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Allicin and Glycyrrhizic Acid Display Antiviral Activity Against Latent and Lytic Kaposi Sarcoma-associated Herpesvirus

Yirui Xie, Ying Chen, Yongzheng Guo, Ying Huang, Biao Zhu✉

Abstract

Kaposi sarcoma-associated herpesvirus (KSHV) triggers the development of Kaposi sarcoma, a skin malignancy that is one of the most widespread defining symptoms in acquired immunodeficiency syndrome patients. KSHV manifests in two distinct cycles, a chronic latent cycle and an acute lytic cycle. Current clinical anti-herpesvirus therapeutic agents are predominantly composed of nucleoside analogues that target viral replication in the lytic cycle only, while KSHV latent genes are at the basis tumorigenesis. Currently, there are no effective therapies targeting latent KSHV infections. Therefore, the aim of this study was to identify putative therapeutic compounds with inhibitory activity against latent KSHV. The KSHV-infected primary effusion lymphoma cell line BC-3 was used to study antiviral activity of glycyrrhizic acid (GA), Allicin, and epigallocatechin-3-gallate (EGCG) against latent and lytic KSHV. Activity of GA, Allicin, EGCG, and the established anti-lytic cycle control compound ganciclovir was quantified by real-time polymerase chain reaction of nuclear and virion KSHV DNA yields after treatment compared with the untreated control. GA and Allicin showed antiviral activity against both latent and lytic KSHV, while EGCG displayed activity against lytic KSHV only. Therefore, GA and Allicin are interesting compounds for further development of anti-KSHV therapy against latent cycle infections.

Keywords: Kaposi sarcoma-associated herpesvirus; BC-3 cells; Allicin; glycyrrhizic acid

Introduction

Kaposi sarcoma-associated herpesvirus (KSHV) is the etiologic agent for the development of Kaposi sarcoma and other KSHV-related diseases in immuno-compromised patients.[1] Although Kaposi sarcoma can be manifested in four distinct epidemiological forms, it is most commonly recognized as an acquired immunodeficiency syndrome-related skin malignancy.[1] After infecting host cells, KSHV can display two different life cycle phases, the latent and the lytic phase. In latent infected cells, the virus genome exists in the cell nucleus as episomal DNA that can persist with expression of only a few latency-associated genes. Following not well defined host-mediated stimulating factors, including the development of immunodeficiency, the lytic phase is induced.[1–3] In the productive lytic phase, the viral genomes replicate in the host nucleus, express most of the viral genes, and produce infectious virions.[4]

The predominant and most successful therapeutic agents for KSHV are nucleoside analogs such as acyclovir, ganciclovir (GCV), zidovudine, and valganciclovir, which inhibit viral replication by inhibition of the DNA polymerase. However, these antiviral drugs only inhibit KSHV and other herpes viruses in the lytic cycle and since they have no effect on the latent infection state, they are ineffective at eliminating latent KSHV from chronically infected hosts.[5–7] So far, there are no effective drugs facilitating inhibition of KSHV latent infections. Due to the absence of efficient evaluation methods, limited development has been made in the area of novel antiviral drugs targeting latent KSHV infections. Despite the many studies related to antiviral compounds from natural plant extracts, there have been relatively few reports detailing the effects of these compounds against KSHV.[8,9]

Several years ago, it was reported that a KSHV ORF26 real-time polymerase chain reaction (PCR) showed excellent sensitivity, specificity, and suitability for dynamics assays, making it suitable for quantification of lytic and latent KSHV.[10] Therefore, in this study an in vitro evaluation system was established using the KSHV-infected primary effusion lymphoma (PEL) cell line BC-3 in combination with real-time PCR to study anti-KSHV effects of the traditional Chinese medicinal drugs Allicin, Glycyrrhizic acid (GA) and Epigallocatechin-3-gallate (EGCG).

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Results

Drug cytotoxicity analysis

To study the therapeutic effects of GCV, Allicin, GA, and EGCG on latent and lytic KSHV infection, their cytotoxicity was first determined, since cell viability likely impacts overall viral DNA yield and; therefore, the experimental outcomes. Cells were exposed to a large concentration range of the drugs and cell viability and proliferation were determined relative to the untreated controls using trypan blue staining and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. The maximum tolerable drug concentrations that did not show major effects on cell viability were 16 μM for GCV, 960 μM for GA, 6 μM for Allicin, and 100 μM for EGCG. Therefore, in subsequent therapeutic experiments these concentrations were set as the maximum dose.

Anti-KSHV efficacy of Allicin, GA, and EGCG

To study the efficacy of Allicin, GA, and EGCG against both latent and lytic KSHV, BC-3 cells containing latent KSHV were stimulated with 12-O-tetradecanoylphorbol-13-acetate (TPA), which induces reactivation of KSHV in latently infected cells, and subsequently treated with Allicin, GA, EGCG, and the established lytic cycle-only therapeutic GCV. Quantification if viral DNA from the nucleus (latent) and virions (lytic) by real-time PCR indeed showed that even the highest GCV concentration (16 μM) did not significantly reduce virus DNA in the nucleus compared with untreated cells (Figure 1A). In contrast, even at the lowest GCV concentration (1 μM), a significant reduction to 51% of the control was observed in virion DNA (Figure 1B), which further reduced at higher GCV concentrations to 25% of the untreated control. Similar results were obtained after treatment with EGCG. Even at the highest EGCG concentration (100 μM), no significant reduction of viral DNA in the nucleus was observed compared with the untreated control (Figure 2A), while at the lowest EGCG concentration (5 μM) virion DNA yields were reduced to 30% of the control (Figure 2B). At the highest EGCG concentration, even a further reduction of virion DNA was observed to 9% of the control. In contrast, Allicin treatment appeared to have an impact on both latent and lytic infections. KSHV DNA yields in the nucleus showed a significant gradual concentration-dependent reduction ranging from 73% at 3 μM to 10% of the untreated control at 60 μM (Figure 3A). Interestingly, Allicin therapy of latent KSHV appeared to be more effective than therapy of lytic KSHV, since virion DNA yield were not reduced at the lower Allicin concentrations (Figure 3B). Only at 15 μM Allicin and higher a significant reduction in virion DNA was observed, reaching 6% of the control at 60 μM. Finally, GA also appeared to show therapeutic effects on both latent and lytic KSHV. Although at the lowest GA concentration, no therapeutic effect was observed for latent KSHV, at 120 μM and higher, a significant reduction in viral DNA yields from the nucleus were observed compared with the untreated control (Figure 4A), reaching 31% of the control for the highest GA concentration (960 μM). Similarly, GA concentrations of 120 μM and higher were required to observe a reduction in virion DNA compared with the untreated control (Figure 4B), with the most potent effect observed at 480 μM GA (11% of the control). Overall, these results indicate that both Allicin and GA display anti-viral activity to both latent and lytic KSHV, while EGCG only displayed activity against lytic KSHV.

Discussion

KSHV chronic infection results in a lifelong disease because there is currently no treatment available to clear the virus in a latent state. After infection of host cells, viral oncogenic proteins are activated, which have profound interactions with the host cell, and cause the observed KSHV pathogenesis. The latent genes are expressed in most KSHV-infected cells and are thought to promote tumorigenesis.[11–14] Therefore, the inhibition of KSHV replication and elimination of KSHV latent infections will likely be the key for the prevention of KSHV-related diseases.

Currently, the most common agents used for treatment of herpesvirus are the nucleoside analogs that inhibit viral replication by inhibiting DNA polymerase. Previous studies analyzing the anti-KSHV activities of acyclovir, GCV and famciclovir on the KSHV-infected B-cell lymphoma cell lines BC-1 and BCBL-1A showed that these drug only inhibited KSHV lytic cycle replication, but had little activity against the latent

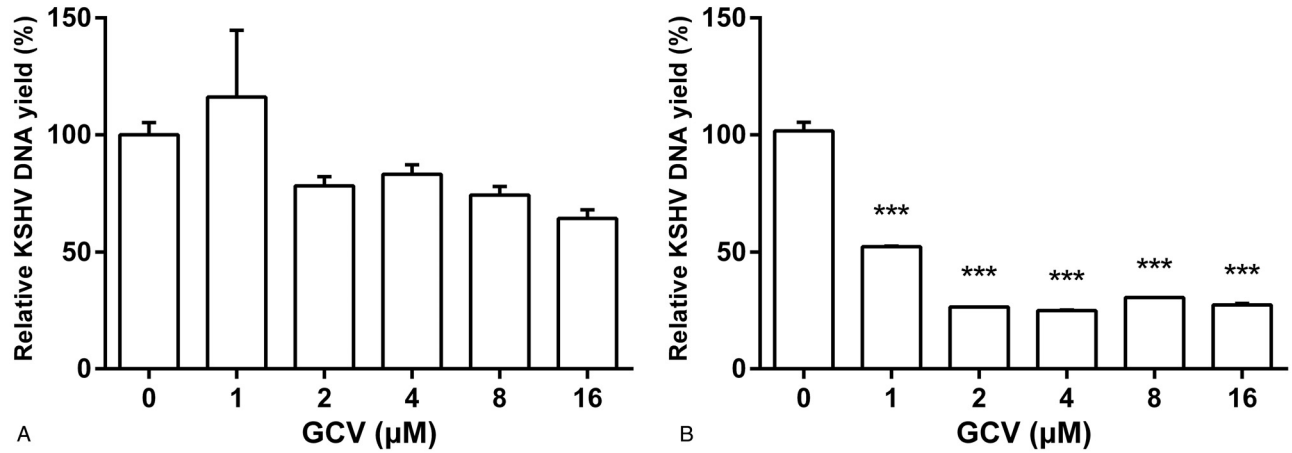

Figure 1. Therapeutic effects of ganciclovir (GCV) against latent and lytic KSHV. BC-3 cells containing KSHV were treated with GCV and viral DNA yields from the nucleus (latent) and virions (lytic) were quantified by quantitative real-time PCR. Graphs express the relative viral DNA yields compared with the untreated control and represent the mean and SD of three biological repeats. A: KSHV nuclear DNA yields after exposure to different concentrations of GCV. B: KSHV virion DNA yields after exposure to different concentrations of GCV. Significant differences compared with the untreated control were identified by one-way ANOVA (GraphPad Prism). $^{***}P < 0.001$. ANOVA: analysis of variance; KSHV: Kaposi sarcoma-associated herpesvirus; PCR: polymerase chain reaction; SD: standard deviation.

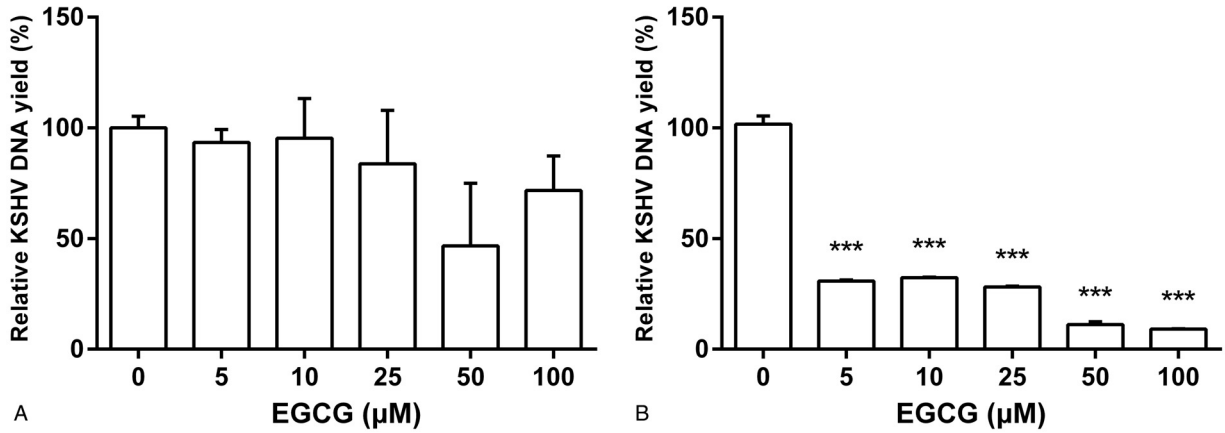

Figure 2. Therapeutic effects of epigallocatechin-3-gallate (EGCG) against latent and lytic KSHV. BC-3 cells containing KSHV were treated with EGCG and viral DNA yields from the nucleus (latent) and virions (lytic) were quantified by quantitative real-time PCR. Graphs express the relative viral DNA yields compared with the untreated control and represent the mean and SD of three biological repeats. A: KSHV nuclear DNA yields after exposure to different concentrations of EGCG. B: KSHV virion DNA yields after exposure to different concentrations of EGCG. Significant differences compared with the untreated control were identified by one-way ANOVA (GraphPad Prism). $^{***}P < 0.001$. ANOVA: analysis of variance; KSHV: Kaposi sarcoma-associated herpesvirus; PCR: polymerase chain reaction; SD: standard deviation.

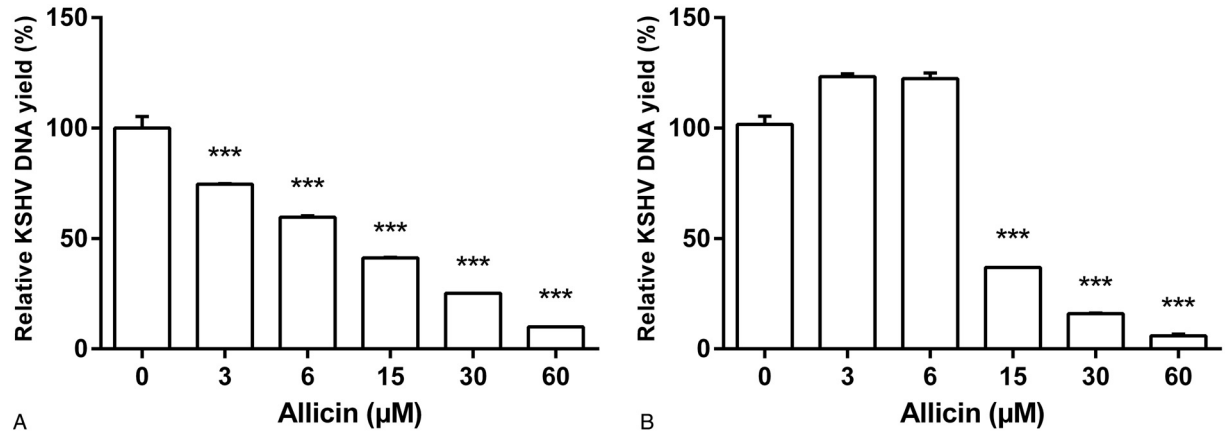

Figure 3. Therapeutic effects of Allicin against latent and lytic KSHV. BC-3 cells containing KSHV were treated with Allicin and viral DNA yields from the nucleus (latent) and virions (lytic) were quantified by quantitative real-time PCR. Graphs express the relative viral DNA yields compared with the untreated control and represent the mean and SD of three biological repeats. A: KSHV nuclear DNA yields after exposure to different concentrations of Allicin. B: KSHV virion DNA yields after exposure to different concentrations of Allicin. Significant differences compared with the untreated control were identified by one-way ANOVA (GraphPad Prism). $^{***}P < 0.001$. ANOVA: analysis of variance; KSHV: Kaposi sarcoma-associated herpesvirus; PCR: polymerase chain reaction; SD: standard deviation.

infection state.[5,6] However, since KSHV latent infections are believed to be critical for development of KSHV-associated tumors,[16] it is essential to develop drugs targeting KSHV latent gene products. A previous study reported that KSHV-infected PEL cells were killed by rapamycin through its effect on autocrine signaling.[17] Therefore, it should be possible to target the latent cycle of KSHV. In this study, we investigated the effects of antiviral compounds from natural plant extracts against KSHV.

KSHV latently-infected PEL-derived BC-3 cells were used to evaluate the effect of the traditional Chinese medicine products GA, Allicin, and EGCG on the KSHV latent and lytic cycle by quantification of cellular and virion DNA yields. The lytic cycle was reactivated with TPA, a well-established and widely used inducer of lytic KSHV in several human PEL cell lines.[2] In BC-3 cells, KSHV is maintained in a latent infection state that can enter into a lytic cycle replication state after TPA stimulation.[15] To reliably and accurately study KSHV inhibition after treatment, quantitative real-time PCR was used as the most reliable method for quantification of cellular and virion KSHV DNA.[8,19]

GA is a triterpenoid compound that has already been shown to inhibit the lytic replication cycle of several herpesviruses.[18] Furthermore, it has been shown that latent infection of B lymphocytes with KSHV could potentially be terminated by GA.[9] GA was able to disrupted latent KSHV infection by down-regulating the expression of latency-associated nuclear antigen and upregulating the expression of viral cyclin. In addition, GA selectively induced cell death of KSHV-infected cells.[9] A clinical study performed by our research team also confirmed that GA displayed anti-KSHV activity.[19] Here, we showed that GA displayed in vitro inhibitory effects against both latent and lytic KSHV.

Fresh garlic extract and other garlic-associated compounds have been shown to display strong antiviral activity against a wide spectrum of viruses, including enveloped viruses (para-

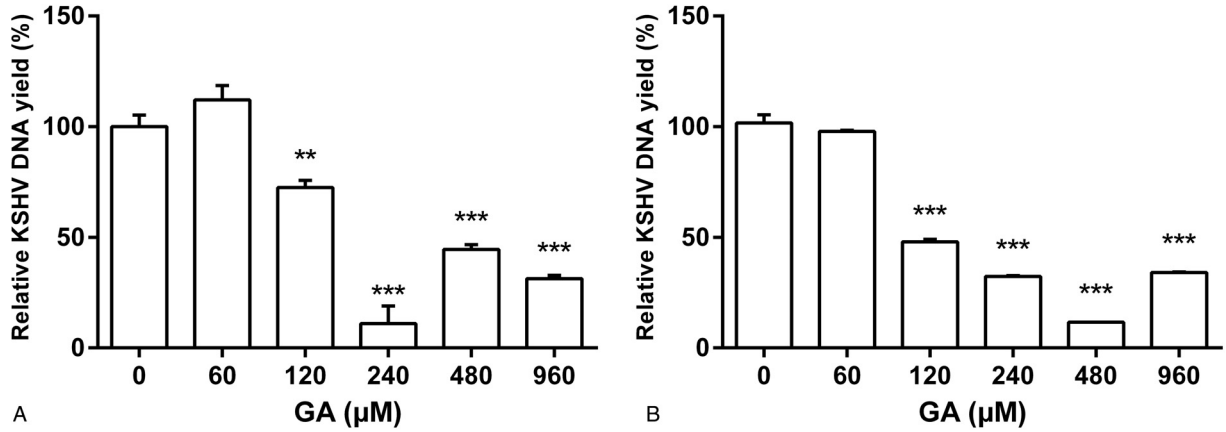

Figure 4. Therapeutic effects of Glycyrrhizic acid (GA) against latent and lytic KSHV. BC-3 cells containing KSHV were treated with GA and viral DNA yields from the nucleus (latent) and virions (lytic) were quantified by quantitative real-time PCR. Graphs express the relative viral DNA yields compared with the untreated control and represent the mean and SD of three biological repeats. A: KSHV nuclear DNA yields after exposure to different concentrations of GA. B: KSHV virion DNA yields after exposure to different concentrations of GA. Significant differences compared with the untreated control were identified by one-way ANOVA (GraphPad Prism). $^{**}P < 0.01$, $^{***}P < 0.001$. ANOVA: analysis of variance; KSHV: Kaposi sarcoma-associated herpesvirus; PCR: polymerase chain reaction; SD: standard deviation.

influenza virus type 3 and vesicular stomatitis virus), herpes simplex virus, non-enveloped viruses (human rhinovirus type 2) and the vaccinia virus.[20] Here, we showed that the garlic-associated compound Allicin also displays strong in vitro inhibitory activity against both latent and lytic KSHV.

EGCG is a major component of extracts from the dry leaves of *Camellia sinensis*. Antiviral properties were previously discovered in EGCG, suggesting that EGCG could be used as an antiviral agent in medications.[21] It was previously shown that EGCG can inhibit the infectivity of both enveloped and non-enveloped viruses at micromolar levels by interrupting viral attachment to the cell membrane.[22] EGCG displayed antiviral activity against a broad range of viruses, including HSV, hepatitis C virus, hepatitis B virus, influenza A virus, adenovirus, VSV, reovirus, mouse cytomegalovirus, and Sindbis virus.[22] Furthermore, supplementation with EGCG inhibited Ebola virus infectivity in human cells through the inhibition of a host protein.[23] Also, it has previously been reported that EGCG can reduce the production of progeny viruses from BCBL-1 and BC-1 cells without stimulating KSHV reactivation.[10] Here, we now established that EGCG only has inhibitory activity against the lytic cycle of KSHV.

In conclusion, our study established antiviral activities of GA, Allicin, and EGCG against KSHV. Importantly, while EGCG only displayed activity against the lytic KSHV cycle, both GA and Allicin displayed activity against latent and lytic KSHV. Particularly Allicin displayed strong activity against latent KSHV at very low concentrations. Therefore, these compounds are excellent candidates for further therapeutic development against both latent and/or lytic KSHV.

Materials and methods

Cell viability and proliferation assays

KSHV latently-infected PEL-derived BC-3 cells purchased from the American Type Culture Collection were used as a model throughout the study. BC-3 cells were cultured in RPMI1640 medium containing 20% fetal bovine serum (Gibco, Grand Island, NY, USA) at 37°C with 5% CO_2. Logarithmic growing BC-3 cells were suspended in RPMI1640 medium supplemented with 20 ng/mL TPA (Sigma Co., St louis, MO, USA) to induce lytic KSHV and incubated of 20 hours. Subsequently, the cells were seeded at a density of 2×10^5 cells/mL in 96-well sterile culture plates (200 μL per well). Cells were exposed to different concentrations of GCV (Sigma Co., St louis, MO, USA), GA (ZBD Pharmaceutical Co., China), Allicin (ZBD Pharmaceutical Co.), EGCG (ZBD Pharmaceutical Co.), and drug-free RPMI1640 control and incubated for 24 hours. Cell viability was determined by trypan blue staining and microscopic quantification. For cell proliferation assays, 20 μL of MTT (5 mg/mL) was added to each well and cells were incubated for 4 hours. Subsequently, the supernatant was removed and dimethyl sulphoxide (DMSO) was added to solubilize the formazan crystals formed in the viable cells. After shaking the resultant mixture for 10 minutes, an ELISA microplate reader (BioTech Company, San Francisco, USA) was used for absorbance measurements at 570 nm.

Isolation of cellular and virion KSHV DNA

Logarithmic growing BC-3 cells were suspended in RPMI1640 medium with 20 ng/mL TPA and incubated of 20 hours. The cells were seeded at a density of 2×10^5 cells/mL in 24-well plates. The cells were subsequently incubated with various concentrations of GCV, GA, Allicin, EGCG, and drug-free RPMI1640 control medium. After 48 hours incubation, medium was replaced with fresh medium containing the drug for incubation of another 48 hours. Subsequently, cells were harvested by centrifugation at $800 \times g$ for 5 minutes. In addition, the resulting supernatant was centrifuged at $5000 \times g$ for 10 minutes and the pellet was suspended in 100 μL of DEPC-treated water. Subsequently, 10 μL of 10 × DNase I Buffer, 20 U of DNaseI (RNANase-Free), and 20 U of RNase inhibitor were added and the mixtures were incubated at 37°C for 30 minutes, followed by incubation at 65°C for 30 minutes to catalyze inactivation of DNaseI. The Viral DNA extraction kit (Sangon Biotech Co., Ltd., Shanghai, China) was finally used to extract cellular KSHV DNA from the BC-3 cells and virion KSHV DNA from the cell culture supernatant fraction according to the manufacturer's instructions.

Quantitative determination of KSHV DNA

Quantification of KSHV DNA was performed as described in details previously,[19] using the fluorogenic Taqman probe FAM-5′-TTCCCCATGGTCGTGCCGC-3′-TAMRA, and the ORF26-specific primers 5′-GCTCGAGTCCAACGGATTG-3′ and 5′-AATGGCGTGCCCCAGTTGC-3′. Standard curves with serial ten-fold dilutions of plasmid DNA ranging from 10^3 to 10^7 copies were generated in parallel with each analysis. Reactions were run on a Chrom4 Automatic real-time PCR instrument (Bio-Rad, Hercules, California, USA) using initial steps of 2 minutes at 50°C and 10 minutes at 95°C, and 35 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Statistical analysis

All graphs represent the mean and standard deviation of three biological independent experiments. Quantitative data were analyzed by one-way ANOVA using GraphPad Prism 5.0 (San Diego, CA, USA).

References

[1] Cesarman E, Damania B, Krown SE, Martin J, Bower M, Whitby D. Kaposi sarcoma. *Nat Rev Dis Prim* 2019;5(1):9.

[2] Lukac DM, Kirshner JR, Ganem D. Transcriptional activation by the product of open reading frame 50 of Kaposi's sarcoma-associated herpesvirus is required for lytic viral reactivation in B cells. *J Virol* 1999;73(11):9348–9361.

[3] Miller G, Heston L, Grogan E, et al. Selective switch between latency and lytic replication of Kaposi's sarcoma herpesvirus and Epstein-Barr virus in dually infected body cavity lymphoma cells. *J Virol* 1997;71:314–324.

[4] Paulose-Murphy M, Ha N-K, Xiang C, et al. Transcription program of human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus). *J Virol* 2001;75(10):4843–4853.

[5] Medveczky MM, Horvath E, Lund T, Medveczky PG. In vitro antiviral drug sensitivity of the Kaposi's sarcoma-associated herpesvirus. *AIDS* 1997;11(11):1327–1332.

[6] Kedes DH, Ganem D. Sensitivity of Kaposi's sarcoma-associated herpesvirus replication to antiviral drugs: Implications for potential therapy. *J Clin Invest* 1997;99(9):2082–2086.

[7] Uldrick TS, Polizzotto MN, Aleman K, et al. High-dose zidovudine plus valganciclovir for Kaposi sarcoma herpesvirus-associated multicentric Castleman disease: a pilot study of virus-activated cytotoxic therapy. *Blood* 2011;117(26):6977–6986.

[8] Curreli F, Friedman-Kien AE, Flore O. Glycyrrhizic acid alters Kaposi sarcoma-associated herpesvirus latency, triggering p53-mediated apoptosis in transformed B lymphocytes. *J Clin Invest* 2005;115(3):642–652.

[9] Tsai CY, Chen CY, Chiou YH, et al. Epigallocatechin-3-gallate suppresses human herpesvirus 8 replication and induces ROS leading to apoptosis and autophagy in primary effusion lymphoma cells. *Int J Mol Sci* 2018;19(1):E16.

[10] Speicher DJ, Johnson NW. Detection of human herpesvirus 8 by quantitative polymerase chain reaction: development and standardisation of methods. *BMC Infect Dis* 2012;12:210.

[11] Ablashi DV, Chatlynne LG, Whitman JE, Cesarman E. Spectrum of Kaposi's sarcoma-associated herpesvirus, or human herpesvirus 8, diseases. *Clin Microbiol Rev* 2002;15(3):439–464.

[12] Moore PS, Boshoff C, Weiss RA, Chang Y. Molecular mimicry of human cytokine and cytokine response pathway genes by KSHV. *Science (80-)* 1996;274(5293):1739–1744.

[13] Nicholas J, Ruvolo VR, Burns WH, et al. Kaposi's sarcoma-associated human herpesvirus-8 encodes homologues of macrophage inflammatory protein-1 and interleukin-6. *Nat Med* 1997;3(3):287–292.

[14] Neipel F, Albrecht JC, Ensser A, et al. Human herpesvirus 8 encodes a homolog of interleukin-6. *J Virol* 1997;71(1):839–842.

[15] Ma W, Galvin TA, Ma H, Ma Y, Muller J, Khan AS. Optimization of chemical induction conditions for human herpesvirus 8 (HHV-8) reactivation with 12-O-tetradecanoyl-phorbol-13-acetate (TPA) from latently-infected BC-3 cells. *Biologicals* 2011;39(3):158–166.

[16] Yan L, Majerciak V, Zheng ZM, Lan K. Towards better understanding of KSHV life cycle: from transcription and posttranscriptional regulations to pathogenesis. *Virol Sin* 2019;34(2):135–161.

[17] Sin SH, Roy D, Wang L, et al. Rapamycin is efficacious against primary effusion lymphoma (PEL) cell lines in vivo by inhibiting autocrine signaling. *Blood* 2007;109(5):2165–2173.

[18] Son M, Lee M, Sung GH, et al. Bioactive activities of natural products against herpesvirus infection. *J Microbiol* 2013;51(5):545–551.

[19] Xie Y, Ruan B, Chen Y, Wu N, Hu M, Zhu B. Kaposi's sarcoma-associated herpesvirus infection in Chinese patients with chronic hepatitis B. *J Med Virol* 2011;83(5):879–8831.

[20] Aviello G, Abenavoli L, Borrelli F, et al. Garlic: empiricism or science? *Nat Prod Commun* 2009;4(12):1785–1796.

[21] Hsu S. Compounds derived from epigallocatechin-3-gallate (EGCG) as a novel approach to the prevention of viral infections. *Inflamm Allergy-Drug Targets* 2015;14(1):13–18.

[22] Colpitts CC, Schang LM. A small molecule inhibits virion attachment to heparan sulfate- or sialic acid-containing glycans. *J Virol* 2014;88 (14):7806–7817.

[23] Patrick Reid S, Shurtleff AC, Costantino JA, et al. HSPA5 is an essential host factor for Ebola virus infection. *Antiviral Res* 2014;109:171–174.