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Fungal metabolite myriocin promotes human herpes simplex virus-2 infection☆

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ABSTRACT

Aims: Myriocin is a fungal metabolite with antiviral activity, including influenza, hepatitis B, and hepatitis C viruses. We investigated whether myriocin has activity against human HSV-2, one of the most prevalent pathogens of sexually transmitted disease.
Main methods: Cell culture systems were used to evaluate myriocin effect on HSV-2 infection. Plaque forming assay and immunoblotting studies were used to determine virus production and viral protein expression, respectively.
Key findings: Myriocin showed no cytotoxic effect at up to 5 μM. Myriocin treatment did not inhibit HSV-2 infection. Instead, the treatment resulted in accelerated replication of HSV-2 and increased titers of infectious virion. The effect was detected at concentrations as low as 3 nM and plateaued at approximately 30 nM. Myriocin at 30 nM increased HSV-2 production by approximately 1.7 logs. Myriocin also promoted HSV-1 infection but required higher concentrations. A time course study revealed that myriocin promoted HSV-2 infection by acceleration of virus replication. Unlike trichostatin A that promotes HSV-2 infection and histone modifications, myriocin treatment did not alter histone modifications. Myriocin is a well characterized inhibitor of sphingolipid biosynthesis pathway. Structurally different inhibitors of the pathway showed no effect on HSV-2 infection. Exogenous sphingolipids did not reverse the effect of myriocin on HSV-2 infection either.
Significance: We found that myriocin promotes HSV-2 replication at nanomolar concentrations with yet unknown mechanisms. Further studies may uncover novel mechanisms regulating HSV replication and targets of myriocin action. This may have potential application in enhancing efficacy of oncolytic HSV for cancer therapy and other diseases.

Introduction

Herpes simplex viruses are double stranded DNA viruses that belong to the Herpesviridae family. There are two serotypes of human herpes simplex viruses, namely HSV-1 and HSV-2. HSV-1 is mainly associated with facial infections with visible cold sores or fever blisters and HSV-2 in general is associated with genital infection and causes genital herpes [21,40], but both strains have the ability to cause infection in either area [3]. HSV can have both lytic and latent infection cycles. In the lytic infection, HSV infects mucosa epithelial cells after direct contact with an infected individual or body fluid from those individuals. The virus replicates with vigorous multiplication of immediate early (IE) genes within the first few hours of infection to "prime" host cells for further expression of viral genes and mobilize cellular transcriptional machinery including the NF-κB pathway for viral genome replication and viral structural protein expression of the early and late phase genes [1,14,27,28]. After initial infection, HSV-1 and HSV-2 are transported along sensory nerves to the sensory nerve cell bodies, where they establish life-long latency of the human host. HSV exits latency periodically and is transported to the body surface where recurrent infection occurs. Several viral genes have been identified as critical for HSV replication [5,35], while few small molecules except trichostatin A (TSA), a well documented inhibitor of histone deacetylases, are known to promote HSV infection [9,10,16,20,25].

Myriocin is a fungal metabolite originally isolated from *Mycelia sterilia* with antibacterial and antifungal activities [18,26]. The compound is an atypical alpha amino acid that also resembles that of sphingosine. Myriocin is a well-characterized natural product with potent activity against serine palmitoyltransferase (SPT), a critical enzyme in *de novo* sphingolipid biosynthesis [7,22,38]. Recent studies show myriocin with potent activity against viral infection of human diseases,

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including influenza virus, HBV, and HCV infection [2,33,34,36]. In this study, we investigated whether myriocin possessed antiviral effect against HSV-2. Unexpectedly, we found that myriocin treatment resulted in increased HSV infection at nanomolar concentrations. Histone deacetylase inhibitors are among the only group of small molecule compounds that have been observed with a similar effect on HSV infection. We found that the effect of myriocin treatment did not affect histone modifications. In addition, the effect seemed to be independent of sphingolipid pathway inhibition. The results are reported here.

Materials and methods

Cells and viruses

African green monkey kidney epithelial Vero cells (ATCC CCL-81) and human neuroblastoma SK-N-SH cells (ATCC HTB-11) were purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China). The cells were cultured at 37 °C in DMEM (high glucose) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Carlsbad, CA), non-essential amino acids and sodium pyruvate in a humidified incubator with 5% CO_2. The HSV-2 strain G was purchased from ATCC (Manassas, VA). HSV-1 strain 8F was kindly provided by Dr. Qihan Li, Institute of Medical Biology, Chinese Academy of Sciences (Kunming, China). The stock viruses were propagated in Vero cells and titrated by using plaque forming assay.

Antibodies and reagents

Myriocin (PubChem CID: 6438394), MTT (methylthiazolyl diphenyltetrazolium bromide), and *D*-sphingosine were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were purchased from Enzo Life Sciences Inc. (Farmingdale, NY) or Cayman Chemicals (Ann Arbor, MI).

Antibodies to viral gD and ICP0 of HSV-1 were purchased from Santa Cruz Biotechnologies (San Cruz, CA), to acetylated histone-3 at Lys9 and Lys14 (Ac-H3)from Millipore (Billerica, MA), to histone-3 (H3) and to Ser10 phosphorylated histone-3 (p-H3) from Beyotime Institute of Biotechnology (Haimen, China). Antibody to GAPDH was purchased from Bioworld Technology (Minneapolis, MN). An antiserum to ICP0 of HSV-2 was prepared using a commercial source (Abmart, Shanghai) by immunizing New Zealand rabbits with a synthetic peptide. IRDye 800 and IRDye 700DX-conjugated secondary antibodies were obtained from Rockland Immunochemicals Inc. (Gilbertsville, PA). HRP-conjugated secondary antibodies were purchased from Sigma-Aldrich.

Cytotoxic assay

MTT assay was used to assay the cytotoxic effect of myriocin on host Vero cells as we previously used [8]. Briefly, cells in triplicates were treated with myriocin for 72 h. At the end of the treatment, MTT was added to each well to a final concentration of 0.5 mg/ml for the measurement of formazan formation, which can be extracted by DMSO and measured at 570 nm on a Versa Max microtiter plate reader (Molecular Devices, Sunnyvale, CA).

Infection assay

Monolayers of Vero cells or SK-N-SH cells were incubated with indicated concentrations of myriocin for 2 h and then uninfected or infected with HSV-2 or HSV-1 at an MOI of 0.3 or as indicated. For experiments that involved drug treatment, the compound was left in the medium once added. We used methods to determine the effect of myriocin on HSV-2 infection. We used MTT method to measure cell viability as a quick assessment of an infection since HSV-2 infection would lead to increased cell death due to cytopathic effect of an infection. We performed plaque forming assays to quantitatively determine infectious virion production by measuring plaque-forming units (PFUs) as we previously described [8]. Briefly, after an infection of an indicated hour, the cells and culture supernatants were collected and then freeze–thawed in liquid nitrogen to release infectious virus. After removal of cellular debris by centrifugation, the samples were series-diluted and used for titration by infecting nearly confluent Vero cells in 24-well plates, with each dilution in triplicate. After adsorption for 1 h, plates were washed and overlaid with DMEM containing 2% FBS and incubated for another 5 days. The plates were fixed with 3% paraformaldehyde and stained with 1% crystal violet for visualization of plaques. Virus titers are expressed as plaque forming units per ml (PFU/ml).

In-cell western assay

Monolayers of Vero cells in 96-well plate were treated as indicated. At 36 h PI, in-cell western assay was performed as described previously [29]. Briefly, cells were fixed with 3% paraformaldehyde for 30 min and permeabilization with 0.2% Triton X-100 for 5 min. After briefly washing

Fig. 1. Effects of myriocin on HSV-2 infection. A. Determination of maximal non-toxic concentration of myriocin in Vero cells. Vero cells in 96-well plates remained untreated or were incubated with myriocin at indicated concentrations for 72 h. DMSO at 0.2% was included as a solvent control since myriocin was dissolved in DMSO as a 1000× stock. Cell viability was determined by MTT assay. Data were presented as average optical densities at 570 nm (OD570 nm) ± standard deviation (SD) of triplicate samples. **B. Pretreatment of Vero cells with myriocin causes more severe cytopathic effect in HSV-2-infected cells.** Vero cells in 24-well plates were untreated or treated with myriocin (Myr) at 30 nM for 2 h prior to the infection. The cells were uninfected or infected with HSV-2 at 0.3 MOI for approximately 36 h. Represented fields of corresponding samples were photographed under an inverted microscope with an objective lens of 20× magnification. Con: untreated and uninfected Vero cells; HSV-2: untreated, but HSV-2 infected; Myr: uninfected but myriocin-treated; H + Myr: HSV-2 infected and myriocin-treated. The experiments were performed independently for at least 3 times. **C. Myriocin treatment enhances infection-associated cell lysis.** Vero cells in triplicate were mock-treated with DMSO or with myriocin (Myr) at indicated concentrations 2 h prior to the infection. The cells remained uninfected or were then infected with HSV-2 at 0.3 MOI for 36 h. Cell viability was then determined using a MTT assay. Open squares: myriocin-treated but uninfected controls (toxicity assay). Filled squares: HSV-2 infected that were treated with varying amounts of myriocin or untreated, which represents HSV-2 infected but untreated control (pointed with an arrowhead). Data are from an independent experiment of three separated studies. ** denotes a $p \leq 0.001$, indicating a significant difference between myriocin-treated and HSV-2 infected samples to that of the infected but untreated controls (myriocin at zero concentration). **D. Myriocin treatment on production of infectious HSV-2.** Vero cells were mock-treated or treated with myriocin at indicated concentrations for 2 h, and then infected with HSV-2 at an MOI of 0.3. The cells and culture supernatants were collected at 36 h PI, and used for titration of infectious virion by a plaque forming assay. The data are presented as mean ± SD of triplicate samples. * and ** indicate statistically significant difference of infectious virion production in myriocin-treated samples compared to that of mock-treated controls (* and ** represent $p \leq 0.01$ and $p \leq 0.001$, respectively). The experiment was performed twice independently. **E. Myriocin treatment on HSV-2 gene expression.** Vero cells were treated with myriocin at varying concentrations 2 h prior to inoculation. The cells were infected with HSV-2 (MOI = 0.3) for 36 h and used for the detection of ICP0 and gD expression by immunoblotting assays. Numeric numbers under ICP0 and gD blots indicate relative intensities of protein expression in those samples compared to that of the infected but untreated controls. GAPDH was used as an internal control for the study. **F. Myriocin treatment on late gene gD expression detected by in-cell western assay.** Vero cells that were untreated or treated with 30 nM myriocin were infected with HSV-2 at MOIs as indicated. The cells were then fixed at 36 h PI with paraformaldehyde and stained with antibody against HSV-2 gD protein or GAPDH as a control, followed by fluorescence dye-labeled secondary antibodies. The experiment was performed twice independently. **G. Myriocin treatment on HSV-2 gene expression determined by RT-PCR.** Vero cells in 12-well plates were untreated or treated with 30 nM myriocin 2 h prior to inoculation. The cells were then infected with HSV-2 for indicated times. Total RNA was harvested and used for the detection of HSV-2 gene expression by RT-PCR. Myriocin treatment resulted in increased detection of HSV-2 *TK* and *gD* gene in corresponding samples. Numeric numbers under *TK* and *gD* gels indicate relative intensities of viral gene expression in the samples. The intensity of the untreated controls with a measureable band was arbitrarily assigned as 1 and used to calculate relative intensities of other samples. The boxed numbers highlight the difference between myriocin-treated and untreated samples. The experiments were performed twice independently.