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Inhibitory effects of piceatannol on human cytomegalovirus (hCMV) *in vitro*

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Human cytomegalovirus (hCMV) is a ubiquitous herpesvirus, which results in the establishment of a latent infection that persists throughout the life of the host and can be reactivated when the immunity is low. Currently, there is no vaccine for hCMV infection, and the licensed antiviral drugs mainly target the viral enzymes and have obvious adverse reactions. Thus, it is important to search for compounds with anti-hCMV properties. The present study aimed to investigate the suppressive effects of piceatannol on hCMV Towne strain infection and the putative underlying mechanisms using human diploid fibroblast WI-38 cells. Piceatannol supplementation prevented the lytic changes induced by hCMV infection in WI-38 cells. Furthermore, piceatannol suppressed the expression of hCMV immediate-early (IE) and early (E) proteins as well as the replication of hCMV DNA in a dose-dependent manner. Moreover, hCMV-induced cellular senescence was suppressed by piceatannol, as shown by a decline in the senescence-associated β-galactosidase (SA-β-Gal) activity and decreased production of intracellular reactive oxygen species (ROS). $p16^{INK4a}$, a major senescence-associated molecule, was dramatically elevated by current hCMV infection that was attenuated by pre-incubation with piceatannol in a dose-dependent manner. These results demonstrated that piceatannol suppressed the hCMV infection via inhibition of the activation of $p16^{INK4a}$ and cellular senescence induced by hCMV. Together, these findings indicate piceatannol as a novel and potent anti-hCMV agent with the potential to be developed as an effective treatment for chronic hCMV infection.

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Introduction

Human cytomegalovirus (hCMV), which belongs to the β-herpesvirus subfamily, is a large, enveloped, double-stranded DNA virus, and its 235 kb genome encodes at least 165 proteins (Stern-Ginossar *et al.*, 2012). hCMV typically causes asymptomatic infections in immunocompetent individuals ubiquitously (Hyde *et al.*, 2010). The hCMV infection is one of the primary factors causing neonatal defects, and also a major cause of sensorineural hearing loss and neurological impairments (Grosse *et al.*, 2008; Hyde *et al.*, 2010; Rolland *et al.*, 2016). Usually, the prevalence of hCMV infection is higher in developing countries and among individuals of lower socioeconomic status (Cannon *et al.*, 2010). It is asymptomatic in the majority of the cases because the virus is maintained in a state of latency or low-level shedding, which is clinically undetectable. Nevertheless, hCMV is a major concern in some specific high-risk groups, and the primary infection or reactivation from latency may be critical, especially in immunocompromised patients, including HIV-infected individuals and transplant recipients (Cannon *et al.*, 2010; Ariza-Heredia *et al.*, 2014). Additionally, it is a vital factor in the etiologies of atherosclerosis, coronary artery restenosis (Speir *et al.*, 1994), and inflammatory bowel diseases (Berk *et al.*, 1985). Therefore, the effective treatment for hCMV infection is urgent to improve the quality of the birth population and the quality of life of immunocompromised individuals.

Currently, three licensed antiviral drugs are used for the prevention and/or treatment of hCMV infection: ganciclovir (VAL), foscarnet (FOS), and cidofovir (CDV). These drugs mainly influence the activity of DNA polymerase by targeting the stages of replication and translation period (Biron, 2006; O'Brien *et al.*, 2008; Campos *et al.*, 2016); however, they do not prevent the viral induction of multiple signal transduction events during the infection cycle (Mar *et al.*, 1983, 1985). The prevalence of drug-resistant and cross-resistant strains of hCMV has severely reduced the effectiveness of these drugs. Moreover, these drugs also effectuate obvious adverse reactions, such as bone marrow suppression, nephrotoxicity, neutropenia, thrombocytopenia, and electrolyte disturbance (Ljungman *et al.*, 2001; Castagnola *et al.*, 2004; Ariza-Heredia *et al.*, 2014). Currently, there is no licensed hCMV vaccine, although some progress towards this goal has been made in recent clinical trials (Anderholm *et al.*, 2016).

Piceatannol (3,3′,4,5′-tetrahydroxy-*trans*-stilbene), a hy-

droxylated analog of resveratrol (3,5,4′-trihydroxy-*trans*-stilbene), is a polyphenolic stilbene phytochemical that is abundantly found in passion fruit (*Passiflora edulis*) seeds, grapes, red wine, and white tea (Matsui *et al.*, 2010; Seyed *et al.*, 2016). Some evidence showed that piceatannol has a broad spectrum of beneficial effects, such as vasorelaxant effect (Sano *et al.*, 2011), Sirt1 induction activity (Kawakami *et al.*, 2014), upregulation of expression of endothelial nitric oxide synthase (eNOS) (Kinoshita *et al.*, 2013), and protection of the skin from ultraviolet B (UVB) radiation (Maruki-Uchida *et al.*, 2013). Supplementation with piceatannol also improves the metabolic health, including insulin sensitivity, blood pressure (BP), and heart rate (HR) (Kitada *et al.*, 2017), which is attributable to its antioxidative, anti-inflammatory, and anticancer activities (Surh and Na, 2016).

Drug repurposing is an emerging strategy for antiviral drug discovery (Nukui *et al.*, 2018; Mercorelli *et al.*, 2019). In a previous study, we established a preliminary screening system for anti-hCMV agents (data not published) and discovered the anti-hCMV activity of piceatannol at the concentration of 10 μM. The present study aimed to further investigate the inhibitory effect of piceatannol on hCMV infection and the underlying molecular mechanisms *in vitro*.

Materials and Methods

Cells, virus, reagents, and antibodies

The human diploid fibroblasts (WI-38) and Towne strain of hCMV were obtained from the American Tissue Culture Collection (ATCC). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco-BRL Life Technologies. Piceatannol was purchased from Sigma-Aldrich and dissolved in dimethylsulfoxide (DMSO) as a 10 mM stock solution and stored at -20°C until use. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) was also obtained from Sigma-Aldrich. BCA protein assay kit was procured from Pierce Companies.

Primary antibodies, including anti-$p16^{INK4a}$ and anti-β-actin were purchased from Santa Cruz Biotechnology. The primary antibody of anti-hCMV immediate-early (IE) proteins and primary antibody anti-hCMV early protein (UL44) were purchased from Virusys Corporation. QIAamp DNA Mini Kit was obtained from Qiagen. iQ SYBR Green Supermix Kit and polyvinylidene fluoride (PVDF) membrane were obtained from Bio-Rad Laboratories. Cocktail and Senescence-associated Beta-galactosidase Staining Kit were purchased from Cell Signaling Technology.

Cell culture, hCMV infection, and piceatannol treatment

WI-38 cells were cultured in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells are considered young at the population doubling (PD) ≤ 32 and became replicative senescent at PD ≥ 50. The cumulative PD was calculated as $\log_2$ (D/D0), where D and D0 were defined as the cell density at the time of harvesting and seeding, respectively. All experiments in this study were performed using young cells between 27 and 32 PD unless indicated otherwise.

The stocks of the Towne strain of hCMV were prepared as described previously (Li *et al.*, 2015; Mao *et al.*, 2016). WI-38 cells (2×10^4 cells/cm^2) were cultured in DMEM with 10% FBS; then, the culture medium was replaced with 0.2% FBS to effectuate serum starvation for 48 h for cell synchronization in G phase of the cell cycle before infection. Then, WI-38 cells were infected with hCMV at the multiplicity of infection (MOI) of 0.01 at 37°C for 2 h and then replaced with fresh DMEM containing 0.2% FBS. Mock-infected controls were exposed to an equivalent volume of mock control medium described above. The cell samples were harvested at indicated time points post-infection.

Piceatannol was added 2 h before virus inoculation, and then, the cells were harvested.

MTT assay

MTT assay was performed as described previously (Mao *et al.*, 2010). WI-38 cells were seeded in 96-well plate for 24 h. Subsequently, the culture medium was replaced with that containing piceatannol at different concentrations for an additional 48 h; the control group was also cultured in the same condition without piceatannol. Then, 20 μl MTT (5 mg/ml) was added to each well and incubated for another 4 h. Finally, the reaction was quenched with 200 μl DMSO, and the absorbance of each well was determined spectrophotometrically at 570 nm on a Bio-Rad microplate reader.

Immunofluorescence staining

WI-38 cells were seeded in 96-well plate, and hCMV infection and piceatannol treatment were performed as described above. Mock cells were used as negative controls, while Foscarnet (200 μg/ml)-treated cells were used as a positive control. The cell samples with hCMV infection with or without piceatannol treatment were harvested at 3 dpi and washed twice with phosphate-buffered saline (PBS) and fixed with cold acetone/methanol (1:1) at -20°C for 30 min before air dry. After blocking with 5% non-fat dry milk in PBST for 1 h at room temperature, the fixed cells were incubated with mouse mAbs to IE protein, followed by FITC-conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories). The stained cells were washed three times with PBST and examined under a Zeiss microscope.

Western blot analysis

hCMV infection and piceatannol treatment were performed as described above. Cell samples of hCMV infection with or without piceatannol treatment were harvested at 3 dpi and lysed with cell lysis buffer containing protease inhibitor cocktail; the mock-infected cells were used as control. The protein concentration of the lysate was determined by the BCA assay. An equivalent of 50 μg of protein extracts was resolved by 12% SDS-PAGE and transferred to a PVDF membrane. After blocking with 5% skimmed milk in PBS with 0.05% tween 20 for 1 h, the membranes were probed overnight with primary antibodies at 4°C, as mentioned above, followed by incubation with HRP-conjugated goat anti-mouse IgG or goat anti-rabbit IgG at 37°C for 1 h. The immunoreactive bands were detected using enhanced chemiluminescence (ECL) method.

qPCR

DNA was extracted from harvested cells using QIAamp DNA Mini Kit. qPCR was performed to determine the copy number of hCMV viral DNA using iQ SYBR Green Supermix kit in a reaction volume of 20 µl containing 10 ng of total DNA. The amplification was carried out on Roche Light Cycler480 Real-Time PCR System Detector was used. The primers targeted to the hCMV *UL123* region were employed as described previously (Leng *et al.*, 2011; Mao *et al.*, 2016): forward 5′-TCTGCCAGGACATCTTTCTC-3′ and reverse 5′-GTGA CCAAGGCCACGACGTT-3′. The amplification cycles were performed according to the manufacturer's instructions: 3 min at 95°C, followed by 40 cycles of 5 sec at 95°C and 30 sec at 60°C. Cellular *β-actin* (forward 5′-CTGGAACGGTG AGGTGACA-3′ and reverse 5′-AAGGGACTTCCTGTAA CAATGCA-3′ was used as a reference gene. Each sample was assayed in triplicate. Parallel mock-infected WI-38 and FOS (200 µg/ml)-treated WI-38 cells were used as blank and positive controls, respectively. The relative differences in the expression levels of the target genes in different samples were obtained by -ΔΔCt method. Firstly, the housekeeping gene (*β-actin*) was used to normalize the Ct values of all treatment and control samples, i.e., ΔCt = Ct (sample) - Ct (housekeeping gene). Secondly, the Ct values of the treated experimental samples were compared to those of the control samples. ΔΔCt = ΔCt (sample) - ΔCt (control samples). The relative expression of the target gene is represented by $2^{-\Delta\Delta Ct}$.

SA-β-Gal staining

SA-β-Gal staining was performed according to the manufacturer's instructions. Briefly, cells were washed with PBS twice and fixed in 0.25% glutaraldehyde for 5 min, followed by incubation in the freshly prepared staining solution without CO_2 at 37°C for 3–16 h. The percentage of SA-β-Gal-positive cells (blue-green cells) out of the total number of cells was estimated. The average percentage was obtained from three independent experiments.

Measurement of intracellular reactive oxygen species (ROS)

The intracellular ROS level was estimated as described previously (Mao *et al.*, 2010). Briefly, cells were digested with trypsin and collected for staining with the ROS probe 2′,7′-dichlorodihydrofluorecein diacetate (H_2DCFDA, 10 µM) for 30 min at 37°C in the dark. The intracellular fluorescence intensity was positively correlated to the intracellular ROS production and measured by flow cytometer.

Statistical analysis

Data are expressed as mean ± SD and analyzed using two-way analysis of variance (ANOVA) by SPSS 19.0 software. $P < 0.05$ was considered statistically significant.

Results

Effect of piceatannol on the viability of WI-38 cells

MTT assay was used to detect the potential cytotoxicity of different concentrations of piceatannol in WI-38 cells (PD30). As shown in Fig. 1B, no significant cytotoxicity was detectable at the concentration of 1–100 µM after 48 h of treatment. The half-maximal cytotoxicity concentration was approximately 348 µM, which was much higher than that in the current experiments. Additionally, no significant morphological changes were detected in hCMV infected group with 20 µM piceatannol treatment as compared to the mock-infected controls for 7 days (Fig. 2A). These results suggested that piceatannol did not exert cytotoxicity on the host cell of hCMV *in vitro* within the concentration range detected (1–100 µM).

Antiviral activity of piceatannol on hCMV

We observed the cytopathic effects in host cells infected with hCMV. As shown in Fig. 2A, WI-38 cells infected with hCMV at MOI of 0.01 demonstrated significant morphological changes and plaque formation at 3 dpi. Until 7 dpi, these developed into significant lytic cytopathic effect, while pretreatment of the cells with 20 µM piceatannol showed similar morphology as the mock control without plaque formation. These results indicated that piceatannol exerted a protective effect on the morphology of WI-38 cells with hCMV infection.

In order to observe the hCMV-specific antigens in the infected cells, we carried out an indirect immunofluorescence assay (Fig. 2B). hCMV IE was mainly distributed in the cell nucleus of infected cells, which was similar with the published data (Burgdorf *et al.*, 2011; Botto *et al.*, 2019). When the cells

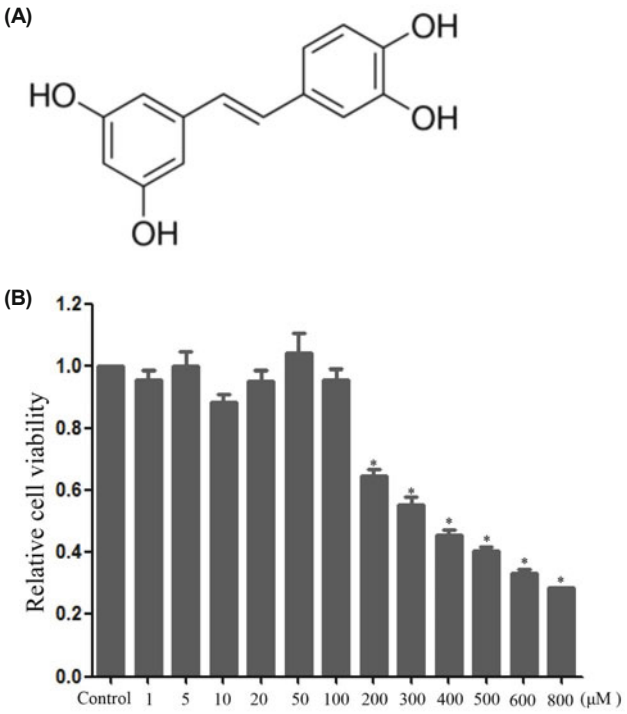

Fig. 1. (A) Chemical structure of piceatannol. (B) Effect of piceatannol on cell viability. WI-38 cells (PD30) seeded in 96-well plates at 3×10^3 cells/well were cultured in DEME-0.2% FBS for 48 h and then treated with piceatannol at indicated concentration for 48 h and then with MTT for 4 h. The reaction was stopped by DMSO and cell viability was determined spectrophotometrically at 570 nm. *$P < 0.001$ vs control.

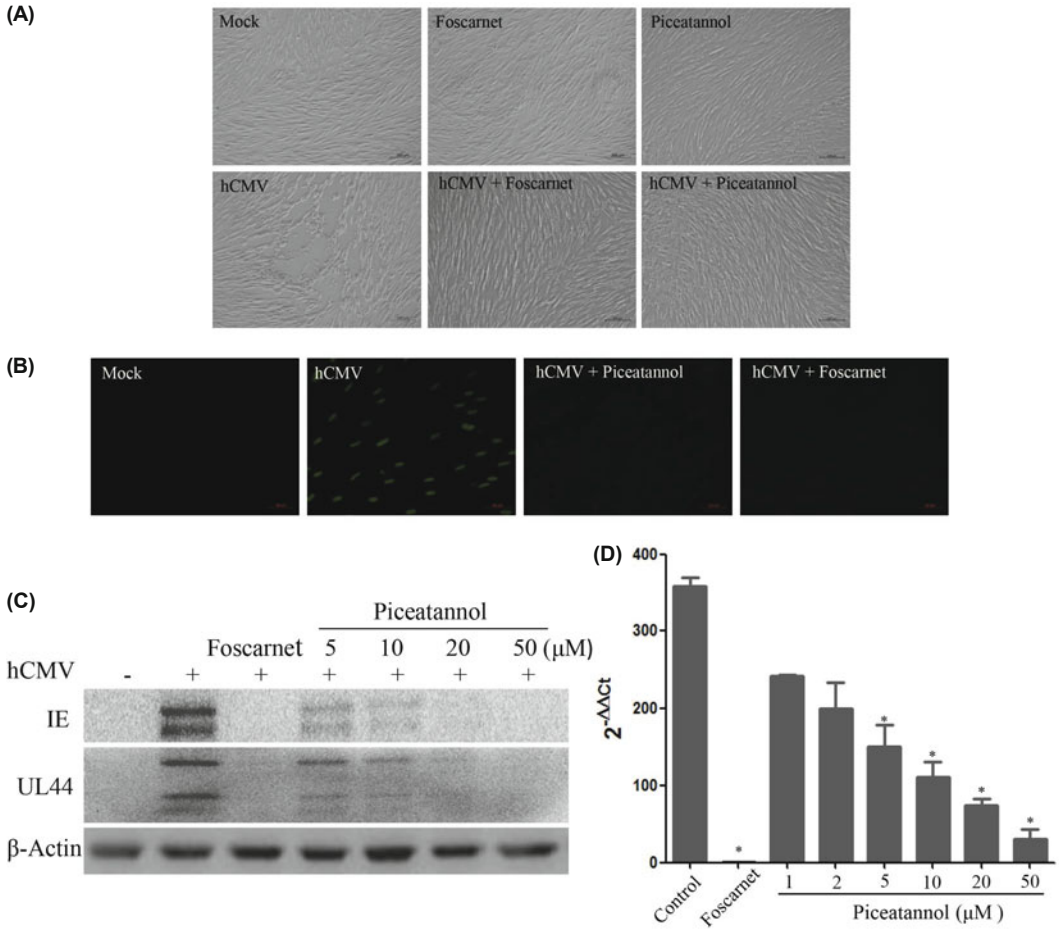

Fig. 2. Antiviral activity of piceatannol on hCMV. Morphological changes (A) and immunofluorescent assay (B) in hCMV infected WI-38 cells with or without pretreatment of piceatannol. WI-38 cells at PD30 were pretreated with piceatannol (20 μM) for 2 h followed by hCMV inoculation at MOI of 0.01 and cultured for additional 7 days (A) and 3 days (B). Representative photographs shown are from three repeated experiments. Foscarnet was used as positive control. (C) Effects of piceatannol on expression of hCMV viral immediate early (IE) and early UL44 protein. Serum starved low passage WI-38 cells (PD30) were treated with piceatannol at the indicated concentrations added 2 h before hCMV inoculation (MOI of 0.01), and then were harvest at 3 days post infection (3 dpi) for protein expression using Western blot analysis. (D) Effects of piceatannol on hCMV viral replication in WI-38 cells. WI-38 cells (PD30) were serum starved and then treated with piceatannol (1 μM, 2 μM, 5 μM, 10 μM, 20 μM, and 50 μM) for 2 h before hCMV inoculation (MOI of 0.01). Cells were harvested at 5 dpi for viral gene determined using qPCR, hCMV treated alone used as control, and Foscarnet used as anti-hCMV positive control. Data were obtained from three independent experiments. * $P < 0.001$ vs Control.

were treated with 20 μM piceatannol, the IE-positive signal could not be detected, indicating a satisfactory anti-hCMV effect of the drug.

Furthermore, we examined the effects of piceatannol on the expression of hCMV IE and early protein (UL44). As shown in Fig. 2C, the 68–72 kDa components of IE decreased to an undetectable level at 72 h after infection at the concentration of 20 μM. UL44 was suppressed significantly at 5 μM concentration and completely inhibited at 50 μM. These results showed that piceatannol significantly suppressed the hCMV protein expression in a dose-dependent manner.

Since piceatannol inhibits IE and E hCMV proteins, hCMV DNA replication occurs directly following the synthesis of viral early proteins (Huang, 1975a, 1975b); thus, we hypothesized that piceatannol blocks viral DNA replication. Next, the viral DNA levels were measured by qPCR in the presence or absence of piceatannol to further confirm the anti-hCMV activity of the drug. As shown in Fig. 2D, hCMV DNA amplification is significantly inhibited by piceatannol, and the half-maximal cytotoxicity concentration was about 5.35 μM. This finding demonstrated that piceatannol has a potent suppressive effect on hCMV infection in host WI-38 cells.

Effects of piceatannol on senescence of WI-38 cells induced by hCMV

WI-38 are host cells of hCMV *in vitro* and have been widely used as a cellular senescence model. Previous studies have shown that hCMV infection induces cellular senescence through IE2 protein, which subsequently elevated the level of $p16^{INK4a}$, a critical senescence-related molecule (Noris *et al.*, 2002; Zannetti *et al.*, 2006; Mao *et al.*, 2016).

To investigate the impact of piceatannol on hCMV-induced activation of molecular mechanisms of senescence, we used SA-β-Gal staining to examine whether hCMV infection would lead to the development of this phenotype in young WI-38

fibroblasts at PD30. As shown in Fig. 3A, WI-38 fibroblasts at PD30 with hCMV infection at MOI of 0.01 showed over 90% SA-β-Gal-positive staining at 3 dpi (Fig. 3A middle panel and B), while less (20%) SA-β-Gal activity was detected in hCMV infection pre-treatment with 20 μM piceatannol (Fig. 3A right panel and B), and mock-infected cells showed only sporadic staining (< 5%) (Fig. 3A left panel and B). These results suggested that piceatannol significantly impedes the senescence phenotype of WI-38 cells, induced by hCMV infection.

Oxidative stress is involved in the process of cellular senescence and hCMV infection (Finkel and Holbrook, 2000; Toussaint *et al.*, 2000; Mao *et al.*, 2010). Herein, we analyzed the effects of piceatannol on the hCMV-induced ROS production. In accordance with our previous study, hCMV infection elevated the ROS production in WI-38 fibroblasts (Mao *et al.*, 2016), while piceatannol reversed this effect with 20 μM showing an optimal effect (Fig. 3C).

Lastly, we investigated the expression level of $p16^{INK4a}$ in hCMV-infected WI-38 cells with or without piceatannol treatment. As shown in Fig. 4, $p16^{INK4a}$ expressed at low level in control WI-38 cells, while the protein level was significantly upregulated with hCMV infection alone but downregulated

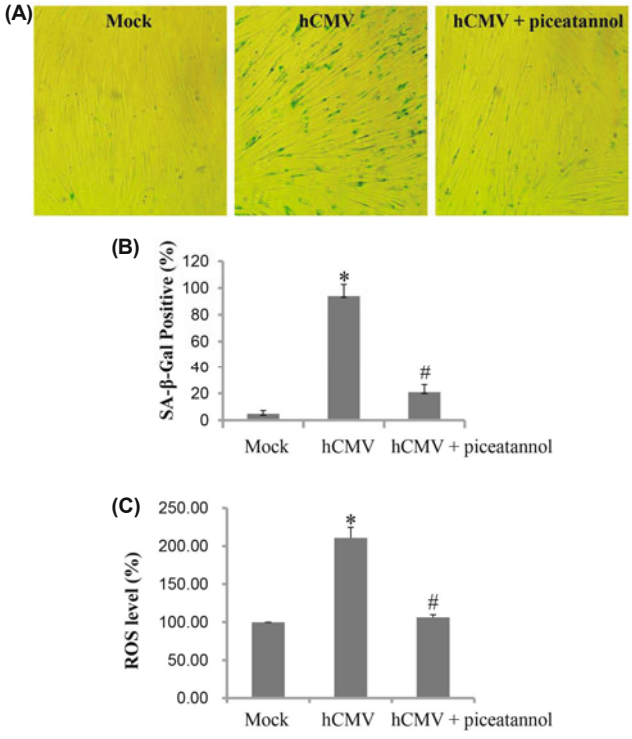

Fig. 3. Effect of piceatannol on hCMV induced cellular senescence and reactive oxygen species production. Young WI-38 cells at PD30 either with Mock infection control, hCMV infection alone at MOI of 0.01, or same hCMV infection with pretreatment of piceatannol (20 μM) were submitted for SA-β-Gal staining or ROS detection at 3 dpi according to the protocol. Representative photos shown were from three repeated experiments (A) and the percentage of SA-β-gal positive cells out of the total cells was counted and the average data was obtained from three independent experiments (B). ROS level was reflecting by the H_2DCFDA mean fluorescent intensity (C). $^{*}P < 0.001$ vs Mock; $^{\#}P < 0.001$ vs hCMV alone.

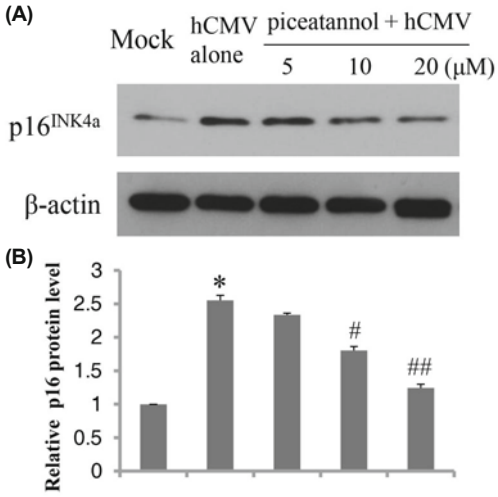

Fig. 4. Effects of piceatannol on hCMV-induced expression of $p16^{INK4a}$ in WI-38 cells. (A) Serum starved low passage WI-38 cells (PD30) were treated by piceatannol at indicated concentration added 2 h before hCMV inoculation (MOI 0.01) and then were harvested at 3 dpi for Western blot analysis. (B) Quantitative analysis of $p16^{INK4a}$ protein levels. The optical density for each ladder was calculated by Image J software. Relative protein levels counted as D1/D0 (the value for Mock was set as 1.0), where D0 and D1 stand for the optical density of β-actin ladder and sample ladder, respectively. Data were obtained from three independent experiments. $^{*}P < 0.001$ versus Mock group; $^{\#}P < 0.01$ versus hCMV alone group; $^{\#\#}P < 0.001$ versus hCMV alone group.

rapidly when hCMV-infected cells were treated with 10 or 20 μM piceatannol. These results demonstrated that piceatannol had a significant suppressive effect on hCMV-induced expression of $p16^{INK4a}$ in a dose-dependent manner.

Taken together, these data demonstrated that hCMV-infection induces the activation of senescence phenotype, including SA-β-Gal-positive staining, enhanced ROS production, and $p16^{INK4a}$ expression, and piceatannol demonstrated a suppressive effect on these phenotypes.

Discussion

This study, for the first time, reports potent suppressive effects of piceatannol on the hCMV infection in human diploid fibroblasts, and the inhibitory effect is further demonstrated to be related to the suppression of hCMV-induced activation of molecular mechanisms underlying senescence and ROS production.

Piceatannol is a polyphenolic stilbene phytochemical. Also, it is a hydroxylated analog of resveratrol and also possesses several biological roles and various mechanisms of action similar to those of resveratrol, such as antioxidative, anti-inflammatory, and anticancer activities (Seyed *et al.*, 2016). Resveratrol has demonstrated the potent antiviral effect for a wide range of animal and human viruses, including both DNA and RNA viruses in the entry, replication, or transcription stage (Yang *et al.*, 2015). A previous study has shown that potent anti-hCMV activity of resveratrol is due to its phenolic hydroxyl group(s) because little or no antiviral activity is observed for other stilbenes and stilbene-like compounds

lacking phenolic hydroxyl groups (Evers *et al.*, 2004). Compared to resveratrol, piceatannol has an additional hydroxyl group in its structure (Fig. 1A), and the slight difference in the chemical structure of piceatannol might be beneficial for its functions, including antiviral activity. We established a drug screening system for anti-hCMV research in a previous study (data not published) and detected the anti-hCMV activity of piceatannol. In this study, the anti-hCMV activity *in vitro* was further confirmed by the fact that piceatannol significantly inhibits the expression of hCMV proteins and viral DNA replication without detectable toxicity in human diploid fibroblast WI-38 cells.

In the previous experiments, we found that piceatannol incubated with hCMV alone could not directly inactivate hCMV (data not shown), and hence, it could be speculated that the anti-hCMV activity of piceatannol depends on the biological signaling events during cell-virus interaction. To create an optimal environment for replication, hCMV needs to activate the appropriate cellular biosynthetic pathways for DNA synthesis by preventing the replication of the host cell DNA via blockage of the cell cycle (Kalejta and Shenk, 2002). As hCMV infection was effectuated in arrested cells, the observed phenotype was ascribed to the activation of the molecular mechanism of senescence. WI-38 cells infected with hCMV became enlarged, acquired a flattened and irregular shape typical of senescent cells, and exhibited an increase in the level of specific markers, such as SA-β-Gal activity, ROS production, and $p16^{INK4a}$ expression (Figs. 3 and 4), which was consistent with that observed in previous studies (Noris *et al.*, 2002; Mao *et al.*, 2016).

Oxidative stress has been a major theory of aging. Accumulation of detrimental molecules, such as ROS in cells, could result in a pattern of cumulative oxidative damage and pose a significant threat to cellular function and organism aging (Finkel and Holbrook, 2000). Several studies have demonstrated that oxidative damage induces cellular senescence and reduction of ROS production protects the cells from developing senescence significantly (Toussaint *et al.*, 2000; Mao *et al.*, 2010). Moreover, accumulating evidence has suggested an essential role for oxidative stress during viral infection (Aubert *et al.*, 2008; McGuire *et al.*, 2011; Tung *et al.*, 2011). hCMV appears to utilize virus-specific mechanisms to protect the cells from the adverse effects of ROS and maintain redox homeostasis (Tilton *et al.*, 2011). ROS enhances hCMV replication through paracrine and autocrine mechanisms, while *N*-acetylcysteine, a common H_2O_2 scavenger can suppress the activation of hCMV replication (Xiao *et al.*, 2015). In this study, we also observed that hCMV with 0.01 MOI increased the ROS production level, while piceatannol suppressed hCMV-induced ROS level (Fig. 3C). Thus, we proposed that piceatannol inhibited the hCMV infection partially through its antioxidant activity.

hCMV gene expression occurs in three temporal phases, designated IE, E, and late stages. The *IE* gene plays a crucial role in the expression and regulation of other viral genes. Of these, IE1 and IE2, especially IE2, elevated the expression of $p16^{INK4a}$ and was primarily responsible for senescence following hCMV infection (Noris *et al.*, 2002). Further studies demonstrated that functional $p16^{INK4a}$ was necessary for hCMV replication, and the effect of functional $p16^{INK4a}$ on hCMV infection occurred in the early phases of infection, i.e., virus adsorption, entry, or uncoating (Zannetti *et al.*, 2006). These findings suggested that hCMV triggers the expression of $p16^{INK4a}$ in the early phases of infection and does not replicate in cells lacking a functional $p16^{INK4a}$, which in turn, demonstrated that hCMV might exploit the p16-pRb axis to stimulate the senescence program favorable for replication (Zannetti *et al.*, 2006). In this study, we found that piceatannol had a potent suppressive effect on hCMV infection and hCMV-induced activation of $p16^{INK4a}$ (Fig. 4); this effect was at least partially responsible for the anti-hCMV activity.

True animal models of hCMV infection are extremely difficult to develop due to its species specificity. However, several attempts have been made to develop models of hCMV infection in animals that recapitulate one or more phases of the viral replication cycle (Gao *et al.*, 2007). Because *guinea pig* cytomegalovirus (GPCMV) and *Rhesus macaques* cytomegalovirus (RhCMV) share sequences and structural homology with hCMV, and have a placental structure similar to that of humans (Hansen *et al.*, 2003; Kanai *et al.*, 2011; Auerbach *et al.*, 2013; Gnanandarajah *et al.*, 2014; Gerna and Lilleri, 2019), GPCMV and RhCMV infections have been indicated as suitable models for the study of hCMV infection. Owing to our experimental purpose and conditions, we will use the *guinea pig* model to carry out the drug safety experiment and the verification experiment of the anti-CMV effect of piceatannol *in vivo* in future studies. Taken together, the data of animal experiments would serve as a guide for the development of piceatannol as a potential anti-hCMV drug.

In summary, the current results indicated that piceatannol had potent anti-hCMV activity *in vitro*, and the mechanism was at least partially related to suppressing the hCMV-induced activation of molecular mechanisms of senescence. The underlying mechanism is yet to be elucidated. This study provided a preliminary theoretical basis for the research and development of piceatannol in anti-hCMV drugs.

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