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Yatein from *Chamaecyparis obtusa* suppresses herpes simplex virus type 1 replication in HeLa cells by interruption the immediate-early gene expression

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Abstract

Inhibitory effects of methanolic extracts from nine Chinese herbs on herpes simplex virus type 1 (HSV-1) replication were studied. By a bioassay-guided fractionation procedure, yatein ($C_{22}H_{23}O_7$; M.W.399) was isolated from *Chamaecyparis obtusa*; yatein significantly suppressed HSV-1 multiplication in HeLa cells without apparent cytotoxicity. To further localize the point in the HSV-1 replication cycle where arrest occurred, a set of key regulatory events leading to the viral multiplication was examined, including viral immediate-early (α) and late (γ) gene expression and DNA replication. Results indicated that levels of glycoprotein B (gB) and gC mRNA expression in HeLa cells were impeded by yatein. Data from polymerase chain reaction showed that replication of HSV-1 DNA in HeLa cells was arrested by yatein. Furthermore, yatein decreased ICP0 and ICP4 gene expression in HeLa cells. Results of an electrophoretic mobility shift assay demonstrated that yatein interrupted the formation of α-*trans*-induction factor/C1/Oct-1/GARAT multiprotein complex. The mechanisms of antiviral action of yatein seem to be mediated, by inhibiting HSV-1 α gene expression, including expression of the ICP0 and ICP4 genes, and by arresting HSV-1 DNA synthesis and structural protein expression in HeLa cells. These results suggest that yatein is an antiviral agent against HSV-1 replication.

Keywords: HSV-1; Yatein; gB; ICP4; α-*Trans*-induction factor

1. Introduction

Herpes simplex virus type 1 (HSV-1) causes a variety of infections in human. Individuals who are with either immunocompromised by AIDS, cancer, or organ transplantation are at increased risk of severity of HSV-1 infection (Honess and Roizman, 1974; Kuo and Lin, 1990; Waggoner-Fountain and Grossman, 2004). Infection with HSV-1 can lead to scarification, which is a major cause of blindness in developing countries (Corey and Spear, 1986). In addition, HSV-1 and HSV-2 have been shown to be factors for spreading of human immunodeficiency virus and causes severe diseases in acquired immunodeficiency syndrome (AIDS) patients (Corey et al., 2004; Mann et al., 1984).

One successful replication cycle of HSV-1 is dependent upon the completion of a number of steps, including virion entry, subsequent expression of viral immediate-early (α) genes such as infected cell protein 0 (ICP0) and ICP4, early (β_1, β_2) genes including DNA polymerase and thymidine kinase, and late (γ_1, γ_2) genes containing glycoprotein B (gB), ICP5 and gC, and unpaired DNA replication (Roizman and Sears, 1996). The initial expression of HSV-1 α genes depends on binding of the α-*trans*-induction factor (αTIF)/C1/Oct-1 multiprotein complex to the TAAGARAT (R = purine; GARAT) sequences of the *cis*-acting site (McKnight et al., 1987). Nucleoside analogues have been extensively investigated in the search for effective antiherpesvirus agents (Darby, 1994). Among those acyclovir is widely used for the systemic treatment of HSV infections. It is a highly selective antiviral agent because it is specifically phosphorylated by viral thymidine kinase in infected cells (Elion et al., 1977; Furman et al., 1979). However, acyclovir-resistant HSV infection in immunocompromised patients such as transplant patients

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and patients with AIDS has recently been observed (Chibo et al., 2004; Coen, 1996; Kimberlin and Whitley, 1996). Therefore, it is desirable to develop new anti-HSV agents that substitute for or complement acyclovir.

Chinese herbs are potential sources of edible and medicinal plants. They are expected to find use as functional foods because of their various biological activities such as immunomodulatory and antitumor functions (Kuo et al., 1997, 2000). More and more people in developing countries utilize traditional medicine for their major primary health care needs (Farnsworth, 1993; Houghton, 1995). However, ethnopharmacology provides scientists with an alternative approach for the discovery of antiviral agents. The polysaccharides (Marchetti et al., 1996), anthraquinones (Sydiskis et al., 1991), triterpenes (Simões et al., 1999), phloroglucinol (Arisawa et al., 1990), flavonoids (Kuo et al., 2002), and catechin derivatives (Ferrea et al., 1993) isolated from medicinal plants have been found to have inhibitory activities against the replication of HSV-1. BAY 57-1293 is a member of thiazolylsulfonamides with potent antiherpetic activity in vitro and in vivo. It is an inhibitor of the HSV helicase-primase (Betz et al., 2002; Kleymann et al., 2002). There has been a promising result for a naturally occurring antiherpetic agent, *n*-docosanol, which has recently completed extensive clinical evaluation and been approved by the U.S. Food and Drug Administration as a topical treatment for herpes labialis (Alrabiah and Sacks, 1996; Pope et al., 1996; Sacks et al., 2001). These findings indicate that natural products are still potential sources in the search for new antiherpetic agents (Boulware et al., 2001; Vanden Berghe et al., 1986).

In the present study, nine Chinese herbs which are widely known in folk medicine for the treatment of viral and bacterial infection were selected for an anti-HSV-1 replication assay. They were *Viburnum luzonicum* (VL), *Ficus pumila* (FP), *Ilex asprella* (IA), *Duchesnea indica* (DI), *Bryophullum pinnatum* (BP), *Radix clerodendri* (RC), *Hibiscus taiwanensis* (HT), *Selaginella leptophylla* (SL), and *Chamaecyparis obtusa* (CB). The methanolic extracts that showed appreciable anti-HSV-1 activity were separated by a bioassay-guided fractionation procedure. The mechanisms of antiviral action of yatein were elucidated in vitro.

2. Materials and methods

2.1. Preparation of crude extracts for Chinese herbs

All nine species of Chinese herbs were purchased from Chinese medicine shops in Taipei, Taiwan, and were identified by Dr. Yueh-Hsiung Kuo and Dr. Yuang-Lian Lin. Each dried Chinese herb (600 g) was extracted with methanol (3× 5 l). After solvent was removed, the crude extracts were dissolved in dimethylsulfoxide (DMSO) to a concentration of 100 mg/ml and stored at 4 °C until use.

2.2. Yatein isolation from C. obtusa

The methanolic extract of the dried leaves of *C. obtusa* was partitioned in succession between H_2O and *n*-hexane, followed by ethyl acetate and *n*-butanol. The bioactive ethyl acetate fraction (320 g) was subjected to silica gel chromatography. Extensive gradient elution was then employed using *n*-hexane, ethyl acetate, and methanol, respectively. The like fractions were combined to give 12 main fractions with monitoring by thin-layer chromatography (TLC) and the solvent was removed under reduced pressure. Each combined fraction was further purified by rechromatography and recrystallization. Yatein was identified from the fraction 6. The nuclear magnetic resonance (NMR) and mass spectrum data of yatein are: ^{1}H NMR ($CDCl_3$) δ: 2.49 (1H, m), 2.51 (1H, dd, J = 13.5, 8.5 Hz), 2.57 (1H, m), 2.62 (1H, dd, J = 13.5, 6.5 Hz), 2.88 (1H, dd, J = 14.0, 6.0 Hz), 2.94 (1H, dd, J = 14.0, 5.0 Hz), 3.83 (9H, s, OCH3), 3.88 (1H, dd, J = 9.1, 7.5 Hz), 4.18 (1H, dd, J = 9.1, 7.0 Hz), 5.93 (1H, s), 6.36 (2H, s), 6.46 (1H, d, J = 1.6 Hz), 6.47 (1H, dd, J = 7.7, 1.6 Hz), 6.70 (1H, d, J = 7.7 Hz); ^{13}C NMR ($CDCl_3$) δ: 35.2, 38.3, 41.0, 46.4, 56.1, 60.8, 65.1, 71.1, 101.0, 106.2, 106.2, 108.2, 108.8, 121.5, 133.3, 136.8, 146.4, 147.9, 153.2, 153.2, 178.5; EIMS m/z (%): 400 (M^+, 79), 385 (2), 265 (4), 264 (4), 251 (5), 219 (7), 182 (62), 135 (100), 91 (9), 77 (22). It was dissolved in DMSO to a concentration of 100 mM and stored at 4 °C until use.

2.3. Cell culture and viruses

HeLa cells were cultured in minimal essential medium (MEM; GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin and incubated at 37 °C in a 5% CO_2 incubator. To prepare HSV-1 (KOS strain) stocks, HeLa cells were infected by HSV-1 at 3 multiplicity of infection (m.o.i.) and harvested at 24 h postinfection (p.i.) and centrifuged at 1500 × *g* (Jouan CR312, Jouan, France), at 4 °C for 20 min. The supernatant was collected and stored at −70 °C for use.

2.4. The plaque reduction assay

The assay followed procedures described previously (Kuo et al., 2001). Acyclovir was used as a positive control. HeLa cells (3.5×10^5/dish) were incubated with 100 plaque forming units (PFU) of HSV-1 and the test extracts or acyclovir (10 μM) were added to the cells at varying concentrations. The viruses were adsorbed for 1 h at 37 °C and 1% methylcellulose was added to each well. After 5 days, the virus plaques formed in HeLa cells were counted by crystal violet staining. The activities of test extracts and acyclovir for inhibition of plaque formation were calculated.

2.5. Determination of cell viability

Approximately 3.5×10^5 HeLa cells were cultured in 25-cm^2 flask and incubated with 0.1% DMSO or various concentrations of yatein for 5 days. Total, viable, and nonviable cell numbers were counted for three times under the microscope with the help of a hemocytometer following staining by trypan blue. The percentage of viable cells was calculated. The cell viability was also

evaluated as lactate dehydrogenase (LDH) release according to the manufacturer's instructions (Kit Roche, MiLan, Italy). LDH activity in milliunits per milliliter, where 1 mU is the amount of enzyme required to transform 0.0167 nM nicotinamide adenine dinucleotide per minute, was determined.

2.6. Extraction of total cellular DNA and RNA

Cellular DNA and RNA were extracted from HeLa cells by the method described previously (Kuo et al., 1993, 2001). HeLa cells (5×10^6) were infected with or without 3 m.o.i. of HSV-1 in the presence or absence of 75 μM yatein and harvested at various time. For DNA extraction, the cells were lysed with 0.2 M Tris–HCl (pH 8.5) containing 100 mM ethylenediaminetetraacetate (EDTA), 100 mM NaCl, 0.5% NP-40, 1% sodium dodecyl sulfate (SDS), and 100 μg/ml proteinase K. To extract cellular RNA, HeLa cells were washed by cold Tris-saline (pH7.4) and then suspended in NDD buffer containing 1% NP-40, 0.5% sodium deoxycholate, and 1% dextran sulfate. The DNA or RNA solutions were extracted with phenol–chloroform and precipitated with 100% cold ethanol, and their concentrations were determined by measuring the optical density at 260 nm.

2.7. Synthesis of first strand cDNA

The method followed procedures described previously (Cone et al., 1991; Kuo et al., 2001). Aliquots of 1 μg of RNA were reverse transcribed with the Advantage™ RT-for-PCR kit from CLONTECH according to the manufacturer's instructions. The reaction mixture was initially incubated at 42 °C for 1 h and then at 94 °C for 5 min to terminate the reaction. Diethyl pyrocarbonate-treated H_2O (80 μl) was added to the tube, and the tube was then stored at −20 °C for use in the polymerase chain reaction (PCR).

2.8. PCR

The method has been described elsewhere (Kuo et al., 2002; Saiki et al., 1985). Briefly, 10 μl of cDNA or total cellular DNA was mixed with 0.75 μM primers, 4 units of Taq polymerase, 10 μl of reaction buffer (2 mM Tris–HCl, pH 8.0; 0.01 mM EDTA; 0.1 mM dithiothreitol; 0.1% Triton X-100; 5% glycerol; and 1.5 mM $MgCl_2$), and 25 μl of water in a total volume of 50 μl. All oligonucleotide primer pairs for HSV-1 were designed from the published data (Kuo et al., 2002). The PCR was done at the following settings of the air thermocycler: denaturing temperature of 94 °C for 1 min, annealing temperature of 53 °C for 1 min, and elongation temperature of 72 °C for 2 min for the first 25 cycles then elongation temperature of 72 °C for 10 min. The amplified products were quantitated using laser scanning densitometer SLR-2D/1D (Biomed Instruments Inc., Fullerton, CA, USA).

2.9. Western blot analysis

The experiment followed procedures described previously (Kuo et al., 2002). The 5×10^6 HeLa cells were infected with HSV-1 at 3 m.o.i. or were not infected in the presence or absence of yatein (75 μM) for various time. Extracted cellular proteins (50 μg) were dissolved in the dissociation buffer (2% SDS, 5% β-mercaptoethanol, 0.05 M Tris–HCl, and 20% glycerol, pH 7.6) and resolved by 10% SDS-PAGE then transferred to nitrocellulose filters. After filters were blocked, they were incubated with mouse monoclonal antibodies (Virusys Corporation, East Coast Biologics, Inc., North Berwick, ME, USA) raised against HSV-1 and α proteins (ICP0 or ICP4). Specific reactive proteins were detected by an enhanced chemiluminescence method employing a rabbit anti-mouse immunoglobulin Ab linked to horseradish peroxidase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

2.10. Electrophoretic mobility shift assay (EMSA)

Oligonucleotide sequences 5′-GATCCCGTGCATGCTA-ATGATATTCTTTGGG-3′ and 3′-GGCACGTACGATTACTA-TAAGAAACCCCTAG-5′, corresponding to consensus αTIF/C1/Oct-1 site (nucleotides −170 to −143) of the ICP0 gene was used as a probe. Nuclear extracts of HSV-1-infected HeLa cells treated with yatein (75 μM) were prepared 4 h after infecting cells at 20 m.o.i. Reaction mixtures (15 μl) contained, together with 8 μg of nuclear extracts, digoxigenin (DIG)-labeled probe, 2 μg of poly(dI–dC), 5% glycerol, 1 mM EDTA, 100 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 10 mM Tris–HCl, pH 7.4. After incubation for 30 min at room temperature, reaction mixtures were applied to 8% polyacrylamide gels in 0.5% Tris borate–EDTA buffer. DIG-labeled DNA–protein complexes were detected by a chemiluminescence method, employing sheep anti-DIG Fab fragments conjugated with alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany).

2.11. Statistical analysis

Data were presented as means ± standard deviation, and the differences between groups were assessed with Student's *t*-test.

3. Results

3.1. Effects of methanolic extracts from nine Chinese herbs on HSV-1 replication

As shown in Table 1, methanolic extracts isolated from nine Chinese herbs were evaluated for their activities in inhibiting HSV-1 plaque formation in HeLa cells. HSV-1 replication was not affected by DMSO treatment. Acyclovir blocked HSV-1 plaque formation in HeLa cells. While methanolic extracts from VL, FP, IA, BP, HT or SL had little effect on HSV-1 replication, 100 μg/ml of CB methanolic extracts significantly suppressed HSV-1 replication with an IC_{50} value of 23.1 ± 9.3 μg/ml. The extracts from DI and RC inhibited the HSV-1 plaque reduction formation to 42.2 ± 11.9 and 38.5 ± 9.2%, respectively. It indicated that CB methanolic extracts had the greatest effect of those assayed.

Table 1
The effects of nine Chinese herbs on HSV-1 replication in HeLa cells

Chinese herbs	Plaque number	Inhibitory activity (%)
Medium	125 ± 29	
DMSO	109 ± 15	
Acyclovir	3.0 ± 2.0	97.2 ± 1.9
VL	99.0 ± 7.0	9.2 ± 6.4
FP	101 ± 14	11.0 ± 8.0
IA	105 ± 7.0	3.7 ± 6.5
DI	63.0 ± 13	42.2 ± 11.9
BP	96.0 ± 8.0	11.9 ± 7.4
RC	67.0 ± 10	38.5 ± 9.2
HT	96.0 ± 10	11.9 ± 9.2
SL	103 ± 7.0	5.5 ± 6.4
CB	5.0 ± 3.0	95.4 ± 2.8

The HeLa cells were grown at 6-cm dishes as a monolayer (3.5×10^5/well). One hundred plaque forming units of HSV-1 was added to each well and the test extracts (100 μg/ml) or acyclovir (10 μM) were added to the cells. After adsorption for 1 h, 1% methylcellulose was added to each well. On the fifth day p.i., the cells were stained with crystal violet and the virus plaques were counted. The inhibitory activities of the various test extracts were calculated.

3.2. Yatein identified from C. obtusa inhibits HSV-1 replication

To extract pure active compounds from the *C. obtusa*, we used the complete isolation process in each chromatographic cycle and finally by HPLC. The structure of compound with strongest activity is shown in Fig. 1A. The name of this bioactive component is yatein ($C_{22}H_{23}O_7$; M.W.399). Both NMR and mass spectrum data for the compound were compatible with the published report (Koulman et al., 2003). The results of plaque reduction assay indicated that the inhibitory effect of yatein on HSV-1 replication was concentration-dependent (Fig. 1B). At 6.25 μM, yatein interrupted HSV-1 replication by 13.9 ± 2.5. The corresponding degrees of inhibition for 12.5, 25, 50, 75, and 100 μM were 15.6 ± 3.0, 32.0 ± 2.0, 83.6 ± 6.9, 100 ± 0.0, and 100 ± 0.0%, respectively, with an IC_{50} value of 30.6 ± 5.5 μM.

3.3. Effects of yatein on viability of HeLa cells

To delineate whether the suppressory effect of yatein on HSV-1 replication was related to cytotoxicity, we examined the viability of HeLa cells after treated with yatein for 5 days. The DMSO did not affect the cell viability. Comparison with control groups, the viability of HeLa cells treated with 25, 50, 75, and 100 μM of yatein did not decrease (Fig. 1C). Moreover, the cytotoxic effect of yatein on HeLa cells was evaluated as LDH release and the data indicated that LDH release from HeLa cells was no difference in the presence or absence of 100 μM yatein for 5 days (3.35 ± 1.8 versus 3.64 ± 2.5 mU/ml). These results demonstrated that inhibitory mechanisms of yatein on HSV-1 replication were not through cytotoxicity.

3.4. Effects of yatein on HSV-1 adsorption

To further elucidate whether yatein inhibition of HSV-1 replication was related to blocking viral adsorption, we examined the

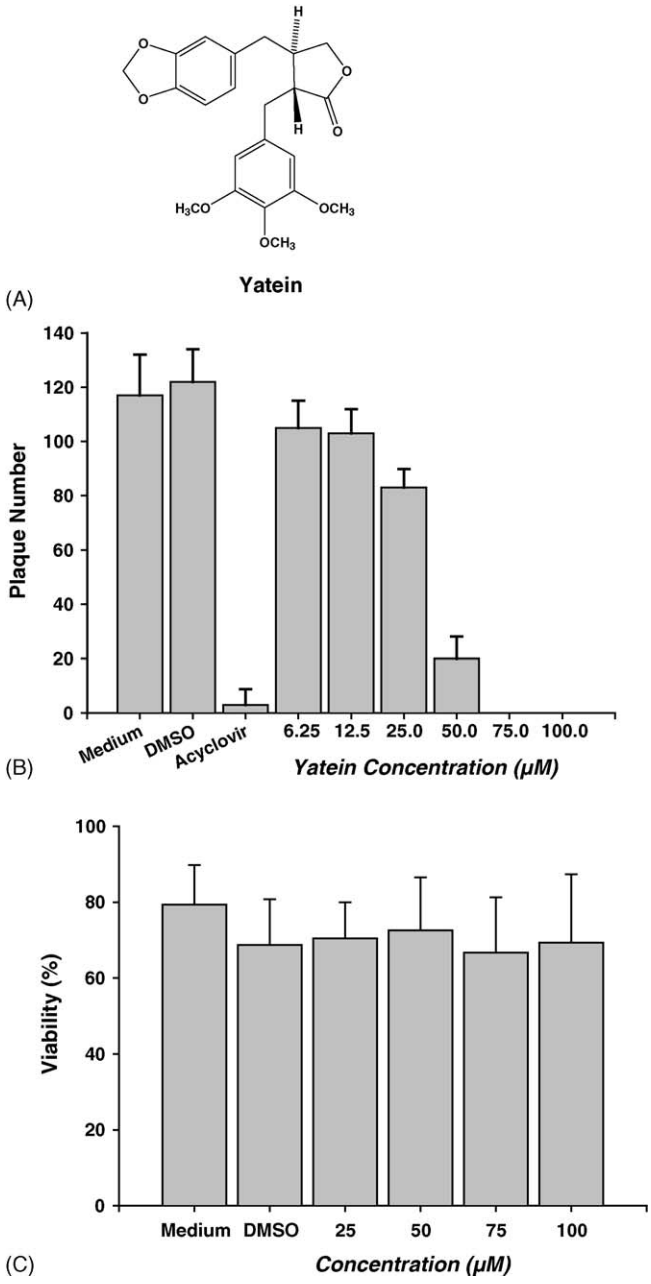

Fig. 1. The yatein structure and its effect on HSV-1 replication and HeLa cells viability. (A) The structure of yatein purifed from *C. obtuse*. (B) Inhibitory effects of 10 μM acyclovir and indicated concentration of yatein on HSV-1 replication were determined by a plaque reduction assay. (C) HeLa cells (3.5×10^5 in 25-cm^2 flasks) were treated with medium, 0.1% DMSO, or 25, 50, 75, and 100 μM yatein for 5 days. Then total, viable, and nonviable cells were counted after stained by trypan blue. Each bar represents the mean ± S.D. of three independent experiments.

effect of its addition at various time. Cell supernatants were collected at 0, 2, 4, 6, 8, 16, 24, and 36 h p.i., and HSV-1 titers were determined by a plaque forming assay. As shown in Fig. 2A, the virus titers in cell supernatants gradually increased at 8 h p.i. and the highest titer was obtained at 24 h p.i. DMSO did not affect the HSV-1 amplification. By contrast, whether 75 μM of yatein was added at the same time as HSV-1 or after HSV-1 adsorption for 1 h, the virus titers in cell supernatants were decreased. The same

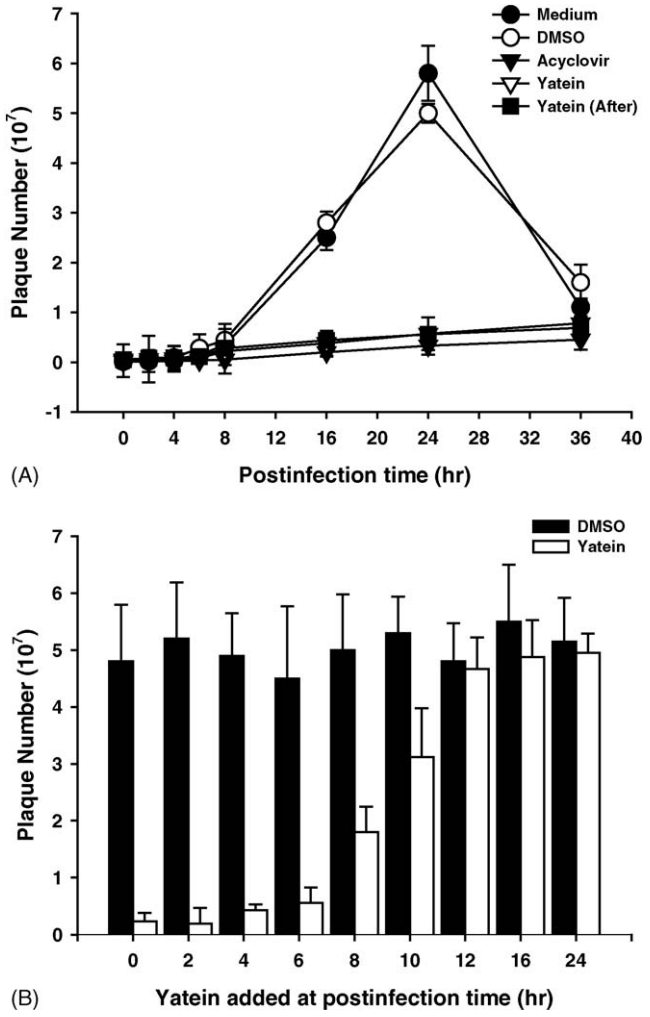

Fig. 2. Yatein kinetically inhibits HSV-1 replication in HeLa. (A) HeLa cells (5×10^6) were infected with 3 m.o.i. of HSV-1 in the presence or absence of acyclovir (10 μM, ▼) or yatein (75 μM). Treatments were as followed: yatein was added with HSV-1 at the same time (▽); and yatein was present after the adsorption period (■). Then cell supernatants were collected at 0, 2, 4, 6, 8, 16, 24, and 36 h p.i. and the viral titers were determined by a plaque forming assay. Each point represents the mean ± S.D. of three independent experiments. (B) HeLa cells (5×10^6) were infected with HSV-1 (3 m.o.i.) and yatein (75 μM) was added at the indicated time. Cell supernatants were collected at 25 h p.i. and HSV-1 titers were determined as described in Section 2. Each bar represents the mean ± S.D. of three independent experiments.

results were obtained in acyclovir-treated cells. These results demonstrated that interruption of HSV-1 adsorption could not be a major factor for yatein blocking HSV-1 replication in HeLa cells.

3.5. Time course analysis of the effect of yatein on HSV-1 replication

Time course experiments were performed to determine at what point in the replication process yatein inhibited HSV-1 replication. Yatein (75 μM) was added with HSV-1 at the same time as HSV-1 (0 h p.i.) or added to the cultures at 2, 4, 6, 8, 10, 12, 16, and 24 h p.i. The cell supernatants were collected at 25 h p.i., and a plaque forming assay was performed. The results indicated that addition of yatein between 0 and 8 h significantly suppressed HSV-1 replication (Fig. 2B). Addition of yatein at 10–24 h p.i. showed only partial or minimal inhibitory effects on HSV-1 replication. The fact that yatein was inhibitory when added between the 0 and 8 h p.i., suggested that the inhibitory effects of yatein might be related to the blocking HSV-1 DNA synthesis and α gene expression necessary for HSV-1 replication during this time frame.

3.6. Yatein attenuated HSV-1 DNA synthesis in HeLa cells

HSV-1 replicates its DNA about at 4 h p.i. or even earlier (Roizman and Sears, 1996). We further defined whether yatein had any effects on HSV-1 DNA synthesis in HeLa cells. After HSV-1 adsorption, the cellular DNA was harvested at 2, 4, 6, and 16 h p.i. and viral DNA was analyzed by PCR. As shown in Fig. 3A, although HSV-1 DNA could not be detected in uninfected HeLa cells (lane 1), the HSV-1 DNA synthesis was increased in HSV-1-infected cells (lanes 2–5). Graphical representation of the ratio of HSV-1 DNA to GAPDH DNA showed that increasing of the signal in HSV-1-infected cells

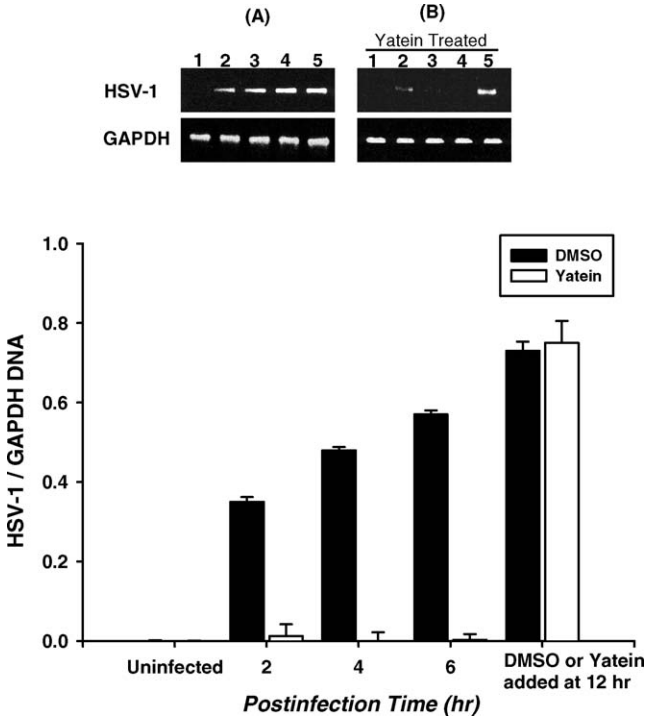

Fig. 3. Effects of yatein on HSV-1 DNA synthesis in HeLa cells detected by PCR. The 5×10^6 cells were infected with or without 3 m.o.i. of HSV-1 in the presence or absence of 75 μM yatein. The cells were harvested at 2, 4, 6, and 16 h p.i. and total DNA was extracted with phenol–chloroform. (A) DNA was extracted from uninfected HeLa cells at 16 h p.i. (lane 1) or from HSV-1-infected cells at 2, 4, 6, and 16 h p.i., respectively (lanes 2–5). (B) DNA was extracted from uninfected HeLa cells treated with yatein at 16 h p.i. (lane 1). Yatein and HSV-1 were added in the cells at the same time and the total cellular DNA was extracted at 2 (lane 2), 4 (lane 3), 6 h p.i. (lane 4), respectively. Lane 5 indicates total cellular DNA extracted from infected cells treated with yatein at 12–16 h p.i. Each band was quantitated by densitometer and the ratio of HSV-1 to GAPDH DNA was calculated. Each bar represents the mean ± S.D. of three independent experiments.

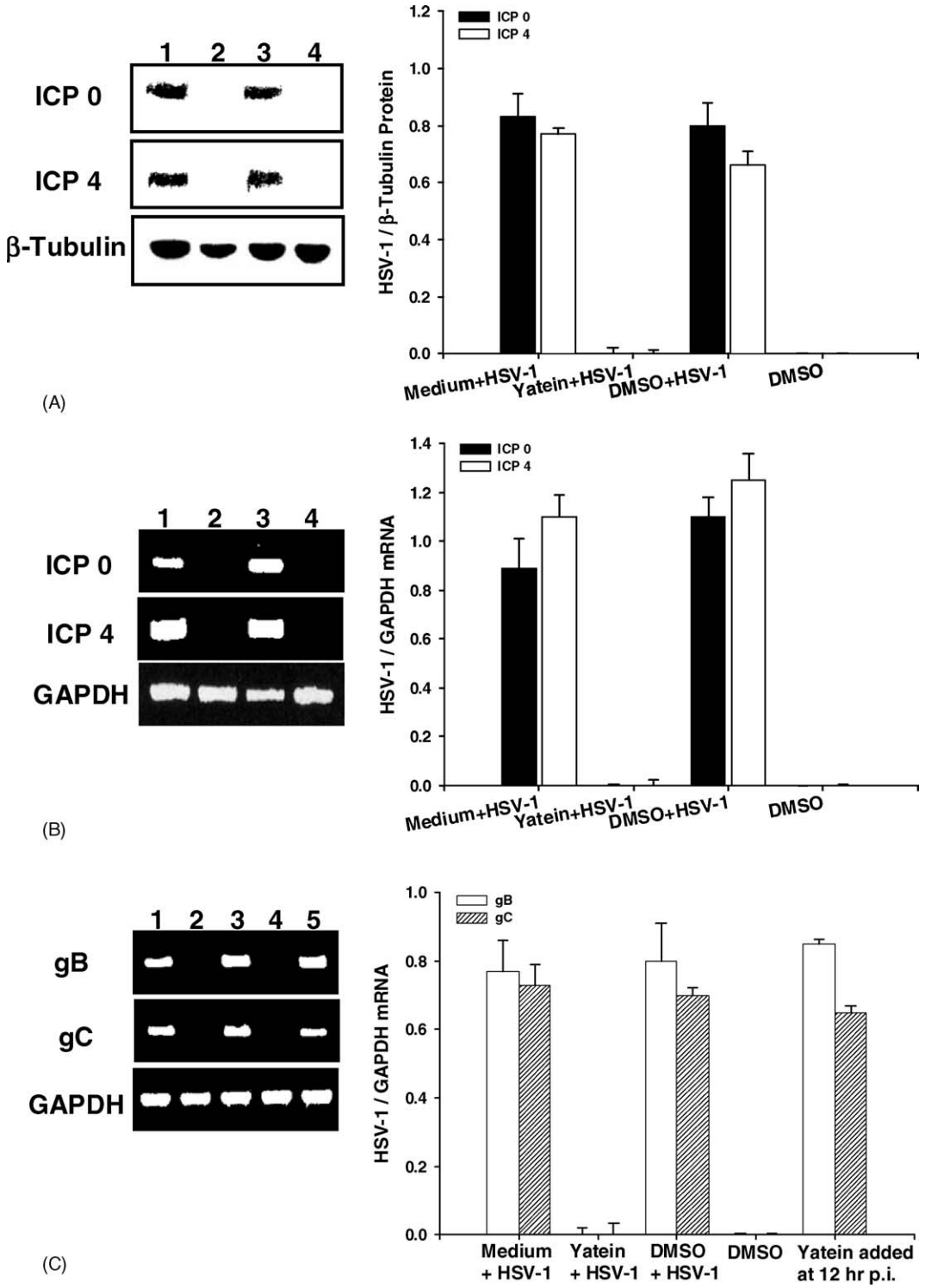

Fig. 4. Effects of yatein on HSV-1 α and γ gene expression in HeLa cells detected by Western blotting and RT-PCR, respectively. HeLa cells (5×10^6) were infected with or without 3 m.o.i. of HSV-1 in the presence or absence of 75 μM yatein. (A) Lysates (50 μg of protein) were collected at 4 h p.i. and run on a 10% SDS-PAGE gel and analyzed by immunoblotting with anti-ICP0 or ICP4 antibody. The total cellular RNA was isolated from HeLa cells at 4 h p.i. (B) or 16 h p.i. (C) and analyzed by RT-PCR. (lanes1 and 3) HSV-1-infected cells treated without or with DMSO; (lane 2) infected cells treated with yatein; (lane 4) uninfected cells; (lane 5) infected cells treated with yatein at 12 h p.i. Bar graph indicates the ratio of ICP0, ICP4, gB, or gC to β-tubulin proteins or GAPDH mRNA, respectively. Each bar represents the mean ± S.D. of three independent experiments.

corresponded to increasing the p.i. time. Although a little HSV-1 DNA was detected at 2 h p.i. (Fig. 3B, lane 2), 75 μM of yatein suppressed viral DNA synthesis in HeLa cells (Fig. 3B, lanes 3 and 4). Additionally, HSV-1 DNA synthesis in HeLa cells was restored when yatein was added to cells at 12–16 h p.i. (Fig. 3B, lane 5). These results suggested that the blocking of HSV-1 replication by yatein was related to impairment of HSV-1 DNA synthesis in HeLa cells.

3.7. Effects of yatein on HSV-1 α gene expression in HeLa cells

As we know, HSV-1 DNA is not synthesized in the absence of competent α proteins such as ICP0 and ICP4 (Roizman and Sears, 1996). To confirm that yatein inhibition of HSV-1 DNA synthesis was related to α gene expression, HeLa cells were infected with HSV-1 or were not infected in the presence or absence of yatein. The total cellular proteins were extracted at 4 h p.i., and ICP0 and ICP4 proteins were determined by Western blot analysis. As shown in Fig. 4A, while uninfected cells expressed virus ICP0 and ICP4 proteins below detectable limits (lane 4), both 110-kDa of ICP0 and 175-kDa of ICP4 were synthesized in HSV-1-infected HeLa cells (lane 1). DMSO did not affect the expression of either protein in HeLa cells (lane 3). However, yatein decreased levels of ICP0 and ICP4 proteins in HSV-1-infected HeLa cells (lane 2). Graphical representation of the ratio of ICP0 or ICP4 to β-tubulin proteins in HeLa cells indicated that signal from infected HeLa cells was significantly impeded by yatein ($P < 0.001$). Furthermore, RT-PCR analysis was applied to prove whether decreasing of ICP0 and ICP4 mRNA expression caused the impairment of protein production in yatein-treated cells. As shown in Fig. 4B, while ICP0 and ICP4 mRNAs were expressed in HSV-1-infected HeLa cells (lanes 1 and 3), yatein significantly decreased the levels of ICP0 and ICP4 mRNAs in the cells (lane 2) ($P < 0.001$). These results suggested that the decreasing of ICP0 and ICP4 proteins by yatein was attributed to reduction of ICP0 and ICP4 mRNA expression in HeLa cells.

Furthermore, HSV-1 γ genes such as gB and gC are dependent on α proteins for expression (Roizman and Sears, 1996). To confirm the suppressory effect of yatein on ICP0 and ICP4 to cause deficient in γ gene expression, we examined gB and gC mRNA expression in HeLa cells treated with yatein. At 16 h p.i., the total cellular RNA was collected from HeLa cells in the presence or absence of 75 μM yatein and was available for reverse transcription-PCR (RT-PCR). As shown in Fig. 4C, neither DMSO nor yatein affected the levels of GAPDH mRNA in HeLa cells. While little expression of gB and gC mRNAs was detectable in uninfected HeLa cells, the levels of both γ mRNAs were significantly increased in HSV-1-infected cells (lanes 1 and 3). By contrast, the level of RT-PCR products for gB and gC mRNA amplified from HSV-1-infected HeLa cells were decreased by yatein (lane 2). Laser densitometry analysis demonstrated that the ratios of gB and gC to GAPDH mRNA were significantly impaired by yatein ($P < 0.001$). The inhibition of gB and gC mRNA expression due to yatein activity was blocked when yatein was added in HeLa cells between 12 and 16 h p.i. (lane 5). We suggest that the decrease of gB and gC mRNAs by yatein was the result of a deficiency of ICP0 and ICP4 proteins in HeLa cells.

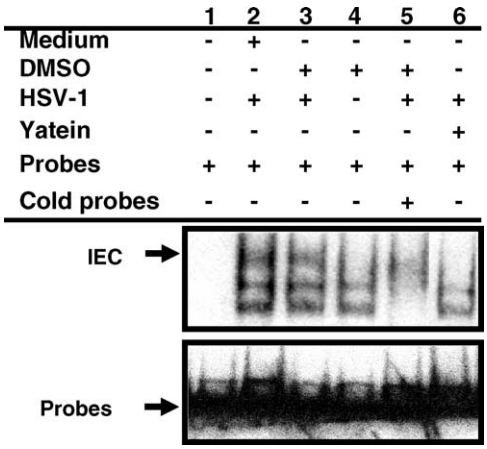

Fig. 5. Effects of yatein on formation of IEC in HeLa cells detected by EMSA. EMSA was performed as described in Section 2. Nuclear extracts from uninfected (lane 4) or HSV-1-infected HeLa cells (lane 2) were incubated with a DIG-labeled GARAT probe. The formation of IEC in virus infected nuclear extracts treated with 0.1% DMSO (lane 3) or 75 μM yatein (lane 6) mixed with the probes were detected. (Lane 1) Results of adding free probes to the reaction; (lane 5) results of adding a 50-fold excess of unlabeled probe to the reaction mixture.

3.8. A novel virus-induced protein–DNA complex reduced by yatein

The formation of αTIF/C1/Oct-1/GARAT multiprotein/DNA complexes is responsible for induction of α genes (McKnight et al., 1987). To determine whether yatein reduction of ICP0 and ICP4 transcripts was related to this initial transcriptional *trans* activation event, the DIG-labeled DNA fragment containing GARAT was incubated with nuclear extracts from yatein-treated HeLa cells. As shown in Fig. 5, with extracts from infected cells, a novel virus-induced protein–DNA complex (immediate early complex; IEC) was detected (lane 2). While DMSO did not influence protein–DNA complex formation (lane 3), a 50-fold excess of unlabeled probe blocked the formation of these complexes (lane 5). The level of IEC was decreased by yatein treatment (lane 6). These results suggested that the reduction by yatein of ICP0 and ICP4 mRNAs might be related to an interruption in the formation of αTIF/C1/Oct-1/GARAT multiprotein/DNA complexes.

4. Discussion

The plaque reduction assay offers a popular system to evaluate the effect of antiviral agents against HSV-1 (Bacon et al., 1996). In this study, nine Chinese herbs were screened by this model then *C. obtusa* was evaluated to contain anti-HSV-1 agents, supporting the validity of its use for pharmacological studies. Results shown here indicated that yatein purified from *C. obtusa* suppressed HSV-1 replication in HeLa cells without significantly reducing the cells viability. The inhibitory effect

of yatein may be attributed to its interference with structural proteins and DNA synthesis, and α gene expression of HSV-1. Hence, the suppressive activity of yatein on the viral replication, might important implications with regard to *C. obtusa* therapeutic activity in microorganisms infection. This is the first report of the antiviral action mechanisms of yatein.

Yatein isolated from *C. obtusa* is a lignan compound (Hwang et al., 2004). Lignans present in many plants and animals and are as biomarkers of healthy foods (Adlercreutz, 1998). There are several evidences to prove their biological functions containing antiviral (Craigo et al., 2000) and antiinflammatory activities (Bastos et al., 2001). Results showing that yatein reduced HSV-1 replication in HeLa cells were compatible with data reported by Medarde et al. (1995). Yatein blockage of HSV-1 replication was unlikely related to DMSO because the cells viability and HSV-1 replication in HeLa cells were not changed by DMSO. Although we did not determine whether yatein bound to virus or destroyed virus structure, a comparison with control groups showed that the HSV-1 infectivity and titer were not significantly decreased when viral particles were treated with 75 μM or 100 μM yatein at 37 °C for 1 h then infected cells (data not shown). Moreover, addition of the drug after viral adsorption produced antiviral activity similar to that when HSV-1 and yatein were added at the same time. Yatein was inhibitory when it was added to cells between the 0- and 8-h p.i. time range. These results suggest that binding of yatein to virion or destruction of HSV-1 structure could not be a major inhibitory factor for virus replication. In the host cells, HSV-1 replication is coordinately regulated and sequentially ordered in a cascade and is believed to proceed as follows: (a) αTIF, a γ protein packaged in the virion, turns on α genes to be transcribed; (b) expression of α genes regulates β genes to be expressed; (c) both α and β gene expression initiate HSV-1 DNA replication; (d) γ genes are synthesized, and then virions are assembled; and (e) HSV-1 is enveloped as it buds through the nuclear membrane (Mackem and Roizman, 1982; Roizman and Sears, 1996; Jones and Roizman, 1979). In the present study, we found that yatein decreased the ICP0 and ICP4 gene expression in HeLa cells. The results of EMSA indicated that the retarded specie IEC was decreased in the GARAT probes incubated with nuclear extracts from yatein-treated HeLa cells. Thus, the possibility that yatein inhibited HSV-1 replication through disturbance of αTIF/C1/Oct-1/GARAT stable complex formation cannot be excluded.

ICP0 has been reported to perform several functions including selection of transcriptional termination sites and stimulation of DNA synthesis. Although ICP0 is not essential for HSV-1 replication in some cell cultures, defects in this gene delay the expression of β and γ genes and impair viral replication (Roizman and Sears, 1996). ICP4 is the major transactivator of HSV-1 genes. Thus, ICP4 and ICP0 play important roles in regulation of β and γ gene expression and are essential for HSV-1 DNA replication (Honess and Roizman, 1975). Moreover, the present data indicated that yatein blocked HSV-1 DNA synthesis in HeLa cells. We suggest that the decrease in HSV-1 DNA synthesis due to yatein is related to impairment of ICP0 and ICP4. Both gB and gC are involved in viral envelope structure and play important roles in viral attachment and penetration (Roizman and Sears, 1996). The evidence demonstrates that ICP4 is required for expression of γ genes (Honess and Roizman, 1974). HSV-1 DNA synthesis is required for gC mRNA expression. The present results indicated that yatein decreased gB and gC mRNA expression in HeLa cells. Thus, we predict that yatein attenuates levels of gB and gC mRNA in HeLa cells, which may be related to impairment of DNA synthesis and ICP4 and ICP0 production.

From the present results, we hypothesize that impairment of HSV-1 multiplication in yatein-treated HeLa cells, was related to (a) decreases in HSV-1 ICP0 and ICP4 gene expression due to yatein, which might be related to disturbance of the formation of αTIF/C1/Oct-1/GARAT multiproteins complexes; (b) inhibition of viral DNA synthesis; (c) interference with gB and gC mRNA synthesis; and (d) no HSV-1 plaque formation in HeLa cells. The expression of the immediate-early gene represents one stage of the HSV-1 replication cycle that could be targeted by a novel antiviral therapy to deliver a significant reduction in virus replication in both acute and latent infections (Roizman and Sears, 1996). While nucleoside analogues have been successful in treating acute infections, they fail to modulate reactivation of latent virus. Thus, small molecules identified from Chinese herbs such as yatein which act as inhibitors of HSV-1 immediate-early gene expression may have the potential to impact clinical disease to a far greater extent than currently marketed nucleosides. Chinese herbs may be used as health foods to prevent HSV-1 from recurrent infection. Future experiments with treatment of HSV-1-infected animals with yatein will be necessary to define whether it can reduce experimental viral infection injury. Moreover, this study not only demonstrates Chinese herbs are potential therapeutic drugs for the viral infection but also supports a model for future protocol design in preclinical studies.

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