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Lupeol impairs herpes simplex virus type 1 replication by inhibiting the promoter activity of the viral immediate early gene α0

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Summary. – Herpes simplex virus type 1 (HSV-1) is an important human pathogenic virus. It is urgent to develop novel antiviral targets because of the limited treatment options and the emergence of drug resistant strains. In this study, we tested the antiviral activity of lupeol, a triterpenoid compound, against HSV-1 and acyclovir (ACV) resistant strains. Lupeol significantly inhibited HSV-1 (F strain) and ACV-resistant strains including HSV-1/106, HSV-1/153, and HSV-1/Blue. Lupeol activity of the HSV-1α0 and α4 promoters, therefore down regulating the expression of the α0, α4, and α27 genes. Collectively, lupeol showed strong antiviral activity against HSV-1 and ACV-resistant strains, and could be a promising therapeutic candidate for HSV-1 pathogenesis.

Keywords: herpes simplex virus 1; lupeol; ACV-resistant strains; promoter

Introduction

Herpes simplex virus (HSV) is a double-stranded DNA virus belonging to the alpha herpes virus subfamily (Liu *et al.*, 2018). There are two types of HSV strains, with HSV-1 causing infection of the oral mucosa and HSV-2 affecting the genital mucosa and causing neonatal infections (Whitley, 2006). In the absence of effective treatment, the mortality rate of HSV-1 infections can reach 70% (Heidary *et al.*, 2014; Wysocka and Herr, 2003). According to the latest report of the World Health Organization, two-thirds of the world's population under the age of 50 are infected with HSV-1, and the incidence of herpes simplex virus encephalitis (HSE) caused by HSV-1 infection is increasing annually. HSV-1 infections were associated with neurodegenerative diseases (e.g. Alzheimer's disease; AD). Intracranial infection caused by HSV-1 was identified as an important factor in the pathogenesis of AD (Ramu *et al.*, 2016) and the brain changes in some patients with HSE were found to be similar to that of AD patients (Ball, 1982). HSV-1 has also been associated with certain types of cancers (e.g., cervical carcinoma and acute lymphocytic leukemia) (Aurelian *et al.*, 1973; Kolokotronis and Doumas, 2006).

Clinically, anti HSV-1 drugs are mainly nucleoside drugs, such as acyclovir (ACV), ganciclovir, valacyclovir, etc., which mainly inhibit viral replication by interfering with viral DNA polymerase. With the widespread use of these drugs, drug-resistant strains have emerged (Chuanasa *et al.*, 2008). In order to cope with the problem of drug-resistant strains, research has focused on newer anti-HSV drugs. The viral DNA polymerase is still a target for some of these studies (Digard *et al.*, 1995). The HSV-1 life cycle includes adsorption and entry into the host cell, intracellular transport to the nucleus, DNA replication, gene transcription, protein synthesis, nucleocapsid assembly and viral release (Coen and Schaffer, 2003). Drugs that theoretically affect any stage in the life cycle could be

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Abbreviations: ACV = acyclovir; HSV = herpes simplex virus; IE = immediate early

inhibitory. For example, benzothiophene PD146626 exerts an inhibitory effect by targeting both the immediate early gene expression of the virus and viral DNA injection into the nucleus (Brideau *et al.*, 2002). The phosphoinositide-dependent kinase 1 (PDK1) inhibitor, BX-795 inhibits HSV replication with impact on the Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) pathway (Tarapore *et al.*, 2013).

Lupeol is a pentacyclic triterpenoid widely found in a variety of fruits (mango, olive, etc.), vegetables (cabbage, green peppers, tomatoes, etc.) and medicinal plants (aloe) (Saleem *et al.*, 2008). Lupeol has strong anti-oxidant and anti-inflammatory effects and has been tested in the context of pathogenic infections, tumors, and diabetes (Adnan *et al.*, 2017; Pavlova *et al.*, 2003; Saleem *et al.*, 2008). Lupeol was found to inhibit liver, rectal, and colon cancer cells (Looker *et al.*, 2015; Su *et al.*, 2017; Wang *et al.*, 2011). The anti-HSV-1 activity of various mono-, tri- and sesquiterpenes have been demonstrated previously (Astani *et al.*, 2010, 2011; Piacentini *et al.*, 2014). However, the specific antiviral mechanism of lupeol has yet not been elucidated. In the present study, we analyzed the antiviral activity of lupeol against HSV-1. The infectivity of HSV-1 was significantly decreased *in vitro* and a mechanism of inhibition by lupeol was identified.

Materials and Methods

Plant materials. Lupeol was isolated from Ganqingqinglan (*Dracocephalum tanguticum* Maxim. var. tanguticum), which was collected in Huangzhong County, Qinghai Province. The isolation and identification of the compound is described in the supplementary information.

Cells and viruses. African green monkey kidney cells (Vero; ATCC CCL81) were propagated in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, USA), 100 U/ml penicillin and 100 U/ml streptomycin at 37°C in 5% CO_2. The human neuroblast-like cell line, HaCaT (ATCC CRL-2266) was cultured at 37°C in 5% CO_2 and cultivated under the same conditions as Vero cells. HSV-1 strain F (ATCC VR733), HSV-1/Blue, a resistant strain of ACV due to the thymidine kinase (TK) mutation (Tarapore *et al.*, 2013) and two ACV-resistant clinical HSV-1 strains (HSV-1/106 and HSV-1/153) were a kind gift from Tao Peng (Guang Zhou Institutes of Biomedicine and Health, Chinese Academy of Sciences). Green fluorescent protein (GFP)-HSV-1 construct, expressing the fluorescent tagged viral protein Us11 was used to evaluate the antiviral activity of the drug treatment (Whitley *et al.*, 2002). HSV-1 Us11 is a multifunctional late protein which regulates the accumulation of RNA species and facilitates HSV-1 replication (Liu *et al.*, 2016). All viruses were propagated in Vero cells and stored at -80°C until further use.

Cytotoxicity. Exponentially growing cells were seeded at 1.0×10^4 cells per well in a 96-well microtiter plate. After the cell confluency reached 90%, various concentrations of lupeol were added to the plate. Three replicates were performed for each treatment. An equal volume of dimethyl sulfoxide (DMSO) was used as the solvent control. After 72 h of cultivation, a colorimetric assay for the determination of cell viability (Cell counting kit-8; CCK-8, Beijing Tianyu Hengtai Technology Co., Ltd. China) was performed. The CCK8 solution (10 µl) was added to each well and incubated for 1–2 h. Absorbance of the solution was measured at 450 nm (Epoch 2; BioTek Instruments, Inc., USA). The half maximal inhibitory concentration (CC_{50}) values were calculated using the PrismPad program (Version5.0, GraphPad Software, USA).

Plaque reduction assay. For antiviral activity the plaque reduction assay (PRA) was used. Exponentially growing cells were seeded at 3.0×10^5 cells per well in a 24-well plate. Upon reaching 100% confluency, the cells were infected with the HSV-1 strain F (50 PFUs/well) for 2 h. The overlay medium consisting of a maintenance medium containing 1% methylcellulose (Sijia Biotech, China) in the presence or absence of lupeol was added to each well. After incubation for 72 h, the cells were fixed with 4% paraformaldehyde for 15 min. Finally, the plates were stained with 1% crystal violet for 20 min and washed with 1x phosphate buffered saline (PBS). The images of the monolayers were acquired using inverted microscope (LEICA 090-134.007-000), plaque counting was performed, and the plaque reduction ratio was calculated.

Time-of-addition assay. To determine the possible step(s) in the viral life cycle targeted by lupeol, a time course study was performed. Vero cells (3.0×10^5 /ml) were seeded into 24-well plates. First, the cells were infected with HSV-1 (50 PFUs/well) at 4°C 1 h. In order to allow HSV-1 adsorption, maintenance solution was replaced after 1 h (0 h group was immediately replaced with the covering solution containing lupeol). After incubation, the medium was changed by covering solution containing the 50 µM lupeol according to the following time points 2, 4, 6, 8, 10, 12 and 24 h, and incubated for 72 h to allow plaque formation.

Quantitative real-time PCR. HSV-1 (multiplicity of infection (MOI) = 5) infected Vero cells were treated with lupeol for 1, 2, 3, and 4 h post infection (p.i.) and total RNA was extracted from cells with TRIzol reagent (Tiangen, China) according to the manufacturer's protocol, and transcribed into cDNA using a PrimeScript reverse transcriptase (RT) reagent kit (Takara, China). The quantitative polymerase chain reaction (qPCR) assay was performed using specific primers (Table 1) and SYBR Premix Ex Taq II kit (Takara) in a CFX96 real-time PCR system (Bio-Rad).

The mRNA levels were standardized against the housekeeping 18s gene in Vero cells using the $2^{-\Delta\Delta CT}$ method with the CFX96 system software (Bio-Rad).

Western blot analysis. To explore the effect of lupeol on the immediate early genes of HSV-1, Vero cells were cultured in culture plates to 80–90% confluence, infected with HSV-1 for 1 h, and treated with or without lupeol. The cells were harvested and lysed in radio-immunoprecipitation assay (RIPA) buffer

Table 1. Primers used in this study

Gene name	Primer sequence
α0F	CCCACTATCAGGTACACCAGC
α4F	CTGCGCTGCGACACCTTTT
α4R	TGCTGCTGCTGTCCACG
α27-F	ACGGTGTTGACCACGATGAG
α27-R	TGGCCGTCAACTCGCAGA
UL47-F	TACGAGGAGGACGACTACCC
UL47-R	ATCCGGACACGGGTAAAACC
IFN-α-F	CTCATACACCAGGTCACGCT
IFN-α-R	AGTGTAAAGGTGCACATGACG
IFN-β-F	ACTGGCTGGAATGAAACCGT
IFN-β-R	GGCACAGCTTCTGTACTCCT
IFN-γ-F	GCTACACACTGCATCTTGGC
IFN-γ-R	CATGTCACCATCCTTTTGCCAG
18S-F	CATGGTGACCACGGGTGAC
18S-R	TTCCTTGGATGTGGTAGCCG

(Beyotime, China) for 30 min on ice, and the protein concentrations were measured with an enhanced bicinchoninic acid (BCA) protein assay kit (Beyotime). The cell lysates were mixed with a 5x sodium dodecyl sulfonate – polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (Beyotime) and boiled for 10 min. Finally, the samples were separated on 8–15% gradient SDS PAGE gel, transferred to a polyvinylidene difluoride (PVDF) membrane (Merck Millipore, Germany), probed with the indicated primary antibodies, and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. All proteins of interest were detected by enhanced chemi-luminescence (Millipore). The band intensity of each protein was calculated using Quantify One software (Bio-Rad, USA) and normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Virus inactivation assay. Vero cells were cultured in 24-well plates (1.5x10^5 cells/well) overnight with mix of HSV-1 (50 PFUs/well) with lupeol-containing culture medium and incubate at 37°C for 2h. The inoculum was removed and a cover layer was added and incubated for three days at 37°C. Following this, the cells were fixed and stained as described above.

Table 2. Antiviral HSV-1/F activity of ACV and lupeol

Compound	CC_{50} (μM)	EC_{50} (μM)	SI (μM)
		HSV-1/F	
ACV	>100	<0.53125	ND
Lupeol	81.13±3.975	4.743±0.511	17.11

Virus attachment assay. Vero cells were cultured in 24-well plates (1.5x10^5 cells/well) overnight, pre-cooled at 4°C for 1 h and washed with cold PBS. Virus inoculum (50 PFUs/well) and 50 μM lupeol were added to the cells and the mixture was incubated at 4°C for 2 h to allow virus attachment to the cells. The inoculum was removed, a cover layer was added and the cells were incubated for three days at 37°C. Following this, the cells were fixed and stained as described above.

Virus penetration assay. Vero cells were cultured in 24-well plates (1.5x10^5 cells/well) overnight, pre-cooled at 4°C for 1 h and washed with cold PBS. HSV-1 (50 PFUs/well) was added and incubated for 2 h at 4°C to allow virus attachment. Following this, the inoculum was removed and the cells were washed with cold PBS. Then different concentrations of lupeol were added and incubated at 37°C for 10 min for analysis of virus penetration. After incubation, PBS (pH = 3) was added into every well for 1 min to inactive the virus. The solution was neutralized with the neutral PBS and PBS was removed. A cover layer was added and the cells were incubated for three days at 37°C. Following this, the cells were fixed and stained as described above.

Fluorescence experiment. Vero cells were cultured in 24-well plates (1.0x10^5 cells/well) until they reached a confluency of 80%. Following a wash with PBS, the cells were infected with 100 μl of virus (MOI = 1 HSV-1/EGFP) inoculum and 100 μl of lupeol solution and incubated for 2 h at 37°C. The mixture was discarded and the cells were washed with PBS. A maintenance solution containing lupeol was added and the cells were incubated for 24 h at 37°C. Fluorescence imaging was performed after 24 h using Fluorescence microscope (OLYMPUSIX71).

Luciferase reporter gene assay. The effect of lupeol on the promoter activity of viral immediate early genes was analyzed using a dual luciferase assay. Briefly, the promoter sequence of viral immediate early gene α0 and α4 was cloned into the luciferase reporter plasmid pGL4.12 [luc2p] (Promega, USA) according to manufacturer's instructions. HSV-1 virion protein 16 (VP16) is a crucial protein involved in the assembly of a transactivation complex on the viral promoters α0 and α4.

Therefore, the exogenous expression of VP16 was used as a positive control and the protein coding sequence of VP16 was cloned into the expression plasmid pcDNA3.1 (pcDNA). Vero cells were transfected with pcDNA3.1(+)-VP16 plasmid (pcDNA-VP16) (250 ng/well) in combination with the pGL4.12 [luc2p]-α0 promoter (p-GLα0) plasmid (250 ng/well) or pGL4.12 [luc2p]-α4 promoter (p-GLα4) plasmid (250 ng/well) using a jetPRIME® kit (PT-114-15; Polyplus Transfection, France). The pRL-TK plasmid (5 ng/well) was transfected as an internal reference. After transfection, the cells were treated with 50 μM lupeol for 2 h. Dual-Luciferase® Reporter assay was performed using a GloMax 20/20 GloMax20/20 instrument (Promega, USA).

Statistical analysis. Data are presented as mean ± SD (standard deviation). Data were analyzed by a one-way analysis of variance (ANOVA) or Student's t test as appropriate, and the level of significance was set at $p < 0.05$ (*), $p < 0.01$ (**), or $p < 0.001$ (***).

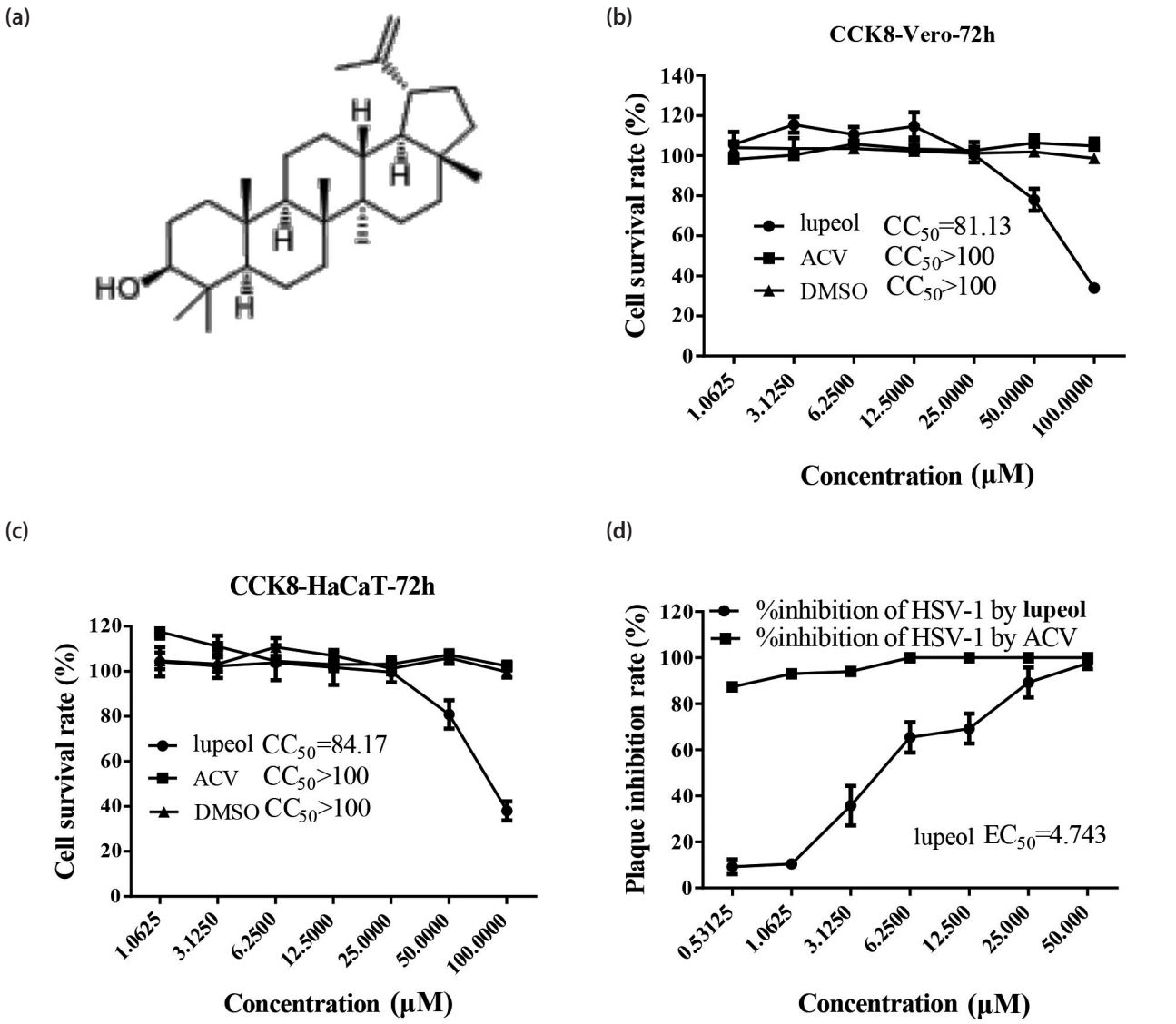

Fig. 1

The toxicities and antiviral activities of lupeol

(a) The chemical structure of lupeol. **(b-c)** Vero and HaCaT cells were treated with lupeol or ACV for 72 h, then cell survival was analyzed by CCK8 assay. **(d)** Anti-HSV-1 activity of lupeol. Vero cells were infected with HSV-1 and lupeol or ACV was added for 2 h, replaced with the covering solution containing lupeol or ACV and cultured for 72 h. The cells were fixed, stained and finally plaque statistics were performed.

Results

Cytotoxicity and comprehensive antiviral activity of lupeol

Vero and HaCaT cells were treated with lupeol (the structure is shown in Fig. 1a) or ACV for 72 h, and the cell survival rate was calculated by the CCK8 assay (Fig. 1b,c). The 50% cytotoxic concentration (CC_{50}) values are shown in Table 2. In addition, the antiviral activity of lupeol was estimated by plaque assay. The results show that lupeol has an antiviral effect when used at a concentration between 12.5 and 50 μM. As expected, the antiviral drug ACV showed excellent antiviral effect at a low concentration of 0.53125 μM (Fig. 1d), and the half maximal effective concentration (EC_{50}) values and selectivity index (CC_{50}/EC_{50}) (SI) are shown in Table 2. Based on the above results, we proved that lupeol strongly inhibits HSV-1 activity at higher concentrations than ACV.

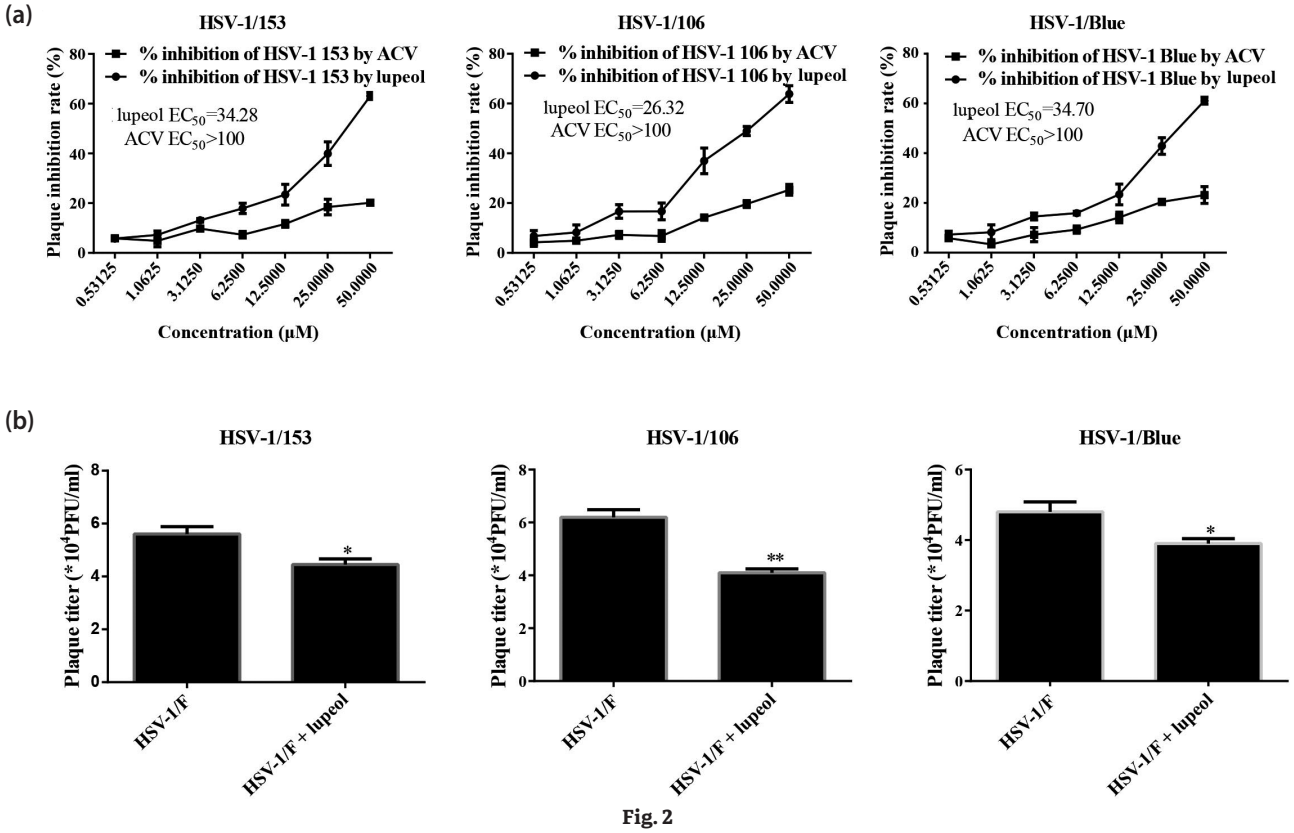

Fig. 2

Detection of the activity of lupeol drug-resistant viruses HSV-1/153, HSV-1/106 and HSV-1/Blue by plaque assay
(a) Vero cells were infected with ACV resistant strains (HSV-1/106; HSV-1/153; HSV-1/Blue) and treated with lupeol or ACV for 2 h, then replaced with the covering solution containing lupeol or ACV and cultured for 72 h. The cells were fixed and stained and finally plaque statistics were performed. **(b)** Vero cells were infected with HSV-1/153; HSV-1/106; HSV-1/Blue (MOI = 1) and lupeol or ACV was added for 2 h, then replaced with the maintenance medium containing lupeol or ACV and cultured for 24 h. Data are mean ± SD (n = 3). *p <0.05; **p <0.01.

Activity of lupeol against ACV resistant strains

In this study we evaluated the antiviral activity of lupeol against three ACV-resistant strains, including HSV-1/Blue, a TK mutant derived from HSV-1, and two clinical strains (HSV-1/106 and HSV-1/153) (Jin *et al.*, 2014). The plaque assay confirmed that ACV has no antiviral activity even at high concentrations (up to 50 μM) (Fig. 2a). However, 50 μM lupeol significantly decreased HSV-1 titer and plaque formation (Fig. 2a,b). The EC_{50} values and SI are shown in Table 3.

Anti-HSV-1 effect of lupeol on HaCaT cells

Antiviral activity experiments were performed using the human keratinocyte cell line (HaCaT). The extracellular and intracellular HSV-1 genomes from HaCaT infected cells were extracted. Our results show that lupeol can significantly decrease HSV-1 DNA copy number (Fig. 3a,b). In addition, we also performed immunofluorescence experiments. First, we infected HaCaT cells with HSV-1/EGFP for 24 h, and then performed fluorescence photography. Fluorescence analysis indicated that lupeol

Table 3. Anti-ACV resistant strain activity of ACV and lupeol

Compound	CC_{50} (μM)	EC_{50} (μM)			SI (μM)		
		HSV-1/106	HSV-1/153	HSV-1/Blue	HSV-1/106	HSV-1/153	HSV-1/Blue
ACV	>100	>100	>100	>100	ND	ND	ND
Lupeol	81.13±3.975	26.32±1.072	34.28±1. 495	34.70±0.916	3.08	2.37	2.34

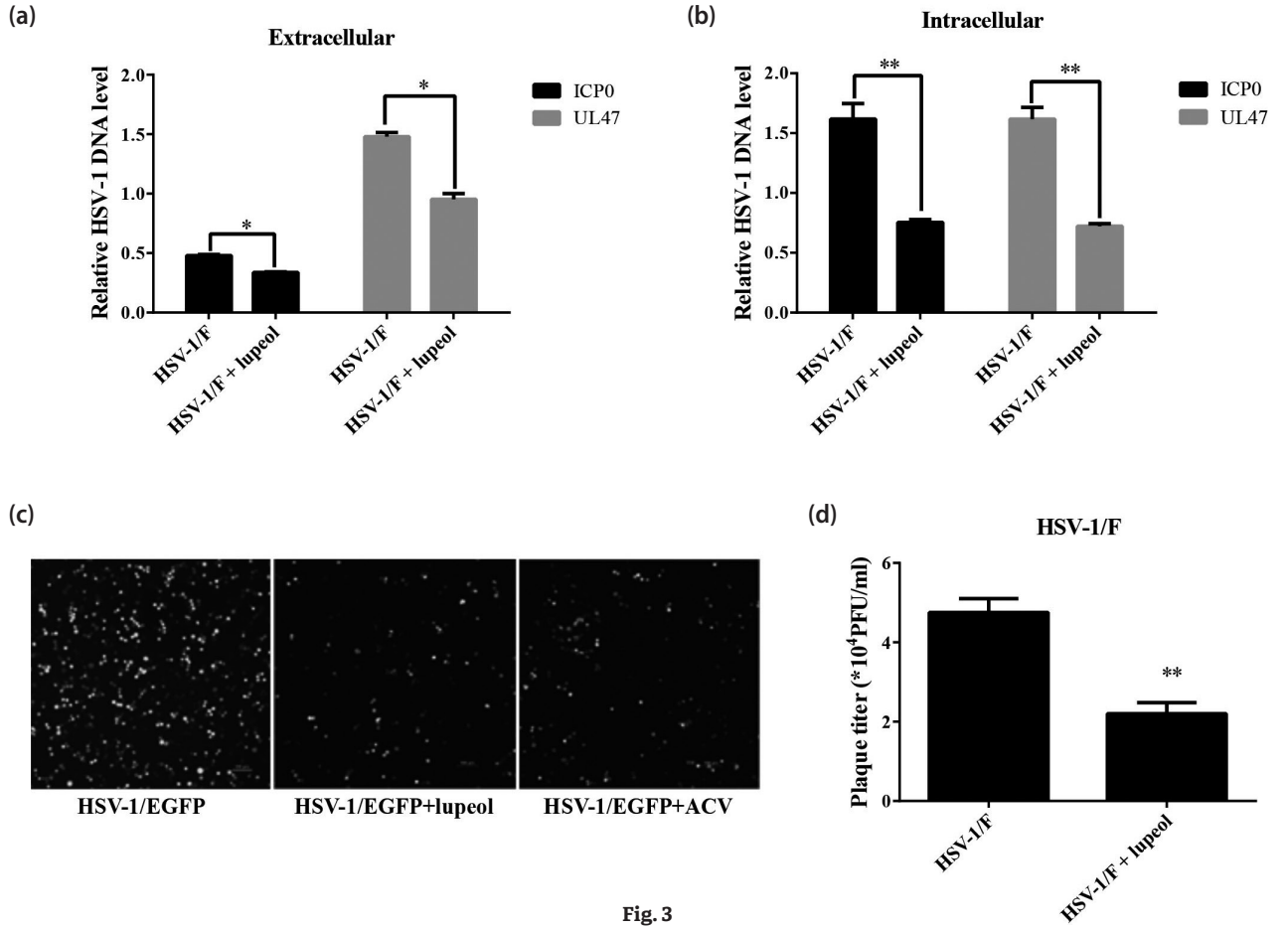

Fig. 3

Lupeol antiviral effect on HaCaT cells

(a-b) HaCaT cells were infected with HSV-1/F (MOI = 1) and treated with 50 μM lupeol for 2 h, then changed to the maintenance medium containing lupeol and cultured for 24 h. The extracellular and intracellular HSV-1 genomes were extracted, and analyzed by qRT-PCR. **(c)** Flourescence of HaCaT cells that were infected with HSV-1/F (MOI = 1) and 50 μM lupeol or 50 μM ACV for 2 h, then changed to the maintenance medium containing lupeol or ACV and cultured for 24 h. **(d)** Titration of HSV-1 in HaCaT cells. HaCaT cells were infected with HSV-1/F (MOI = 1) and 50 μM lupeol for 2 h, then changed to the maintenance medium containing lupeol and cultured for 24 h. Data are mean ± SD (n = 3). *p <0.05; **p <0.01.

can significantly decrease the fluorescence intensity of GFP-tagged HSV-1 similar to ACV (Fig. 3c). Furthermore, lupeol significantly decreased the HSV-1 titer (Fig. 3d). In summary, the results indicate that lupeol (50 μM) has anti-HSV-1 activity in HaCaT cells.

Lupeol has antiviral effect in the early stage

To find out the possible stage of viral life cycle inhibited by lupeol, we performed the time-of-addition assay. The results show that lupeol exerts its inhibitory effect at 0–2 h post-infection (Fig. 4b). However, lupeol does not affect HSV-1 inactivation, attachment and penetration (Fig. 4c,d). In addition, we also tested the expression of interferon (IFN) in the immediate early stage (Fig. 4e). The results show that lupeol has no significant effect on IFN expression suggesting that lupeol perhaps interferes with the viral early replication process.

Effect of lupeol on immediate early gene expression

To further analyze the effect of lupeol on HSV-1 replication cycle, we determined the expression of three immediate early (IE) gene products. The results showed that the expression of the IE genes α0, α4, and α27, are significantly downregulated (Fig. 5a). Western blot analysis showed that after 4 h of virus infection, lupeol could significantly decrease the levels of IE proteins ICP0, ICP4, and ICP27 (Fig. 5b,c).

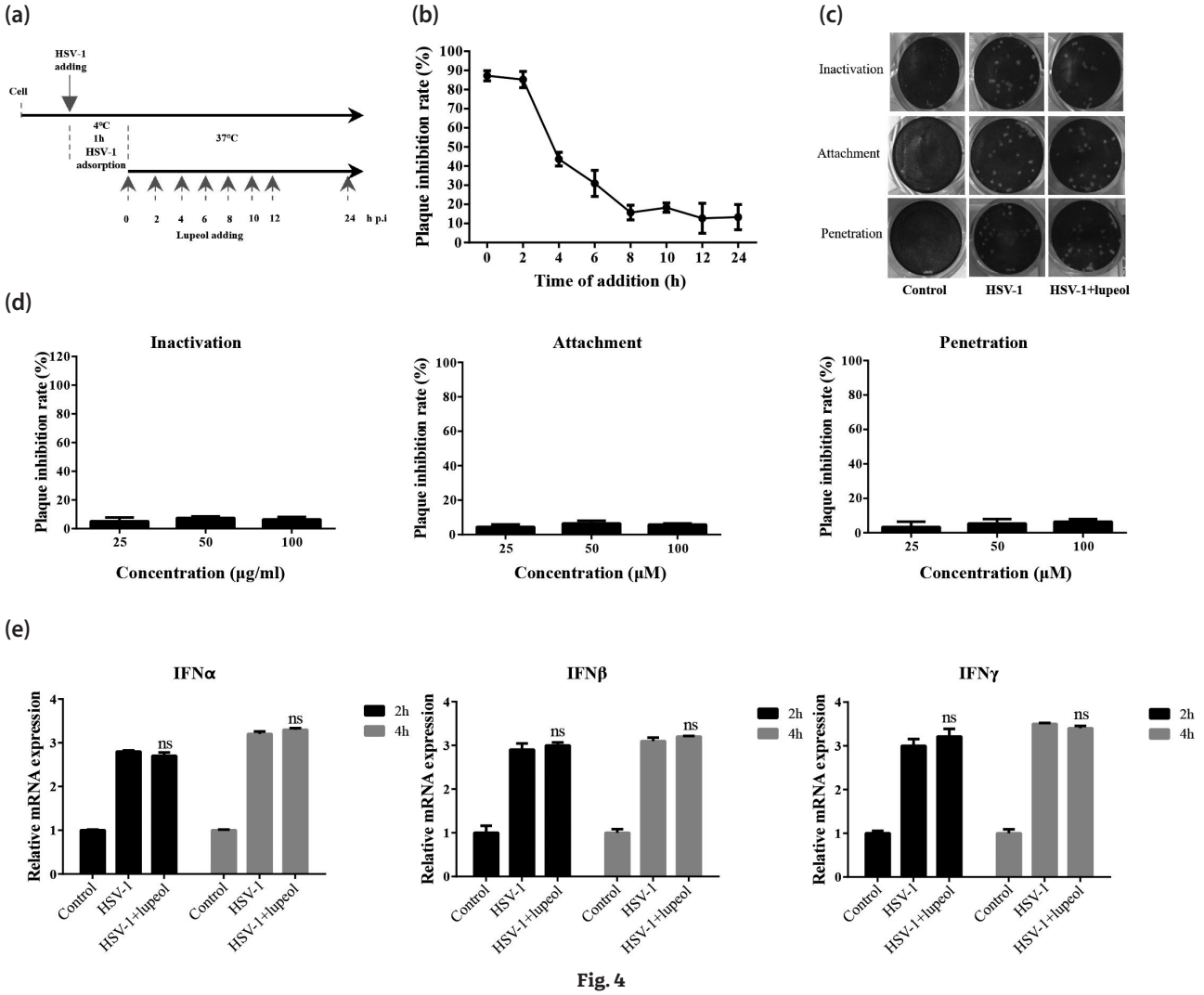

Fig. 4

Lupeol has antiviral effect in the early stage of HSV-1 replication

(a) The diagram of time-addition assay. **(b)** Inhibitory effect of lupeol at various time points, post-infection (0–24 h) with HSV-1 (50 PFU/well), determined by the plaque reduction assay. **(c)** Inactivation assay. Cells were treated with different concentrations of lupeol and infected with HSV-1 (50 PFU/well) for 2 h. The coating medium containing lupeol was added for 72 h. Plaque assay was carried out. Attachment assay. Cells were prechilled at 4°C for 1h, then challenged with HSV-1 (50 PFU/well) in absence or presence of different concentrations of lupeol and kept for 2 h at 4°C and then the plaque assay was carried out. Penetration assay. The cells were pre-cooled 4°C for 1 h, and then HSV-1 was left to adsorb for 2 h at 4°C . The cells were treated with lupeol and incubated for 20 min at 37°C to facilitate viral penetration. The extracellular virus was inactivated by PBS (pH 3.0) for 1 min and then the plaque assay was carried out. **(d)** Graph depicts the statistics of **(c)** graph, indicating the plaque inhibition rate. **(e)** Vero cells were treated with HSV-1(MOI = 5) and lupeol for 2 h and 4 h. IFN mRNA expression level by qRT-PCR was determined. Each bar represents the mean ± SD of three independent experiments.

Lupeol down regulates immediate early transcriptional events

The results so far clearly demonstrate that treatment of infected cells with lupeol inhibits HSV-1 infection at 2 h p.i., and substantially decreases the IE viral gene expression (Fig. 5a,b). We hypothesized that the inhibitory effect may occur at the level of the gene regulation at the promoter. Using the dual luciferase assay, we evaluated the promoter inhibition activity of lupeol. HSV-1 tegument protein VP16 promotes the formation of a transactivation complex, which binds to the promoters of IE genes to initiate their gene expression (Siveen *et al.*, 2014). The VP16 induced luciferase activity in the pcDNA3.1(+)-VP16 and pGL-α0-co-transfected cells was suppressed by lupeol (Fig. 6a). In addition, we used the same method to detect the effect of lupeol on the α4 promoter and found that lupeol can significantly inhibit luciferase activity in the

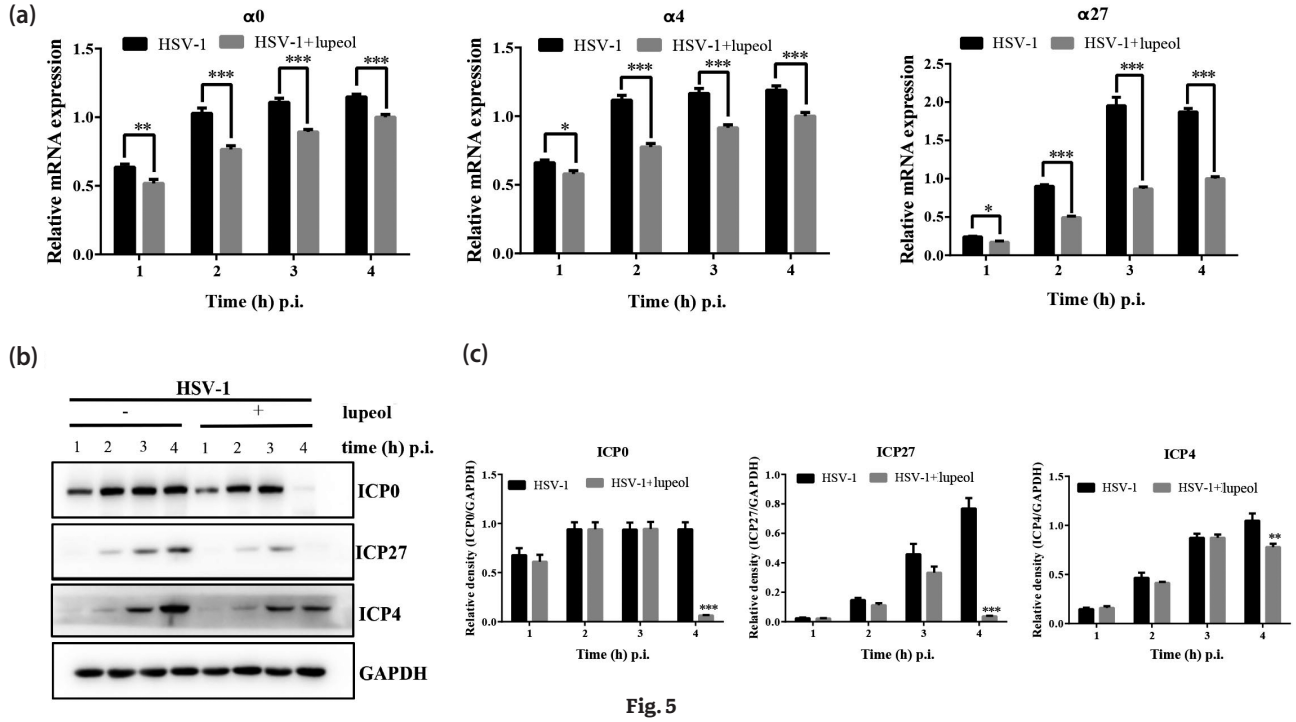

Fig. 5

Effect of lupeol on HSV-1 gene and protein expression

(a) HSV-1(MOI = 5) infected cells were treated with lupeol for 1–4 h p.i. Total RNA was extracted and qRT-PCR of α0, α4 and α27 was performed. **(b)** Western blot of the ICP0, ICP27 and VP16 expression. **(c)** Densitometric analysis for all western blot bands. GAPDH served as a loading control. Data are mean ± SD (n = 3). **p <0.01; ***p <0.001 versus HSV-1-treated group.

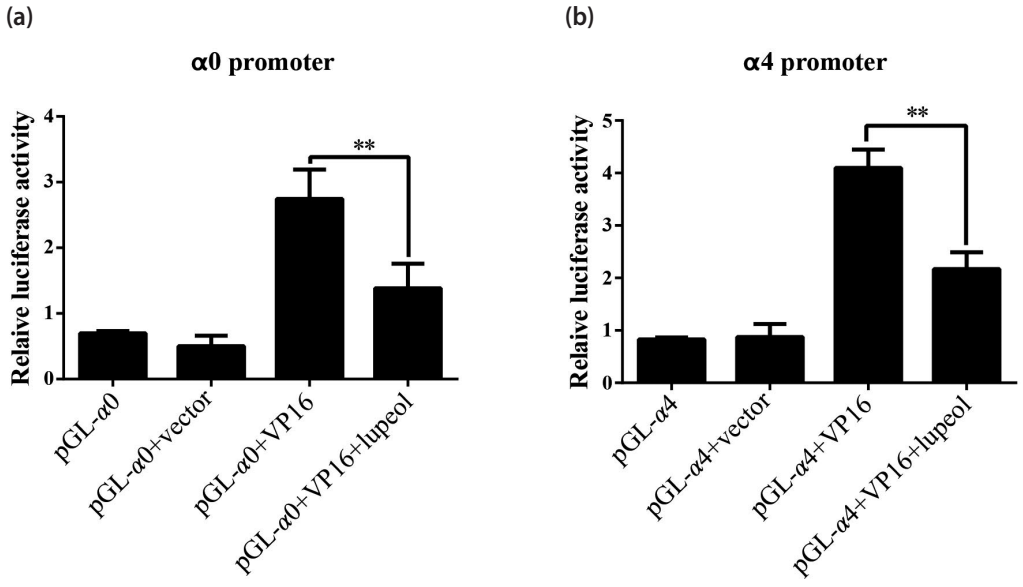

Fig. 6

Lupeol inhibits the activity of the HSV-1 α promoter

(a-b) Vero cells were transfected with indicated plasmid combinations for 24 h, the cell lysates were then subjected to luciferase activity assay. The cells transfected with pGL-α0 plasmids (pGL-α4 plasmids) or co-transfected with pGL-α0 and pcDNA plasmids (pGL-α4 and pcDNA plasmids) were used as negative controls. Data are mean ± SD (n = 3). **p <0.01.

pcDNA3.1(+)-VP16 plasmid and pGL-α0-transfected group (Fig. 6b). These results confirmed that lupeol suppressed the immediate early gene expression partly by inhibiting its promoter activity.

Discussion

Lupeol is a pentacyclic trialkyl compound with known antiviral, anti-cancer, antioxidant and anti-inflammatory effects (Adnan *et al.*, 2017; Parvez *et al.*, 2018; Siveen *et al.*, 2014). However, the mechanism of viral inhibition remains unclear. In this study, lupeol showed low toxicity to Vero cells despite the high concentration (50 μM) used. The cell survival rate was greater than 80% at this concentration. The plaque assay showed that lupeol had significant antiviral activity at high concentrations against ACV sensitive (25 μM and 50 μM) and resistant (50 μM) strains. The observed antiviral activity is not cell line or type dependent as demonstrated by the effects seen in Vero (primate kidney) and HaCaT (human keratinocyte) cells. The results from detecting the copy numbers of HSV-1 genome in the supernatant and intracellular virus showed that lupeol can significantly inhibit the replication of HSV-1. In addition, lupeol can also significantly inhibit the HSV-1 / EGFP fluorescence intensity and titer experiments also proved that lupeol can significantly inhibit the activity of HSV-1. We have demonstrated that lupeol has good antiviral activity using different experimental methods, different virus strains and different cells, which has considerable research value.

We further investigated the mechanism by which lupeol could inhibit HSV-1 and showed that lupeol affected viral early infection events not related to inactivation, attachment or penetration of the virus. HSV-1 genes are divided into immediate early (α), early (β) and late genes (γ) based on the timing of gene expression. Our results indicated that lupeol acts in the IE stage. In the presence of lupeol, the mRNA expression of α0, α4, and α27 and correspondingly, the levels of protein products ICP0, ICP4, and ICP27 also decreased significantly at 4 h p.i. The downregulation of the IE gene expression was linked to transcriptional inhibition at the level of the IE promoter.

The expression of IE genes requires the viral VP16-HCF-1-Oct-1 complex to stimulate its promoter activity independent of DNA replication (Kim *et al.*, 2012). Future studies will address how lupeol inhibits promoter activity. We speculate that the interaction between the VP16-HCF-1-Oct-1 complex could be a target for inhibition by lupeol.

In addition, the anti-inflammatory effects of lupeol have been linked to the regulation of IFN and interleukins (IL), and the NF-kB signaling pathway (Kangsamaksin *et al.*, 2017). It would be interesting to verify whether lupeol inhibition of HSV-1 activity involves the NF-kB signaling pathway.

In summary, our results demonstrate that lupeol inhibits HSV-1 by down regulating the IE gene promoter activity. Further studies are warranted to address the translational value of this drug as an alternative option for anti-HSV therapy.

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Supplementary information is available in the online version of the paper.

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