Wogonin inhibits in vitro herpes simplex virus type 1 and 2 infection through modulating cellular NF-κB and MAPK pathways

Ying Chu (chy7994@163.com)
Affiliated Wujin Hospital of Jiangsu University

Xiaowen Lv
Department of Pediatrics, Affiliated Hangzhou First People's Hospital, Zhejiang University School of Medicine

Longfeng Zhang
Clinical Laboratory, Affiliated Hospital of Jiangsu University

Xingli Fu
Health Science Center, Jiangsu University

Siwei Song
Department of Cardiology, Renmin Hospital of Wuhan University

Airong Su
Central Laboratory, The Second Affiliated Hospital of Nanjing Medical University

Deyan Chen
Center for Public Health Research, Medical School, Nanjing University

Lianhong Xu
Clinical Laboratory, Wujin Hospital Affiliated with Jiangsu University, Wujin Clinical College of Xuzhou Medical University

Yongfang Wang
Clinical Laboratory, Wujin Hospital Affiliated with Jiangsu University, Wujin Clinical College of Xuzhou Medical University

Zhiwei Wu
Center for Public Health Research, Medical School, Nanjing University

Zhihua Yun
Clinical Laboratory, Wujin Hospital Affiliated with Jiangsu University, Wujin Clinical College of Xuzhou Medical University

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Abstract

Background: Wogonin, a naturally flavonoid-like chemical compounds, has exhibited anti-inflammatory, anti-tumor, anti-viral, neuroprotective, and anxiolytic effects through modulating a variety of signaling pathways including PI3K-Akt, p53, nuclear factor κB (NF-κB) and mitogen-activated protein kinases (MAPK). In this study, its antiviral effect against herpes simplex virus type 1 and 2 (HSV-1 and HSV-2) replication has been investigated. Results: The evidence showed that wogonin could suppress HSV-1 and -2-induced cytopathogenic effect (CPE) and reduce viral RNA transcription, protein synthesis, and titers of infectious virion particles formation in a dose-dependent manner. Time-of-drug-addition assay demonstrated that wogonin acted as a viral post-entry inhibitor. It was also found that wogonin significantly reduced HSV-induced NF-κB and MAPK pathways activation, which were proved to be significant for viral replication previously. Conclusions: Our results suggested that anti-herpes effect of wogonin may be mediated by its modulation of cellular NF-κB and JNK/p38 MAPK pathways, implying its potential application as an anti-HSV agent.

Background

Herpes simplex virus (HSV) type 1 and 2, the two serotypes of Herpesviridae family [1], were the most prevalent human pathogens causing watery blisters in the skins or mucosa. HSV-1 mainly infects oral epithelial tissues and can cause herpes labialis and devastating encephalitis [2], while HSV-2 mainly infects the genital mucosa and is proven as the risk factor to certain sexually transmitted diseases such as human immunodeficiency virus type 1 (HIV-1) [3–5]. Due to unavailability of cures or vaccines to prevent HSV infection, antiviral treatment was the only way to suppress the primary and recurrent infection. Approved anti-HSV medications, such as acyclovir, penciclovir and valacycovir, are the specific inhibitors of herpesvirus DNA polymerase, whereas clinical evidence showed that they would give rise to the emergence of drug resistant mutants [6]. So it is urgent to identify novel anti-HSV agents or compounds with different mechanisms to overcome viral drug resistance. And natural products and traditional Chinese herbs are the alternative sources for it.

Wogonin (5, 7-dihydroxy-8-methoxyflavone), a flavonoid-like compound, is derived from the root of a traditional Chinese medical herb Huang-Qin (Scutellaria baicalensis Georgi). S. baicalensis has been widely used for clinical treatment of various diseases such as hepatitis, hypertension, diarrhea, common cold, and inflammation [7]. As one of the bioactive constituents in the extract of Scutellaria Radix, wogonin has been found to possess anti-inflammatory, anti-tumor, anti-viral, neuroprotective, and anxiolytic effects in recent relevant studies [7]. For it antiviral activity investigation, previous reports mainly focused on its inhibitory effects on respiratory syncytial virus [8], hepatitis B virus [9, 10], and varicella-zoster virus [11] replication. Wogonin exerted anti-HBV activity by inhibiting HBV antigen HBsAg secretion and reducing HBV DNA level in vitro, as be confirmed in vivo by animal model of DHBV-positive duck and HBV-transgenic mice [9, 10]. Although anti-viral activity of wogonin was found, its precise mechanism has not been fully elucidated.
In this paper, we demonstrated that wogonin can inhibit the HSV replication in vitro and impede viral gene expression in a dose-dependent manner. We also provided evidence that the inhibitory effect of wogonin is depended on its modulation of host cellular NF-κB and JNK/p38 MAPK pathways. This finding demonstrated that wogonin may be as a potential candidate for anti-HSV treatment in clinical practice.

Results

Wogonin inhibit HSV-1/2 replication in vitro

Wogonin, 5,7-dihydroxy-8-methoxyflavone, is an O-methylated flavonoid compound primarily isolated from Chinese herbal plants *S. baicalensis* and *S. barbata*. (The chemical structure of wogonin was showed in Fig. 1A). HSV-2 infection in HEC-1-A cells would cause significant visible cytopathogenic effect (CPE). So we designed to observe whether wogonin could inhibit CPE formation in HSV-2-infected cell culture primarily. The monolayers of HEC-1-A cells were pre-incubated with 100 µM wogonin for 30 min, and then infected with HSV-2 (G) (moi = 1) for 24 hrs. The optical inverted microscope observation demonstrated that it might prevent HSV-2-induced CPE formation in HEC-1-A cell cultures (Fig. 1B). To better understand its suppressive effect on infectious viral particle formation, HEC-1-A cells were infected with HSV-1 (HF) and HSV-2 (G) (moi = 1), while treated with serial concentrations of wogonin. After 24 hrs p.i., intracellular infectious viral particles were released by freezing and thawing cycles, and the yields of virion production was determined by titration of the plaque forming unit (PFU). The results illustrated that wogonin could suppress virion production of both HSV-1 (Fig. 1C) and HSV-2 (Fig. 1D) in a dose-dependent manner, that in turn could block viral replication.

Furthermore, we confirmed the anti-HSV-1/2 effect of wogonin through monitoring glycoprotein D (gD) expression level reduction while treated with serial concentrations of wogonin. HSV gD is a representative of HSV late gene product and could indicate the protein expression and viral replication. As shown in Fig. 2A and B, wogonin could impede HSV-1/2 gD mRNA transcription in a dose-dependent manner, which was determined via qPCR. In parallel, we also investigated gD protein expression level via western Blot to confirm the inhibitory effect of wogonin on HSV replication. The inhibitory effect of wogonin on HSV gD protein expression level was consistent with that of mRNA expression results in both HEC-1-A and Vero cells (Fig. 2C and D).

We also evaluated the cytotoxicity of wogonin to exclude the possibility that anti-HSV-1/2 activity of wogonin was correlated with the direct cytotoxicity effect. The results shown in Fig. 2E illustrated that wogonin had low cytotoxicity to both HEC-1-A and Vero cell lines, with 50% cytotoxicity concentrations (CC₅₀s) greater than 200 µM, which were significantly higher than its viral inhibitory dosage. It was demonstrated that wogonin could inhibit HSV-1/2 viral replication in vitro while showed low cytotoxicity to HSV permissive cells.

Wogonin Blocked Hsv Life Cycle At Post-entry Step
To explore the anti-viral mechanism of wogonin, we should identify which stage of HSV viral life cycle was blocked by wogonin addition. The time-of-drug-addition assay was simple and clear approach to provides information on the potential target of wogonin for HSV. As shown in Fig. 4, wogonin and two other drugs, acyclovir and dextran sulfate were dispensed into HSV-2-infected HEC-1-A cells at different indicated time point. Acyclovir is an acrylic purine nucleoside analog, which was well-known as a highly potent inhibitor of herpes virus thymidine kinase [18]. Dextran sulfate was as a viral entry inhibitor for HSV [19]. These two drugs with different antiviral action modes were as the “references”. It was found that wogonin and acyclovir inhibited the HSV-2 replication from 0 to 8 hrs p.i. and dextran sulfate, a viral attachment and entry inhibitor, lost its inhibition from 0 to 2 hrs p.i. We concluded that wogonin might act after viral entry, and the certain post-entry stage of HSV life cycle would be blocked by wogonin.

**Wogonin Impeded Hsv Ie Genes Expression**

HSV immediate-early (IE) genes were key for viral gene transcription and protein expression. After entering into a host cell, IE genes, including Infected cell polypeptide 27 (ICP27), Infected cell polypeptide 4 (ICP4) and Infected cell polypeptide 0 (ICP0) act in part to upregulate early (E) and late (L) genes [20]. Therefore, we also investigated its effect upon representative of immediate-early (IE) viral genes expression. ICP4 is a major viral transcription factor of HSV. In fact, wogonin can suppress the ICP4 protein expression in a time-course manner, and the results showed that ICP4 protein expression was completely inhibited at 4 hrs p.i. and significantly inhibited at 8 and 12 hrs p.i. (Fig. 4A). Its effect on ICP0 protein expression was also evaluated, and similar results were shown in Fig. 4B. ICP27, contributing to nuclear export of viral mRNAs, could also be blocked completely by wogonin in from 8 to 12 hrs p.i. We also employed HSV-1/blue recombinant virus with ICP4 promoter-driven lacZ reporter gene to confirm our conclusion. As shown in Fig. 4C, wogonin inhibited ICP4 promoter-driven lacZ gene expression in a dose-dependent manner. It was concluded that wogonin might inhibit HSV replication through interfering with the viral IE genes expression and functions.

**Wogonin Attenuated Hsv-2-induced Nf-kb Activation**

Previous studies have proven that HSV-induced persistence activation of NF-κB pathway is prerequisite for viral replication and host cell survival at the early stage of HSV life cycle [21–23]. So we attempted to determine whether wogonin would influence the HSV-2-induced NF-κB activation. Firstly, we employed NF-κB luciferase reporter system to investigate it. As shown in Fig. 5A,

HSV-2 infection would activate IκB-mediated luciferase expression, and wogonin could attenuate this effect in a dose-dependent manner. In comparison, a NF-κB specific inhibitor-MG132 could completely inhibit virus-stimulated NF-κB activation. IκB-α degradation was also a distinct marker to indicate NF-κB pathway activation, and we also evaluated IκB-α amount in HSV-2 infection HEC-1-A cells untreated or treated with wogonin or MG132. As shown in Fig. 5B, HSV-2-stimulated IκB-α degradation were blocked by wogonin or MG132 addition. In addition, p65 nuclear translocation was also investigated after virus
infection via imaging, which is often recognized as an indication of NF-κB activation. Wogonin could inhibit HSV-stimulated p65 nuclear translocation and viral gD expression in HEC-1-A cells simultaneously. Taken together, it was concluded that wogonin might inhibit NF-κB activation, and then result in inhibition of HSV IE gene expression. As shown in Fig. 5C, wogonin could inhibit p65 nuclear translocation and viral gD expression in HSV-2-infected HEC-1-A cells simultaneously. Taken together, these results suggest that wogonin might inhibit HSV-2-induced NF-κB activation, and result in inhibition of HSV replication.

**Wogonin Suppressed Hsv-2-induced Mapk Activation**

Cellular JNK and p38 MAPK pathways were required for HSV viral protein expression and facilitated the viral replication [24, 25]. Our earlier work also verified that JNK and p38 MAPK pathways could be stimulated by HSV-2 infection [14]. Therefore, we investigated the inhibitory effect of wogonin on these two pathways activation. And the results showed that wogonin attenuated the phosphorylation of the p38 and JNK stimulated by virus infection (Fig. 6A). The phosphorylation of c-Jun and ATF-2 was also studied. c-Jun is the downstream substrate of JNK, and ATF-2 is the common substrate of both JNK and p38 MAPK. These two markers were simultaneously suppressed by wogonin after HSV-2 infection (Fig. 6B and C).

AP-1-luciferase reporter system was utilized to evaluate whether wogonin could inhibit HSV-2-induced AP-1 activation, which is the major downstream transcription factor of JNK/p38 MAPK pathway. As shown in Fig. 6D, it was elucidated that wogonin inhibited AP-1 activation in a dose-dependent manner, and two inhibitors SP600125 (a potent JNK antagonist) and SB203580 (a potent p38 antagonist) also attenuated the HSV-2-stimulated AP-1 activation. It was concluded that wogonin might attenuate HSV-2-induced JNK/p38 MAPK activation to interfere with viral replication.

**The Synergistic Effects Of Wogonin And Acyclovir Against Hsv-2**

Combined drugs with synergistic effect will provide the possibility of clinical application. To evaluate the synergism of the two drugs, the anti-HSV-2 activity of individual drugs and combination of two drugs was determined by in-cell western assay. As shown in Fig. 7, the combination index (CI) was 0.792, which indicated a moderate synergism of wogonin and acyclovir in combination, suggesting potential beneficial effect when using wogonin and acyclovir together.

**Discussion**

Traditional Chinese herbal medicine has long been used to prevent and treat viral infectious diseases in China and other oriental countries, and is an important source for antiviral agents screening [26]. As a mainly pharmacological ingredient in Scutellaria Radix, wogonin exerts an inhibitory effect on some human viruses, including respiratory syncytial virus, hepatitis B virus and varicella-zoster virus [8–11]. In
this study, we demonstrated that wogonin showed inhibitory activity on HSV infection in permissive cell lines (Fig. 1 and Fig. 2). According to a time-of-drug-addition assay, wogonin inhibits HSV infection at a post-entry step from viral entry to viral genomic DNA replication (Fig. 3). Furthermore, for exploring the inhibition mechanism of wogonin, its effect on HSV IE gene expression was studied, and the results illustrated that the treatment of wogonin would attenuate expression level of ICP0, ICP4 and ICP27 proteins, which would be prerequisite for viral E and L gene transcription and expression (Fig. 4). Our findings suggested that wogonin would mediate to suppress HSV IE gene expression, then interfering with HSV downstream early and late gene expression.

It was hypothesized that certain host cellular pathways will affect viral IE gene promoter transcriptional activity, and thus influence viral E and L genes expression. We further studied the effect of wogonin on host cellular signaling pathways and found that wogonin suppressed the HSV-2-stimulated NF-κB pathway activation, exhibiting suppressive effect on the degradation of IκB-α and p65 nuclear translocation. Previous studies showed that NF-κB is necessary for preventing the host cell from apoptosis during the early stage of HSV infection [21, 27]. And also, it is a key regulator of cellular events including immune modulation, inflammatory, and anti-apoptotic responses [28]. In fact, NF-κB pathway is also significant for certain pathogens invading and infection. Many human viruses have evolved to utilize host cellular NF-κB pathway, such as hepatitis B virus [29], hepatitis C virus [30], HIV-1 [31], respiratory syncytial virus [32] and Epstein-Barr virus [33]. HSV was also reported to activate NF-κB pathway to facilitate its replication [34, 35]. Due to its role in HSV viral replication, modulating host cellular NF-κB signaling pathway seems to be an alternative approach to prevent virus infection [36].

Beside the interaction between HSV and NF-κB signaling pathway, MAPK pathways may also participate in life cycle of certain virus infection. Previous study showed that rotavirus could stimulate JNK/p38 signaling pathways in permissive cell lines and would enhance virus replication [37]. Varicella-zoster virus (VZV) was also reported to stimulate JNK/p38 MAPK signaling pathways, and this pathway activation is correlated with viral replication and gene expression [38]. Mclean et al. and Zachos et al. reported that HSV-induced JNK/p38 MAPK pathway activation would lead to activating a series of cellular genes expression, further help to activate viral transcription and DNA replication [24, 25]. In this study, wogonin inhibited the activation of AP-1 with dose-dependent manner and downregulated HSV-induced phosphorylation of c-Jun and ATF-2, the two components of transcription factor AP-1. Although wogonin slightly increased the level of phosphorylated p38 in mock-infected cells, it exhibited inhibitory effect on HSV-induced p38 phosphorylation in viral-infected cells (Fig. 6A). Besides, wogonin could suppress HSV-induced JNK phosphorylation in a dose-dependent manner. Based on these observations, we postulated that wogonin may act as an inhibitor on HSV-induced JNK/p38 MAPK activation and inhibited HSV infection.

Previous studies showed that combination treatment of acyclovir with another drug with a different antiviral mechanism may increase anti-HSV activity in vitro and in vivo [39, 40]. Our data demonstrated that wogonin has moderate synergistic effect with acyclovir against HSV-2 replication in vitro (Fig. 7).
Therefore, combination treatment of wogonin and acyclovir may be therapeutic potential for HSV infection.

Conclusions

In this paper, we investigated the anti-viral mechanism of wogonin on HSV-1/2 infection and found that wogonin significantly suppressed HSV-2-induced NF-κB and JNK/p38 MAPK activation. Thus, we conclude that inhibitory effect of wogonin on these signaling pathways may account for anti-HSV activity. However, further study is required to clarify the correlation between IE genes and HSV-induced signaling pathways in detail. Due to its low cytotoxicity and long history of medical use, wogonin may be a potential anti-herpes drug candidate for further study as an alternative treatment.

Methods

Reagents, cell lines, plasmids and viruses

Wogonin and acyclovir were got from the National Institutes for Food and Drug Control in China (Beijing, China). SB203580, SP600125, and MG132 were purchased from Beyotime Biotechnology Institute (Haikou, Hainan, China). Alexa Fluor 488 goat anti-mouse IgG (H+L), DAPI, DRAQ5 and SYBR green real-time PCR reagent were got from Life Technologies, Thermo Fisher Scientific (Carlsbad, CA, USA). IRDye 680 goat-anti-rabbit and IRDye 800 goat-anti-mouse were obtained from LI-COR (Lincoln, NE, USA). Antibodies specific for HSV gD-1/2, HSV-1 ICP0, HSV-1 ICP4, HSV-1 ICP27, JNK2, p38, GAPDH, and RIPA lysis buffer were purchased from Santa Cruz (Santa Cruz, CA, USA). p65, p-p38, p-c-Jun, p-JNK1/2, p-ATF-2, IκB-α antibodies were from Cell Signaling Technology (Beverly, USA). Bright-Glo luciferase assay system was purchased from Promega (Madison, WI, USA).

Vero, HEC-1-A cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). NF-κB-luc and AP-1-luc reporter plasmid was purchased from Clontech (Palo Alto, CA, USA). HSV-1(HF), HSV-1/blue and HSV-2 (G) were propagated and titrated on Vero cells as described [12].

In vitro viral inhibition assay

In vitro viral inhibition of wogonin was determined via titrating infectious virions according to previously described method [13]. Briefly, confluent HEC-1-A cells in 96-well plates were pretreated with serial concentrations of wogonin for 30 min, and then infected with HSV-1 or HSV-2 (multiplicity of infection [moi] = 1). At 24 hrs post-infection (p.i.), the culture medium was replaced with fresh medium. The infected cells were frozen and thawed with three cycles for releasing the virions. Viral titration was quantified by counting the numbers of plaques on confluent Vero monolayer cells.

In vitro Cytotoxicity assay
The *in vitro* cytotoxicity was determined using the commercial CCK-8 kit (Dojindo, Kumamoto, Japan) according to the manufacturer’s instructions. Briefly, 2 × 10^4 cells per well were seeded into 96-well plates and cultured for 24 h, then serially diluted compound were dispensed in triplicate. After 24 hrs incubation, 10 µl CCK-8 reagent was added into each well, and the plates were incubated at 37 °C for 3 h. The absorbance at 450 nm was measured using a TECAN Infinite M200 microplate reader (Männedorf, Switzerland), and cell viability was plotted as the percent viable cells of the mock-treated control cells.

**Western Blot And In-cell Western Assay**

The method was described previously [14]. Cells were lysed using RIPA lysis buffer on ice for 30 min and then centrifuged at 12,000 × g for 10 min at 4 °C to collect the supernatants. Total protein concentrations were determined using BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). After separated via SDS-PAGE, electroluted onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), membrane blocking using Odyssey blocking buffer (LI-COR), and primary and secondary antibodies incubation, protein bands were visualized via Odyssey Infrared Imager (LI-COR).

In-cell Western assay was performed in 96-well plate. Cells were fixed with 4% paraformaldehyde for 20 min at room temperature (RT) and permeabilized by five washes in 0.1% Triton X-100 in PBS with 5 min for each wash. Cell monolayers were blocked for 90 min in blocking buffer, 4% nonfat dry milk in PBS-T (phosphate-buffered saline, 0.1% Tween-20), and then incubated with primary antibodies diluted into blocking buffer (1:200) for 2 hrs at RT. After washing with PBS-T, cell layers were stained in IRDye IgG (1:1500) for 1 hr. The plate was rinsed and scanned in Odyssey Infrared Imager. Relative protein expression level was normalized against DRAQ5.

**Time-of-drug-addition Assay**

The Time-of-drug-addition assay was carried out via measuring HSV-2 glycoprotein D (gD) protein expression level, which could indicate viral replication efficiency. HEC-1-A cells were seeded in 96-well plate and infected with HSV-2 (moi = 1). Wogonin or other drugs with known inhibitory mechanisms were dispensed at different time points. Viral gD level was determined via in-cell western assay at 24 hrs p.i. as described previously [15].

**Cell Transfection And Luciferase Assay**

HEC-1-A cells were transiently transfected with luciferase reporter plasmids using Lipofectamine 2000 transfection reagent (Life Technologies, Thermo Fisher Scientific). The relative luminescence units (RLUs) were determined using Bright-Glo luciferase assay system (Promega, Madison, WI, USA). Briefly, HEC-1-A cells were seeded into 96-well plates. When the confluence reaching ~ 90%, cells were transfected with 100 ng NF-κB or AP-1 luciferase reporter plasmids. Cells were subsequently cultured for 24 hrs and then
treated with inhibitors for another 24 hrs. The luminescence signal was monitored by GloMax-96 microplate luminometer (Promega, Madison, USA).

**Rna Extraction And Real-time Pcr**

Total RNA was extracted using TRIzol reagent (Life Technologies, Thermo Fisher Scientific) according to manufacturer’s instructions. Complementary DNA (cDNA) was reverse-transcribed using ReverTra Ace qPCR RT kit (TOYOBO, Osaka, Japan). Real-time PCR was performed in triplicate on ABI Prism 7300 Sequence Detection System using the SYBR Green PCR Master Mix (Life Technologies). The sequences of primers used in this study was as follow: HSV-1 gD (Forward: 5’-AGCAGGGGTTAGGGAGTTG-3’; Reverse: 5’-CCATCTTGAGAGAGGCATC-3’); HSV-2 gD (Forward: 5’- CCAATACGCCTTAGCAGACC − 3’; Reverse: 5’- CACAGTGATCGGGATGCTGG − 3’); human GAPDH (Forward: 5’- TGCACCACCAACTGCTTAGC − 3’; Reverse: 5’- GGCATGGACTGTGGTCATGAG − 3’). Messenger RNA (mRNA) transcription levels were standardized against housekeeping gene GAPDH.

**Hsv-1/blue Assay**

HSV-1/blue assay was performed as described, but modified [16]. Confluent HEC-1-A cells in 96-well plate were pre-incubated with serial diluted drugs for 30 min at 37°C. Cells were infected with HSV-1/blue (moi = 1). Cells were lysed with 1% NP-40 in DMEM 12 hrs p.i. Cell lysates from each well were then transferred into a new Costar 96-well flat plate. β-gal substrate solution-chlorophenol red-β-D-galactopyranoside (CPRG) were added into each well. Absorbance at 570 nm was measured after 1 hr using a TECAN Infinite M200 microplate reader.

**Immunofluorescence Staining And Confocal Microscopy**

HEC-1-A cells were seeded onto Φ10mm glass coverslips which were placed in a 24-well plate. The cells growing on the coverslips were rinsed with PBS and then fixed with 4% paraformaldehyde for 15 min at RT, following the permeabilization with 0.2% Triton X-100 for 15 min. The coverslips were blocked with 1% BSA in PBS for 30 min at RT. Target biomarkers were immunolabeled using the respective primary antibodies and followed by Alexa Fluor 488 IgG. Nuclei were visualized by staining with DAPI. Images were acquired using an Olmpus FluoView FV10i confocal microscope (Tokyo, Japan).

**Drug Synergistic Analysis**

Firstly, we employed the in-cell western assay to determine the inhibitory efficiency for HSV-2 gD expression of wogonin and acyclovir in HEC-1-A cells, respectively. And the 50% maximal effective concentrations (EC50s) of the two compounds were calculated. The compounds combinations were investigated with a fixed molar concentration ratio, which was optimized to give the greatest synergism
with a range of serial dilutions. The combination index (CI) of the two drugs was calculated by CalcuSyn software (Biosoft, Cambridge, UK), and the method was referred to Chou and Talalay [17]. The synergy was indicated according to the CI values and scored as follows: CI < 0.1, very strong synergism; CI = 0.1 to 0.3, strong synergism; CI = 0.3 to 0.7, synergism; CI = 0.7 to 0.85, moderate synergism; CI = 0.85 to 0.90, slight synergism; CI = 0.9 to 1.1, nearly additive synergism; and CI = > 1.1, antagonism.

**Abbreviations**

DAPI
4',6-diamidino-2-phenylindole; HBV: hepatitis B virus; DHBV: duck hepatitis B virus; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; AP-1: activator protein 1; ATF-2: activating transcription factor 2

**Declarations**

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Not applicable.

**Author Contributions**

Y.C., Z.W., and Z.Y. conceived and designed the study. Y.C., X.Lv., L.Z., and S.S. performed the antivirus experiments. A.S., and D.C., performed the Western-blotting experiments. X.F., L.X., and Y.W., assisted with data analysis. Y.C., and X.Lv., interpreted the results and wrote the paper. Z.W., and Z.Y., edited the English language. All authors read and approved the final manuscript.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**
Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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**Figures**
Figure 1

Wogonin inhibit HSV-induced CPE and viral infectious particles formation in vitro. (A) The chemical structure of wogonin. (B) Wogonin inhibited HSV-2-induced CPE formation. HEC-1-A cells were mock-infected or infected with HSV-2 (G) (moi=1) in the presence or absence of wogonin (100 μM). The images were captured 24 hrs p.i. under an optical inverted microscope. (C and D) Wogonin inhibited the formation of intracellular HSV-1/2 infectious viral particles. Confluent HEC-1-A cells were pre-treated with serial concentrations of wogonin prior to infection with HSV-1 (HF) or HSV-2 (G) (moi=1) for 24 hrs. The infectious viral particles were released from the cell cultures by three cycles of freezing and thawing, and viral infectivity was titrated by measuring the PFUs as described. Titrations of HSV-1/2 infectious virions were shown as the means ± the standard deviations of results of three separate experiments.
Figure 2

Wogonin inhibited HSV late gene expression. (A and B) Wogonin inhibited HSV-1/2 gD mRNA transcription in HEC-1-A cells. Cells were pre-treated with serial concentrations of wogonin for 30 min, and then infected with HSV-1 (HF) or HSV-2 (G) (moi=1). HSV gD-1/2 mRNA transcripts level was determined via real-time PCR 24 hrs p.i. (C and D) Wogonin suppressed HSV-1/2 gD protein expression in HEC-1-A cells (C) and Vero cells (D). Cells were treated with wogonin prior to infection with HSV-1 or HSV-2.
gD protein expression level was determined via western blot 24 hrs p.i. (E) The cytotoxic effect of wogonin on HEC-1-A and Vero cells was investigated by CCK-8 colorimetric assay after 48 hrs compound exposure. All experiments were performed three times. The representative results were shown. Data are mean values (±SD) of triplicate determinations.

Figure 3

Wogonin inhibited HSV infection at a post-entry step. HEC-1-A cells were infected with HSV-2 (moi=1) and treated with wogonin (100 μM), acyclovir (50 μg/ml) or dextran sulfate (100 μg/ml) at indicated time points. Viral infection level was represented by gD-2 expression as determined by In-cell western 24 hrs p.i. Data represent mean values (±SD) of triplicate determinations from three dependent experiments.
Wogonin inhibited HSV immediate early gene expression. (A to B) HEC-1-A cells were either mock-treated or treated with wogonin (100 μM) and then infected with HSV-1 (moi=1). Cells were lysed at each time point. The protein expression levels of ICP4, ICP0 (A) and ICP27 (B) were determined via western blot and normalized by GAPDH. (C) Wogonin inhibited HSV-1/blue ICP4 promoter-driven lacZ gene expression in a dose-dependent manner. HEC-1-A cells were pre-treated with serial concentrations of wogonin 30 min prior to infection with HSV-1/blue (moi=1). The β-Gal activity was measured as described 12 hrs p.i. Data represent mean values (±SD) of triplicate determinations from three dependent experiments.
Figure 5

Wogonin attenuated HSV-stimulated NF-κB activation. (A) HEC-1-A cells were transfected with NF-κB-luc reporter plasmid. The cells were mock-treated or treated with indicated concentrations of wogonin or...
MG132 (5μg/ml) prior to mock-infected or infected with HSV-2 (moi=1). The relative luciferase activity was determined after 24 hr treatment. Data represent mean values (±SD) of triplicate determinations from three dependent experiments. (B) Wogonin prevented virus-induced IκB-α degradation. HEC-1-A cells were mock-infected or infected with HSV-2 (moi=1) in the absence or presence of wogonin (50 and 100 μM) or MG132 (5μg/ml). IκB-α levels were visualized 24 hrs p.i. by western blot. (C) Wogonin reversed HSV-2-induced p65 nuclear translocation. HEC-1-A cells were mock-infected or infected with HSV-2 (moi=1) in the presence or absence of wogonin (100 μM). The p65 translocation was determined via immunofluorescence assay 24 hrs p.i.

**Figure 6**

Wogonin inhibited HSV-2-induced JNK and p38 MAP kinase activation. (A to C) Wogonin inhibited HSV-2-induced JNK and p38 MAPK phosphorylation. HEC-1-A cells were mock-infected or infected with HSV-2 (moi=1) in the presence or absence of wogonin (100 μM). JNK, p38 MAP kinase, and their phosphorylated forms and the downstream p-c-Jun, p-ATF-2 were determined by Western blot 24 hrs p.i. (D) Wogonin inhibited HSV-2-induced AP-1 activation. HEC-1-A cells were transfected with AP-1-luc reporter plasmid and then the cells were mock-treated, treated with serial concentrations of wogonin, SB203580 (20 μM) or SP600125 (20 μM) prior to mock-infected or infected with HSV-2 (moi=1). SB203580 and SP600125, p38/MAP kinase and JNK inhibitors, respectively, were used as controls. The relative luciferase activity was determined after 24 h. Data represent mean values (±SD) of triplicate determinations from three dependent experiments.
Figure 7

Wogonin exerted moderate synergistic effect with acyclovir against HSV-2 infection. The effective concentrations for inhibition of HSV-2 infection by a compound alone and in combination were plotted in two curves. The CI values were calculated using CalcuSyn software. CI value was evaluated according as described. Data represent mean values (±SD) of triplicate determinations from three dependent experiments.