Antiviral activity of PHA767491 against human herpes simplex virus in vitro and in vivo

Jue Hou, Zili Zhang, Qiang Huang, Jun Yan, Xiaohu Zhang, Xiaoliang Yu, Guihua Tan, Chunfu Zheng, Feng Xu and Sudan He

Abstract

Background: Herpes simplex virus (HSV) is a common human pathogen that causes a variety of diseases, including oral-labial, genital lesions and life-threatening encephalitis. The antiviral nucleoside analogues such as acyclovir are currently used in anti-HSV therapies; however, clinical overuse of these drugs has led to the emergence of drug-resistant viral strains. Hence, there is an urgent need to develop new anti-HSV agents.

Methods: To identify novel anti-HSV-1 compounds, we screened the LOPAC small scale library of 1280 bioactive compounds to identify inhibitors of HSV-1-induced necroptosis. Further experiments including western blot analysis, Q-PCR analysis and immunohistochemistry were performed to explore the antiviral mechanism of the compounds.

Results: Here, we identified PHA767491 as a new inhibitor of HSV. PHA767491 potently blocked the proliferation of HSV in cells, as well as HSV induced cell death. Further, we found that PHA767491 strongly inhibited HSV infection post viral entry. Moreover, PHA767491 reduced the expression of viral genes required for DNA synthesis including UL30/42 DNA polymerase and UL5/8/52 helicase-primase complex. The essential immediate early (IE) genes such as ICP4 and ICP27 are critical for the expression of the early and late genes. Of note, PHA767491 inhibited the expression of all IE genes of both HSV-1 and HSV-2. Importantly, PHA767491 reduced viral titers in the tissues from the mice infected with HSV-1. Consistently, immunohistochemistry analysis showed that PHA767491 dramatically attenuated expression of viral protein gB in the livers.

Conclusions: Taken together, PHA767491 has potent anti-HSV activity by inhibiting viral replication both in vitro and in mouse model. Thus, PHA767491 could be a promising agent for the development of new anti-HSV therapy.

Keywords: HSV, Viral replication, PHA767491, Anti-HSV, Immediate early gene

Background

Herpes simplex virus (HSV) is a group of common human pathogen that causes a variety of clinical manifestations including oral-labial and genital lesions, keratitis and life-threatening encephalitis [1–3]. After primary infection, HSV usually establishes a latent infection in sensory neurons throughout the entire life of the host.

This latent infection can be reactivated, resulting in recurrent diseases. HSV is divided into two serotypes, HSV type 1(HSV-1) and type 2(HSV-2), that are the major causes of oral-labial and genital herpes, respectively. In the world, above 80% adults and 20% adults are infected with HSV-1 and HSV-2, respectively. Immuno-compromised patients lean to HSV infection and suffer recurrence [4–7].

HSV is enveloped double-strand DNA virus possessing a large genome of around 150k nucleotides. The HSV genome encodes approximately 80 proteins. HSV-1 and HSV-2 share around 80% sequence identity in the protein-coding region. After the HSV virus enters the cytoplasm of the infected cell, the viral genome
HSV, plaque forming assay and western blot assay were used to test the effect of compounds to suppress HSV-1 replication in mouse model. To identify novel anti-HSV-1 compounds, we screened more than 1000 compounds for some antiviral drugs by using the model in which HSV-1 directly induced necrosis of L929. To test the effect of compounds to suppress HSV, plaque forming assay and western blot assay were performed. We further explored the antiviral mechanism of the compounds by using the experiments including Q-PCR analysis, immunofluorescent staining and immunohistochemistry analysis.

Viruses and reagents
HSV-1 KOS strain was from Dr. Sandra K. Weller. (University of Connecticut Health Center) and GFP-labeled HSV-1 F strain was from Dr. Chunfu Zheng (Soochow University). LOPAC small scale library of 1280 bioactive compounds, LPS and Poly (I:C) were purchased from Sigma Aldrich. Necrostatin-1 was purchased from Alexis Biochemicals. Z-VAD were purchased from WuXi AppTec. The smac mimetic compound were from Dr. Xiaodong Wang (National institute of biological sciences).

Antibodies
The following antibodies were used: anti-VP16 monoclonal antibody (ab110226; Abcam), anti-gB monoclonal antibody (ab6505; Abcam), anti-β-actin monoclonal antibody (Sigma-Aldrich), anti-P65 polyclonal antibody (F0514; Santa Cruz), secondary antibody (Sigma-Aldrich), anti-gB monoclonal antibody (ab110226; Abcam), anti-CP27 (Soochow University). LOPAC small scale library of 1280 bioactive compounds, LPS and Poly (I:C) were purchased from Sigma Aldrich. Necrostatin-1 was purchased from Alexis Biochemicals. Z-VAD were purchased from WuXi AppTec. The smac mimetic compound were from Dr. Xiaodong Wang (National institute of biological sciences).

Antiviral activity assay
L929 Cells were seeded into 96-well plates at the density of 8 × 10^4. L929 cells were pretreated with compounds (10 μM) for 1 h and then were infected with HSV-1 (MOI = 2) for addition 18 h. Cell viability was determined by using Cell Titer-Glo Luminescent cell viability assay kit (Promega) according to the product instructions. Antiviral activities of compounds were calculated as a percentage of viability of control.

Western blot analysis
Cell pellet was collected by centrifugation at 13000 × g for 1 min and resuspended in lysis buffer (20 mM Tris–HCl, PH 7.4, 150 mM NaCl, 1% glycerol, 1% Triton X-100, 1 mM Na3VO4, 25 mM β-glycerolphosphate, 0.1 mM PMSF, a complete protease inhibitor set (Roche). The re-suspended cell pellet was vortexed for 20 s and lysed on ice for 20 min. Cell lysates were centrifuged at 13000 × g for 20 min at 4°C. The supernatants were collected and subjected to western blot analysis. The proteins was detected by using appropriate antibody.

Methods
Study design
To identify novel anti-HSV-1 compounds, we screened more than 1000 compounds for some antiviral drugs by using the model in which HSV-1 directly induced necrosis of L929. To test the effect of compounds to suppress HSV, plaque forming assay and western blot assay were performed. We further explored the antiviral mechanism of the compounds by using the experiments including Q-PCR analysis, immunofluorescent staining and immunohistochemistry analysis.

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Cell infected with GFP-labeled virus imaging
Cell were seeded into the 6-well plates at a density of 3 × 10^6/well and were infected with GFP-labeled HSV-1 (MOI = 2) for 8h. The images were obtained with a Leica DMLLED inverted microscope.

Q-PCR analysis
Total RNA was extracted with TRIzol (Invitrogen) and reverse-transcribed into cDNA according to the procedure of RevertAid First Strand cDNA kit (Thermo). Gene expression was detected by qPCR analysis with SYBR Green PCR Master Mix (Applied Biosystems). The results were analyzed by Applied Biosystems 7500 Fast Real Time PCR System. The sequence of the PCR primers used in Q-PCR analysis were shown in Table 1.

Drug treatment and mice infection
DMSO or PHA767491 were diluted with sterile phosphate-buffered saline (PBS). Eight-week-old RIP3 KO mice were pretreated with DMSO or PHA767491 (25mg/Kg) via i.p. injection for 1h. Then the mice were infected with HSV-1 with 2 × 10^7 plaque forming units by i.p. injection. Mice were sacrificed 48 h after the HSV-1 injection and the Livers and spleens of mice were excised for virus titer test or immunohistochemistry analysis.

Determination of viral titers in organs of mice infected with HSV-1
Livers and spleens of mice infected with HSV-1 were ground to homogenates. The homogenates were thawed and frozen three times to fully release the viral particle. Then the homogenates were spin at 600 × g for 5 min. The supernatant were collected and analyzed by plaque forming assay in vero cells.

Immunohistochemistry analysis
Liver sections from DMSO or PHA767491 pretreated mice were processed by paraffin embedding and stained with anti-gB antibody. To detect the replication of HSV-1, the slices were visualized with DAB (Genetech).

Immunofluorescent staining
Cells were seeded in culture plates. After the TNF-α treatment, cells were washed with PBS, followed by fixation in 4% paraformaldehyde for 10 min. Then the cells were washed three times with PBS followed by incubation with 0.25% Triton X-100 in PBS for 10 min. The cells were blocked for 30 min with 5%BSA in PBS and stained with primary and secondary antibody successively. Nuclei was stained with DAPI. Images ware captured with a Leica confocal microscope.

Table 1 List of PCR primers used in Q-PCR analysis

| HSV-1 ICP0-F | CCTGTCGCCTTACGTAACAC |
| HSV-1 ICP0-R | CCAATGTTCCCGCTGCCTTCC |
| HSV-1 ICP4-F | CTATATGACGTCGCTGCCAGG |
| HSV-1 ICP4-R | CGTCTGACGTGTCCTGTCTCTG |
| HSV-1 ICP6-F | GAGCCCGTCTGAGGAGG |
| HSV-1 ICP6-R | AGTCAAACGTCTGCCTGGAG |
| HSV-1 ICP22-F | GAAATCTCCGATGCCACCGA |
| HSV-1 ICP22-R | TCTGGGGTTTCCAAGGTAAC |
| HSV-1 ICP27-F | CCGAGCCTCTATCGCACTTT |
| HSV-1 ICP27-R | GTCCGATAATGGGTCCTCG |
| HSV-1 GB-F | GGACATCAAGGCAGG |
| HSV-1 GB-R | TTCTCCTGAAGACCACGCC |
| HSV-1 ICP47-F | TACCAGATACGGGGG |
| HSV-1 ICP47-R | ATAAAAAGGCGCGTGAAGAC |
| HSV-1 UL5-F | GATGAGCTACGAGTTCG |
| HSV-1 UL5-R | CCCTCAAGGGAGTTCCCCTCC |
| HSV-1 UL8-F | ATTTTAGTGGCGGGAGG |
| HSV-1 UL8-R | CGTTAATACTAGCACCGG |
| HSV-1 UL9-F | GCAGAACGCTAGCATTAC |
| HSV-1 UL9-R | GGTTACCCGGGAAACAG |
| HSV-1 UL42-F | TTCCCCGTACACCCTT |
| HSV-1 UL42-R | CTGCAAAACACAGTGACCC |
| HSV-1 UL52-F | GCCAAGCAGCATACATT |
| HSV-1 UL52-R | GAAAGTGGTTAGCTCCCGCA |
| HSV-2 VP16-F | CCAGTGGGCCTGCTGTTTC |
| HSV-2 VP16-R | CGTTTGTTCACGTAAAGGC |
| HSV-2 ICP0-F | CAGAAGTGGTGCCAGAGAAG |
| HSV-2 ICP0-R | GTCCGTCACAACAGCTCTT |
| HSV-2 ICP22-F | CATGAGGTACGAGTTCG |
| HSV-2 ICP22-R | CCCCCGTACACCCTT |
| HSV-2 ICP27-F | GCTTGGATACGTCTGCA |
| HSV-2 ICP27-R | GTGCTGGCAGTCTCAG |
| HSV-2 ICP47-F | GCCCGGAGATCTGATAAAGG |
| HSV-2 ICP47-R | CAAAGGAGTGGTGAGAC |
| GAPDH-F | CATACAGGAAATGAGCCTTG |
| GAPDH-R | ACAGGAGTGGTGAGAC |

Statistical analysis
Data of antiviral activity are represented as the mean ± SD of duplicates. All experiments were repeated at least twice with same results. Significance of difference was evaluated by T tests (Graphpad prism software).

Results
Screening of small-molecule inhibitors against HSV-1
Our previous study has shown that HSV-1 infection naturally triggers programmed necrosis (termed necroptosis)
in mouse fibroblast L929 cells [26]. This process requires effective HSV-1 replication. So compounds with anti-HSV-1 activities can cause reduced necroptosis and increased cell viability of HSV-1-infected L929 cells. To identify novel anti-HSV-1 compounds, we screened the LOPAC small scale library of 1280 bioactive compounds to identify inhibitors of HSV-1-induced necroptosis. PHA767491 was identified as one of the most effective hits that significantly inhibited HSV-1 induced necrosis (Fig. 1a and b). Further, we confirmed that PHA767491 efficiently blocked HSV-1 induced necrosis in a dose-dependent manner, with an estimated IC50 value of 1.86 μM (Fig. 1c). Next, to evaluate the effect of PHA767491 on HSV-1 proliferation in cells, L929 cells were treated with PHA767491 prior to infection with GFP-labeled HSV-1. As shown in Fig. 1d, PHA767491 potently reduced the proliferation of GFP-labeled HSV-1 in cells, indicating an antiviral activity of PHA767491 against HSV-1.

### PHA767491 does not block TNF-α induced or TLR-induced necrosis

It is well known that necroptosis can be induced by the activation of death receptors or Toll-like receptors (TLR) [27–31] in addition to HSV-1 infection. We found that PHA767491 did not inhibit TNFR induced necroptosis in L929 cells (Fig. 2a) or TLR-induced necroptosis in macrophages (Fig. 2b), suggesting that PHA767491 does not affect common signaling molecules in the necroptosis pathway. These results suggest that PHA767491 blocks HSV-1 proliferation.

### PHA767491 reduces viral titers and expression of HSV-1 proteins in various cell lines

To further confirm the antiviral activity of PHA767491 against HSV-1, we analyzed viral titer and viral proteins in HSV-1 infected cells in the presence of PHA767491. Using plaques forming assay, we clearly clarified that PHA767491 decreased the viral titer of HSV-1 in mouse L929 and MEFs (Fig. 3a and b). The similar inhibitory effect of PHA767491 on HSV-1 viral titer was observed in human cervical cancer HeLa and glioblastoma T98G cells (Fig. 3c and d). In addition, western blotting analysis showed that PHA767491 efficiently blocked the expression levels of the envelop glycoprotein gB and viral ribonucleotide reductase large subunit ICP6 in all of these examined mouse and human cell lines (Fig. 3e and f). Taken together, these results demonstrate that PHA767491 efficiently reduces HSV-1 viral proteins and viral titers.

### PHA767491 blocks HSV-1 infection post viral entry

Next, we investigate whether PHA767491 blocks HSV-1 infection at viral entry or post viral entry. L929 cells were infected with HSV-1 for 1h. After the entry of HSV-1 into the cells, viruses in the medium were washed away and then cultured cells in fresh medium containing DMSO or PHA767491 for additional 8h (Fig. 4a). Notably, PHA767491 efficiently blocked expression of viral proteins such as gB and ICP6 in multiple cell lines after virus entry (Fig. 4b, c and d). Consistently, PHA767491 significantly reduced HSV-1 proliferation.
induced necrosis even after HSV-1 entry (Fig. 4e). Thus, PHA767491 inhibited HSV-1 infection post virus entry.

PHA767491 inhibits the expression of immediate-early viral genes
Studies have shown that HSV-1 replication is regulated by some viral proteins such as UL30/42 DNA polymerase and UL5/8/52 helicase-primase complex [11–14]. We evaluated the expression of these genes in the presence of PHA767491. Quantitative PCR analysis showed that PHA767491 remarkably reduced expression of UL5, UL8, UL29, UL30, UL42 and UL52 in the HSV-1 infected cells (Fig. 5a). It is known that HSV immediate early (IE) genes regulate the expression of early and late viral genes. We further examined the effect of PHA767491 on the essential HSV-1 IE genes expression including ICP0, ICP4 and ICP27. As shown in Fig. 5b, the expression levels of ICP0, ICP4, ICP22, ICP27 and ICP47 were greatly inhibited in cells treated with PHA767491. These results suggest that PHA767491 inhibits HSV-1 replication through the suppression of immediate early gene expression.

PHA767491 suppresses HSV-2 IE gene expression, viral replication and HSV-2 induced necrosis
To further address whether PHA767491 could block HSV-2 replication, cells were pretreated with DMSO or PHA767491 and infected with HSV-2. Notably, HSV-2 proteins including VP16 and gB were totally inhibited by...
PHA767491 at the concentration of 3μM (Fig. 6a). Moreover, we found PHA767491 caused the inhibition of HSV-2 IE gene expression (Fig. 6b). Consistently, PHA767491 significantly blocked HSV-2 induced cell death (Fig. 6c). Thus, PHA767491 is a novel agent against both HSV-1 and HSV-2 viral replication.

**PHA767491 significantly attenuates HSV-1 replication in mouse model**

We have previously shown that HSV-1 infection triggers necroptosis in non-natural host mouse cells, but not in natural host human cells [26, 32, 33]. Thus, RIP3-deficient mouse is a more relevant model for the study of HSV-1 infection in vivo. To assess the effect of PHA767491 on HSV-1 replication in mouse model, we injected RIP3-deficient mice with vehicle or PHA767491 followed by HSV-1 infection. Compared with vehicle treatment, PHA767491 reduced viral titer in livers and spleens from RIP3-deficient mice infected with HSV-1 (Fig. 7a and b). Further immunohistochemistry analysis confirmed that PHA767491 dramatically attenuated expression of viral protein gB in the livers (Fig. 7c). These results indicate that PHA767491 exhibits promising therapeutic activity against HSV-1 infection in mouse model.

**Discussion**

Uncontrolled HSV infection always lead to some severe diseases such as gingivostomatitis [2, 34, 35] and encephalitis [36–38]. In the current study, we demonstrate...
PHA767491 as a new anti-HSV agent that potently blocks viral replication of HSV-1 and HSV-2. Our in vivo study revealed a strong inhibition of HSV-1 production by PHA76749 in the mice infection model. Thus, PHA767491 could be a promising agent for the development of new anti-HSV therapy.

One complete cycle for HSV replication need a series of steps including viral attachment and penetration, viral replication, and viral release. Intervention in these processes provides effective protection against HSV infection. Theaflavin digallate has shown to prevent HSV-1 from entering into host to achieve the antiviral activity [39]. Nucleoside
analogue such as acyclovir, penciclovir and foscarnet interfere with the viral DNA synthesis to attenuate HSV infections [18–20]. In this study, we found PHA767491 remarkably reduced the expressions of HSV-1 proteins including ICP6 and gB and viral titer in multiple cell lines even after the entry of HSV into the host cells. Similar inhibition of HSV-2 infection by PHA767491 was observed at the post-viral entry stage. These results demonstrate that PHA767491 effectively blocks HSV replication.

HSV replication is regulated by viral proteins. At least several viral proteins are required for viral DNA synthesis, including proteins encoded by UL5, UL8, UL29, UL30, UL42 and UL52 genes. We found PHA767491 strongly inhibited the expression of these genes. HSV IE genes are expressed earliest after infection without the requirement of viral protein synthesis [3]. The essential IE genes such as ICP4 and ICP27 are critical for the efficient expression of the early and late genes. Of note, we found that PHA767491 significantly impaired the expression of all IE genes. The levels of ICP27 RNA and protein were very low or undetectable in the presence of PHA767491. Therefore, PHA767491 disrupts HSV replication by intervening in IE gene expression or upstream signaling molecules regulating IE gene expression.

In addition to viral proteins, NF-κB signals and MAPK signals of host cells are critical for virus replication at the early stage of infection [40–43]. The natural product harmine has been shown to inhibit HSV replication by downregulating both NF-κB and MAPK pathways [44]. However, in our study, PHA767491 did not affect the NF-κB activation (Additional file 1: Figure S1 A and B). Furthermore, we found that PHA767491 had no effects on the activations of ERK, p38 and JNK, three critical kinases of MAPK (Additional file 1: Figure S1 C, D and E). Thus, PHA767491 exhibits anti-HSV activity without affecting cellular NF-κB and MAPK pathways.

Conclusion
In this study, we identified PHA767491 as a new inhibitor of HSV. PHA767491 potently blocked the proliferation of HSV in various human and mouse cells, including HSV-1 and HSV-2. Moreover, PHA767491 significantly reduced the expression of viral genes required for DNA synthesis including UL30/42 DNA polymerase and UL5/8/52 helicase-primase complex. Moreover, PHA767491 also inhibited the expression of all IE genes of both HSV-1 and HSV-2. Importantly, PHA767491 reduced viral titers in the tissues from RIP3-deficient mice infected with HSV-1. Consistently, immunohistochemistry analysis showed that PHA767491 dramatically attenuated expression of viral protein gB in the livers. Further studies will be required to understand the precise molecular mechanism through which PHA767491 controls the expression of IE genes.

Additional file

Additional file 1: Figure S1. PHA767491 has no effect on NF-κB and MAPK activation. (DOCX 526 kb)

Abbreviations
BMM: Bone marrow derived macrophage; HSV: Herpes simplex virus; LPS: Lipopolysaccharide; MEF: Mouse embryonic fibroblast; Poly (I:C): Polyinosine acid cytidine acid; RIP3: Receptor-interacting protein kinase 3; TLR: Toll like receptor; TNFR: Tumor necrosis factor receptor; ZVAD: Z-val-ala-asp-(o-methylated)-fluoromethylketone

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Availability of data and materials
All data contained in the article and additional file.

Authors’ contributions
HSD, XF and ZZL contributed to experimental design and data analysis. ZXH and ZCF provided important reagents and were involved in the data analysis. HJ, ZZL, HQ, YJ, YXL and TGH carried out the experiments. ZZL and HJ made critical revisions in response to reviewers’ comments. The manuscript draft was written by HJ and ZZL and was revised by HSD. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval
All animal experiments were performed in accordance with protocols by the Institutional Animal Care and Use committee at Soochow University.

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Author details
1Lycus Tang Hematology Center, Collaborative Innovation Center of Hematology, Jiangsu Institute of Hematology, Soochow University, Suzhou, China. 2Jiangsu Key Laboratory of Preventive and Translational Medicine for Geriatric Diseases, Soochow University, Suzhou, China. 3Department of emergency medicine, First Affiliated Hospital, Soochow University, 1 Shizhi Rd, Suzhou, Jiangsu, China. 4Jiangsu Key Laboratory of Translational Research and Therapy for Neuro-Psycho-Diseases and College of Pharmaceutical Sciences, Soochow University, Suzhou, China. 5Institutes of Biology and Medical Sciences, Soochow University, Suzhou, China.

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