Antiviral Research 100 (2013) 75–83

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Antiviral activity of angelicin against gammaherpesviruses

Hye-Jeong Cho [1], Seon-Gyeong Jeong [1], Ji-Eun Park [1], Jin-Ah Han [1], Hye-Ri Kang, Dongho Lee, Moon Jung Song *

Department of Biosystems and Biotechnology, Division of Biotechnology, College of Life Sciences and Biotechnology, Korea University, Seoul 136-713, Republic of Korea

ARTICLE INFO

Article history:
Received 31 January 2013
Revised 13 July 2013
Accepted 15 July 2013
Available online 25 July 2013

Keywords:
Antiviral activity
EBV
KSHV
MHV-68
Angelicin
Furocoumarins

ABSTRACT

Human gammaherpesviruses including Epstein–Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV) are important pathogens as they persist in the host and cause various malignancies. However, few antiviral drugs are available to efficiently control gammaherpesvirus replication. Here we identified the antiviral activity of angelicin against murine gammaherpesvirus 68 (MHV-68), genetically and biologically related to human gammaherpesviruses. Angelicin, a furocoumarin naturally occurring tricyclic aromatic compound, efficiently inhibited lytic replication of MHV-68 in a dose-dependent manner following the virus entry. The IC_{50} of angelicin antiviral activity was estimated to be 28.95 μM, while the CC_{50} of angelicin was higher than 2600 μM. Furthermore, incubation with angelicin efficiently inhibited 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced lytic replication of human gammaherpresviruses in both EBV- and KSHV-infected cells. Taken together, these results suggest that MHV-68 can be a useful tool to screen novel antiviral agents against human gammaherepsviruses and that angelicin may provide a lead structure for the development of antiviral drug against gammaherpesviruses.

1. Introduction

Gammaherpesviruses such as Epstein–Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV) are important human pathogens as they are known to cause various kinds of malignancies. EBV is related to Burkitt's lymphoma, nasopharyngeal carcinoma, posttranplantation lymphoproliferative disease, Hodgkin's lymphoma, and non-Hodgkin's lymphoma, and sporadic cancers of the gastrointestinal tract (Rickinson and Kieff, 2007), while KSHV is associated with all forms of Kaposi's sarcoma (KS), such as classic, endemic, posttransplant, and AIDS-associated epidemic KS, primary effusion lymphoma and multicentric Castleman's disease (Boshoff et al., 1997; Cesarman and Knowles, 1997; Moore et al., 1996; Nador et al., 1996). Acute infection of EBV in adolescences results in self-limiting infectious mononucleosis, although it may also lead to severe and sometime fatal disease in immunocompromised patients.

Like other herpesviruses, gammaherpesviruses exhibit two distinct phases of the life cycle: they undergo lytic replication in epithelial cells and establish life-long latency in lymphocytes. Latently infected lymphocytes periodically become permissive for virus replication near an epithelium, nucleate a focus of epithelial replication, and enable the virus to complete its life cycle by disseminating the particles within or among the hosts (Pellett and Roizman, 2007). Although latent infection is thought to be critical for gammaherpesvirus-associated tumors, lytic replication is also important for the development of the tumors as evidenced in cases where a risk of KS was decreased in AIDS patients treated with antiviral compounds (Glesby et al., 1996; Martin et al., 1999; Mocroft et al., 1996).

Antiviral treatments for gammaherpesvirus infection are usually limited since commonly used antiviral drugs against herpesviruses, such as acyclovir or ganciclovir (GCV), are inefficient in eliminating latent gammaherpesviruses from chronically infected hosts. In recent years, there have been several reports to show that induction of latently-infected gammaherpesviruses to lytic replication could be therapeutically beneficial for gammaherpesvirus-associated tumors, especially in conjunction with antiviral drugs (Daibata et al., 2005; Feng et al., 2004). However, due to the lack of efficient virus replication system, the development of novel antiviral drugs against human gammaherpesviruses has been impeded. Murine gammaherpesvirus-68 (MHV-68 or γHV-68) is considered to be an important model system for the study of replication and pathogenesis of human gammaherpesviruses (Simas and Efstathiou, 1998; Virgin and Speck, 1999). Unlike human gammaherpesviruses, a number of cell lines are available to support robust lytic replication of MHV-68 and a recombinant MHV-68 expressing EGFP or firefly luciferase provides an amenable

* Corresponding author. Address: Rm. 307, West Building, College of Life Sciences and Biotechnology, Korea University, 1 Anam-dong 5-ga, Seongbuk-gu, Seoul 136-713, Republic of Korea. Tel.: +82 2 3290 3019; fax: +82 2 3291 3012.
E-mail address: moonsong@korea.ac.kr (M.J. Song).
[1] These authors contributed equally to this work.

0166-3542/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.antiviral.2013.07.009

screening system for antiviral agents using diverse libraries of chemicals or natural plant extracts (Cho et al., 2008 Hwang et al.,. 2008; Kang et al., 2012; Wu et al., 2001). Thus, MHV-68 allows us to examine the antiviral effects of screening hits on viral *de novo* infection, while human gammaherpesviruses are used to test their antiviral effects on reactivation or induced lytic replication from latently infection. Despite many studies on antiviral compounds from natural plant extracts (Arav-Boger, 2009; Curreli et al., 2002; Nichols et al., 2011), there have been relatively few reports on such compounds against gammaherpesviruses.

Angelicin is an angular furocoumarin that naturally occurs in the seeds of *Psoralea corylifolia*, the roots of *Angelica archangelica* and the family of *Umbelliferae* (or *Apiaceae)* plants, belonging to the class of psoralens, photosensitizers used for the treatment of various skin diseases together with long wavelength UV (UVA) irradiation (Bordin et al., 1991; Viola et al., 2009). Angelicin was shown to increase γ-globin mRNA in human erythroid cells, so that it can be used to treat thalassemia and sickle cell anemia (Bianchi et al., 2009; Gambari and Fibach, 2007; Lampronti et al., 2009). In this report, we screened a laboratory collection of 116 compounds isolated from diverse natural products for their antiviral activity against MHV-68 and showed that angelicin efficiently inhibited replications of gammaherpesviruses including KSHV and EBV. Structually related coumarin compounds also manifested antiviral activity against gammaherpesviruses. Our results suggest that MHV-68 can be a useful tool to identify novel antiviral agents against human gammaherepsviruses in a highthroughput screening system. Angelicin may serve as a structural lead for antiviral drugs against human gammaherpesviruses.

2. Materials and methods

2.1. Cell cultures and virus infection

BHK21 (baby hamster kidney fibroblast cell line) and Vero (green monkey kidney cell line) cells were cultured in complete Dulbesco's modified Eagle's medium containing 10% fetal bovine serum (HyClone) and supplemented with penicillin and streptomycin (10 units/ml, HyClone). BC-3 and BCBL-1 cells are KSHV-positive, EBV-negative cell lines, while B95.8 and Raji cells are EBV-positive, KSHV-negative B cells. BC-3G, a reporter cell line derived from BC-3 cells contains a strong RTA-responsive element driving destabilized EGFP expression. These cells were cultured in complete RPMI1640 medium containing 10% fetal bovine serum (Welgene, Republic of Korea) and supplemented with penicillin and streptomycin (10 units/ml, HyClone). MHV-68 virus was originally obtained from the American Type Culture Collection (VR1465). A reporter virus expressing the enhanced green fluorescence protein (MHV-68/EGFP) was generated by conventional homologous recombination as previously described (Wu et al., 2001). The viruses were grown in BHK21 cells and tittered by plaque assays, using Vero cells overlaid with 1% methylcellulose (Sigma) in normal growth media. After 5 days of infection, the cells were fixed and stained with 0.2% crystal violet in 20% ethanol. Plaques were then counted to determine the titers.

2.2. Chemicals

Angelicin and psoralen were originally isolated from *Psoralea corylifolia* and angelicin was later purchased from Sigma. Two coumarin compounds, 7-hydroxycoumarin (7-HC), and 5,7-dihydroxychromone (5,7-DHC), were isolated from *Cudrania tricuspidata*. The stock solution was prepared by dissolving the compound in dimethyl sulfoxide (DMSO) and diluted to the appropriate concentrations in culture medium. Ganciclovir (GCV) purchased from Sigma was dissolved in 10 mM Tris–HCl, pH 7.5.

2.3. Cell-based antiviral screening

BHK21 cells (1×10^4/well) were seeded in 96-well plates, infected with MHV-68/EGFP at a multiplicity of infection (MOI) of 0.05, and daily monitored for cytopathic effects (CPEs) and EGFP expression up to 3–4 days. Tested compounds were added to the cells at 3 h prior to virus infection as well as during 1 h of virus adoption. After removal of virus inoculum, tested compounds were newly added to the fresh medium for the indicated time. DMSO or methanol was used as negative controls and GCV as a positive control. These antiviral screenings were independently repeated three times with three concentrations (4, 20, and 100 μg/ml) of 116 tested compounds and the compounds only with consistent antiviral activity and no cytotoxicity were identified as candidates with antiviral activities.

2.4. Western blot analysis and antibodies

For western blot analysis, cells lysates were resolved by SDS–PAGE and transferred to polyvynilidene fluoride membrane. The membranes were probed with primary antibodies against MHV-68 ORF45 (1:500), ORF65 (M9) (1:500), KSHV RTA (1:500), or EBV EA-D (Novocastra Laboratories Ltd., 1:500) to detect virus lytic replication. A monoclonal antibody to α-tubulin (Sigma, 1:2000) was used to re-probed as a loading control. Goat anti-rabbit or goat anti-mouse IgG conjugated with horseradish peroxide secondary antibody (Santa Cruz) was detected by EPD Western blot detection kit (ELPIS, Republic of Korea) and the signals were detected and analyzed using LAS-4000, a chemiluminescent image analyzer (Fujifilm).

2.5. Real-time quantitative PCR

Genomic DNAs including viral DNAs were isolated from the harvested cells by a standard method of phenol chloroform extraction and ethanol precipitation (Lee et al., 2007). Real-time PCR of the whole genomic DNA (100 ng) was performed in triplicate on iCycler iQ multicolor real-time PCR detection system (Bio-Rad) as a 20 μl reaction mixture using ORF 56-specific primers with SYBR green I (Invitrogen) (Song et al., 2005) to quantitate the copy number of MHV-68 viral genomic DNAs. To quantitate EBV genomic DNAs, EBV OriLyt locus- and actin-specific primers were used (Lin et al., 2008): EBV OriLyt (EBV OriLyt-F, 5′-TCGCTTTCTTTTAT CCTCTTTTTG-3′, EBV OriLyt-R, 5′-CCCAACGGGATAAAATGACA-3′) and actin (Actin-F, 5′-ATTGCCGACAGGATGCAGAA-3′; Actin-R, 5′-GCTGATCCACATCTGCTGGAA-3′). For KSHV viral loads, ORF57 locus- and GAPDH-specific primers are used as previously reported (Brown et al., 2005; Prichard et al., 2009): KSHV ORF57 (KSHV ORF57-F, 5′-TGGACATTATGAAGGGCATCCTA-3′; KSHV ORF57-R, 5′-CGGGTTCGGACAATTGCT-3′) and GAPDH (GAPDH-F, 5′-GAAGGTGAAGGTCGGAGT-3′; GAPDH-R, 5′-GAAGATGGTGATGG-GATTTC-3′). These realtime PCRs of the KSHV or EBV genomic DNAs (50 ng) were performed in triplicate on iCycler iQ multicolor Real-time PCR detection system (Bio-Rad) and the results were analyzed on Optical system software (Bio-Rad) using actin or GAPDH as an internal control. Real-time PCRs were run at 50 °C for 2 min and 45 cycles at 95 °C for 10 s, 58 °C for 15 s, and 72 °C for 20 s, followed by melting curve analysis.

2.6. RNA extraction and RT-Q-PCR analysis

Total RNAs were extracted from cultured cells using TRI reagents (Molecular Research Center) according to the manufac-

turer's instructions. The cDNAs were synthesized using a RevertAid First strand cDNA synthesis (Fermentas, Republic of Korea) with random hexamers. The synthesized cDNAs were subjected to RT-Q-PCR analysis using viral transcript-specific primers including RTA, ORF57, ORF29, and ORF73 or cellular β-actin specific primers as described elsewhere (Lee et al., 2007). RT-Q-PCR analysis using EBV transcript-specific primers including BMRF1 or cellular β-actin specific primers is described elsewhere (Cho et al., 2008).

2.7. Promoter reporter analysis

For promoter activity analysis, 293T cells (1.2×10^5/well) were seeded in 24-well plates 24 h before transfection. A total of 650 ng of plasmid DNA per well was co-transfected using polyethylenimine (Sigma), and treated with angelicin at 1 h post-transfection. A luciferase reporter plasmid containing the upstream 210 bp-sequence of RTA cloned into pGL3-basic (pGL3-RTAp) was a kind gift from Dr. Ebrahimi at Liverpool University, UK. The plasmid DNA mixture included the luciferase reporter plasmid pGL3-RTAp (50 ng), pCMV-β-gal (80 ng), and Flag-mRTA (0.04–10 ng). The plasmid pCMV2-flag (260–269.96 ng) was added as a filler DNA to make 650 ng of total DNA. The luciferase assay system (Promega) was used according to manufacturer's instructions. The cells were washed with 1 × PBS and lysed with the 1 × passive lysis buffer 48 h after transfection. After one cycle of freeze–thaw, the cells were centrifuged to separate cell debris. Cell lysates (20 μl) were added to luciferase substrate buffer (40 μl) and luciferase activities were measured by Victor 3 (Perkin Eimer). Cell lysates (20 μl) were added to β-galactosidase substrate (30 μl) and incubated for 20 min. The activities of β-galactosidase were measured by PowerWave XS (Bio-Tek) and used as an internal control.

2.8. Plaque reduction assays

To determine IC_{50} for MHV-68 antiviral activity, plaque reduction assay using MHV-68 WT was performed as previously described with minor modification (Harper, 2000). Briefly, Vero cells were seeded at 5×10^4 cells/well in 12 well plates. Next day, the cells were incubated with MHV-68 (~100 pfu/well) for 90 min and the overlay media of DMEM containing 10% FBS and 0.6% methylcellulose was added upon removal of the virus inoculum. Tested compounds were diluted at the indicated concentrations into the normal growth media for pre-treatment (3 h prior to virus adoption) or the overlay media for post-treatment and added into the cells. After 5 days of incubation, the cells were fixed and stained with 0.2% crystal violet in 20% ethanol. Plaques were then counted and IC_{50} value was determined as the quantity of angelicin required to reduce the plaque number by 50% using Prism 6 (GraphPad Software Inc.).

2.9. Cytotoxicity assays

The cytotoxicity of angelicin was assessed via the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, as previously described (Cho et al., 2008). Briefly, 10^4 cells were seeded in 100 μl of complete medium into each well of 96-well plates and incubated with various concentrations of angelicin in a volume of 2 μl for 24 h. After treatment, 10 μl of MTT solution (5 mg/ml in 1 × PBS) was added to each well and the mixtures were incubated for an additional 3 h at 37 °C. The plates were then centrifuged, the supernatants were discarded, and 100 μl of DMSO was added to each well. After the crystals were dissolved, the amount of reduced MTT was measured at 570 nm.

3. Results

3.1. Antiviral activity of angelicin against MHV-68

A laboratory collection of 116 compounds isolated from natural products were screened at concentrations of 4, 20, and 100 μg/ml for antiviral activity against MHV-68/EGFP at an MOI of 0.05 in BHK21 cells. MHV-68/EGFP expresses the green fluorescence protein upon virus infection, serving as a reporter system to monitor virus replication under a fluorescence microscope. Angelicin, a furocoumarin compound, was consistently identified with strong antiviral activity from three independent screenings, and further examined for its inhibitory effect on gammaherpesvirus replication in this study. To confirm the antiviral effect of angelicin against MHV-68, BHK21 cells were treated with various doses of angelicin (3, 7.5, 15, and 30 μg/ml) before and after MHV-68/EGFP virus infection (MOI = 0.05) and analyzed for virus replication at 4 days post-infection (Fig. 1). The infected cells treated with media alone (N.T.) or DMSO were included as negative controls, while the infected cells with ganciclovir (GCV) were used as a positive control. Consistent with our screening results, angelicin treatment dose-dependently reduced EGFP expression (Fig. 1A). When further examined for viral protein expression, angelicin treatment exhibited inhibitory effects on late gene protein expressions such as ORF45 (tegument protein) and ORF65 (small capsid protein) (Fig. 1B). Angelicin also decreased viral genome replication in a dose-dependent manner, as analyzed by realtime PCR (Fig. 1C). In addition, angelicin treatment inhibited the mRNA expression of RTA, an immediate early (IE) gene of gammaherpesviruses in a dose-dependent fashion (Fig. 1D). These results confirm dose-dependent antiviral activity of angelicin against MHV-68 and indicate that our screening system was effective in identifying agents with antiviral activity.

3.2. Angelicin acts early in the MHV-68 replication cycle

To further investigate which stage of virus replication may be inhibited by angelicin, Vero cells were incubated with angelicin either before (pre-treatment) and/or after (post-treatment) virus infection and analyzed using plaque reduction assays (Fig. 2A and B). The same concentration of GCV (20 μg/ml) was used as a positive control. A representative picture of plaque reduction assays is shown in Fig. 2A. Plaque numbers in DMSO treatment were set as 100% and relative plaque formation (%) was calculated as shown in Fig. 2B. Pre-treatment of angelicin exhibited no effect on plaque formation of MHV-68, whereas post-treatment alone was as effective as both pre- and post-treatments in reducing plaque formation. Given that GCV showed similar patterns of inhibition, the results suggest that angelicin may inhibit MHV-68 replication after virus entry.

Our previous results showed that angelicin treatment reduced RTA IE gene expression at the transcript level (Fig. 1D). To test whether this is due to a direct effect on RTA expression or a secondary effect from reduced virus replication, we measured the RTA promoter (pGL3/RTAp-211) activity when induced by the RTA expression plasmid (FLAG-mRTA) in the absence or presence of angelicin using luciferase reporter assays (Fig. 2C). Angelicin treatment reduced transactivation of the RTA promoter in a dose-dependent manner when activated by RTA itself (autoactivation), suggesting that angelicin may directly affect RTA IE gene expression. Taken together, these results suggest that angelicin may inhibit gammaherpesvirus replication at an early stage of virus infection following virus entry.

3.3. IC_{50} and CC_{50} of angelicin

To determine the antiviral efficacy of angelicin against gammaherpesviruses, we performed plaque reduction assays using

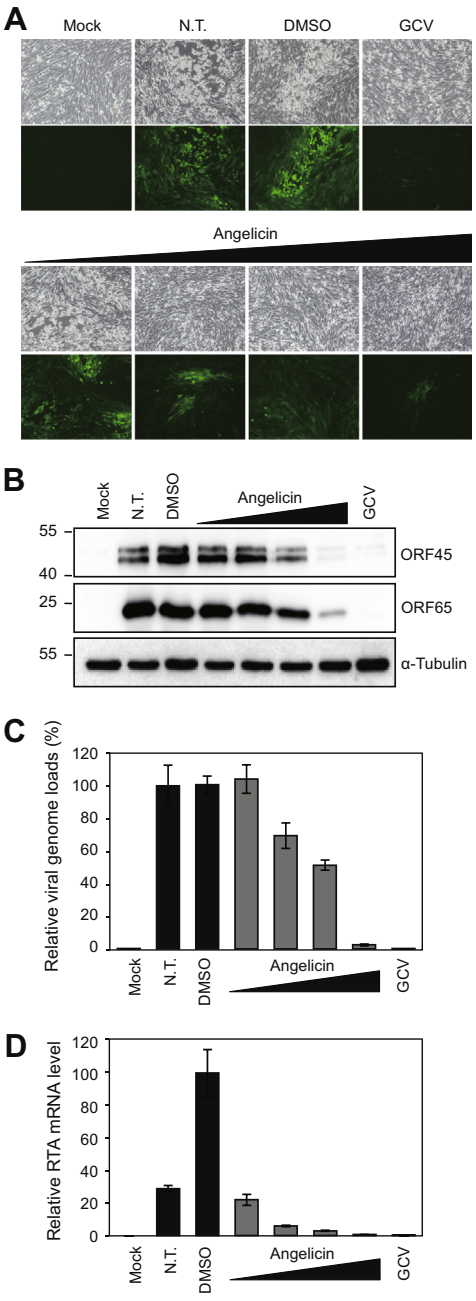

Fig. 1. Angelicin inhibits MHV-68 virus replication. BHK-21 cells infected with MHV-68/EGFP at MOI of 0.05 were treated by increasing doses of angelicin (3, 7.5, 15, and 30 μg/ml) and analyzed for viral replication at 4 days post-infection. No treatment (N.T.) and DMSO treatment were served as negative controls, while ganciclovir (GCV, 20 μg/ml) was used as a positive control. (A) Effect of angelicin on EGFP expression during MHV-68/EGFP infection. The cells were monitored under a fluorescence microscope and shown are the representative pictures of GFP expression and bright-fields. (B) Effect of angelicin on viral protein expression. The cells were harvested and analyzed by immunoblotting with antibodies against anti-ORF45 (a tegument protein) and anti-ORF65 (a small capsid antigen). Tubulin was used as a loading control. (C) Effect of angelicin on viral genome replication. Total genomic DNAs were extracted from the harvested cells and the copy numbers of viral genome were measured by real-time PCR. Shown are the relative viral genome loads in triplicate of genomic DNA (100 ng) when the viral genome copy number in DMSO-treated cells was set as 100%. (D) Effect of angelicin on a viral transcript. The total RNAs were extracted and subjected to RT-Q-PCR. The levels of an immediate early gene, RTA were measured and normalized with those of the actin transcript.

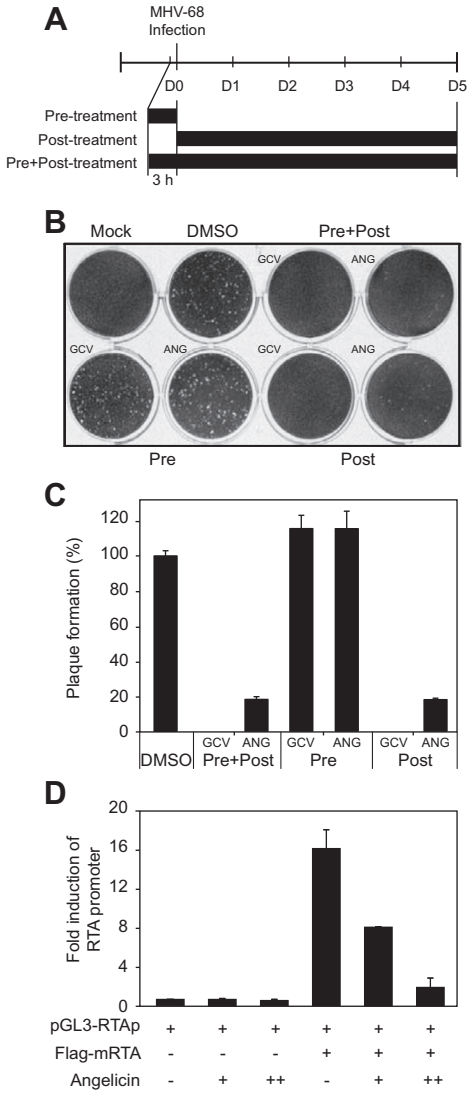

Fig. 2. Angelicin inhibits the early step of virus replication after virus entry. (A and B) Effects of angelicin on MHV-68 plaque formation. Plaque reduction assays were performed using WT MHV-68. A schematic diagram is shown for plaque reduction assays (A). Vero cells were treated with angelicin (20 μg/ml) either before (pre-treatment) or after (post-treatment) virus infection and the number of plaques was counted at day 5. GCV (20 μg/ml) was used as a positive control. A representative picture of plaque reduction assays is shown (B). The plaque numbers in DMSO treated samples were set as 100% and relative plaque numbers (%) were calculated in angelicin-treated samples (B). (C) Inhibition of the RTA promoter activity by angelicin. The luciferase reporter plasmid containing the RTA promoter (pGL3-RTAp) was co-transfected into 293T cells with the RTA expression plasmid (Flag-mRTA). At 1 h post-transfection, the cells were incubated with increasing doses of angelicin (0, 30, and 60 μg/ml). At 48 h post-transfection, the cells lysates were assayed for luciferase activity. The β-gal activity was used to normalize the luciferase activity as an internal control. Three independent experiments were performed and similar results were obtained.

wild-type MHV-68 at the concentrations of angelicin ranging from 0.1 to 90 μg/ml (0.54 to 483.3 μM) and determined the concentration required to inhibit MHV-68 replication by 50% (IC_{50}). Plaque numbers in DMSO treatment were set as 100% and relative plaque formations were calculated and shown with standard deviation (Fig. 3A). The IC_{50} of angelicin against MHV-68 was estimated to be 5.39 μg/ml (28.95 μM) (Fig. 3A).

To evaluate the cytotoxicity of angelicin, the concentration of the compound that decreased cell viability to 50% (CC_{50}) of DMSO control was determined in Vero cells by incubating with various concentrations of angelicin ranging from 0.1 to 500 μg/ml (0.54 to 2685.86 μM). The relative cell viability was analyzed based on

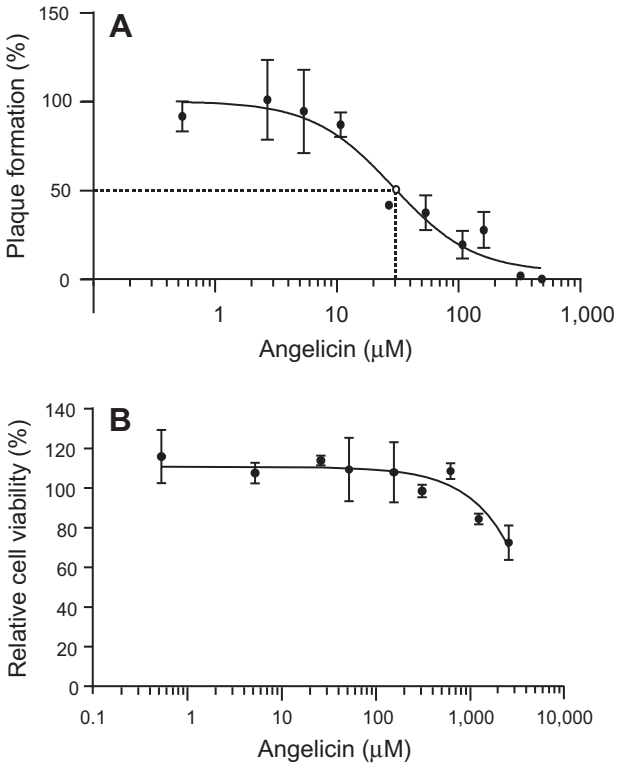

Fig. 3. IC_{50} and CC_{50} of angelicin. (A) Determination of IC_{50} of angelicin. The antiviral activity (IC_{50}) of angelicin was determined by plaque reduction assays. Vero cells were incubated with increasing amounts of angelicin ranging from 0.1 to 90 μg/ml (0.54 to 483.3 μM) after MHV-68 infection. Plaque numbers of angelicin-treated samples were measured in triplicate and compared with those of DMSO-treated samples. Data are represented as mean ± standard deviation. (B) Determination of CC_{50} of angelicin. The cytotoxicity (CC_{50}) of angelicin was determined by MTT assay in Vero cells. The cells were incubated with various doses of angelicin ranging from 0.1 to 500 μg/ml (0.54 to 2685.86 μM) for 24 h and relative cell viability was determined after normalizing with the values in DMSO treated samples. Data are represented as mean ± standard deviation of triplicates.

the DMSO control by the MTT assays (Fig. 3B). The results indicated that angelicin did not show significant cytotoxicity in the tested cells and CC_{50} could not be calculated since relative cell viability did not reach 50% even at the highest concentration tested. The cell viability at the maximal concentration of angelicin (500 μg/ml, 2685.86 μM) is 72.5% as shown in Fig. 3B.

3.4. Antiviral activity of angelicin against human gammaherpesviruses

MHV-68 (γ2 herpesvirus) is genetically and biologically related to human gammaherpesviruses such as EBV (γ1 herpesvirus) and KSHV (γ2 herpesvirus). Next, we sought to examine the effects of angelicin on virus replication of KSHV and EBV. Since KSHV and EBV are latently infected in various tumor cells, we analyzed the effects of angelicin on lytic reactivation of latently infected tumor cells when induced by TPA. When Raji, a EBV positive Burkitt's lymphoma-derived tumor cell line or B95.8, an EBV producing B cell line derived from a cotton-top tamarin (*Saguinus oedipus*) was induced for lytic replication by TPA together with sodium butyrate (NaB) or TPA alone, respectively, in the presence of angelicin, EA-D early gene expression was efficiently inhibited in both cell lines as seen in western analysis (Fig. 4A and B). Inhibition of EA-D expression by angelicin was also confirmed in immunofluorescence assays and intracellular staining (Fig. 4C–E). Relative viral genome loads were strongly inhibited by angelicin treatment in B95.8 cells, although induction folds were relatively low due to the short exposure time to TPA (Fig. 4F). The level of the BMRF1 early lytic transcript was also reduced in angelicin treated B95.8 and Raji cells in a dose-dependent manner (Fig. 4G and H), suggesting that angelicin efficiently inhibits lytic replication of EBV.

The BC-3G cell line derived from KSHV-infected BC-3 cells for its expression of potent lytic promoter-driven destabilized EGFP upon RTA induction, has proven to be an efficient system for monitoring KSHV reactivation and lytic replication in real time PCR (Cho et al., 2008; Yu et al., 2007). When treated with angelicin, the number of TPA-induced GFP-positive BC-3G cells was significantly reduced in a dose-dependent manner, compared with that in mock-treated cells (Fig. 5A). Consistent with this result, the immediate early gene, RTA protein expression was significantly inhibited by angelicin treatment in BC-3G cells (Fig. 5B). The inhibitory effects of angelicin were also confirmed in BCBL-1, another KSHV-positive cell line (Fig. 5B). Although increase in viral genome levels were relatively low in TPA alone treated cells at 24 h post-induction, angelicin reduced relative viral genome loads in a dose-dependent manner when compared with mock-treated BC-3G cells (Fig. 5C), suggesting that angelicin exhibits the potent antiviral activity against KSHV. Taken together, our results suggest that angelicin of the potent antiviral activity against MHV-68 also strongly inhibits lytic replication of human gammaherpesviruses.

3.5. Structure specificity of a coumarin ring in antiviral activity against gammaherpesviruses

Since angelicin contains a coumarin ring structure, we examined the effects of other coumarin-containing structural homologues isolated from natural products, such as psoralen (an isomer of angelicin), 7-hydroxycoumarin (7-HC), and 5,7-dihydroxychromone (5,7-DHC) (Fig. 6A). Although angelicin was most potent in blocking MHV-68 in plaque reduction assays, psoralen, 7-HC, and 5,7-DHC also exhibited significant antiviral activity against MHV-68 in a dose-dependent manner (Fig. 6B). Next, EBV- or KSHV-infected cells were treated with angelicin, psoralen, and 7-HC upon reactivation and analyzed for virus lytic replication. Due to the limited amount of 5,7-DHC, it was not included in the following studies. In both B95.8 and Raji cells, psoralen and 7-HC were as effective as angelicin, if not more, in inhibiting EBV lytic replication (Fig. 6C). Similarly, antiviral activities of psoralen and 7-HC against KSHV were as potent as that of angelicin in BC-3G cells (Fig. 6D). These results suggest the structural specificity of a coumarin ring commonly found in these compounds in inhibiting lytic replication of gammaherpesviruses.

4. Discussion

Although human gammaherpesviruses are important pathogens associated with various cancers, the development of novel antiviral drugs against human gammaherpesviruses has been limited due to lack of efficient screening systems. In this study, we performed cell-based antiviral screenings of over 100 compounds isolated from natural products using MHV-68 closely related to human gammahrepsviruses and identified angelicin, a furocoumarin compound of a strong antiviral activity against MHV-68 with little cytotoxicity. The IC_{50} of angelicin against MHV-68 was determined to be 28.95 μM, while the CC_{50} was estimated to be higher than 2,685 μM. Importantly, angelicin was also potent in inhibiting lytic replication of both EBV and KSHV, human gammaherpesviruses upon reactivation. Furthermore, other related compounds commonly containing the coumarin ring exhibited antiviral activity against MHV-68, EBV, and KSHV in a dose-dependent manner. To our knowledge, this is first to report that MHV-68 can provide a

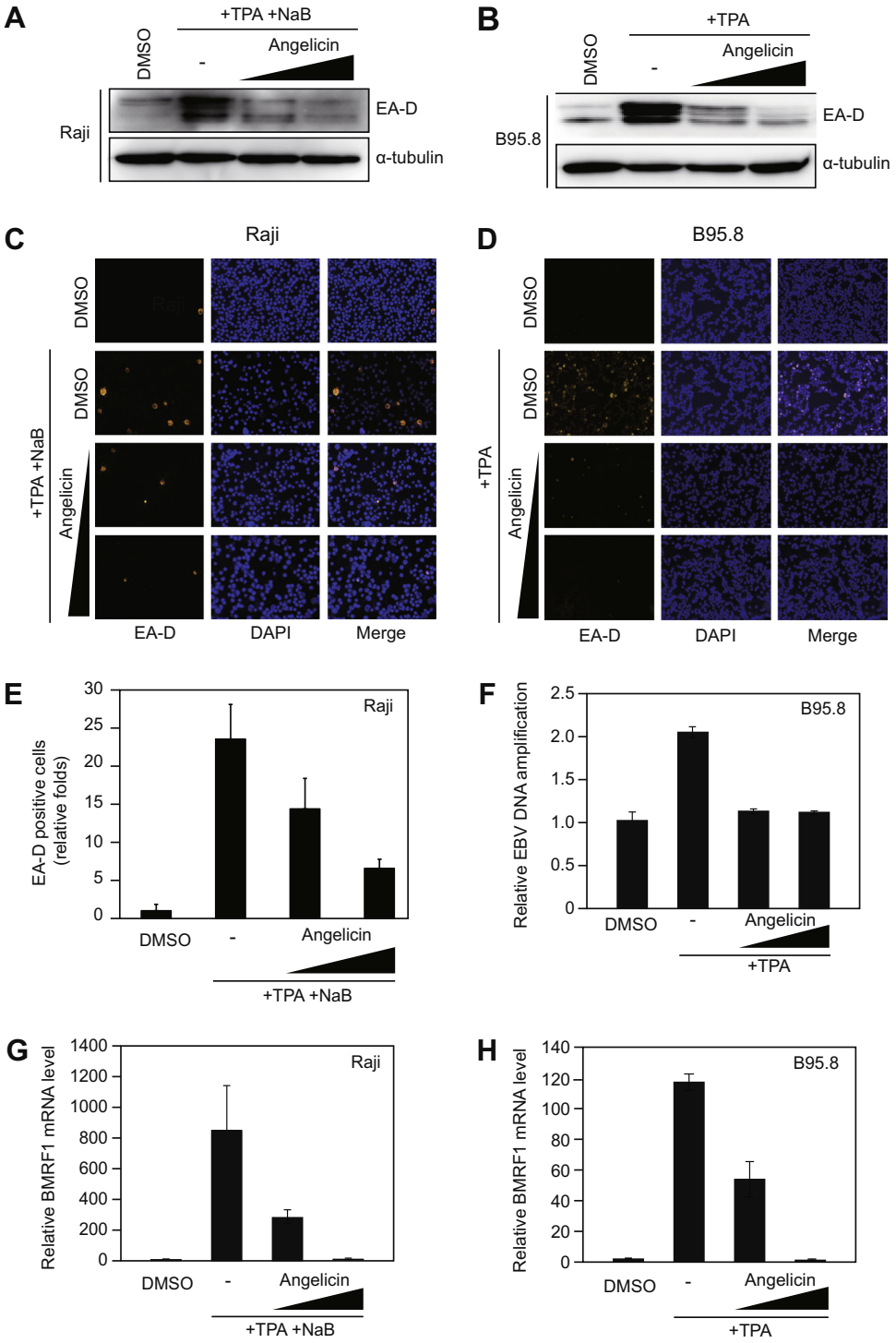

Fig. 4. Antiviral activity of angelicin against EBV. (A–D) Effect of angelicin on lytic protein expression. The EBV-infected Raji or B95.8 cells were treated with 30 and 60 μg/ml of angelicin upon lytic induction by TPA + NaB or TPA alone for 24 h. The cell lysates were analyzed for EBV lytic protein expression by immunoblotting (A and B), immunofluorescence assays (C and D) or intracellular staining (E) using anti-EA-D antibody in indicated cells. Anti-α-tubulin was used as a loading control (A and B), while DAPI was used for nuclear staining (D and E). Relative fold increases for % of EA-D positive cells were shown with standard error of mean in triplicate (E). (F) Effect of angelicin on viral genome loads. B95.8 cells were treated as described above. Total genomic DNA was extracted and relative viral genome loads were measured by realtime PCR using ori-Lyt locus specific primers. Actin-specific primers were used to normalize the level of viral genome as an internal control. (G and H) Effect of angelicin on a lytic gene transcript. The levels of viral BMRF1 transcript were measured by RT-Q-PCR from Raji or B95.8 cells induced by TPA + NaB or TPA, respectively, in the presence of angelicin (30 and 60 μg/ml). Cellular β-actin primers were used as an internal control.

screening system to identify novel antiviral agents against human gammaherpesviruses.

In addition to angelicin, resveratrol, a polyphenolic natural product, was also identified among the compounds that consistently showed antiviral activity with little cytotoxicity (data not shown). Consistent with our finding, resveratrol has been reported to inhibit replication of herpesviruses such as herpes simplex virus 1 (HSV-1), HSV-2, Varicella Zoster virus (VZV), and human cyto-

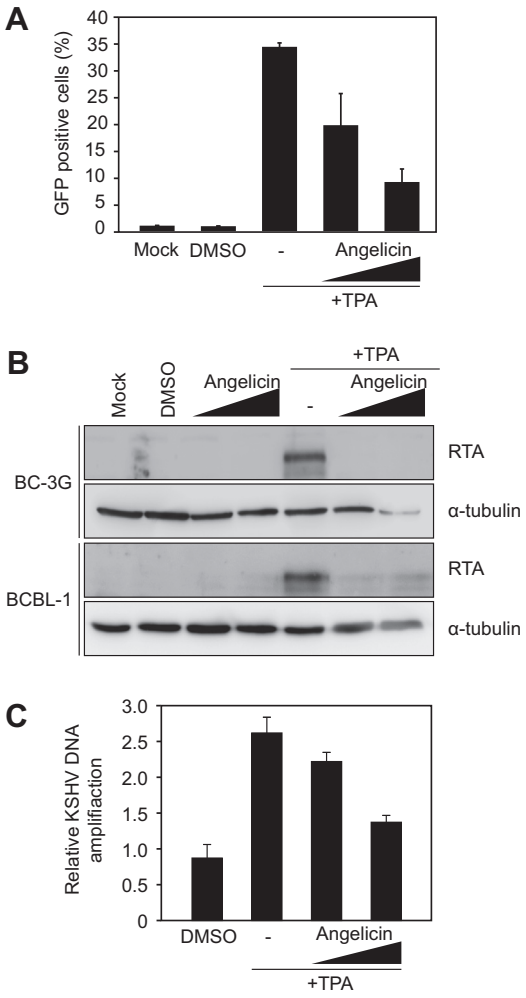

Fig. 5. Antiviral activity of angelicin against KSHV. (A) Effect of angelicin on lytic induction of KSHV. The BC-3G cells contain the destabilized EGFP reporter gene driven by a strong lytic viral promoter. The viral promoter is activated by RTA expression upon reactivation of KSHV. The BC-3G cells were treated with TPA and angelicin (30 and 60 μg/ml) for 24 h and analyzed by flow cytometry. Data is shown as the mean value of GFP-positive cells (%) ± standard deviation of triplicates. (B) Effect of angelicin on KSHV lytic gene expression. BC-3G and BCBL-1 cells were treated with angelicin (30 and 60 μg/ml) in the absence or presence of TPA for 24 h and analyzed for RTA protein expression. Anti α-tubulin was used as a loading control. (C) Effect of angelicin on KSHV viral genome loads. Total genomic DNA was prepared from the BC-3G cells treated as above and subject to realtime PCR using ORF57 locus-specific primers to measure relative viral genome loads. GAPDH gene was used as an internal control.

megalovirus (HCMV) (Campagna and Rivas, 2010; Docherty et al., 1999; Evers et al., 2004). Furthermore, resveratrol was recently reported to inhibit EBV lytic cycle (Espinoza et al., 2012; De Leo et al., 2011, 2012; Yiu et al., 2010) and KSHV reactivation (Dyson et al., 2012), validating our antiviral screening system as a screening tool to identify agents against human gammaherpesviruses. Besides, identification of angelicin with antiviral activity against gammaherpesviruses led us to test other structurally related compounds, including its structural isomer, psoralen. Our results showed that psoralen and two other coumarin containing compounds all showed significant antiviral activity against MHV-68 and that psoralen and 7-HC strongly inhibit the lytic cycle of human gammaheperpesviruses. Although not all antiviral hits from screenings with MHV-68 are likely effective in both EBV and KSHV, our results certainly provides an example that one can identify a lead structure for the development of antiviral drug against gammaherpesviruses via taking advantage of efficient MHV-68 replication system. Since IC_{50} of angelicin was in a micromolar range and relatively high to be a real candidate for clinical use as it is, further studies on structure–activity relationship should be warranted using angelicin as a structural lead to develop an optimized compound with the potent antiviral activity and low cytotoxicity.

Angelicin belongs to furocoumarin compounds and is a structural isomer of psoralen. Furocoumarins are natural plant constituents, bearing a coumarin ring structure attached to a furan ring. They occur in various plant families such as *Apicaceae* and *Umbelliferae*, in *Facabeae* and in citrus plants such as *Rutaceae* (Wagstaff, 1991). These plants produce furocoumarins as their protectants against microorganisms. Furocoumarins are tricyclic compounds with photosensitizing properties. They can reversibly intercalate into nucleic acids and, under UVA irradiation, form covalent C4-cycloadducts with 5,6 double bond of pyrimidine bases, mainly thymines (Bordin et al., 1991; Viola et al., 2009). The ability of furocoumarins to photoreact with nucleic acids has been applied in photochemotherapy to treat skin hyperproliferative disorders, i.e. psoriasis, vitiligo, and mycosis fungoides (Viola et al., 2009). In addition, some furocoumarins are shown to inhibit drug metabolism in a UV light-independent manner, known as a 'grapefruit juice effect' (Guo and Yamazoe, 2004). However, angelicin is an angular furocoumarin structurally distinct from planar furocoumarins and not found in grapefruit juice (Messer et al., 2012). Previously, furocoumarin compounds were reported to inactivate some RNA and DNA viruses mainly under UVA (340–400 nm) treatments that induced their covalent photobinding to viral and/or host nucleic acids (Hanson et al., 1978; Coppey et al., 1979). Since the photosensitizing ability of furocoumarins usually accompanies phototoxicity, treatment of angelicin or psoralen plus UVA at higher concentrations often resulted in severe cytotoxicity, rendering less viral infectivity against HSV-1 (Coppey et al., 1979). To contrast, it is important to note that our results indicated strong antiviral activity and yet little cytotoxicity of furocoumarin compounds without irradiation (Fig. 3B). Similarly, a previous study also reported no or little cytotoxicity of angelicin without or even with UV irradiation (Raquet and Schrenk, 2009). We showed that two other coumarin compounds such as 7-hydroxycoumarin and 5,7-dihydroxychromone, inhibited lytic replication of gammaherpesviruses without UV irradiation. Recently, a novel angelicin derivative 6a was shown to inhibit influenza virus (H1N1) induced cytopathic effect in Madin-Darby canine kidney cell culture in low micromolar range using a cell-based HTS without UVA irradiation and a further optimized lead structure exhibited IC_{50} to be in a nanomolar range (Yeh et al., 2010). Thus, the antiviral effects of angelicin and other related compounds appear to be independent of their photoreactivity.

Although detailed molecular mechanisms underlying antiviral activity of angelicin need further study, our results showed that angelicin pre-treatment had no effect, while post-treatment alone was sufficient for antiviral effect against MHV-68. Angelicin treatment inhibited autoactivation of the RTA promoter, suggesting that angelicin may function during the early phase of virus infection (Fig. 2). Angelicin also inhibited RTA protein expression of KSHV, when co-treated with TPA in BC-3G and BCBL-1 cells (Fig. 5). These results suggest that angelicin may have inhibitory effects on reactivation in addition to those on lytic replication of gammaherpesviruses. Whether or not angelicin shares common viral or cellular targets to inhibit virus lytic replication of gammaherpesviruses remains to be elucidated.

Taken together, our data indicate that angelicin, a furocoumarin compound isolated from natural plants is able to inhibit lytic replication of both murine and human gammaherpesviruses during the early stage of *de novo* infection and/or reactivation. Angelicin treatment down-regulates expression of viral gene transcripts as

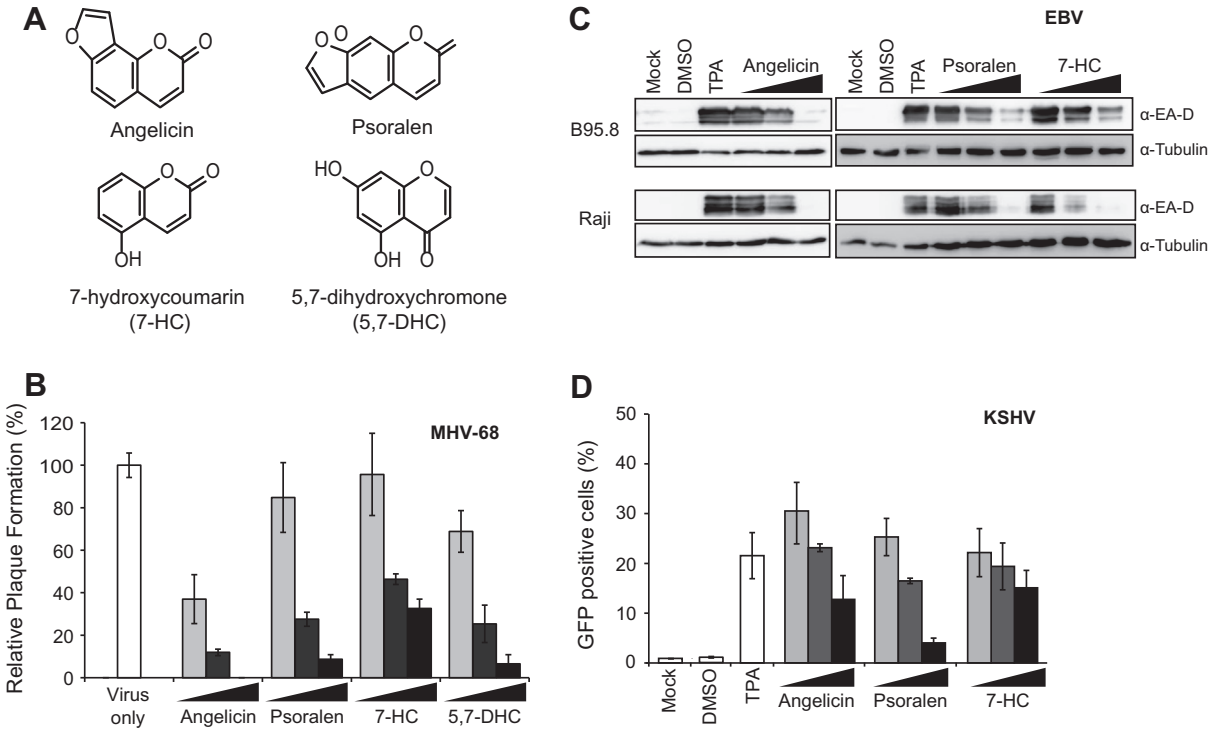

Fig. 6. Antiviral effects of structural homologues of angelicin against gammaherpesviruses. (A) Chemical structures of angelicin, psoralen, 7-hydoxycoumarin (7-HC) and 5,7-dihydroxychromone (5,7-DHC). Psoralen, a furocoumarin, is a structural isomer of angelicin and 7-HC and 5,7-DHC contains a coumarin ring. (B) Antiviral effects of the structural homologues against MHV-68. Plaque reduction assays were performed in Vero cells with increasing doses (10, 30, and 60 μg/ml) of the compounds following MHV-68 infection. Data are represented as mean ± standard deviation. (C) Antiviral effects of the structural homologues against EBV. B95.8 and Raji cells were treated with angelicin, psoralen, or 7-HC (10, 30, and 60 μg/ml) for 24 h in the presence of TPA or TPA + NaB, respectively. The cells were harvested and analyzed for lytic protein expression using anti-EA-D antibody. Anti α-tubulin was used as a loading control. (D) Antiviral effects of the structural homologues against KSHV. BC-3G cells were treated with angelicin, psoralen, or 7-HC (10, 30, and 60 μg/ml) for 24 h in the presence of TPA. The cells were harvested and analyzed for percentage of GFP-positive cells using flow cytometry. Data is shown as the mean value of GFP-positive cells (%) ± standard deviation.

well as genome replication, leading to reduction of plaque formation. Antiviral effect of other related compounds may also provide a molecular basis to further develop a potent antiviral agent against gammaherpesviruses.

Acknowledgements

This study was supported by a Korea University Grant, a Grant of the Korea Healthcare technology R&D Project, Ministry of Health, Welfare & Family Affairs, Republic of Korea (A091021), the National Research Foundation of Korea (NRF) Grant funded by the Korean Government (MEST) (2008-2005321) and Bio-industry Technology Development Program, Ministry for Food, Agriculture, Forestry and Fisheries (2011–2013), Republic of Korea to M.J.S.

References

Arav-Boger, R., 2009. Treatment for Kaposi sarcoma herpesvirus: great challenges with promising accomplishments. Virus Genes 38, 195–203.
Bianchi, N., Zuccato, C., Lampronti, I., Borgatti, M., Gambari, R., 2009. Fetal hemoglobin inducers from the natural world: a novel approach for identification of drugs for the treatment of {beta}-thalassemia and sickle-cell anemia. Evidence-Based Complementary and Alternative Medicine: eCAM 6, 141–151.
Bordin, F., Dall'Acqua, F., Guiotto, A., 1991. Angelicins, angular analogs of psoralens: chemistry, photochemical, photobiological and phototherapeutic properties. Pharmacology & Therapeutics 52 (3), 331–363.
Boshoff, C., Whitby, D., Talbot, S., Weiss, R.A., 1997. Etiology of AIDS-related Kaposi's sarcoma and lymphoma. Oral Diseases 3 (Suppl. 1), S129–S132.
Brown, H.J., McBride, W.H., Zack, J.A., Sun, R., 2005. Prostratin and bortezomib are novel inducers of latent Kaposi's sarcoma-associated herpesvirus. Antiviral Therapy 10, 745–751.
Campagna, M., Rivas, C., 2010. Antiviral activity of resveratrol. Biochemical Society Transactions 38, 50–53.
Cesarman, E., Knowles, D.M., 1997. Kaposi's sarcoma-associated herpesvirus: a lymphotropic human herpesvirus associated with Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. Seminars in Diagnostic Pathology 14, 54–66.
Cho, H.J., Yu, F., Sun, R., Lee, D., Song, M.J., 2008. Lytic induction of Kaposi's sarcoma-associated herpesvirus in primary effusion lymphoma cells with natural products identified by a cell-based fluorescence moderate-throughput screening. Archves of Virology 153, 1517–1525.
Coppey, J., Averbeck, D., Moreno, G., 1979. Herpes virus production in monkey kidney and human skin cells treated with angelicin or 8-methoxypsoralen plus 365 nm light. Photochemstry and Photobiology 29 (4), 797–801.
Curreli, F., Cerimele, F., Muralidhar, S., Rosenthal, L.J., Cesarman, E., Friedman-Kien, A.E., Flore, O., 2002. Transcriptional downregulation of ORF50/Rta by methotrexate inhibits the switch of Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8 from latency to lytic replication. Journal of Virology 76, 5208–5219.
Daibata, M., Bandobashi, K., Kuroda, M., Imai, S., Miyoshi, I., Taguchi, H., 2005. Induction of lytic Epstein–Barr virus (EBV) infection by synergistic action ofrituximab and dexamethasone renders EBV-positive lymphoma cells moresusceptible to ganciclovir cytotoxicity in vitro and in vivo. Journal of Virology 79 (9), 5875–5879.
De Leo, A., Arena, G., Stecca, C., Raciti, M., Mattia, E., 2011. Resveratrol inhibits proliferation and survival of Epstein Barr virus-infected Burkitt's lymphoma cells depending on viral latency program. Molecular Cancer Research 9 (10), 1346–1355.
De Leo, A., Arena, G., Lacanna, E., Oliviero, G., Colavita, F., Mattia, E., 2012. Resveratrol inhibits Epstein Barr Virus lytic cycle in Burkitt's lymphoma cells by affecting multiple molecular targets. Antiviral Research 96 (2), 196–202.
Docherty, J.J., Fu, M.M., Stiffler, B.S., Limperos, R.J., Pokabla, C.M., DeLucia, A.L., 1999. Resveratrol inhibition of herpes simplex virus replication. Antiviral Research 43, 145–155.
Dyson, O.F., Walker, L.R., Whitehouse, A., Cook, P.P., Akula, S.M., 2012. Resveratrol inhibits KSHV reactivation by lowering the levels of cellular EGR-1. PLoS One 7 (3), e33364.
Espinoza, J.L., Takami, A., Trung, L.Q., Kato, S., Nakao, S., 2012. Resveratrol prevents EBV transformation and inhibits the outgrowth of EBV-immortalized human B cells. PLoS One 7 (12), e51306.

Evers, D.L., Wang, X., Huong, S.M., Huang, D.Y., Huang, E.S., 2004. 3,4′,5-Trihydroxy-trans-stilbene (resveratrol) inhibits human cytomegalovirus replication and virus-induced cellular signaling. Antiviral Research 63, 85–95.
Feng, W.H., Hong, G., Delecluse, H.J., Kenney, S.C., 2004. Lytic induction therapy for Epstein–Barr virus-positive B-cell lymphomas. Journal of Virology 78, 1893–1902.
Gambari, R., Fibach, E., 2007. Medicinal chemistry of fetal hemoglobin inducers for treatment of beta-thalassemia. Current Medicinal Chemistry 14, 199–212.
Glesby, M.J., Hoover, D.R., Weng, S., Graham, N.M., Phair, J.P., Detels, R., Ho, M., Saah, A.J., 1996. Use of antiherpes drugs and the risk of Kaposi's sarcoma: data from the Multicenter AIDS Cohort Study. The Journal of Infectious Diseases 173, 1477–1480.
Guo, L.Q., Yamazoe, Y., 2004. Inhibition of cytochrome P450 by furocoumarins in grapefruit juice and herbal medicines. Acta Pharmacologica Sinca 25, 129–136.
Hanson, C.V., Riggs, J.L., Lennette, E.H., 1978. Photochemical inactivation of DNA and RNA viruses by psoralen derivatives. Journal of General Virology 40 (2), 345–358.
Harper, D.R., 2000. Determination of viral infectivity: HSV, VZV, and CMV. Methods in Molecular Medicine 24, 119–127.
Hwang, S., Wu, T.-T., Tong, L.M., Kim, K.S., Martinez-Guzman, D., Colantonio, A.D., Uittenbogaart, C.H., Sun, R., 2008. Persistent gammaherpesvirus replication and dynamic interaction with the host in vivo. Journal of Virology 82 (24), 12498–12509.
Kang, H.-R., Cho, H.-J., Kim, S., Song, I.H., Lee, T.S., Hwang, S., Sun, R., Song, M.J., 2012. Persistent infection of a gammaherpesvirus in the central nervous system. Virology 423 (1), 23–29.
Lampronti, I., Bianchi, N., Zuccato, C., Dall'acqua, F., Vedaldi, D., Viola, G., Potenza, R., Chiavilli, F., Breveglieri, G., Borgatti, M., Finotti, A., Feriotto, G., Salvatori, F., Gambari, R., 2009. Increase in gamma-globin mRNA content in human erythroid cells treated with angelicin analogs. International Journal of Hematology 90, 318–327.
Lee, S., Cho, H.J., Park, J.J., Kim, Y.S., Hwang, S., Sun, R., Song, M.J., 2007. The ORF49 protein of murine gammaherpesvirus 68 cooperates with RTA in regulating virus replication. Journal of Virology 81, 9870–9877.
Lin, T.P., Chen, S.Y., Duh, P.D., Chang, L.K., Liu, Y.N., 2008. Inhibition of the epstein–barr virus lytic cycle by andrographolide. Biological & Pharmaceutical Bulletin 31, 2018–2023.
Martin, D.F., Dunn, J.P., Davis, J.L., Duker, J.S., Engstrom Jr., R.E., Friedberg, D.N., Jaffe, G.J., Kuppermann, B.D., Polis, M.A., Whitley, R.J., Wolitz, R.A., Benson, C.A., 1999. Use of the ganciclovir implant for the treatment of cytomegalovirus retinitis in the era of potent antiretroviral therapy: recommendations of the International AIDS Society-USA panel. American Journal of Ophthalmology 127, 329–339.
Messer, A., Raquet, N., Lohr, C., Schrenk, D., 2012. Major furocoumarins in grapefruit juice II: phototoxicity, photogenotoxicity, and inhibitory potency vs. cytochrome P450 3A4 activity. Food and Chemical Toxicology 50, 756–760.
Mocroft, A., Youle, M., Gazzard, B., Morcinek, J., Halai, R., Phillips, A.N., 1996. Anti-herpesvirus treatment and risk of Kaposi's sarcoma in HIV infection. Royal Free/Chelsea and Westminster Hospitals Collaborative Group. AIDS 10, 1101–1105.
Moore, P.S., Kingsley, L.A., Holmberg, S.D., Spira, T., Gupta, P., Hoover, D.R., Parry, J.P., Conley, L.J., Jaffe, H.W., Chang, Y., 1996. Kaposi's sarcoma-associated herpesvirus infection prior to onset of Kaposi's sarcoma. AIDS 10, 175–180.
Nador, R.G., Cesarman, E., Chadburn, A., Dawson, D.B., Ansari, M.Q., Sald, J., Knowles, D.M., 1996. Primary effusion lymphoma: a distinct clinicopathologic entity associated with the Kaposi's sarcoma-associated herpes virus. Blood 88, 645–656.
Nichols, L.A., Adang, L.A., Kedes, D.H., 2011. Rapamycin blocks production of KSHV/HHV8: insights into the anti-tumor activity of an immunosuppressant drug. PLoS One 6, e14535.
Pellett, P.E., Roizman, B., 2007. The family herpesviridae: a brief introduction. Fields Virology, vol. 2. Lippincott Williams & Wilkins, fifth ed..
Prichard, M.N., Quenelle, D.C., Hartline, C.B., Harden, E.A., Jefferson, G., Frederick, S.L., Daily, S.L., Whitley, R.J., Tiwari, K.N., Maddry, J.A., Secrist 3rd, J.A., Kern, E.R., 2009. Inhibition of herpesvirus replication by 5-substituted 4′-thiopyrimidine nucleosides. Antimicrobial Agents and Chemotherapy 53, 5251–5258.
Raquet, N., Schrenk, D., 2009. Relative photomutagenicity of furocoumarins and limettin in the hypoxanthine phsophoribosyl transferase assay in V79 cells. Chemical Research in Toxicology 22, 1639–1647.
Rickinson, A.B., Kieff, E., 2007. Epstein-barr virus. In: Fields Virology, 5th ed, vol 2. Lippincott Williams & Wilkins, Hagerstown, MD.
Simas, J.P., Efstathiou, S., 1998. Murine gammaherpesvirus 68: a model for the study of gammaherpesvirus pathogenesis. Trends in Microbiology 6, 276–282.
Song, M.J., Hwang, S., Wong, W.H., Wu, T.T., Lee, S., Liao, H.I., Sun, R., 2005. Identification of viral genes essential for replication of murine gamma-herpesvirus 68 using signature-tagged mutagenesis. Proceedings of the National Academy of Sciences of the United States of America 102, 3805–3810.
Viola, G., Salvador, A., Vedaldi, D., Dall'Acqua, F., Bianchi, N., Zuccato, C., Borgatti, M., Lampronti, I., Gambari, R., 2009. Differentiation and apoptosis in UVA-irradiated cells treated with furocoumarinderivatives. Annals of the New York Academy of Sciences 1171, 334–344.
Virgin, H.W., Speck, S.H., 1999. Unraveling immunity to gamma-herpesviruses: a new model for understanding the role of immunity in chronic virus infection. Current Opinion in Immunology 11, 371–379.
Wagstaff, D.J., 1991. Dietary exposure to furocoumarins. Regulatory Toxicology and Pharmacology 14, 261–272.
Wu, T.T., Tong, L., Rickabaugh, T., Speck, S., Sun, R., 2001. Function of Rta is essential for lytic replication of murine gammaherpesvirus 68. Journal of Virology 75, 9262–9273.
Yeh, J.Y., Coumar, M.S., Horng, J.T., Shiao, H.Y., Kuo, F.M., Lee, H.L., Chen, I.C., Chang, C.W., Tang, W.F., Tseng, S.N., Chen, C.J., Shih, S.R., Hsu, J.T., Liao, C.C., Chao, Y.S., Hsieh, H.P., 2010. Anti-influenza drug discovery: structure-activity relationship and mechanistic insight into novel angelicinderivatives. Journal of Medicinal Chemistry 53 (4), 1519–1533.
Yiu, C.Y., Chen, S.Y., Chang, L.K., Chiu, Y.F., Lin, T.P., 2010. Inhibitory effects of resveratrol on the Epstein–Barr virus lytic cycle. Molecules 15, 7115–7124.
Yu, F., Harada, J.N., Brown, H.J., Deng, H., Song, M.J., Wu, T.T., Kato-Stankiewicz, J., Nelson, C.G., Vieira, J., Tamanoi, F., Chanda, S.K., Sun, R., 2007. Systematic identification of cellular signals reactivating Kaposi sarcoma-associated herpesvirus. PLoS Pathogens 3, e44.