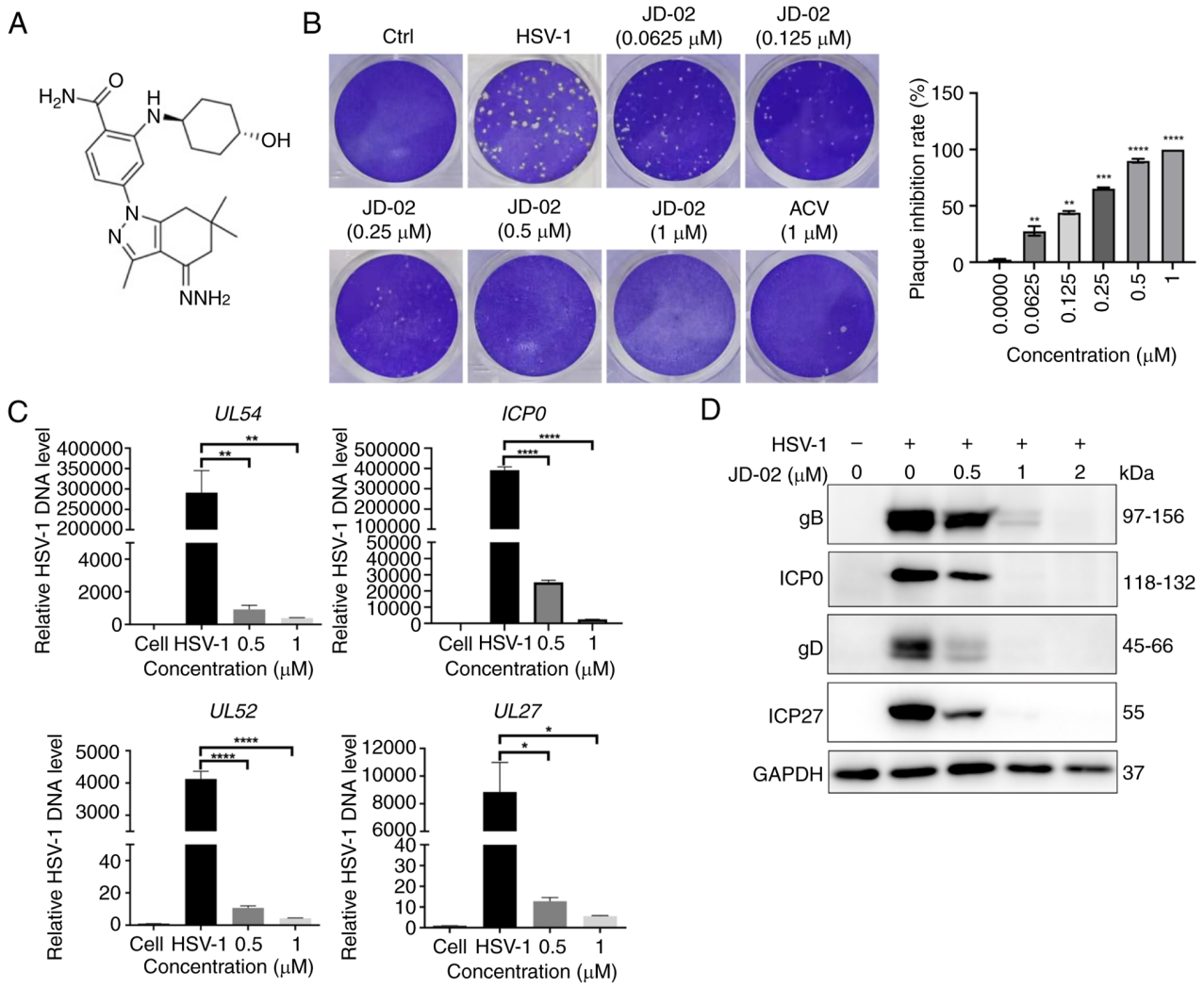


Figure 1. JD-02 inhibits HSV-1 infection. (A) Chemical structure of JD-02. (B) Viral plaque assay of Vero cells infected with HSV-1 (multiplicity of infection 1) and treated with JD-02 (0.5 μM) or ACV (0.5 μM). (C) Reverse transcription-quantitative PCR analysis of the DNA copy number of viral genes in HaCaT cells infected with HSV-1 and treated with JD-02 for indicated concentration. Data are presented as the mean ± SEM (n=3). *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001, compared with the HSV-1 control group. (D) Western blot analysis of viral proteins. ACV, acyclovir; HSV, Herpes Simplex Virus.

superior inhibitory effects on HSV-1/F strains' infection compared with AT533 (IC_{50}=0.23 μM) (Fig. 3D). The DNA copy number analysis further confirmed that JD-02 actively suppresses HSV-1/153 (Fig. 3E) and HSV-1/Blue (Fig. 3F) in HaCaT cells. These findings suggest that JD-02 is capable of inhibiting both normal and ACV-resistant HSV-1 infection,

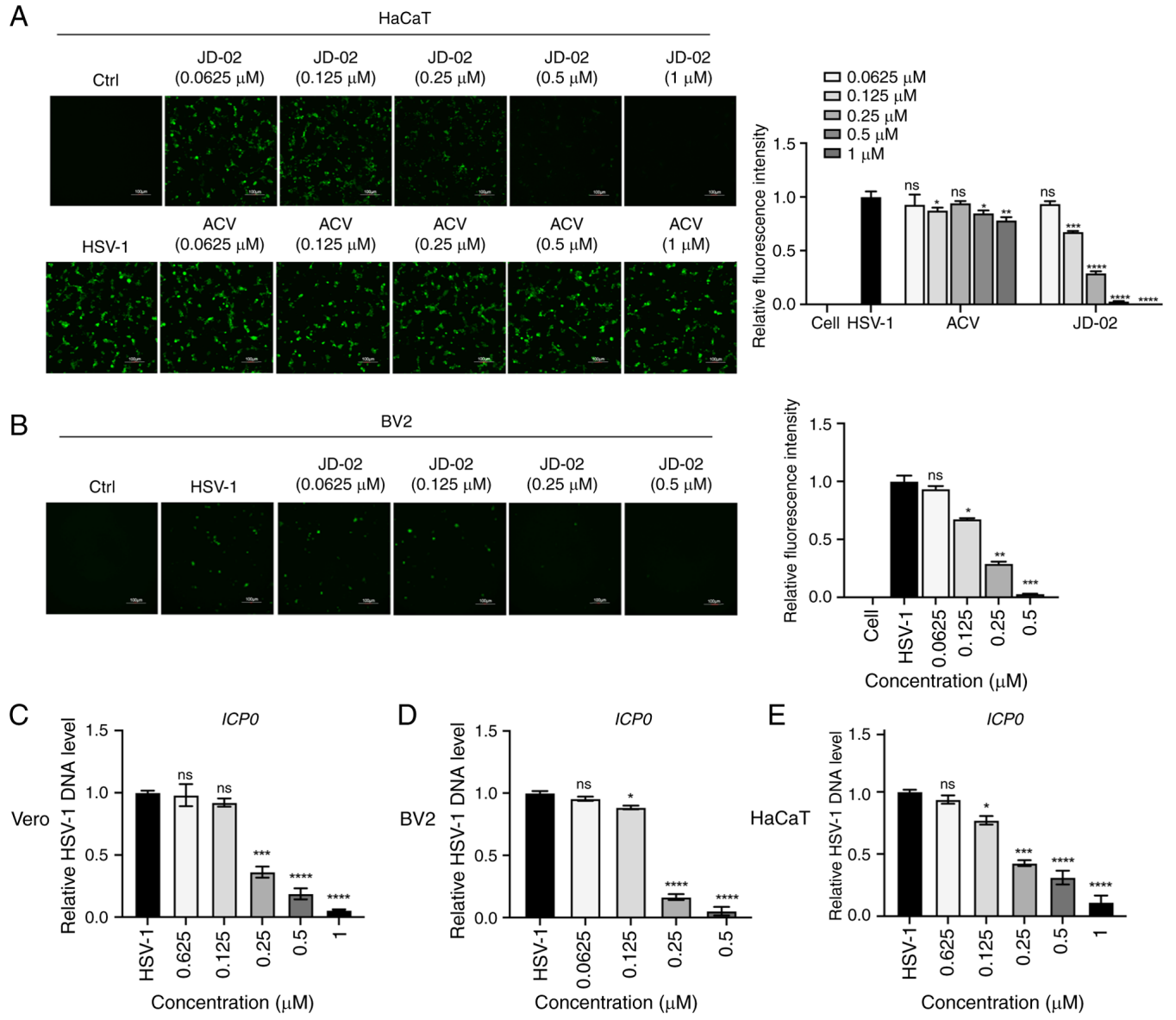


Figure 2. JD-02 inhibits the infection of EGFP-tagged HSV-1. (A) The fluorescence intensity of HaCaT cells infected with HSV-1-EGFP (MOI=0.1) with the treated with JD-02 (indicated concentration) or ACV (indicated concentration) for 24 h was observed by fluorescence microscopy and analysis using ImageJ software (version 1.54f; National Institutes of Health). Scale bar, 100 μm. (B) The fluorescence intensity of BV2 cells infected with HSV-1-EGFP (MOI=0.1) and treated with JD-02 (indicated concentration) for 24 h was observed by fluorescence microscopy and analysis using ImageJ. Scale bar, 100 μm. (C-E) The DNA copy numbers of *ICP0* in (C) Vero cells, (D) BV2 cells and (E) HaCaT cells infected with HSV-1 and treated with JD-02 (indicated concentration) were analysed by reverse transcription-quantitative PCR. Data are presented as the mean ± SD (n=3). *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. MOI, multiplicity of infection; ACV, acyclovir; HSV, Herpex Simplex Virus; ns, not significant.

indicating that its mechanism of action against HSV-1 may differ from that of ACV.

JD-02 alleviation of HSV-1 induced HSE in vivo. To assess the anti-HSV-1 efficacy of JD-02 *in vivo*, male BALB/c mice aged 5 weeks-old were nasally infected with HSV-1 (Fig. 4A). JD-02, ACV and a placebo (0.9% saline) were administered intraperitoneally. The results indicated that both JD-02 and ACV effectively reversed weight loss in mice (Fig. 4B), enhanced survival rates (Fig. 4C), and mitigated symptoms associated with HSE, such as eye swell (Fig. S2A), hair loss (Fig. S2B) and hydrocephalus (Fig. S2C). Treatment with JD-02 significantly reduced the expression levels of inflammatory cytokines, specifically IL-1β and TNF-α, in brain tissue (Fig. 4D) and decreased the DNA copy number of the viral genes UL52 and UL54 in brain tissue (Fig. 4E). Additionally, immunohistochemistry staining of brain tissues revealed a minimal presence of viral particles in the JD-02 and ACV treatment groups (Fig. 4F). H&E staining of various tissues and organs showed that JD-02 administration did not induce any damage to mouse organs (Fig. S3). In conclusion, JD-02 demonstrated potent anti-HSV-1 activity *in vivo* and effectively ameliorated HSV-1-induced neurotropic infection and neuroinflammation.

JD-02 plays a role in the early phase of the HSV-1 life cycle. Subsequently, the potential antiviral mechanisms of JD-02 were investigated. Given that the host's primary defense against

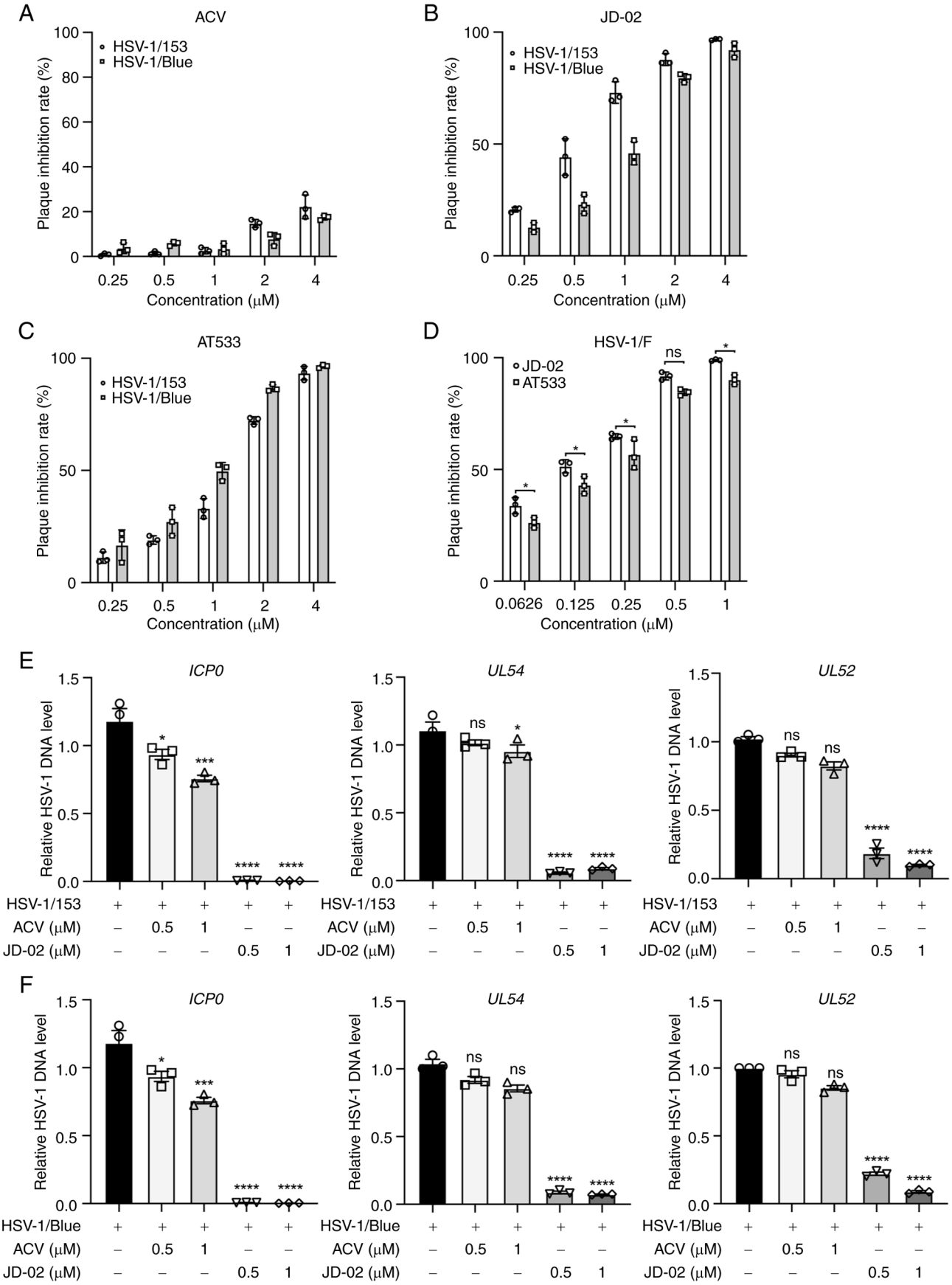


Figure 3. JD-02 inhibits ACV-resistant HSV-1 strains infection. (A-C) Viral plaque assays were performed to assess the effects of (A) ACV, (B) JD-02 and (C) AT533 for indicated concentration on the infection of ACV-resistant HSV-1/153 and HSV-1/blue in HaCaT cells. (D) Viral plaque assays were performed to evaluate the comparative effects of JD-02 and AT533 on HSV-1/F infection in HaCaT cells. (E and F) The DNA copy numbers of the viral genes *ICP0*, *UL54* and *UL52* were detected by reverse transcription-quantitative PCR following the infection with (E) HSV-1/153 and (F) HSV-1/Blue (MOI 0.1). Data are presented as the mean ± SD (n=3). *P<0.05, ***P<0.001, ****P<0.0001 compared with the HSV-1 control group. ACV, acyclovir; HSV, Herpes Simplex Virus; ns, not significant.

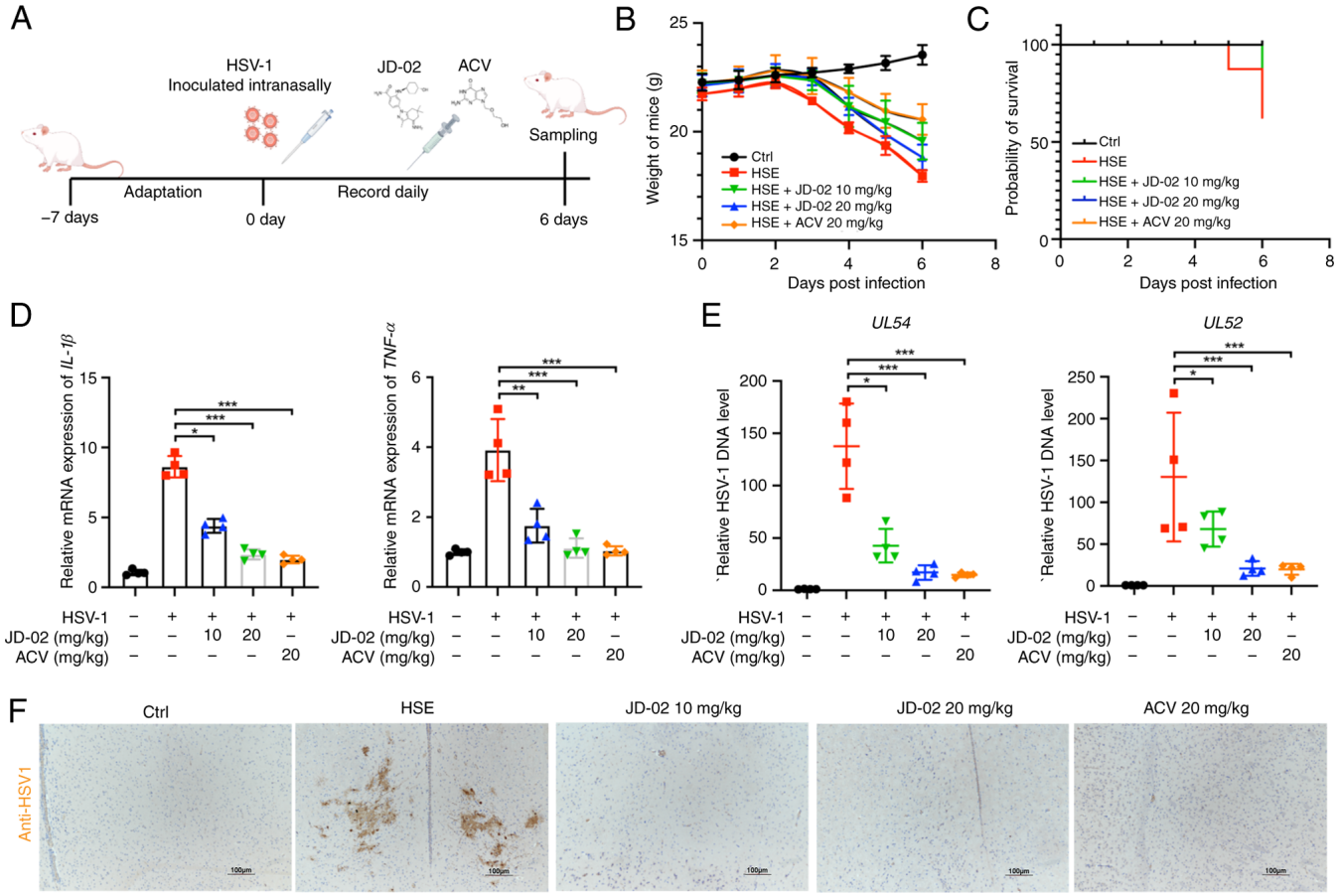


Figure 4. JD-02 ameliorates viral encephalitis *in vivo*. (A) Schematic diagram showing the administration of JD-02 or ACV in the HSE mice model. (B) Relative body weights of mice were monitored for 6 consecutive days after infection (n=10). (C) The survival rate of Ctrl and HSE mice. (D) The mRNA expression of *IL-1β* and *TNF-α* in the brain tissues of Ctrl and HSE mice. (E) The DNA copy numbers of viral genes *UL52* and *UL54* in the brain tissues (n=4). (F) Representative immunohistochemical images for the whole brain in Ctrl, HSE, JD-02 and ACV with HSV-1 infection for 6 days by staining an antibody against HSV-1. Scale bars, 100 μm. Data are presented as the mean ± SD (n=4). *P<0.05, **P<0.01 and ***P<0.001 compared with the HSV-1 group. ACV, acyclovir; HSE, Herpes Simplex Encephalitis; HSV, Herpes Simplex Virus.

viral infections involves the activation of the antiviral immune response, it was assessed whether JD-02 could activate this response to inhibit HSV-1 infection. The RT-qPCR analyses indicated that JD-02 administration led to a suppression of IFNB1 expression (Fig. S4A) as well as CXCL10 expression (Fig. S4B). JD-02 also significantly reduced the expression levels of inflammatory cytokines, including IL-1β, IL-6, and TNF-α (Fig. S4C-E). These findings suggest that the antiviral activity of JD-02 is not mediated through modulation of the host immune response.

To evaluate JD-02 impact on viral protein expression and viral gene copy number, time-of-addition experiments were utilized (Fig. 5A). The western blot indicated that JD-02 significantly inhibited viral protein expression within 6 h (Fig. 5B) corroborated by RT-qPCR results (Fig. 5C). Furthermore, consistent findings were obtained in the evaluation of JD-02's impact on the infection efficiency of EGFP-labeled HSV-1, as assessed through fluorescence microscopy (Fig. S5A) and virus plaque assays (Fig. S5B). These results suggest that JD-02 exerts its antiviral effects during the initial stages of HSV-1 infection. To investigate whether JD-02 directly targets viral particles, viral plaque assay was performed. It was found that JD-02 neither directly inactivated HSV-1 virions (Fig. 5D) nor affected the viral adsorption (Fig. 5E) and penetration processes (Fig. 5F). Moreover, immunofluorescence analysis showed that JD-02 treatment did not alter HSV-1 nuclear entry (Fig. 5G). Consequently, JD-02 exerts its antiviral effect not by modulating the host immune response but by affecting the early stages of HSV-1 infection.

JD-02 suppresses genes linked to HSV-1 replication. Next, the potential impact of JD-02 on viral immediate-early genes and the expression of genes associated with early replication of HSV-1 was assessed. The expression levels of the viral immediate-early genes UL54 (Fig. 6A) and ICP0 (Fig. 6B) were quantified and the findings indicated that JD-02 did not influence the expression of these immediate-early genes within 2- or 4-h post-treatment. Western blot analysis further demonstrated that JD-02 did not alter the expression of viral proteins ICP27 and ICP0 (Fig. 6C). However, additional analysis revealed that JD-02 significantly suppressed the expression of viral replication-related genes UL30 (Fig. 6D) and UL42 (Fig. 6E). To further investigate, Flag-tagged UL30 and HA-tagged UL42 plasmids were overexpressed in HaCaT cells, followed by treatment with JD-02. Western blot analysis indicated that JD-02 effectively inhibited the protein expression level of UL30 (Fig. 6F). It is noteworthy that JD-02 did not decrease the protein levels of UL42 (Fig. 6G). This

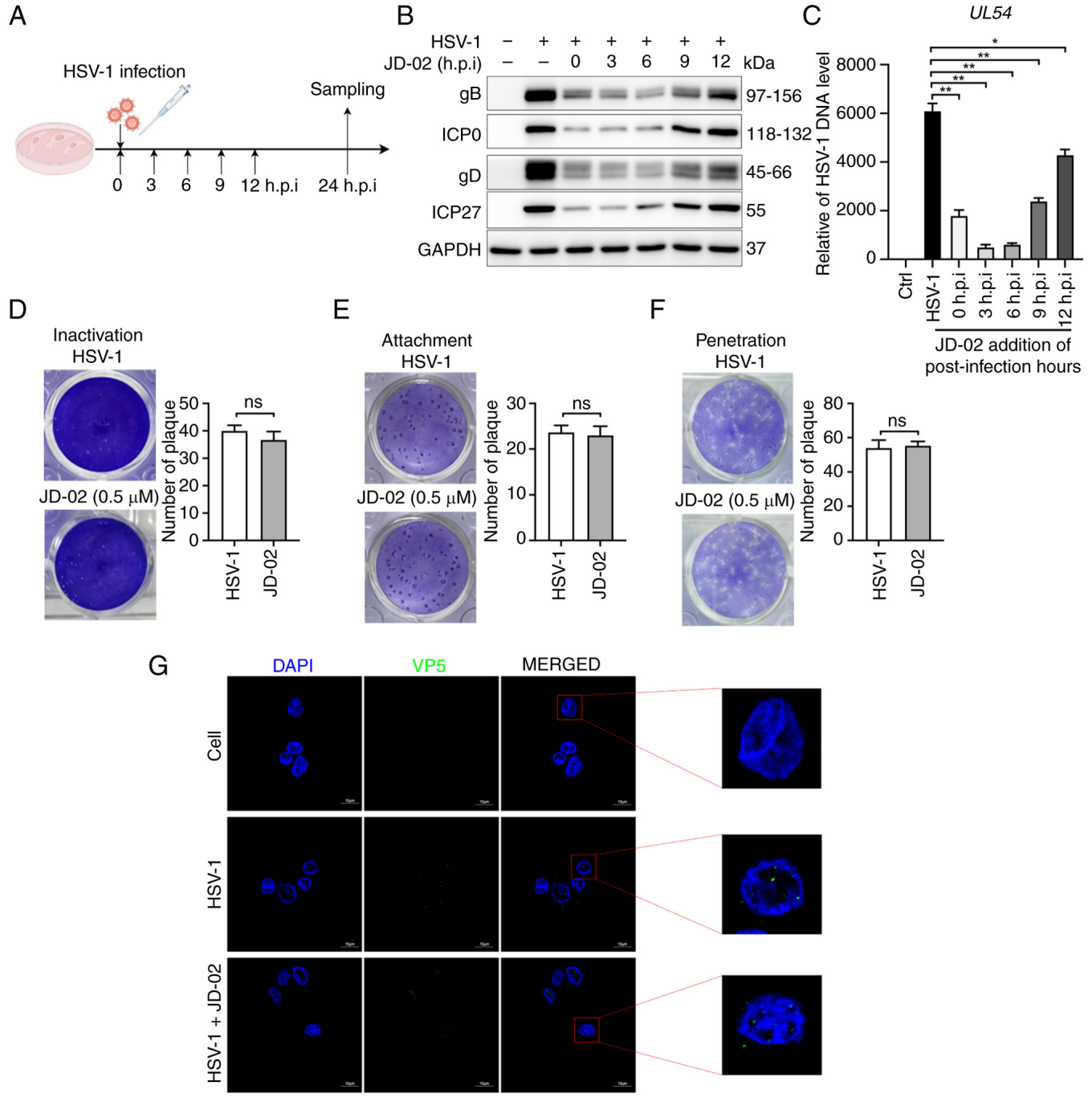


Figure 5. JD-02 inhibits HSV-1 early infection. (A) Schematic representation of the time-of-addition assay. (B) Western blot analysis of viral proteins (gB, ICP0, gD and ICP27) from HaCaT cells infected with HSV-1 and treated with JD-02 (0.5 μM) for indicated time. (C) The DNA copy number of *UL54* from HaCaT cells with HSV-1 infection and treated with JD-02. (D-F) Viral plaque assay detecting JD-02 effect on (D) viral inactivation, (E) viral attachment and (F) viral penetration. (G) Immunofluorescence analysis of the distribution of the viral protein VP5 (green) within the cytoplasm and nucleus (DAPI, blue). Scale bar, 10 μm. Data are presented as the mean ± SD (n=3). *P<0.05 and **P<0.01 compared with the HSV-1 group. HSV, Herpes Simplex Virus; ns, not significant.

observation suggests that the regulatory mechanism of JD-02 on UL42 requires further investigation. The aforementioned results further prove that JD-02 exerts its anti-HSV-1 function by interfering with the early viral infection events.

JD-02 inhibits HSV-1 replication by suppressing Raf/MEK/ERK signaling pathway. Viruses, as obligate parasites, necessitate a host for successful infection and replication. HSV-1, a DNA virus, has been demonstrated to exploit the MAPK-ERK signaling pathway to facilitate its replication and proliferation (31). Given that BRAF is a client protein of Hsp90, the potential impact of JD-02 on the RAF-MEK-ERK signaling cascade was explored. Through western blot analysis, both in the presence and absence of JD-02 treatment, it was observed that HSV-1 infection activates the BRAF/MEK/ERK signaling pathway, resulting in a significant increase in the phosphorylation levels of MEK and ERK (Fig. 7A). Treatment with JD-02 significantly inhibited

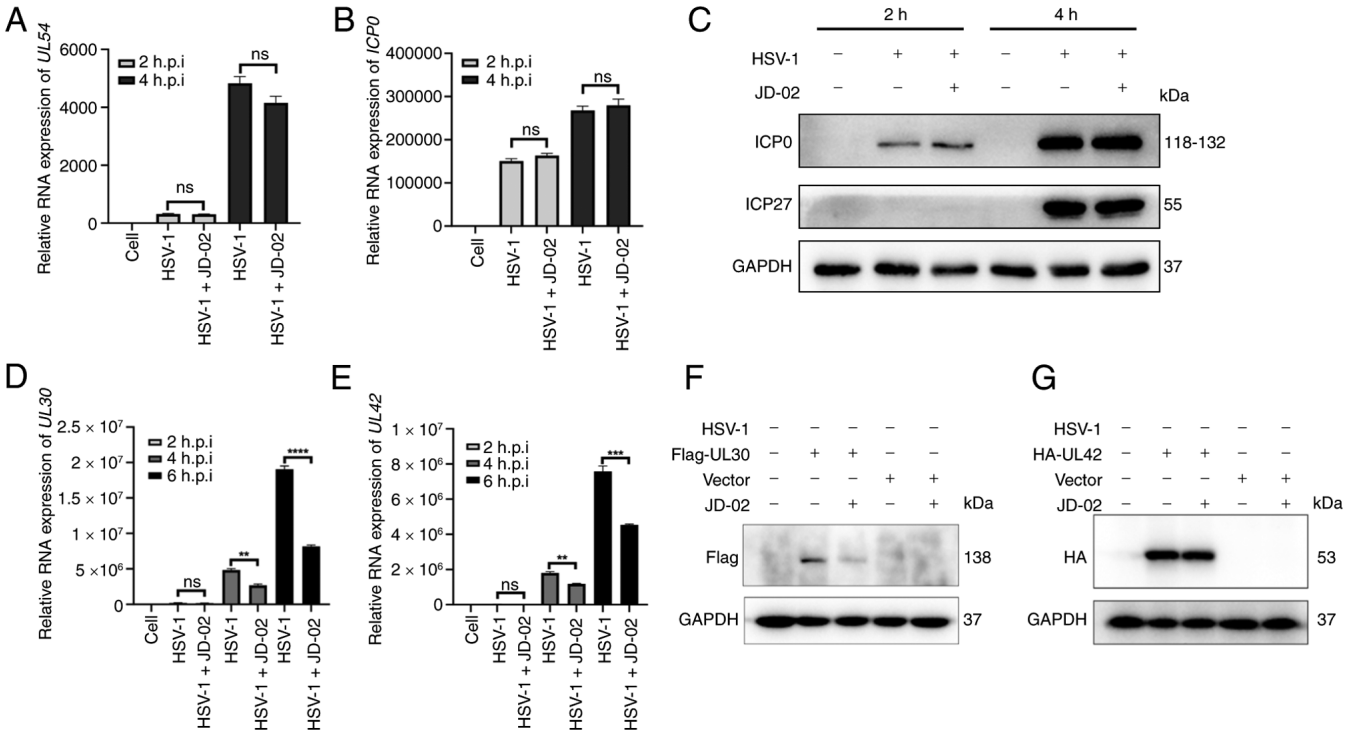


Figure 6. JD-02 reduces the expression of the DNA replication gene UL30. (A and B) RT-qPCR analysis of the DNA copy number of (A) *UL54* and (B) *ICP0* in HaCaT cells infected with HSV-1 (MOI=2) for 2 or 4 h and co-treated with JD-02 (0.5 μM). (C) Western blot assay of ICP0 and ICP27 expression in HaCaT cells infected with HSV-1 (MOI=2) and co-treated with JD-02 (0.5 μM) for 2 and 4 h. (D and E) RT-qPCR analysis of the DNA copy number of viral genes (E) *UL30* and (E) *UL42* in HaCaT cells infected with HSV-1 and co-treated with JD-02 for indicated hours. (F) HaCaT cells were transfected with either Flag tagged vector or Flag tagged UL30 for a duration of 24 h. Subsequently, the cells underwent treatment with JD-02 (0.5 μM) for an additional 12 h, followed by analysis via western blotting. (G) HaCaT cells were transfected with either HA-tagged vector or HA-tagged UL42 for a duration of 24 h. Subsequently, the cells underwent treatment with JD-02 (0.5 μM) for an additional 12 h, followed by analysis via western blotting. Data are presented as the mean ± SD (n=3). **P<0.01, ***P<0.001 and ****P<0.0001 compared with the HSV-1 group. RT-qPCR, reverse transcription-quantitative PCR; HSV, Herpes Simplex Virus; MOI, multiplicity of infection; ns, not significant.

the activation of the BRAF/MEK/ERK pathway induced by HSV-1 infection. Additionally, when various concentrations of JD-02 were applied to HaCaT cells not infected with HSV-1, JD-02 was found to downregulate the phosphorylation levels of BRAF, MEK and ERK, without affecting ERK protein expression (Fig. 7B). These findings suggest that JD-02 may inhibit HSV-1 infection in host cells by suppressing the activation of the RAF/MEK/ERK signaling pathway.

To further elucidate the role of the ERK signaling pathway in HSV-1 infection, siRNA was utilized to suppress ERK expression and western blot analyses were conducted to assess the efficacy of siRNA in suppressing ERK expression in HaCaT cells (Fig. S6). Subsequently, RT-qPCR analysis demonstrated that ERK inhibition led to a significant reduction in the DNA copy number of the HSV-1 viral gene *UL52* (Fig. 7C), a result that was corroborated by western blotting (Fig. 7D). Furthermore, HSV-1-infected HaCaT cells were treated with the MEK/ERK inhibitors U0126 and JD-02, respectively, which resulted in a significant decrease in the expression levels of the viral genes *UL30*, *UL54* and *UL52* (Fig. 7E). Additionally, western blotting indicated that treatment with U0126 significantly diminished the expression of viral proteins as well as phosphorylated ERK (p-ERK) (Fig. 7F). Furthermore, the viral gene UL30 was exogenously overexpressed and using U0126 still markedly inhibited viral protein expression (Fig. 7G). These findings provide compelling evidence supporting the hypothesis that JD-02 exerts its antiviral effects against HSV-1 through the inhibition of the RAF/MEK/ERK signaling pathway.

Discussion

Considering the high prevalence of HSV-1 infection, the absence of an effective vaccine, and the frequent emergence of drug-resistant strains, the development of novel and efficacious therapeutic agents against HSV-1 is imperative for enhancing current clinical treatments for HSV-1-associated diseases (10,32). The present study explored the efficacy of a novel HSP90 inhibitor (33), JD-02, a benzamide derivative, which demonstrated significant inhibitory effects on both standard HSV-1 and ACV-resistant strains. Moreover, JD-02 exhibited reduced cytotoxicity compared with the conventional HSP90 inhibitor AT533 and mitigated disease symptoms in HSE mouse models. These findings suggest its potential as a promising HSP90 inhibitor for the development of therapeutic agents targeting HSV-1-related diseases.

HSV-1, a prototypical neurotropic virus, can cause HSE and establishing lifelong latent infections by targeting the CNS (34). This poses a considerable threat to the health and survival of neonates and immunocompromised individuals (12). Presently, nucleoside analogs that inhibit viral DNA polymerases constitute the mainstay of therapeutic

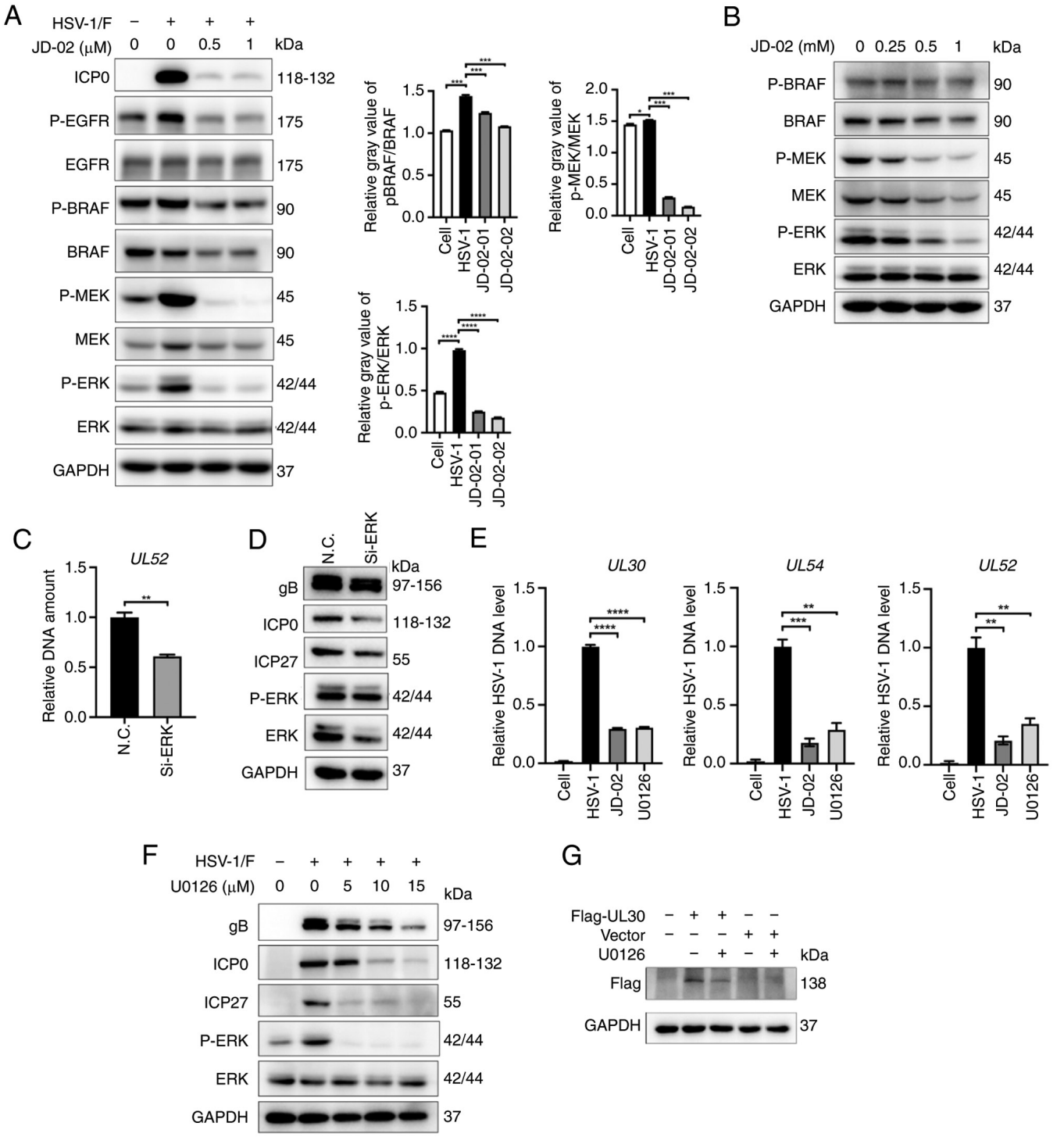


Figure 7. JD-02 inhibits HSV-1 replication by suppressing the Raf/MEK/ERK signaling pathway. (A) Western blot analysis was conducted to assess the effects of HSV-1 (MOI=0.1) infection on the protein levels of BRAF, MEK and ERK, with and without treatment using JD-02. (B) Treatment with JD-02 at the specified concentration influences the protein levels of BRAF, MEK and ERK in HaCaT cells over a 12-h period. (C and D) HaCaT cells were subjected to transfection with either N.C. siRNA or ERK siRNA for a period of 48 h. Subsequently, the cells were infected with HSV-1 (MOI=0.1) for an additional 24 h. The DNA copy number of the viral gene UL54, as well as the viral protein expression of gB, ICP0, ICP27, ERK and p-ERK were evaluated. (E) The DNA copy numbers of the viral genes *UL30*, *UL52* and *UL54* in HaCaT cells infected with HSV-1 (MOI=0.1) and subsequently treated with either JD-02 (1 μM) or U0126 (10 μM) for 24 h, were quantified using reverse transcription-quantitative PCR. (F) Western blot analysis of viral proteins (gB, ICP0 and ICP27), ERK and p-ERK expression in HaCaT cell infected with HSV-1 (MOI=0.1) and treated with U0126 for indicated concentration. (G) Western blot analysis of UL30 overexpression in HaCaT cells treated with U0126. Data are presented as the mean ± SD (n=3). *P<0.05, **P<0.01, ***P<0.01 and ****P<0.0001 compared with the HSV-1 group. HSV, Herpes Simplex Virus; MOI, multiplicity of infection; N.C., negative control; siRNA, small interfering RNA; p-, phosphorylated.

interventions. Nonetheless, the extensive utilization of these antiviral agents has led to an increase in the prevalence of drug-resistant mutations (35), underscoring the urgent need for the development of novel therapeutic compounds (36). HSP90 is a crucial molecular chaperone implicated in various stages of the herpesvirus life cycle (14,37). Numerous studies

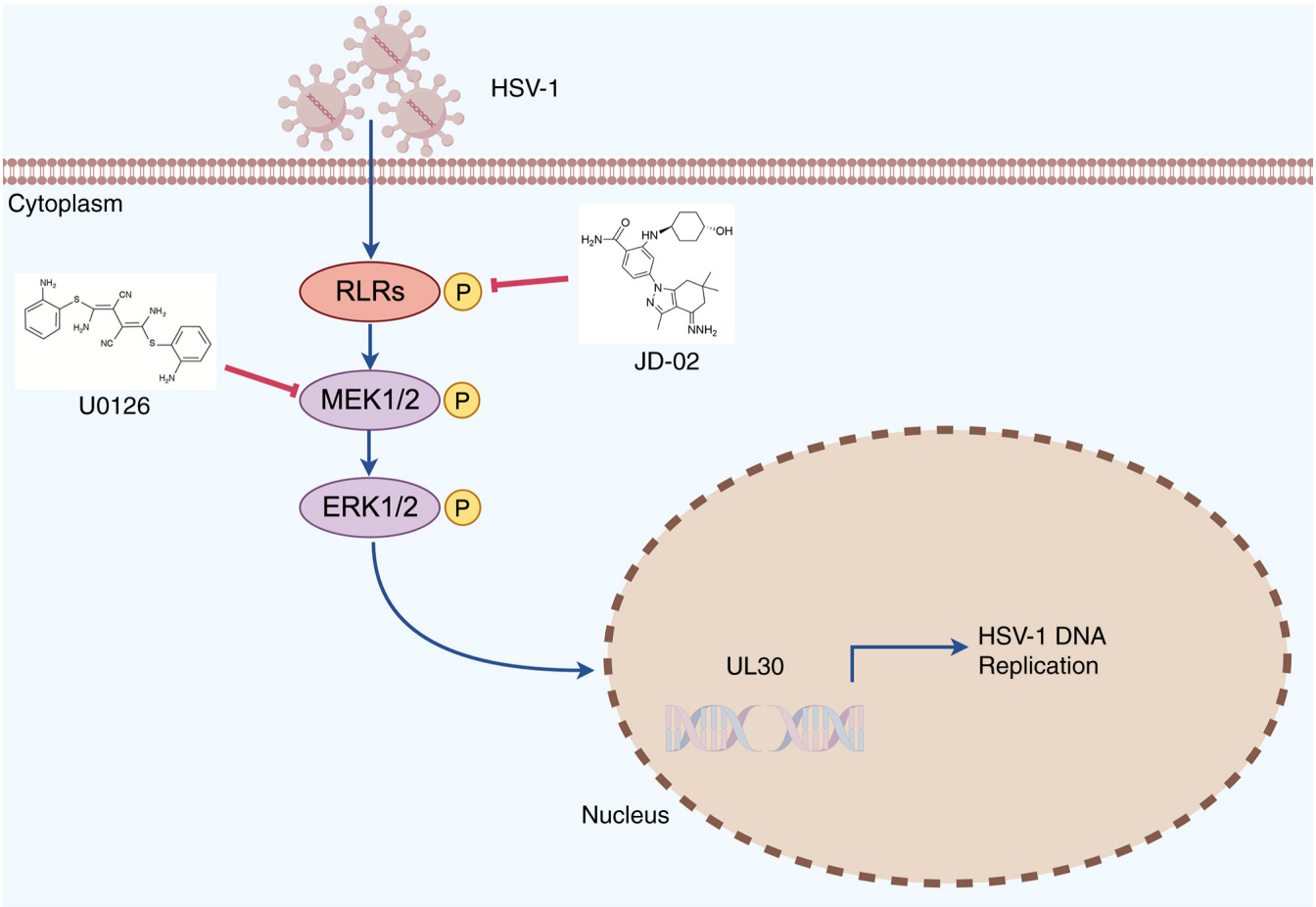


Figure 8. Schematic representation of the mechanism by novel Hsp90 inhibitor JD-02 inhibits HSV-1 infection. HSV, Herpes Simplex Virus.

have validated HSP90 as a viable target for anti-herpesvirus therapies (14,27,38,39). However, challenges such as the toxicity of inhibitors have resulted in clinical trials falling short of expectations. Previous advancements in research have led to the progressive refinement of HSP90 inhibitors. Notably, the orally bioavailable HSP90 inhibitor SNX-5422 has demonstrated efficacy in inhibiting the replication of SARS-CoV-2 (40). The HSP90 inhibitor Pimitespib has received clinical approval in Japan (41), as documented in the literature, underscoring the significance of HSP90 as a critical drug target. In the authors' prior research, a series of benzoyl analogs were synthesized, including SNX-2112 and AT533, which demonstrated efficacy against HSV-1 infection, thereby suggesting the promising potential of HSP90 inhibitors as antiviral agents (27,42). In the present study, six compounds were designed based on the benzoyl core structure. These compounds have been previously identified as novel HSP90 inhibitors capable of inhibiting colorectal cancer growth (33). Furthermore, the findings of the present study revealed that JD-02 exhibits superior cytotoxicity compared with the HSP90 inhibitor AT533. In the HSE model mice, the *in vivo* antiviral efficacy of JD-02 is comparable to that of the nucleoside analog ACV.

In the present study, JD-02, a novel inhibitor of Hsp90, demonstrated significant inhibitory effects against both common HSV-1 and ACV-resistant strains, HSV-1-blue and HSV-1-153. At equivalent concentrations, JD-02 exhibited superior *in vitro* inhibitory activity against HSV-1 compared with ACV. JD-02 significantly reduced the copy numbers of virus-related genes (*UL54*, *UL52*, *ICP0* and *UL27*) as well as the expression levels of virus-related proteins (gB, ICP0, gD, ICP27 and UL30). To elucidate the mechanism underlying JD-02's antiviral action, its potential antiviral pathways were explored. The experimental results indicated that JD-02 does not directly target HSV-1 and does not significantly influence HSV-1 adsorption or penetration. Further investigation revealed that JD-02 exerts its anti-HSV-1 activity by inhibiting the RAF/MEK/ERK signaling pathway, which is crucial for cell proliferation, differentiation and survival (43). BRAF, a client protein of HSP90, plays a significant role in HSV-1 infection of the host (44). Consistent with previous findings, HSV-1 infection significantly enhances the activation of this signaling pathway (17,45). The present research demonstrated that JD-02 effectively inhibits the activation of the RAF/MEK/ERK signaling pathway, as evidenced by a significant reduction in the phosphorylation levels of BRAF, MEK and ERK following JD-02 treatment. Furthermore, the current findings revealed that the ERK inhibitor U0126 also suppresses HSV-1 infection, suggesting that ERK may serve as a critical target for anti-HSV-1 strategies. This warrants further investigation.

It is noteworthy that therapeutics targeting host factors present several advantages over those that directly target viral components. Such drugs often exhibit broad-spectrum

antiviral activity and are less prone to inducing drug resistance. This is particularly significant given the frequent emergence of resistance in viruses subjected to treatments targeting specific viral elements, posing a substantial challenge in clinical settings. The documented resistance of HSV and the resistance to remdesivir or nirmatrelvir caused by SARS-CoV-2 mutations both demonstrate this issue (46,47). However, the likelihood of HSV-1 developing resistance to JD-02 through mutation is remarkably low. The absence of drug-resistant viral strains following the use of HSP90 inhibitors in HSV-1 infections underscores the critical role of HSP90 as a host factor essential for the viral lifecycle (38), rendering it a promising therapeutic target. Consequently, the development of novel HSP90 inhibitors with reduced toxicity, building upon traditional HSP90 inhibitors, holds significant potential for HSV-1 treatment and merits further investigation.

In conclusion, the present research demonstrated that JD-02, a novel HSP90 inhibitor, effectively suppresses HSV-1 replication and mitigates HSE symptoms via the RAF/MEK/ERK signaling pathway (Fig. 8). Notably, JD-02 shows reduced toxicity and enhanced viral inhibition efficacy compared with the conventional HSP 90 inhibitor AT533. Furthermore, JD-02 exhibits the capacity to inhibit HSV-1 infection in ACV-resistant strains. These findings propose that JD-02, as a novel HSP90 inhibitor, holds promise for development as an antiviral therapeutic agent for HSV-1 infection-related diseases.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YW and JX conceptualized the study. XiaohW, YZ and JL performed the experimental studies. XiaohW, JL and XiaoW validated data. YZ and JX wrote the manuscript. JL and XiaoW revised the manuscript. KZ and ZR were involved in the conduction of animal experiments. YW and JX was responsible for study supervision and funding acquisition. YZ, YW and JX confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Animal experiments were conducted in accordance with laboratory animal management guidelines. This study was conducted according to the ethical policies and procedures approved by the Animal Care and Use Committee of Jinan University (approval no. 20200402-07; Guangzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that have no competing interests.

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