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Research Article

Ginsenoside Rg5, a potent agonist of Nrf2, inhibits HSV-1 infection-induced neuroinflammation by inhibiting oxidative stress and NF-κB activation

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ABSTRACT

Background: Herpes simplex virus type 1 (HSV-1), known to latently infect the host's trigeminal ganglion, can lead to severe herpes encephalitis or asymptomatic infection, potentially contributing to neurodegenerative diseases like Alzheimer's. The virus generates reactive oxygen species (ROS) that significantly impact viral replication and induce chronic inflammation through NF-κB activation. Nuclear factor E2-related factor 2 (Nrf2), an oxidative stress regulator, can prevent and treat HSV-1 infection by activating the passive defense response in the early stages of infection.

Methods and results: Our study investigated the antiviral effects of ginsenoside Rg5, an Nrf2 activator, on HSV-1 replication and several host cell signaling pathways. We found that HSV-1 infection inhibited Nrf2 activity in host cells, induced ROS/NF-κB signaling, and triggered inflammatory cytokines. However, treatment with ginsenoside Rg5 inhibited ROS/NF-κB signaling and reduced inflammatory cytokines through NRF2 induction. Interestingly, the Nrf2 inhibitor ML385 suppressed the expression of NAD(P)H quinone oxidoreductase 1(NQO1) and enhanced the expression of KEAP1 in HSV-1 infected cells. This led to the reversal of VP16 expression inhibition, a protein factor associated with HSV-1 infection, thereby promoting HSV-1 replication.

Conclusion: These findings suggest for the first time that ginsenoside Rg5 may serve as an antiviral against HSV-1 infection and could be a novel therapeutic agent for HSV-1-induced neuroinflammation.

1. Introduction

Herpes simplex virus type 1 (HSV-1) primarily infects the mouth, pharynx, face, eyes, and central nervous system (CNS), and is a highly contagious virus that affects approximately 60 %–95 % of adults worldwide [1–3]. Notably, HSV-1 encephalitis leads to severe inflammation and neurological dysfunction in the CNS, presenting various clinical symptoms such as cognitive dysfunction, aphasia, seizures, and personality changes [4]. Despite the use of antiviral drugs, most patients with HSE suffer from severe neurological sequelae [5]. While antiviral research on HSV-1 is active, there is a lack of research on the treatment and mechanisms of neurological diseases caused by this infection.

Recent studies have shown that oxidative stress induced by viral infection is one of the major pathogenic mechanisms and disturbs the balance of the redox cycle in cells, resulting in inflammatory responses and tissue damage [6–8]. The ROS produced during HSV-1 infection play a vital role in viral replication [9]. They are also implicated as a secondary mechanism driving tissue damage in the context of viral encephalitis [6]. Therefore, the cytopathic effects observed during viral encephalitis aren't solely attributable to viral replication; host-mediated secondary damage mechanisms, including oxidative stress, also contribute [6,10,11]. Chronic exposure to HSV-1, leading to persistent inflammation and neuronal damage, may heighten the risk of chronic inflammatory brain diseases linked to neurodegeneration. Consequently, the development of antiviral therapies targeting oxidative stress holds promise in mitigating nerve damage caused by HSV-1 infections.

Nuclear factor E2-related factor 2 (Nrf2) is a well known central transcription factor responsible for orchestrating the cellular response to oxidative stress. It plays a crucial role in determining viral susceptibility, managing virus-associated inflammation, and aiding in immune clearance [7,12,13]. In stress-free conditions, Nrf2 is typically expressed at low levels due to its interaction with Kelch-like ECH-associated protein 1 (KEAP1). This interaction leads to Nrf2's ubiquitination and subsequent degradation. However, when cellular stress signals are triggered, Nrf2

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Abbreviations

HSV-1	Herpes simplex virus-1
IE	Immediate early
CNS	Central nervous system
ROS	Reactive oxygen species
NQO1	NAD(P)H quinone oxidoreductase 1
Nrf2	Nuclear factor E2-related factor 2
KEAP1	Kelch-like ECH-associated protein 1
HO-1	heme oxygenase-1
AMPK	AMP-activated protein kinase
AREs	antioxidant response elements
PPI	protein-protein interaction

dissociates from KEAP1, relocates to the nucleus, binds to DNA sequences known as antioxidant response elements (AREs), and activates protective genes like heme oxygenase-1 (HO-1) and NQO1, shielding cells from the detrimental effects of various stresses [8]. Previous research in mice has demonstrated that Nrf2 deficiencies can result in severe airway inflammation, chronic obstructive pulmonary disease, pulmonary fibrosis, asthma, and enhanced susceptibility to infections [14,15]. This underscores Nrf2's role in bolstering the host's defense against a wide array of stimuli, including infections. However, despite these insights, the precise involvement of Nrf2 in the development of brain diseases resulting from HSV infection, particularly in the context of natural products, remains unclear. Additionally, research concerning the antiviral effects and the mechanisms underlying Nrf2 activity in this context is currently lacking.

Ginsenoside Rg5 (Fig. 1A) is a type of ginsenoside, which is a class of natural compounds found in the roots of the ginseng plant (Panax ginseng). Ginsenosides are often referred to as the active ingredients of ginseng and are known for their various pharmacological effects [16,

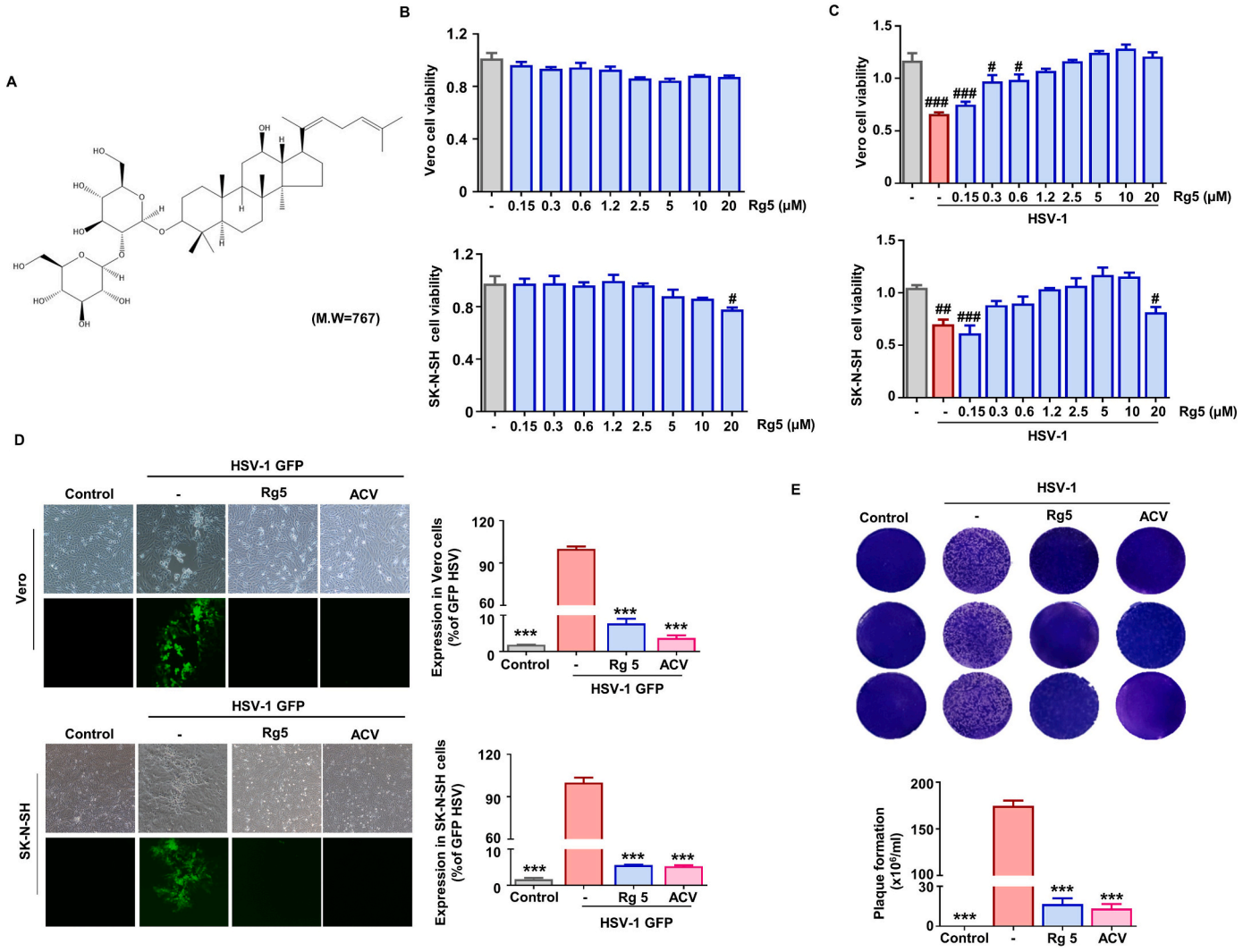

Fig. 1. Ginsenoside Rg5's effect on HSV-1 infection in Vero and SK-N-SH cells. (A) The chemical structure of ginsenoside Rg5. (B) Vero (top) and SK-N-SH (bottom) cells (1×10^4 cells/ml) were exposed to varying concentrations of ginsenoside Rg5. (C) Vero (top) and SK-N-SH (bottom) cells were infected with HSV-1 strains (MOI = 0.1) for 2 h, followed by treatment with ginsenoside Rg5 at the indicated concentrations for 48 h. Cell viability was assessed using CCK-8 assays. (D) Vero (top) and SK-N-SH (bottom) cells were infected with HSV GFP (MOI = 2) for 2 h, then treated with a 10 μM concentration of ginsenoside Rg5 or ACV for 48 h. HSV GFP expression levels were analyzed using fluorescence microscopy (left) and flow cytometry (right). (E) The replication-inhibitory effect of ginsenoside Rg5 during HSV-1 infection was assessed using the plaque method. Vero cells were treated with a 10 μM concentration of ginsenoside Rg5 or ACV after infection with an HSV-1 strain (MOI = 0.1). Cells were cultured at 37 °C for 4 days and stained with crystal violet (top). The average number of plaques is shown (bottom). The data represent the mean ± SEM of three independent experiments. #P < 0.05, ##P < 0.01, ###P < 0.001 vs. Control: ***P < 0.001 vs. HSV-1.

17]. It exhibits various biological activities such as antidermatitic, anti-allergic, platelet anti-aggregating, and radical scavenging [18–21]. However, the antiviral efficacy of ginsenoside Rg5 against HSV-1 infection in neuronal cells and its detailed mechanism remain unreported. This study aims to elucidate the antiviral effect of ginsenoside Rg5 and its mechanism as an Nrf2 activator in brain cells affected by HSV-1 infection. For the first time ever, we investigated the antiviral inhibitory effect of ginsenoside Rg5 in Vero and SK-N-SH cells during HSV-1 infection. Furthermore, we unveiled the underlying molecular mechanism by which ginsenoside Rg5 suppresses ROS and inflammatory factors through Nrf2 activity in human neuroblastoma cells. Lastly, we performed molecular docking to ascertain whether ginsenoside Rg5 acts as an activator of Nrf2.

2. Material and methods

2.1. Materials

In this study, we utilized two cell lines: the Vero epithelial cell line (ATCC, USA, No. CCL-81) and the SK-N-SH human neuroblastoma cell line (ATCC, No. HTB-11). The Vero cells were cultured in Dulbecco's Minimum Essential Medium (DMEM, Hyclone, Logan, UT, USA) supplemented with 10 % fetal bovine serum (FBS, Gibco, NY, USA) and 1 % antibiotics (Gibco). The SK-N-SH cells were cultured in Eagle's Minimum Essential Medium (Hyclone) medium containing 10 % FBS and 1 % antibiotics. The HSV green fluorescent protein (HSV GFP) strain was procured from Imanis Life Sciences (Rochester, MN, USA), and the KBPV-VR-733 (HSV-1 strain) was sourced from the Korea Pathogenic Virus Bank. ginsenoside Rg5 (Sigma-Aldrich, USA, CAS number: 186763-78-0) and ML385 (Sigma-Aldrich, CAS number: 846557-71-9) were diluted to the desired concentrations through a series of dilutions in dimethyl sulfoxide (DMSO, SIGMA).

2.2. Cell viability assay

In brief, Vero and SK-N-SH cells were seeded and exposed to varying concentrations of ginsenoside Rg5, either with or without HSV-1 strains (MOI = 0.1), for a duration of 48 h. Cell viability was evaluated using the CCK-8 assay on a 96-well culture plate, following the method described previously [22].

2.3. HSV-1 GFP expression analysis

The antiviral effect of ginsenoside Rg5 was confirmed using HSV-1 GFP (MOI = 2) as described in a previous study [22]. Expression of HSV GFP was observed using a fluorescence microscope (Nikon ECLIPSE Ti-U, Nikon Co., Japan), and fluorescence expression was quantified using a CytoFLEX flow cell counter (Beckman Coulter Inc., Pasadena, CA, USA) and FlowJo software.

2.4. Plaque reduction assay

Vero cells were infected with HSV-1 strains (MOI = 0.1) for 2 h at 37 °C and then treated with ginsenoside Rg5 at a concentration of 10 μM in 2x complete DMEM containing 1.5 % agarose and cultured for 4 days. Cells were then stained with 1 % crystal violet solution and the resulting plaques were quantified. To compare the effects of drugs, ACV was used as a positive control.

2.5. Nuclear protein extraction and electrophoretic mobility shift assay (EMSA)

Cells were infected with HSV-1 (MOI = 0.1) for a period of 2 h, after which they were treated with ginsenoside Rg5 at concentrations of 5 or 10 μM for 48 h. Subsequently, the nuclei were extracted. The nuclear extracts were isolated using the NE-PER nuclear and cytoplasmic extraction reagent (ThermoFisher Scientific, MA, USA), following the manufacturer's instructions. These extracts were stored at −80 °C until further use. An EMSA was performed using the DIG Gel Shift Kit (Roche), as per the manufacturer's instructions. The nuclear proteins were hybridized with a double-stranded, DIG-labeled oligonucleotide probe containing the consensus binding site for NF-κB: 5′- TTGTTA-CAAGGGACTTTCCGCTG GGGACTTTCCAGGGAGGCGTGG -3′ (the NF-κB binding sites are indicated in bold).

2.6. Measurement of the accumulation of ROS

The intracellular ROS levels and mitochondrial superoxide were assessed using 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) and MitoSOX Red reagents, respectively. SK-N-SH cells were infected with HSV-1 strains (MOI = 0.1) for a duration of 2 h, after which they were treated with ginsenoside Rg5 at concentrations of 10 μM for 48 h. The cells were then treated with 10 μM H2DCFDA (Thermo Fisher Scientific, Waltham, MA, USA) or 5 μM MitoSOX Red (Thermo Fisher Scientific), incubated at 37 °C for 30 min, and observed under a fluorescence microscope (Nikon). The amount of ROS was subsequently measured using flow cytometry (Beckman Coulter, Inc.).

2.7. Western blot analysis

Whole-cell extracts were prepared by lysing cells with RIPA buffer (Thermo Scientific, Waltham, MA, USA), as outlined in a previous study [23]. The extracted proteins were then separated using 10 % SDS-PAGE and transferred to PVDF membranes (Merck Millipore, Burlington, MA, USA) using an electric current. These membranes were subsequently immunoblotted with the primary and secondary antibodies mentioned earlier. The primary antibodies used were against COX-2, IL-6, TNF-α, p-p65, p65, *p*-IκBα, IκBα, Nrf2, HO-1, NQO1, and KEAP1, *p*-AMPKα, AMPKα, β-actin, anti-rabbit IgG, and anti-mouse IgG. From Abcam, Cambridge, UK, we used iNOS, VP16, and gB. From Virusys, Sykesville, MD, we used ICP0, ICP27, ICP4, and Nectin-1. Lastly, goat anti-mouse IgG was purchased from Santa Cruz Biotechnology Inc.

2.8. Real-time PCR

Total RNA was isolated using Trizol reagent as per the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Real-time PCR was evaluated according to previously described methods [22]. Real-time PCR was conducted on cDNA using specific primers for NFE2L2, SQSTM1, GCLM, and GAPDH. The primer sets used for each gene were as follows: NFE2L2, 5-TTCCCGG TCACATCGAGAG-3 and 5-TCCTGTTGCATACCGTCTAAATC-3; GCLM, 5-TGTCTTGGAATGC ACTGTATCTC-3 and 5-CCCAGTAAGGCTGTAAATGCTC-3; SQSTM1, 5-GACTACGACTTGTG TAGCGTCTCGCC-3 and 5-AGTGTCCGTGTTT-CACCTTCC-3; GAPDH, 5-TATGAGAGCTGGGGAATGGGA-3 and 5-ATGGCATGGACTGTGG TCTG-3. For relative quantification, the crossing point (Cp) values for COX-2, iNOS, IL-6, TNF-α, NFE2L2, SQSTM1, and GCLM were normalized to the Cp value of GAPDH.

2.9. In silico docking simulation and pharmacophore analysis of ginsenoside Rg5 to KEAP1

Due to the absence of structural information on the full-length of KEAP1 from X-ray crystallography, its predicted three-dimensional structure was obtained from the AlphaFold Protein Structure Database (model ID: AF-Q14145-F1, available at https://alphafold.ebi.ac.uk/entry/Q14145). To elucidate the mechanism by which ginsenoside Rg5 functions as a KEAP1 inhibitor activating Nrf2, we conducted *in silico* docking simulation using an open-source software AutoDock Vina 1.1.2 (The Scripps Research Institute, La Jolla, CA) integrated with UCSF Chimera 1.17. 3 [24]. The binding affinities of ginsenoside Rg5 to both the KEAP1 Kelch domain (PDB code: 1ZGK) and BTB (bric-a-brac,

tramtrack, broad complex) domains (PDB code: 7EXI) were investigated to explore ginsenoside Rg5's potential as a protein-protein inhibitor disrupting Nrf2:KEAP1 binding and/or as an electrophilic compound inhibiting the assembly of the E3 ligase complex. The binding affinities were determined based on the lowest energy score in the docking simulation. The molecular interactions between Rg5 and each KEAP1 domain were visualized on a 2D diagram using BIOVIA Discovery Studio Visualizer v20.1.0.19295 (Dassault Systèmes, Vélizy-Villacoublay, France).

2.10. Statistical analysis

All experimental results were performed in triplicate each and are expressed as the mean ± SEM derived from three individual experiments. Additionally, our experimental results were professionally evaluated using GraphPad Prism 8.0 for graphical representation and

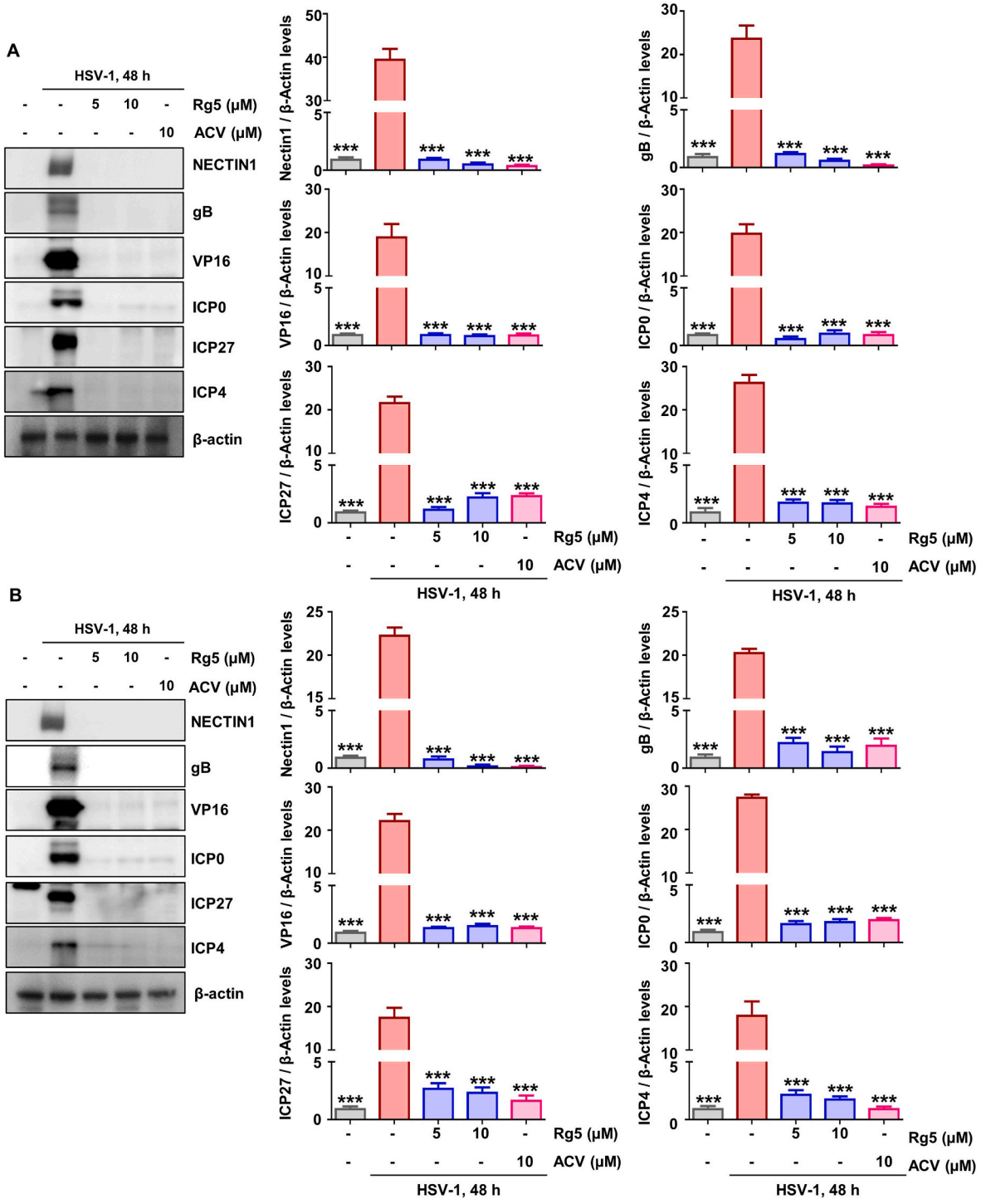

Fig. 2. Ginsenoside RG5's effect on HSV-1 proteins expression in infected cells. (A) Vero cells and (B) SK-N-SH cells were infected with an HSV-1 strain (MOI = 0.1) for 2 h, followed by treatment with 5 and 10 μM of ginsenoside Rg5 for 48 h. ACV, used as a positive control group, was administered at a concentration of 10 μM to compare its antiviral effect. The protein expression of genes involved in HSV-1 infection was measured using Western blot analysis, with β-Actin serving as a control to confirm equal sample loading. The data represent the mean ± SEM of three independent experiments. ***$P < 0.001$ vs. HSV-1.

statistical analysis, employing one-way analysis of variance and Tukey's honest significant difference test. The differences were considered statistically significant when the p-values were less than 0.05.

3. Results

3.1. Ginsenoside Rg5 inhibits cytotoxicity and viral replication caused by HSV-1 infection in vero and SK-N-SH cells

As depicted in Fig. 1B, ginsenoside Rg5 exhibited no toxic effects up to a concentration of 10 μM in both Vero and SK-N-SH cells when treated for 48 h at various concentrations. Moreover, ginsenoside Rg5 treatment for 48 h was shown to restore cell viability up to a concentration of 10 μM, counteracting the cytotoxicity caused by HSV-1 infection (Fig. 1C). Subsequent experiments therefore investigated the antiviral effect and mechanism of ginsenoside Rg5 at concentrations up to 10 μM. The antiviral effect of ginsenoside Rg5 on GFP expression was determined using fluorescence microscopy and flow cytometry, utilizing the HSV-1GFP virus. The results confirmed that the increase in GFP expression due to HSV infection was suppressed by ginsenoside Rg5 in both Vero cells (left) and SK-N-SH cells (right) (Fig. 1D). The antiviral effect of ginsenoside Rg5 was further assessed by measuring the production of infectious HSV-1 virions *in vitro* in HSV-1-infected Vero cells using a plaque assay. The results confirmed the anti-HSV-1 effect by demonstrating a reduction in the production of HSV-1 virions during ginsenoside Rg5 treatment (Fig. 1E). Therefore, ginsenoside Rg5 is a safe and effective antiviral agent against HSV-1 *in vitro*, protecting cells from HSV-1 infection at subtoxic concentrations.

3.2. Ginsenoside Rg5 reduced the expression of HSV-1 proteins in HSV-1-infected cells

HSV-1 causes lytic infection in mucosal epithelial cells and is expressed in a transient cascade. HSV-1 glycoproteins are necessary for HSV-1 entry and cell fusion [25,26]. Immediate early (IE) proteins begin to be expressed as soon as viral DNA enters the nucleus. These proteins stimulate the expression of early (E) proteins, which are largely involved in viral DNA replication [27]. In this context, we sought to determine whether ginsenoside Rg5 inhibits these related proteins during lytic infection in two cell types: Vero (Fig. 2A) and SK-N-SH (Fig. 2B) cells.

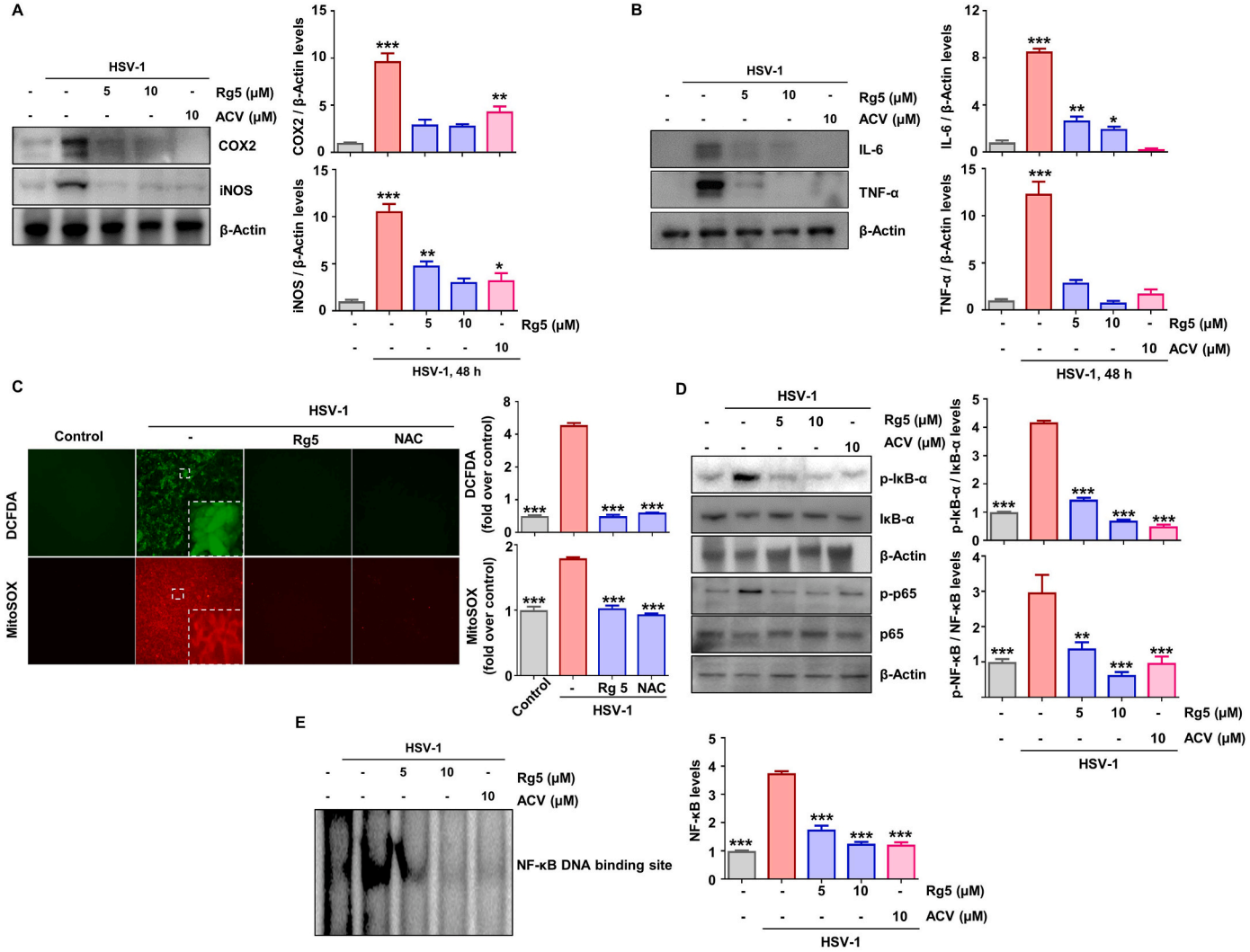

Fig. 3. Ginsenoside Rg5's effect on proinflammatory genes and inflammatory cytokines in HSV-1-Infected SK-N-SH cells. SK-N-SH cells were infected with an HSV-1 strain (MOI = 0.1) for 2 h, followed by treatment with 5 μM and 10 μM of RG5 for 48 h. (A) COX-2, iNOS (B) IL-6 and TNF-α were measured at protein levels. (C) DCFDA (top) was used to measure intracellular ROS levels, and MitoSOX (bottom) red reagent was used to measure mitochondrial peroxide. ROS levels were then measured using fluorescence microscopy (100×) (right) and flow cytometry (left). (D) Western blot analysis was performed with anti-phospho IκBα, IκBα, anti-phospho p65 and p65 antibodies, with β-actin serving as an internal control. (E) DNA binding analysis of NF-κB was performed using EMSA. The data represent the mean ± SEM of three independent experiments. ***P < 0.001 vs. HSV-1.

First, the expression levels of Nectin-1 and gB, an HSV envelope glycoprotein, were found to be suppressed by ginsenoside Rg5. Additionally, the expression levels of IE genes such as VP16, ICP0, ICP4, and ICP27 were also reduced by ginsenoside Rg5. These results suggest that ginsenoside Rg5 exhibits its antiviral activity by inhibiting cell entry through the suppression of Nectin-1 and GB expression, and by reducing the level of IE gene expression through the suppression of the VP16 factor.

3.3. Ginsenoside Rg5 suppressed the expression of proinflammatory genes and inflammatory cytokines in SK-N-SH cells infected with HSV-1

In the progression of viral encephalitis caused by HSV-1, the COX-2 and iNOS by PGE2 are key inflammatory factors [28,29]. We evaluated the effect of ginsenoside Rg5 on the expression of COX2 and iNOS in SK-N-SH cells infected with HSV-1. Ginsenoside Rg5 significantly reduced both COX2 and iNOS protein (Fig. 3A) expression at concentrations of 5 and 10 μM. Furthermore, since overexpression of COX-2 and iNOS induces inflammation during HSV-1 infection [30], we measured the expression of inflammatory factors TNF-α and IL-6 at protein levels. We found that the protein levels of TNF-α and IL-6 increased during HSV-1 infection, but ginsenoside Rg5 suppressed these inflammatory factors (Fig. 3B). Our results suggest that ginsenoside Rg5 suppresses neuroinflammation caused by HSV-1 infection.

3.4. Ginsenoside Rg5 inhibits NF-κB-dependent production of ROS induced by HSV-1 infection in SK-N-SH cells

In our study, we examined the production of ROS in HSV-stimulated SK-N-SH cells to understand the role of redox responses. We observed that ginsenoside Rg5 treatment inhibited the inflammatory activity induced by viral infection. To quantify intracellular ROS levels resulting from the virus infection, we utilized fluorescence microscopy and flow cytometry with H2DCFDA and Mito SOX. Our findings revealed that virus infection led to a significant increase in ROS production in SK-N-SH neuroblastoma cells, which was effectively suppressed by ginsenoside Rg5 treatment (Fig. 3C). Furthermore, ROS activity has been previously linked to the enhancement and prolongation of cytokine-induced NF-κB activation [31]. Considering the well-established fact that HSV-1 infection induces NF-κB activation across various cell types [6,32]. Therefore, we investigated whether IκBα and p65 were constitutively phosphorylated in HSV-1-infected cells. Our findings indicated that both IκBα and p65 (Fig. 3D) exhibited constitutive phosphorylation upon HSV-1 infection. However, we also observed that ginsenoside Rg5 treatment effectively inhibited the phosphorylation of both IκBα and p65 in infected cells. Subsequently, we assessed the impact of ginsenoside Rg5 on constitutive NF-κB activation in HSV-1-infected SK-N-SH cell lines. Through EMSA investigation, we confirmed that ginsenoside Rg5 treatment at a concentration of 10 μM completely inhibited NF-κB downregulation induced by HSV-1 (Fig. 3E).

3.5. Nrf2 expression by ginsenoside Rg5 inhibited HSV-1 infection protein levels

To determine whether Rg5 is an activator of Nrf2, we examined protein expression over time at 10 μM, a concentration that has antiviral effects. Ginsenoside Rg5 increased the protein expression of Nrf2, HO-1, and NQO-1 in SK-N-SH cells in a time-dependent manner (Fig. 4A). Next, we confirmed the protein expression of VP16, one of the HSV-1 infection factors, and Nrf2 at 24 h and 48 h after HSV-1 infection. The results showed that Nrf2 expression was suppressed and VP16 increased during 24 h and 48 h of HSV-1 infection, while Nrf2 activity and VP16 suppression were confirmed in ginsenoside Rg5 (Fig. 4B).

3.6. Ginsenoside Rg5 inhibits viral replication through activating the Nrf2 pathway

Given the previous results that showed ginsenoside Rg5 induces Nrf2 to suppress HSV-related protein expression, we analyzed the mRNA expression levels of Nrf2 target genes in HSV-1-infected cells. It was found that the expression of Nrf2-regulated genes NFE2LE, SQSTM1, and GCLM decreased in HSV-1-infected cells. However, this decrease was reversed by ginsenoside Rg5 (Fig. 5A). While the exact mechanism by which oxidative stress induces Nrf2 activation remains unclear, previous studies have suggested that downstream proteins such as Nrf2 and HO-1 can be enhanced by the activation of upstream kinases like AMP-activated protein kinase (AMPK) [33]. In this study, we observed that VP16 expression was increased during HSV-1 infection, but AMPKα phosphorylation and Nrf2 activity were inhibited (Fig. 5B). On the other hand, we confirmed that AMPKα phosphorylation was increased and Nrf2 protein expression was induced by ginsenoside Rg5 treatment during HSV-1 infection, while Keap1 expression was suppressed (Fig. 5B).

3.7. ML385, an Nrf2 inhibitor, promoted viral replication, but ginsenoside Rg5, an activator of Nrf2, inhibited viral replication

To further assess ginsenoside Rg5's antiviral effectiveness, especially as an Nrf2 activator, we conducted a protein expression analysis using ML385, a known Nrf2 inhibitor. In uninfected cells, we confirmed the modulation of Nrf2 by both ginsenoside Rg5 and ML385. However, in HSV-1 infected cells, the effects were strikingly different. Under the influence of ginsenoside Rg5, Nrf2 and NQO1 expression increased, and the expression of KEAP1 was suppressed, resulting in the absence of VP16 expression (Fig. 5C). Conversely, ML385, unlike ginsenoside Rg5, failed to induce Nrf2 activity and led to increased VP16 expression (Fig. 5C). These observations highlight that inhibiting Nrf2 expression effectively diminishes the cellular antioxidant capacity. They also underscore the pivotal role of the Nrf2-ARE pathway in the cellular defense against viral infections.

3.8. Ginsenoside Rg5 activates the Nrf2-mediated antioxidant response by inhibiting the degradation of Nrf2 through the blockade of Nrf2:KEAP1 interaction

Indeed, Nrf2 expression is typically maintained at a basal level through its interaction with cysteine-rich KEAP1, which acts as an E3 ligase adapter [34,35]. This interaction leads to the formation of an E3 ligase complex comprised of Cullin3 and RBX1 proteins (CUL3/RBX1) within the ubiquitin proteasome system. This results in Nrf2 degradation and ensures its homeostatic regulation. However, under oxidative stress conditions, cysteine residues within KEAP1, particularly the highly reactive Cys151, respond to reactive oxygen species and electrophiles. This weakens the binding affinity of KEAP1 for Nrf2 [35–37]. As a result, activated Nrf2 translocates into the nucleus and upregulates the expression of cytoprotective genes related to antioxidation and detoxification. In clinical trials, Nrf2 activators that target KEAP1 to enhance cytoprotective effects are primarily being developed. These include protein-protein interaction (PPI) inhibitors that disrupt the binding between Nrf2 and the KEAP1 Kelch domain, as well as electrophilic compounds that interact with the KEAP1 BTB (bric-a-brac, tramtrack, broad complex) domain. Particularly important is Cys 151, which plays a pivotal role in the assembly of the E3 ligase complex [35].

In this study, we conducted *in silico* docking simulations to investigate the binding affinity of ginsenoside Rg5 to the KEAP1 Kelch and BTB domains, which play essential roles in the degradation and activation of Nrf2(Fig. 6). The results indicated that ginsenoside Rg5 displayed a higher binding affinity toward the Kelch domain compared to the BTB domain, with binding energy scores (ΔG) of 8.0 kcal/mol and −7.2 kcal/mol, respectively. This suggests that ginsenoside Rg5 is more likely to

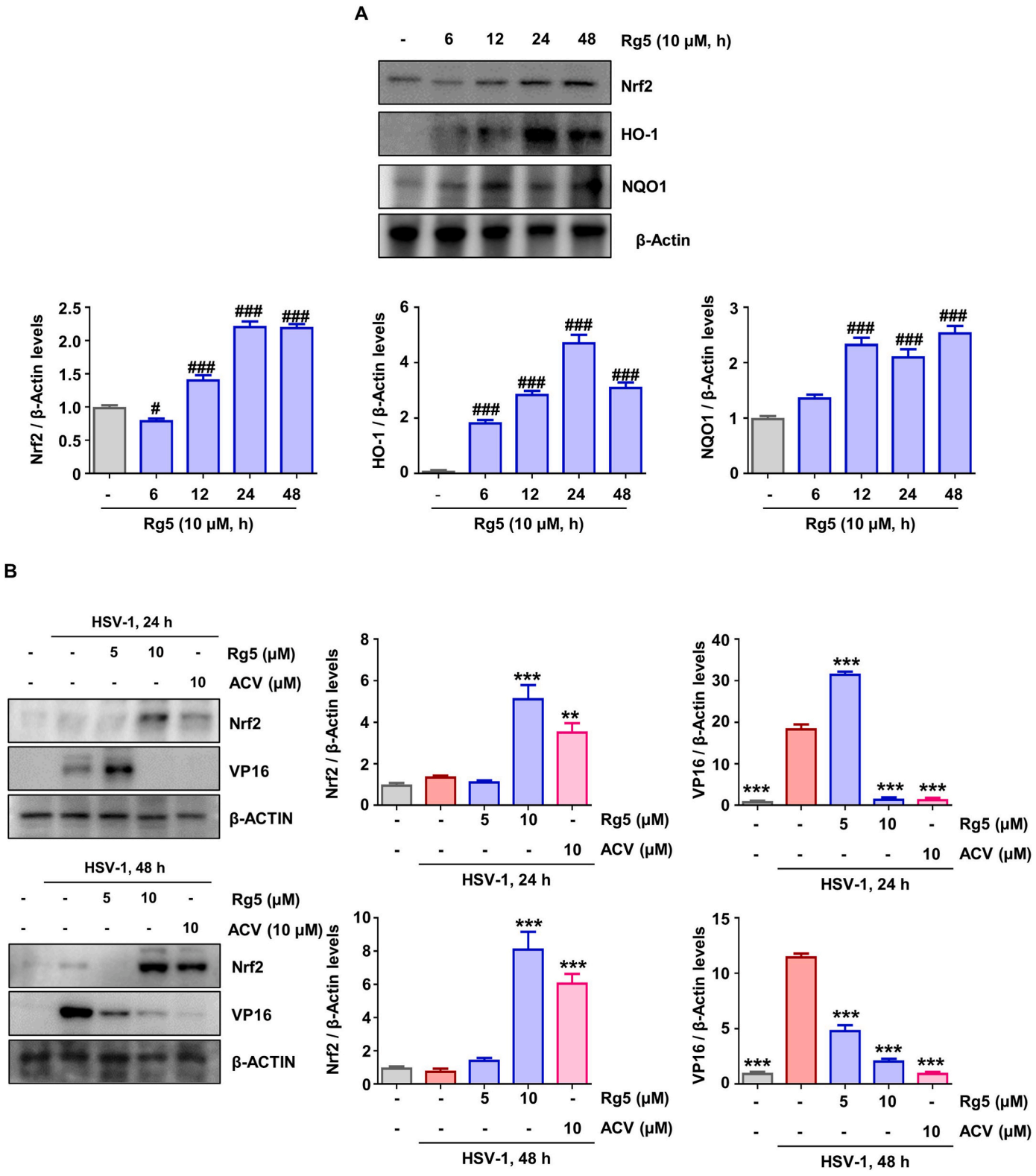

Fig. 4. Ginsenoside Rg5's effect on Nrf2 expression in inhibiting HSV-1 infection protein levels. (A) SK-N-SH cells were treated with 10 μM ginsenoside Rg5 at various times and protein, expression of Nrf2, HO-1, and NQO1 was confirmed through Western blotting. (B) SK-N-SH cells were infected with HSV-1 and then treated with ginsenoside Rg5 at a concentration of 5 or 10 μM for 24 h (top) and 48 h (bottom). Protein expression for Nrf2 and VP16 was analyzed. The data represent the mean ± SEM of three independent experiments: #P < 0.05, ##P < 0.01, ###P < 0.001 vs. control; **P < 0.01, ***P < 0.001 vs. HSV-1.

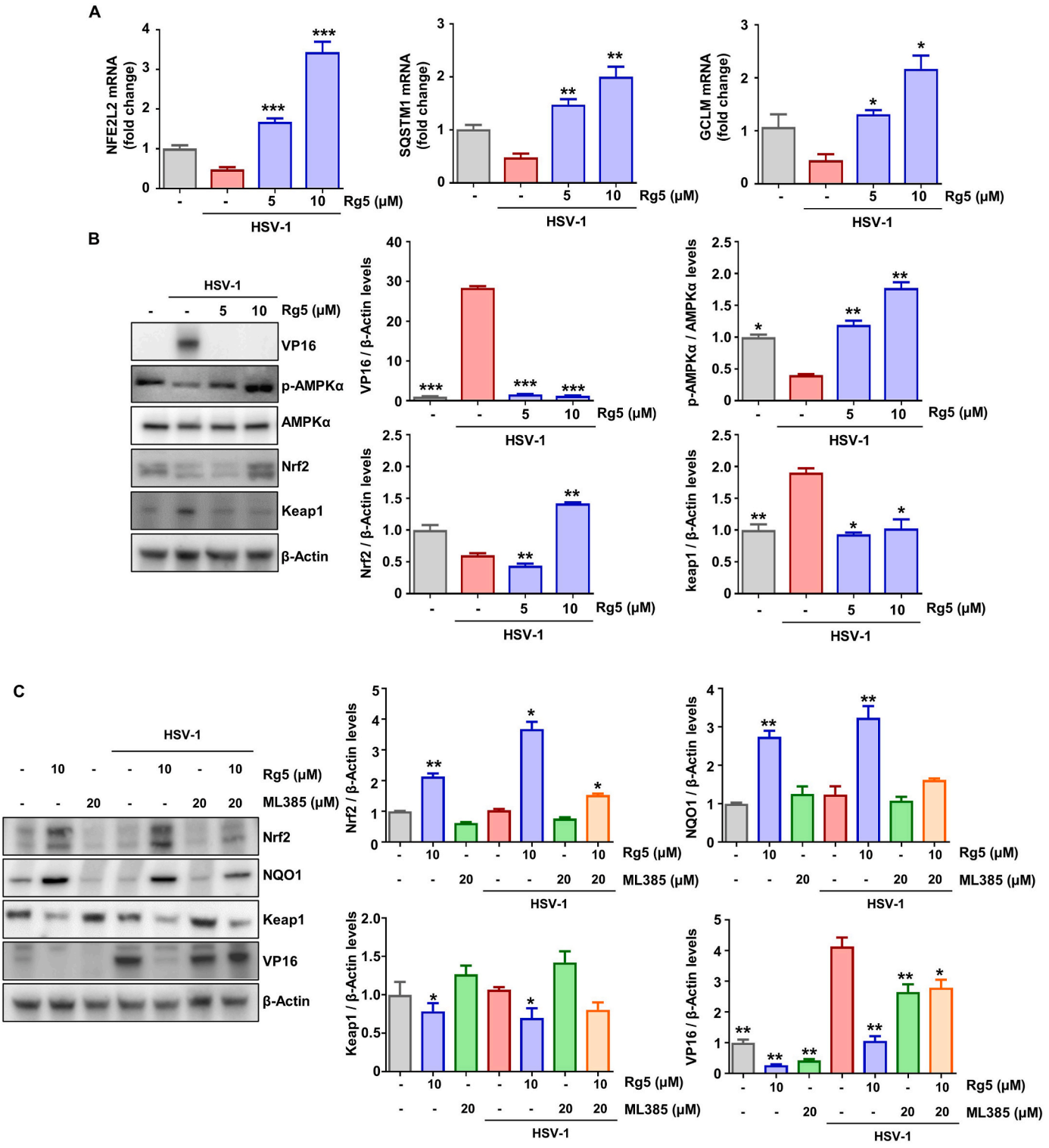

Fig. 5. Ginsenoside Rg5's Effect on Nrf2 induction via AMPK phosphorylation in HSV-1 infected cells. SK-N-SH cells were infected with an HSV-1 strain (MOI = 0.1) for 2 h, followed by treatment with 5 μM and 10 μM of ginsenoside Rg5 for 48 h. (A) The mRNA levels of NFE2L2, SQSTM1, and GCLM were measured using real-time PCR. (B) Protein expression of VP16, AMPK, NRF2, and Keap1 was measured when treated with different concentrations of ginsenoside Rg5 during HSV-1 infection. (C) SK-N-SH cells were infected with an HSV-1 strain (MOI = 0.1) for 2 h, then treated with 10 μM ginsenoside Rg5 and 20 μM Nrf2 inhibitor ML385 for 48 h. Expression of Nrf2, HO-1, NQO1, and the viral protein VP16 was confirmed by Western blot analysis. The data represent the mean ± SEM of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 vs. HSV-1.

function as a PPI inhibitor, targeting the Kelch domain, rather than acting as an electrophile (Fig. 6A and Supplementary Fig. 1). Furthermore, the binding of Nrf2 to KEAP1 hinges on critical interactions involving residues within the Nrf2 Neh domain, particularly 79Glu-Thr-Gly-Glu82 (high affinity) and 29Asp-Leu-Gly31 (low affinity), which interact with their corresponding counterparts within the KEAP1 Kelch domain, Cys434 and Cys489. In the docking modes of ginsenoside Rg5, pharmacophore analysis unveiled its potential to disrupt the binding

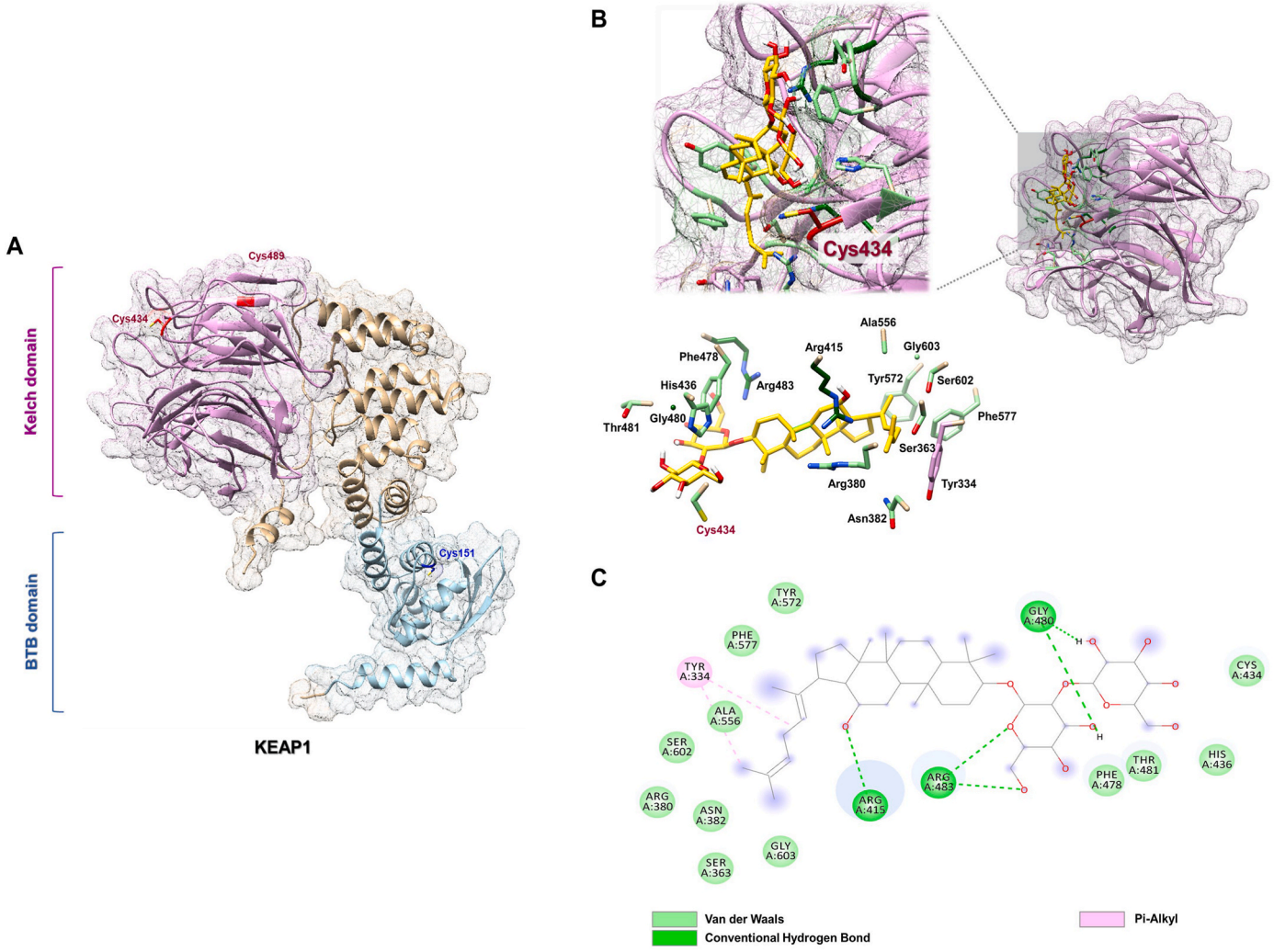

Fig. 6. Molecular docking simulation of ginsenoside Rg5's for Kelch-like ECH-associated protein 1 (KEAP1) Kelch domain. (A) The 3-dimensional structure of KEAP1 was predicted using AlphaFold (model ID: AF-Q14145-F1, available at https://alphafold.ebi.ac.uk/entry/Q14145), with an average model confidence (pLDDT) of 89.95. The *N*-terminal region of the model structure from Met1 to Phe52, which showed significantly low pLDDT scores, was truncated. (B) Ginsenoside Rg5 (depicted in yellow) was docked onto the KEAP1 Kelch domain (PDB code: 1ZGK), and (C) pharmacophore analysis revealed that ginsenoside Rg5 binds to the Kelch domain by interacting with amino acid residues, including the key amino acid Cys434 in NRF2:KEAP1 binding.

between Nrf2 and KEAP1 through more favorable interactions with the Kelch domain adjacent to Cys434, rather than Cys489 (Fig. 6B). These interactions involved the formation of five conventional hydrogen bonds, twelve van der Waals interactions, and two pi-alkyl interactions (Fig. 6C). Conversely, our docking simulation of ginsenoside Rg5 with the BTB domain exhibited no interaction with Cys151, which is a key residue involved in the binding of CUL3 with the KEAP1 BTB domain (Supplementary Fig. 1).

4. Discussion

The significant findings of this study are that ginsenoside Rg5 (i) exerts antiviral effects upon HSV-1 infection, (ii) inhibits neuroinflammation caused by oxidative stress induced by HSV-1 infection, and (iii) upregulation of Nrf2. It was confirmed that silencing of Nrf2 promoted viral infection while inhibiting HSV-1 replication [7]. Our findings indicate that ginsenoside Rg5 significantly reduced the expression of HSV-1 proteins, including Nectin, gB, VP16, and IE genes (ICP0, ICP27, etc.), in Vero and SK-N-SH cells even after HSV entered host cells. These results demonstrate that ginsenoside Rg5 effectively blocks HSV replication.

Asymptomatic recurrent reactivation of the central nervous system in HSV-1-infected humans can lead to progressive deterioration of neurological function [38], and HSV-1 is now considered a risk factor for the development of chronic neuroinflammation and is also a significant factor causing neuropathy [30]. To assess the anti-inflammatory effects of ginsenoside Rg5, we evaluated the transcription of proinflammatory genes (iNOS, COX-2) and the release of proinflammatory and anti-inflammatory cytokines [39]. We confirmed that ginsenoside Rg5 significantly reduced the protein and mRNA expression of COX2 and iNOS in SK-N-SH cells infected with HSV-1. Furthermore, the expression of TNF-α and IL-6 was also reduced through inhibition of these proinflammatory factors. Inflammation increases the level of ROS [40], which can cause oxidative stress. It is well known that oxidative stress activates NF-κB [32,41], a key transcription factor in the development of chronic inflammation. We observed ROS production and induction of NF-κB activation during viral infection in neuroblastoma cells, SK-N-SH. However, we confirmed that ROS and NF-κB induced during infection were completely suppressed by ginsenoside Rg5 treatment. Brain damage caused by HSV-1 infection activates the immune inflammatory response in the brain, causing immunopathological brain damage. The direct impact of the virus on nerve cell damage is also a very important aspect [42]. Therefore, this suggests that ginsenoside Rg5 can alleviate immunopathological damage caused by inflammation by inhibiting

inflammation caused by HSV-1.

Nrf2 stands out as a key transcription factorin dealing with oxidative stress by regulating downstream cellular antioxidant responses. Recent research has unveiled that the Nrf2-ARE pathway not only regulates oxidative stress but also plays a role in regulating inflammatory and antiviral responses [43]. Overexpression of Nrf2 has been associated with reduced viral replication, decreased expression of influenza A nucleoprotein, and HSV-1 expression [7,43,44]. By activating the NRF2 gene, ginsenoside Rg5 was observed to suppress the expression of VP16, a critical factor in HSV-1 infection, while also increasing NQO1 expression and suppressing Keap1 levels. Conversely, the use of the Nrf2 inhibitor ML385 resulted in an increase in VP16, an indicator of enhanced HSV-1 infectivity. Previous studies have indicated that Nrf2 becomes activated in the early stages of HSV-1 infection, playing a pivotal role in regulating antioxidant responses. Conversely, the silencing of Nrf2 appears to promote viral replication. This study's findings suggest that ginsenoside Rg5 not only regulates oxidative stress but also exerts control over inflammation and antiviral responses during HSV-1 infection by activating the Nrf2-ARE pathway [7]. This research introduces the novel idea that ginsenoside Rg5 can be an effective drug against neuroinflammation through its antiviral actions against HSV-1 infection, achieved by targeting the Nrf2 pathway. Under normal conditions, Nrf2, constitutively expressed, forms a complex with KEAP1, undergoing ubiquitination/degradation processes and thereby maintaining basal expression levels. In constrast, under conditions of oxidative stress, NRF2 becomes activated by dissociating from KEAP1 and the KEAP1-bound E3 ligase complex. This activation leads to the upregulation of antioxidant response elements [35–37]. Notably, *in silico* docking simulations of ginsenoside Rg5 with KEAP1 suggest that ginsenoside Rg5 has the potential to activate NRF2-mediated antioxidant responses. It appears to function as a PPI inhibitor, thereby acting as an NRF2 activator. This occurs through direct interaction with the KEAP1 Kelch domain, particularly Cys434, preventing NRF2 from binding to KEAP1. In contrast, ginsenoside Rg5 does not seem to act as an electrophilic compound by interacting with the KEAP1 BTB domain, specifically Cys151. This domain is responsible for CUL3 binding, facilitating NRF2 degradation (Fig. 6 and Supplementary Fig. 1). The result indicated that Rg5 may contribute to the suppression of viral replication by interfering with the interaction between Nrf2 and KEAP1, consequently preventing Nrf2 ubiquitination/degradation and facilitating Nrf2 activation, which warrants further investigation in future studies.

5. Conclusions

Our research indicates that ginsenoside Rg5 can inhibit viral replication in neuroblastoma cells *in vitro* during HSV-1 infection. Interestingly, when NRF2 was inhibited, the expression of the VP16 protein increased, suggesting that the antiviral activity of ginsenoside Rg5 could be partially compromised. Furthermore, ginsenoside Rg5 treatment was found to decrease the expression of virus-induced inflammatory cytokines *in vitro*. It also protected cells from HSV-1 infection *in vivo* by reducing viral titers and suppressing inflammatory cytokine expression. Collectively, these findings suggest that the antiviral and anti-inflammatory effects of ginsenoside Rg5 are linked to its ability to activate the Nrf2 signaling pathway. This implies that ginsenoside Rg5 could potentially serve as an Nrf2 agonist in the treatment of viral diseases.

CRediT authorship contribution statement

Buyun Kim: Investigation, Methodology, Validation, Funding acquisition, Visualization, Writing – original draft, Writing – review & editing. **Young Soo Kim:** Investigation, Validation, Methodology, Visualization, Writing – original draft. **Wei Li:** Resources, Data curation, Validation, Writing – review & editing. **Eun-Bin Kwon:** Investigation, Methodology, Validation, Writing – review & editing. **Hwan-Suck Chung:** Resources, Writing – review & editing. **Younghoon Go:** Investigation, Methodology, Funding acquisition, Supervision, Writing – review & editing. **Jang-Gi Choi:** Methodology, Supervision, Validation, Writing – review & editing, Funding acquisition, All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2024.01.006.

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