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

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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

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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-