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Regulation of herpes simplex virus type 1 replication in Vero cells by *Psychotria serpens*: relationship to gene expression, DNA replication, and protein synthesis

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Abstract

Inhibitory effects of ethanolic extracts from seven Chinese herbs on herpes simplex virus type 1 (HSV-1) replication were investigated. From a bioassay-guided fractionation procedure, PS-A-6 was isolated from *Psychotria serpens* (*P. serpens*), which suppressed HSV-1 multiplication in Vero cells without apparent cytotoxicity. Time-of-addition experiments suggested that the inhibitory action of PS-A-6 on HSV-1 replication was not through blocking of virus adsorption. In an attempt to further localize the point in the HSV-1 replication cycle where arrest occurred, a set of key regulatory events leading to viral multiplication was examined, including viral gene expression, DNA replication, and structural protein synthesis. The results indicated that gB mRNA and protein expression in Vero cells were impeded by PS-A-6. Southern blot analysis showed that HSV-1 DNA replication in Vero cells was arrested by PS-A-6. In addition, PS-A-6 decreased thymidine kinase (tk) and ICP27 mRNA expression in the cells. The mechanisms of antiviral action of PS-A-6 seem to be mediated, at least in part, through inhibition of early transcripts of HSV-1, such as tk and ICP27 mRNAs, arresting HSV-1 DNA synthesis and gB gene expression in Vero cells. Plans are underway for the isolation of pure compounds from PS-A-6 and elucidation of their mechanism of action.

Keywords: DNA replication; gB proteins; HSV-1; ICP27; tk mRNA; PS-A-6

1. Introduction

Herpes simplex virus type 1 (HSV-1) is an enveloped DNA virus, which causes a variety of infections in human. After primary infection, HSV-1 establishes latency in sensory and autonomic neurons innervating the mucosal tissues where primary infection takes place, and is reactivated by the proper stimulus to cause recurrence (Roizman and Sears, 1996). The period of recurrence is irregular (Kuo and Lin, 1990). Individuals who are either immunocompromised or have can-

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cer are in danger of recurrent HSV-1 infections (Logan et al., 1971; Kuo et al., 1987). The recipients of organ transplantation are at a high risk for increased severity of HSV-1 infection (Pass et al., 1978). Infection with HSV-1 can lead to life-threatening encephalitis and ocular infections that result in corneal inflammation and scarification. This scarification is a major cause of blindness in developing countries (Corey and Spear, 1986). In addition, HSV-1 has been shown to be a factor for spreading of human immunodeficiency virus and causes severe diseases in acquired immunodeficiency syndrome patients (Mann et al., 1984; Chen et al., 1998).

Nucleoside analogs have been extensively investigated in the search for effective antiherpetic 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 (tk) in infected cells (Furman et al., 1979; Elion et al., 1997). However, it has recently been observed that the acyclovir-resistant HSV infection has come from immunocompromised patients such as transplant patients and patients with AIDS (Coen, 1996; Kimberlin and Whitley, 1996). Therefore, it is of interest to develop new anti-HSV agents that substitute for or complement acyclovir.

Chinese herbs are potential sources of useful edible and medicinal plants. They are expected to find use as a function food because of their various biological activities such as immunomodulatory and antitumor functions (Kuo et al., 1997, 1998). More and more people in developing countries utilize traditional medicine for their major primary health care needs (Farnsworth, 1993; Houghton, 1995). However, ethopharmacology 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), and catechin derivatives (Ferrea et al., 1993) isolated from medicinal plants are found to have inhibitory activities against the replication of HSV-1. A promising result of a naturally occurring antiherpetic agent was given by *n*-docosanol which is undergoing phase III clinical trials in patients (Alrabiah and Sacks, 1996; Pope et al., 1996). Furthermore, our previous results indicates that flavanones isolated from *Limonium sinense* have anti-HSV-1 activity (Lin et al., 2000). These findings show that natural products are still potential sources in the search for new antiherpetic agents.

In the present study, seven Chinese medicinal herbs, which are widely known in folk medicine for the treatment of viral and bacterial infection, were selected for anti-HSV-1 replication assay. The herbs were *Ampelopsis cantoniensis* (AC), *Melilotus indicus* (MI), *Verbena bonariensis* (VB), *Buddleia asiatica Lour* (BA), *Ruta graveolens* (RG), *Tadehagi triquetrum* (TT), and *Psychotria serpens* (PS). The ethanolic extracts that showed appreciable anti-HSV-1 activity were separated by a bioassay-guided fractionation procedure. The effects of active extracts on HSV-1 DNA, RNA, and proteins synthesis in Vero cells were evaluated. The cytotoxic activities of active extracts were also reported. The mechanisms of antiviral action of active components were elucidated in vitro.

2. Materials and methods

2.1. Source of the seven Chinese herbs

All seven species of Chinese herbs were purchased from Chinese medicine shops in Taipei. The plants were identified by Mr Jun-Chih Ou, resident medicinal plant expert of The National Research Institue of Chinese Medicine.

2.2. Preparation of crude extracts of the seven Chinese herbs

Dried plant material (600 g) was extracted with ethanol (5 l × 3). The solvent was removed under reduced pressure. The solid residue from each crude extract was dissolved in dimethylsulfoxide (DMSO) to a concentration of 100 mg/ml and stored at 4°C until use.

2.3. Fractionation of P. serpens ethanolic extracts

The sample, absorbed on silica gel (sample/adsorbent (v/v) = 1/8), was subjected to dry flash column chromatography. Sufficient hexane was passed through the column to expel all of the air. Extensive gradient elution was then employed using hexane methyl chloride, acetone, and methanol to yield four fractions (PS-H, PS-C, PS-A, and PS-M). The active PS-A fraction was further purified by rechromatography. Elution was started with *n*-hexane, and the polarity of solvents was increased in a stepwise manner in the following sequence: *n*-hexane–ethyl acetate (EtOAc), EtOAc, EtOAc–MeOH and finally MeOH. Altogether, six subfractions PS-A-1 to PS-A-6 were obtained.

2.4. Cell culture

Vero cells were used for the plaque assay. They were cultured as a monolayer in minimal essential medium (MEM; GIBCO, Grand Island, NY) supplement with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin. As the cells became confluent, they were subcultured following dispersing with 0.01% trypsin–ethylenediaminetetraacetate (EDTA). All the cells were incubated at 37°C in a 5% CO_2 incubator. The mycoplasma contamination was checked by Mycotect™ kit in Vero cells (Mycotect™ kit, Life Technology, Gaitherburg, MD). The cells were free from mycoplasma contamination.

2.5. Preparation of HSV-1

HSV-1 (KOS strain) stocks were prepared in Vero cells. The cells were infected by HSV-1 at 0.1 multiplicity of infection (m.o.i.) and harvested at 20 h postinfection (p.i.) and centrifuged at 3000 rpm (Jouan; CR312) at 4°C for 20 min. The supernatant was collected and stored at −70°C for use.

2.6. The plaque reduction assay

The method followed procedures described previously (Kuo et al., 1993). Acyclovir was utilized as a positive control. One day prior to virus infection, the Vero cells were grown in culture dishes (6 cm in diameter; Cellstar) as a monolayer (3.5×10^5/well). One hundred plaque forming units (PFU) of HSV-1 was added to each well and the test extracts or acyclovir were added to the cells at varying concentrations or at different times. The plate was incubated at 37°C, 5% CO_2 and the viruses were adsorbed for 1 h. Then 1% methyl cellulose was added to each well. After 5 days, the cells were stained with crystal violet and the virus plaques formed in Vero cell cultures were counted. The inhibitory activities of test extracts or acyclovir on HSV-1 replication were calculated as follows:

$$\text{Inhibitory activity (\%)} = \frac{\text{Number of plaques}_{(\text{Control-Experiment})}}{\text{Number of plaques}_{(\text{Control})}} \times 100.$$

2.7. Southern blot analysis

The total DNA was extracted from Vero cells by the modified method of Maniatis et al. (1982). The 5×10^6 cells were infected with or without 0.1 m.o.i. HSV-1 in the presence or absence of 20 μg/ml PS-A-6. The cells were harvested at 16 h p.i. and lysed with 0.2 M Tris–HCl (pH 8.5) containing 100 mM EDTA, 100 mM NaCl, 0.5% NP-40, 1% sodium dodecyl sulfate (SDS), and 100 mg/ml proteinase K. The DNA solution was extracted with phenol–chloroform. The extracted total DNA was digested by the *Eco*RI restriction enzyme. Then, 20 μg of digested total DNA was run on a 0.8% agarose gel and then transferred to a nitrocellulose filter. The filter was prehybridized for 4 h at 68°C in 6 × SSC, 0.5% SDS, 5 × Denhardt's solution, 100 μg/ml denatured salmon sperm DNA, and 1 mM EDTA. Hybridization was done in the same buffer containing 1×10^7 cpm/ml of deoxycytidine 5′-[α-^{32}P] triphosphate-labeled HSV-1 tk cDNA for 24 h at 68°C with agitation. The nitrocelluose filter was then rinsed once with 2 × SSC and 0.5% SDS at room temperature and twice with 0.1 × SSC. After washing, the paper was exposed to X-ray film (Kodak).

The hybrid band was detected on the film. Each band was quantitated using a laser scanning densitometer SLR-2D/1D (Biomed Instruments Inc., Fullerton, CA).

2.8. Extraction of total cellular RNA

The total cellular RNA was extracted from Vero cells by a method described previously (Kuo et al., 1998). The 5×10^6 cells were infected with or without 0.1 m.o.i. HSV-1 in the presence or absence of 20 μg/ml PS-A-6. After infection, Vero cells were collected at various time and washed by cold Tris–saline containing 25 mM Tris, pH7.4, 130 mM NaCl, 5mM KCl then suspended in NDD buffer containing 1% NP-40, 0.5% sodium deoxycholate, and 1% dextran sulphate. After centrifugation, the supernatants were extracted with a phenol–chloroform mixture. The extracted RNA was precipitated with 100% cold ethanol. The total cellular RNA was pelleted by centrifugation and redissolved in diethyl pyrocarbonate (DEPC)-treated H_2O. The concentration of the extracted RNA was calculated by measuring the optical density at 260 nm.

2.9. Northern blot analysis and dot blot analysis

In Northern blot analysis, a 15 μg sample of total cellular RNA was resolved on 6.66% formaldehyde agarose gel and then transferred to nitrocellulose filter (Schleicher & Schuell, Germany) with 20 × standard saline citrate (SSC) containing 3M NaCl and 0.3 M sodium citrate. In dot blot analysis, 10 μg of total cellular RNA was directly adsorbed on nitrocellulose filter. The filter was prehybridized at 42°C for 4 h (prehybridization solution of 0.1% sodium pyrophosphate, 0.25 mg/ml denatured salmon sperm DNA, and 50% deionized formamide). Deoxycytidine 5′-[α-^{32}P] triphosphate-labeled tk cDNA or ICP27 cDNA was then added to the same solution and incubated at 42°C for 12 h. The filter was washed with 0.1 × SSC at room temperature for 5 min, and then washed by 0.1X SSC and 0.1% SDS at 50°C for 20 min. Finally, the filter was exposed to X-ray film (Kodak).

2.10. Synthesis of first strand complementary deoxyribonucleic acid

Aliquots of 1 μg of RNA were reverse-transcribed using the Advantage™ RT-for-PCR kit from CLONTECH according to the manufacturer's instructions. Briefly, 1 μg RNA in 12.5 μl of DEPC-treated H_2O was mixed with 20 μM of oligodeoxythymidine (oligo $dT)_{18}$, and heated at 70°C for 10 min, then quick-chilled on ice. The following reagents were added to the tube: 6.5 μl of concentrated synthesis buffer (50 mM Tris–HCl, pH8.3; 75 mM KCl; 3mM $MgCl_2$; 0.5 mM dNTPs; and 0.5 unit RNase inhibitor), and 200 U of the Moloney murine leukemia virus (MMLV) reverse transcriptase. The reaction was initially incubated at 42°C for 1 h, and then at 94°C for 5 min to terminate the reaction. DEPC-treated H_2O (80 μl) was added to the tube and then stored at −20°C for use in the PCR.

2.11. PCR

The PCR was performed in an air thermocycler according to the manufacturer's instructions as described previously (Saiki et al., 1985). Briefly, 10 μl of cDNA was mixed with 0.75 μM primers, 4 units of Taq polymerase, 10 μl of reaction buffer (2 mM Tris–HCl, pH8.0; 0.01 mM EDTA; 0.1 mM dithiothreitol, DDT; 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. The primer pairs for the gB mRNA were designed from the pub-

Table 1
Nucleotide sequence of the primers used for amplification of HSV-1 gB in Vero cells

Primers	Sequence	Predicted size (bp)
GB	5′:CTG GTC AGC TTT CGG TAC GA	341
	3′:CGT TTG TCG ACG TGC TGG AC	
β-Actin	5′: TTG AGA CCT TCA ACA CCC	
	3′: CTC TAC TGA AGC TTT TCG ACT	1300

lished HSV-1 cDNA sequence data (Table 1) (Cone et al., 1991). The PCR was done at the following setting 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 30 cycles then elongation temperature of 72°C for 10 min. Following the reaction, the amplified product was taken out of the tubes and run on 2% agarose gel. Each band was quantitated using laser scanning densitometer SLR-2D/1D (Biomed Instruments Inc., Fullerton, CA).

2.12. Western blot analysis

The density of Vero cells was adjusted to 5×10^6 cells/ml before use. Cell suspension (1 ml) was divided into a six-well flat-bottomed plate (Greiner, Germany) with or without 0.1 m.o.i. HSV-1. 20 μg/ml of PS-A-6 was added to the cells. The plates were incubated in 5% CO_2-air humidified atmosphere at 37°C for 24 h. Cells were harvested and washed one time with phosphate-buffered saline (pH 7.2) containing 0.5 mM EDTA; and lysed in 20 mM Tris-HCl, 30 mM $Na_4P_2O_7$, 50 mM NaF, 5 mM EDTA (pH 7.2) containing 0.5% Triton X-100, 1 mM dithiothretiol, 10 mg/ml leupetin, 5 μg/ml aprotinin, and 10 mM β-glycerophosphate. The lysates were cleared of insoluble material by centrifugation. Proteins (20 μg) were dissolved in the dissociation buffer (2% SDS, 5% β-mercaptoethanol, 0.05 M Tris–HCl, and 20% glycerol, pH7.6) and boiled for 5 min. Then proteins were resolved by 10% SDS–PAGE and transferred to nitrocellulose filters. After blocking the filters with a solution containing 1% bovine serum albumin (BSA), the filers were incubated with mouse monoclonal antibody raised against gB proteins (C65151M, BIØDESIGN). Specific reactive proteins were detected by an enhanced chemiluminescence method, employing a rabbit anti-mouse Ig Ab linked to horseradish peroxidase (Amersham).

2.13. The effect of PS-A-6 on Vero cell growth

The method was modified from previously described (Yang et al., 1999). The DNA, RNA, and protein being synthesized in the growth cells will be labeled with tritiated thymidine, tritiated uridine, and tritiated leucine, respectively. In analysis of total cellular protein synthesis, the cells were preincubated with leucine-free MEM medium for 1 day. Then density of Vero cells was adjusted to 2×10^6 cells/ml and cells were cultured with MEM medium containing 2% FCS. Cell suspension (100 μl) was divided into a 96-well flat-bottomed plate (Nunc 167008, Nunclon, Raskilde, Denmark) with or without various concentration of PS-A-6. The plates were incubated in 5% CO_2-air humidified atmosphere at 37°C for 5 days. Subsequently, tritiated thymidine, tritiated uridine, or tritiated leucine (1 μCi/well, NEN) was added into each well, respectively. After a 16 h incubation, the cells were harvested on glass fiber filters by an automatic harvester (Dynatech, Multimash 2000, Billingshurst, UK). Radioactivity in the filters was measured by scintillation counting.

2.14. Determination of cell viability

Approximately 3.5×10^5 Vero cells were cultured in 25 cm^2 flask and incubated with 0.1% DMSO, or various concentration of PS-A-6 isolated from *P. serpens* for 5 days. Total, viable, and non-viable cell numbers were counted under the microscope with the help of a hemocytometer following staining by trypan blue. The percentage of viable cells were calculated:

$$\text{Viability } (\%) = \frac{\text{Viable cell number}}{\text{Total cell number}} \times 100.$$

2.15. Statistical analysis

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

3. Results

3.1. PS-A-6 isolated from P. serpens inhibit HSV-1 replication

As shown in Fig. 1, ethanolic extracts isolated from seven Chinese herbs were evaluated for their

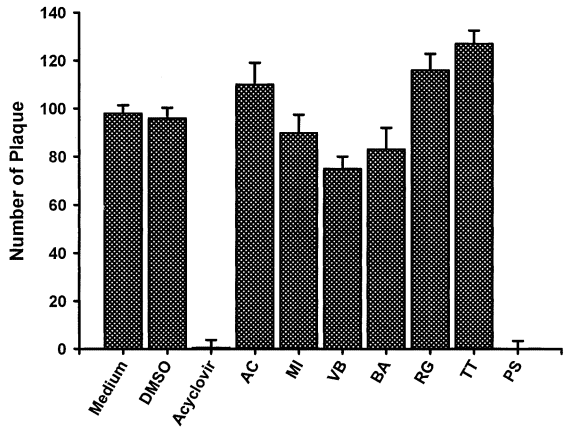

Fig. 1. Effects of ethanolic extracts from seven Chinese herbs on HSV-1 replication in Vero cells. Vero cells (3.5×10^5) were grown in 6 cm-dishes as a monolayer. One hundred PFU of HSV-1 was added to each well and 100 μg/ml of each extract or 10 μM of acyclovir were added to cells. After adsorption for 1 h, 1% methyl cellulose was added to each well. On the 5th day p.i., the cells were stained with crystal violet and the virus plaques formed in the Vero cell cultures were counted. Each bar represents the mean of three independent experiments.

inhibitory activities on the HSV-1 plaque formation in Vero cells, respectively. The HSV-1 replication was not affected by DMSO treatment. The acyclovir blocked HSV-1 plaque formation in Vero cells. While AC, MI, VB, BA, RG, and TT ethanolic extracts had little effects on HSV-1 replication, 100 μg/ml of PS ethanolic extracts significantly suppressed HSV-1 replication. Furthermore, the HSV-1 inhibitory effects of subfractions separated from PS were determined (Table 2). The results indicated that PS-A-6 extracted from PS-A had the highest suppressory activity. The inhibitory effects of PS-A-6 on HSV-1 replication were concentration dependent (Fig. 2A). The IC_{50} of PS-A-6 was significant lower than those of PS-A and PS (Table 2).

3.2. Effects of PS-A-6 on Vero cells viability and growth

To delineate whether the suppressory effect of PS-A-6 on HSV-1 replication was related to cytotoxicity, we examined the viability of Vero cells after treated with PS-A-6 for 5 days. Comparison with control groups, the viability of Vero cells treated with 10, 20 or 50 μg/ml of PS-A-6 was not significantly decreased (Fig. 2B). Results indicated that even at 200 mg/ml, PS-A-6 had no direct cytotoxicity on Vero cells (data not shown). The therapeutic index for PS-A-6 is higher than 20 (EC_{50}/IC_{50}). The DMSO did not affect the cell viability. Additionally, effects of PS-A-6 on Vero cell growth were determined by tritiated thymidine, tritiated uridine, and tritiated leucine uptake methods. As shown in Fig. 3, the DNA, RNA, and protein synthesis in PS-A-6 treated and untreated Vero cells were no difference. These results demonstrated that inhibitory mechanisms

Table 2
The inhibitory activities of various subfractions from *P. serpens* on HSV-1 replication

Chinese herbs	Fractions	Inhibitory activity (%) 50 μg/ml	Subfractions	Inhibitory activity (%) 25 μg/ml	IC_{50} (mg/ml)
PS					58.3 ± 4.6
	PS-H	-10.0 ± 5.5			
	PS-C	20.5 ± 4.6			
	PS-A	100 ± 3.9			27.8 ± 3.8
	PS-M	33.2 ± 7.4			
			PS-A-1	-20.8 ± 6.6	
			PS-A-2	18.0 ± 9.3	
			PS-A-3	-42.9 ± 7.2	
			PS-A-4	-30.0 ± 10.0	
			PS-A-5	15.5 ± 4.8	
			PS-A-6	100 ± 5.5	10.0 ± 2.8

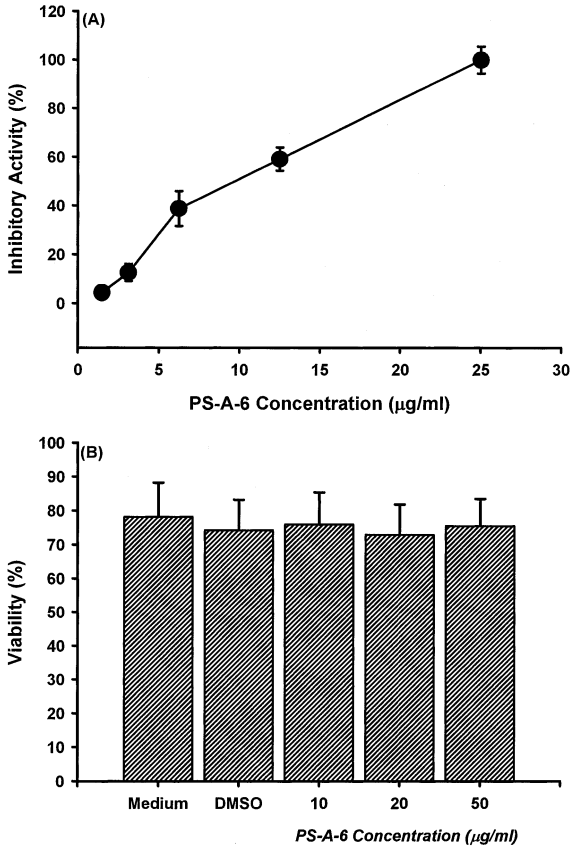

Fig. 2. Suppressory effects of PS-A-6 on HSV-1 replication and the viability of Vero cells treated with the PS-A-6. (A) Inhibitory effects of PS-A-6 on HSV-1 replication were determined as described in Section 2.13 and Section 2.14. The inhibitory activity was calculated as indicated formula. Each point represents the mean of three independent experiments. (B) Vero cells (3.5×10^5) were treated with medium, 0.1% DMSO, or various concentration of PS-A-6 for 5 days. Then total, viable, and non-viable cell number were counted after staining by trypan blue. Each bar represents the mean of three independent experiments.

of PS-A-6 on viral replication were not through cytotoxicity and blocking of Vero cells growth.

3.3. The effect of PS-A-6 on HSV-1 adsorption

To further elucidate whether PS-A-6 inhibited HSV-1 replication was related to blocking viral adsorption, we examined the effect of its addition at various time. Then cell supernatants were collected at 0, 2, 4, 8, 16, 24, 36, and 48 h p.i. and the viral titers were determined by the plaque forming assay. As shown in Fig. 4, virus titers in cell supernatants gradually increased at16 h p.i. and continued to increase at 48 h p.i. By contrast, when 20 μg/ml of PS-A-6 was added with HSV-1 at the same time or added after HSV-1 adsorption, virus titers in cell supernatants were de-

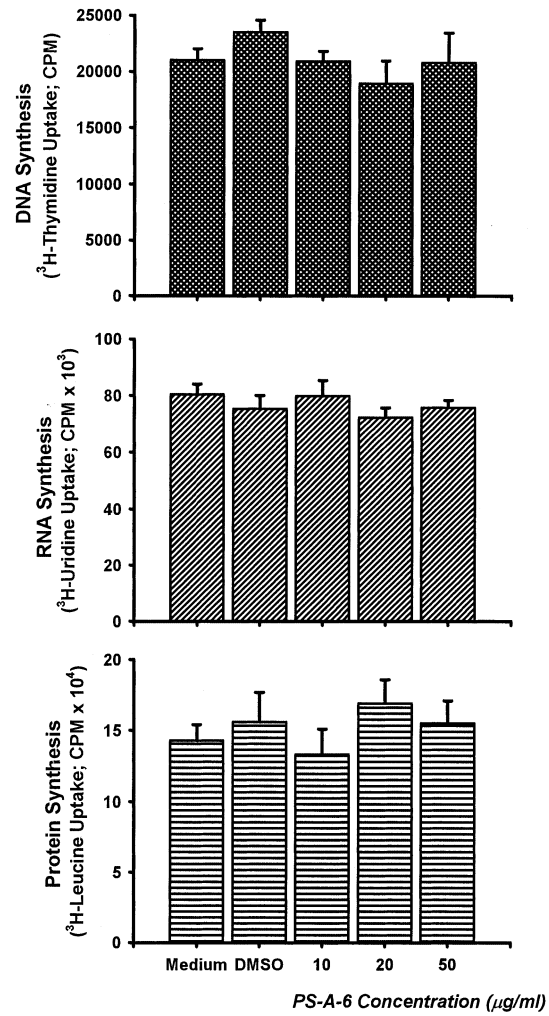

Fig. 3. Effects of PS-A-6 on Vero cells growth. Vero cells (2×10^5/well) were treated with or without the indicated concentration of PS-A-6 for 5 days. The DNA, RNA, and protein synthesized in the growth cells were detected by tritiated thymidine, tritiated uridine, or tritiated leucine uptake (1 μCi/well), respectively. After a 16 h incubation, the cells were harvested by an automatic harvester then radioactivity was measured by a scintillation counting. Each bar represents the mean of three independent experiments.

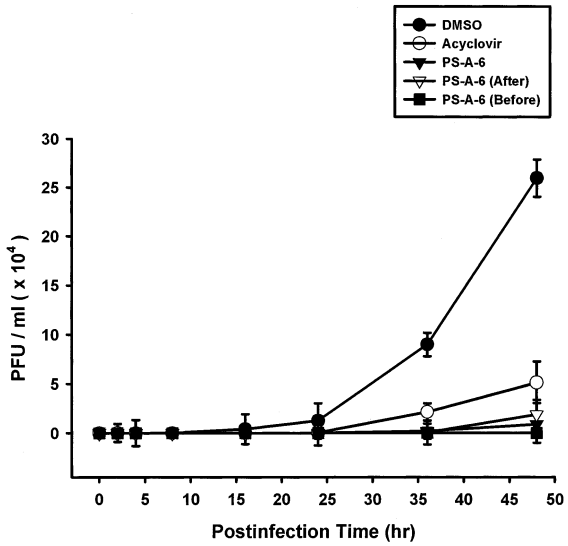

Fig. 4. Kinetics of inhibition of HSV-1 replication by PS-A-6. Vero cells (5×10^6) were infected with 0.1 m.o.i. of HSV-1 in the presence or absence of acyclovir (10 μM) or PS-A-6 (20 μg/ml). Treatments were as follows: PS-A-6 was added with HSV-1 at the same time (PS-A-6); PS-A-6 was continually present after the adsorption period (after); and PS-A-6 was present for 2.5 h and removed before the adsorption phase (before). Then cell supernatants were collected at 0, 2, 4, 8, 16, 24, 36, and 48 h p.i. and the viral titers were determined by the plaque forming assay. Each point represents the mean of three independent experiments.

creased. The same results were obtained in acyclovir treated cells. In addition, the pretreatment of Vero cells with 20 μg/ml of PS-A-6 for 2.5 h before infection still caused inhibition in virus yield. These results demonstrated that PS-A-6 did not affect HSV-1 adsorption to host cells.

3.4. Effects of PS-A-6 on HSV-1 gB proteins and mRNA expression in Vero cells

To further analyze the mechanisms of PS-A-6 action, we examined HSV-1 gB proteins in Vero cells after treated with PS-A-6 (20 μg/ml) for 24 h. As shown in Fig. 5A, while uninfected cells expressed little gB proteins (Lane 2), the 116 kd gB proteins could be detected in HSV-1 infected Vero cells (Lane 1). The PS-A-6 suppressed gB protein synthesis in the cells (Lane 3). Graphical representation of the relative absorbance values of gB proteins signal indicated that little signal was expressed in the cells treated with PS-A-6. The total cellular RNA was collected at various time after viral adsorption and gB mRNA was determined with reverse transcriptase-polymerase chain reaction (RT-PCR). As shown in Fig. 5B, the gB mRNA expression in Vero cells gradually increased after HSV-1 infection. But PS-A-6 decreased the level of gB mRNA. The ratio of gB mRNA to β-actin mRNA was calculated. The PS-A-6 significantly blocked gB mRNA synthesis in Vero cells. These results suggested that the decreasing of gB proteins was attributed to deficiency of gB mRNA expression in Vero cells.

3.5. The effect of PS-A-6 on HSV-1 DNA synthesis

We further defined whether PS-A-6 has any effect on HSV-1 DNA replication. After HSV-1 adsorption, the cellular DNA was harvested at 16 h p.i. and viral DNA was analyzed by Southern blot method (Fig. 6A). The cellular β-actin DNA could be detected in Vero cells treated with or without PS-A-6. Although the strong signal for HSV-1 DNA (5.5 Kb) was detected in HSV-1 infected cells by tk cDNA probe (Lanes 1 and 2), PS-A-6 (20 μg/ml) inhibited the viral DNA synthesis in Vero cells (Lane 4). The results of the laser densitometry analysis demonstrated that PS-A-6 significantly decreased the ratio of HSV-1 DNA to β-actin DNA (Fig. 6B).

3.6. The tk mRNA expression in PS-A-6 treated Vero cells detected by Northern blot analysis

To elucidate whether PS-A-6 suppressed HSV-1 β gene expression such as tk gene, the cells were infected with or without HSV-1 in the presence or absence of 20 μg/ml PS-A-6 then total cellular RNA was extracted at 8 h p.i. As shown in Fig. 7A, the 3.4 Kb tk mRNA was expressed in HSV-1 infected Vero cells (Lane 2) and not expressed in uninfected cells (Lane 3). The level of tk mRNA was impeded by PS-A-6 treatment (Lane 1). However, both DMSO and PS-A-6 did not affect β-actin mRNA expression in the cells. The ratio of absorbance value of the tk mRNA to β-actin

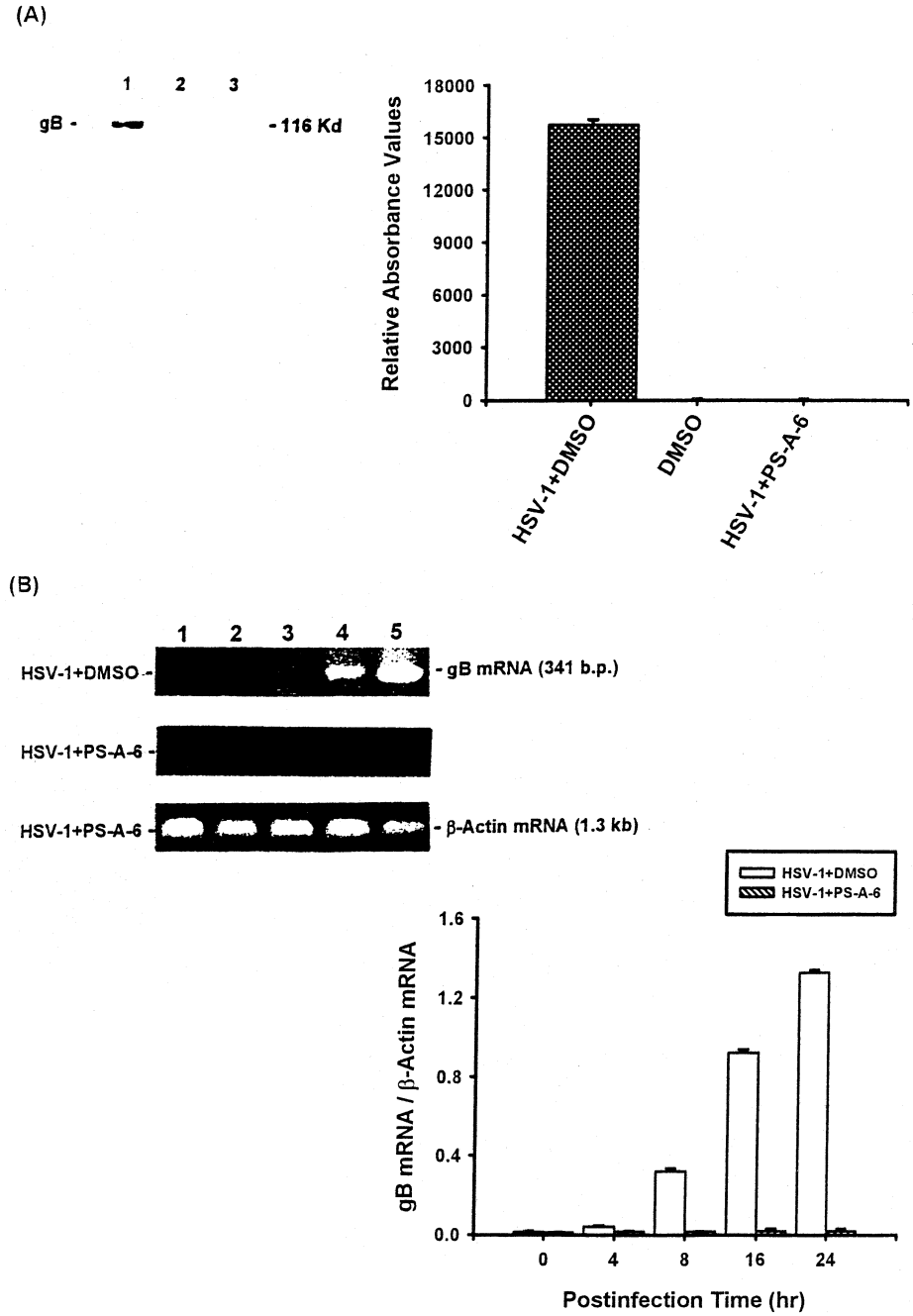

Fig. 5. Effects of PS-A-6 on gB protein synthesis and mRNA expression in Vero cells detected by Western blot analysis and RT-PCR. Vero cells (5×10^6) were infected with or without 0.1 m.o.i. of HSV-1 in the presence or absence of 20 μg/ml PS-A-6. (A) Lysates (20 μg of protein) were collected at 24 h p.i. and run on a 10% SDS–PAGE gel and analyzed by immunoblotting with anti-gB antibody. (Lane1) HSV-1 infected Vero cells, (Lane 2) uninfected cells, (Lane 3) HSV-1 infected cells treated with PS-A-6. Bar chart representing the relative absorbance values of the gB protein signal. Each bar represents the mean of three independent experiments. (B) The total cellular RNA was isolated from the cells at 0 (Lane 1), 4 (Lane 2), 8 (Lane 3), 16 (Lane 4), and 24 (Lane 5) h p.i., respectively. The RT-PCR was done as described in Section 2.11. Following the reaction, the amplified product was run on a 2% agarose gel. Each band was quantitated by densitometer and the ratio of gB mRNA to β-actin mRNA was calculated. Each bar represents the mean of three independent experiments.

mRNA signal is shown in Fig. 7B. The ratio value of PS-A-6 treated cells was lower than that of untreated cells.

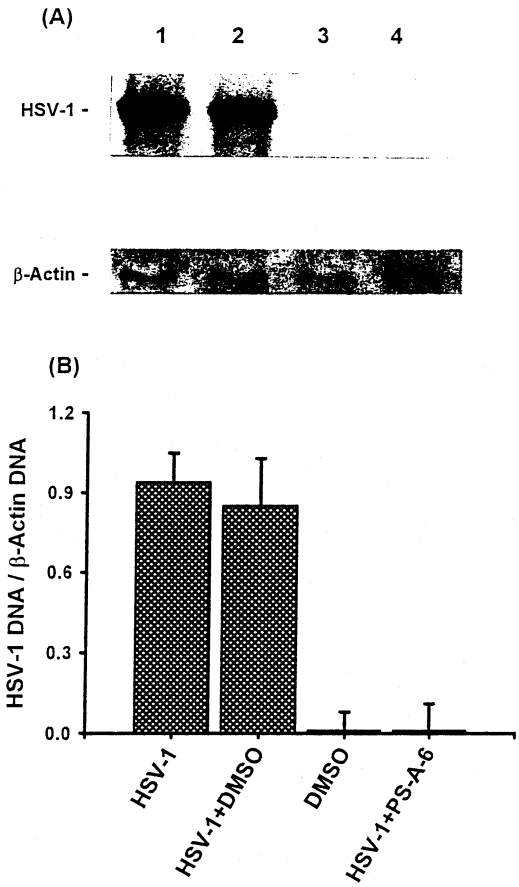

Fig. 6. PS-A-6 affected HSV-1 DNA synthesis in Vero cells detected by Southern blot analysis. The 5×10^6 cells were infected with or without 0.1 m.o.i. HSV-1 in the presence or absence of 20 μg/ml PS-A-6. The cells were harvested at 16 h p.i. and total DNA was extracted with phenol–chloroform. *Eco*RI (20 μg) digested DNA was run on a 0.8% agarose gel and then transferred to nitrocellulose filter. The filter was hybridized with ^{32}P-labeled HSV-1 tk cDNA or β-actin cDNA for 24 h at 68°C with agitation. After washing, the paper was exposed to X-ray film (Kodak). (Lane1) HSV-1 infected cells, (Lane 2) HSV-1 infected cells treated with DMSO, (Lane 3) uninfected cells, (Lane 4) HSV-1 infected cells treated with PS-A-6 (A). Each band was quantitated by densitometer and the ratio of HSV-1 DNA to β-actin was calculated. Each bar represents the mean of three independent experiments (B).

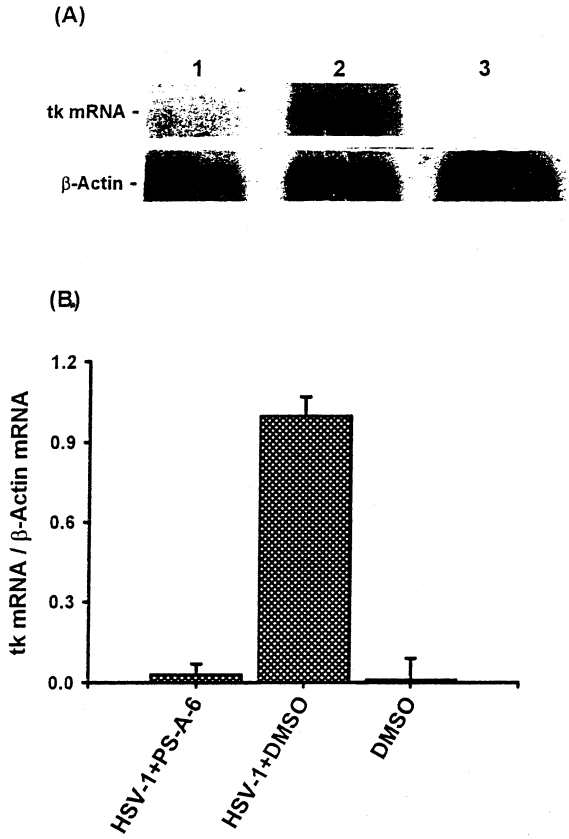

Fig. 7. Effects of PS-A-6 on tk gene transcription in Vero cells detected by Northern blot analysis. Vero cells (5×10^6) were infected with or without 0.1 m.o.i. of HSV-1 in the presence or absence of 20 μg/ml PS-A-6. Then total cellular RNA was isolated from the cells at 8 h p.i. and analyzed in 6.66% formaldehyde agarose gel and hybridized with ^{32}P-labeled tk cDNA or β-actin cDNA. (Lane1) HSV-1 infected cells treated with PS-A-6, (Lane 2) HSV-1 infected cells treated with DMSO, (Lane 3) uninfected cells (A). Each exposed band was quantitated by densitometer and the ratio of tk mRNAs to β-actin mRNA was calculated. Each bar is the mean of three independent experiments (B).

3.7. Effects of PS-A-6 on HSV-1 DNA synthesis and ICP27 mRNA expression in Vero cells infected with 3 m.o.i. of HSV-1

To confirm inhibitory actions of PS-A-6 on HSV-1 replication in the first round of infection, Vero cells were infected with 3 m.o.i. of HSV-1 and viral DNA synthesis and ICP27 mRNA expression were determined. Results of PCR analysis showed that total cellular DNA was harvested

at 0–6 h p.i. and HSV-1 DNA synthesis could be detected at 4–6 h p.i. (Fig. 8A). By contrast, PS-A-6 suppressed HSV-1 DNA synthesis. As shown in Fig. 8B, although background levels of ICP27 mRNA were detected in uninfected Vero cells by the dot-blot analysis, the ICP27 mRNA expression in Vero cells gradually increased after HSV-1 infection. But PS-A-6 attenuated the level of ICP27 mRNA. Results of the laser densitometry analysis demonstrated that PS-A-6 significantly decreased the ratio of HSV-1 ICP27 mRNA to β-actin mRNA.

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 our study, seven Chinese herbs were screened by this model then *P. serpens* was evaluated to contain antiherpetic agents, supporting the validity of its use for pharmacological studies. Results shown here indicated that PS-A-6 extracted from *P. serpens* suppressed HSV-1 multiplication in Vero cells without significantly reducing the cells viability and growth. The inhibitory effect of PS-A-6 may be attributed to its interference with gB protein synthesis, DNA replication, tk and ICP27 mRNA transcriptions of HSV-1. Hence, the suppressive activity of PS-A-6 on the viral replication, might have important implications with regard to *P. serpens* therapeutic activity in microorganisms infection. Although, the observation indicates that *P. serpens* includes antitumor principles (Lee et al., 1998). This is the first report of *P. serpens* for antiviral activity.

The results showed that inhibitory functions of PS-A-6 maintained to 48 h p.i.. The 48 h assay may be a better model for infection in vitro than the 24 h assay because multiple rounds of replication can occur. The PS-A-6 blocking HSV-1 replication was unlikely to be related to DMSO, because the cell viability and HSV-1 replication in Vero cells were not changed by DMSO. However, PS-A-6 did not decrease Vero cell viability whether the cell viability was stated in terms of % viable cell per treatment, total cell numbers or control untreated (data not shown). The morphology and characteristics of Vero cells treated by PS-A-6 or other Chinese herb extracts were similar, suggesting that inhibitory effects of PS-A-6 were not related to the pH, osmolarity, or other physiology variables in different preparations (data not shown). We did not check whether PS-A-6 bound to virus, but results of electron microscopy observation indicated that PS-A-6 did not change the morphology of HSV-1 (data not shown). Thus, we suggest that the inhibitory actions of PS-A-6 may not be related to direct binding of viral particles. In the present study, residual PS-A-6 and HSV-1 in the supernatants were not removed after viral adsorption. We have compared the viral titers in samples with or without PS-A-6 and the data showed that PS-A-6 did not affect results of viral titration (data not shown).

However, pretreatment of cells with PS-A-6 or adding the drug after viral adsorption, produced antiviral activity similar to that when HSV-1 and PS-A-6 were added at the same time. These results suggest that binding of PS-A-6 to virion or host cells could not be an inhibitory factor of virus replication. In addition, the pretreatment of Vero cells with PS-A-6 for 24 h (which was then removed before infection) still caused inhibition in virus yield (data not shown). The possibility that PS-A-6 bound to certain membrane molecules of the host cell which were different from the receptor, resulting in interference with virus penetration into the host cells, such as the virus–cell fusion, was not related to the inhibitory action of PS-A-6. Although in the multiple cycle of HSV-1, we could not distinguish whether PS-A-6 blocked HSV-1 adsorption in the second round of replication. However, effects of PS-A-6 on HSV-l tk and ICP27 mRNA expression were determined at 8 h p.i. and 0–4 h p.i., respectively. PS-A-6 decreased the level of gB mRNA in Vero cells at 0–8 h p.i.. Results from PCR also indicated that PS-A-6 decreased viral DNA synthesis in Vero cells infected with 3 m.o.i. of HSV-1. These inhibitory actions of PS-A-6 were observed in one replication cycle of HSV-1. And the inhibitory effects of PS-A-6 on HSV-1 replication may be related to decreased levels of immediate-early gene and early

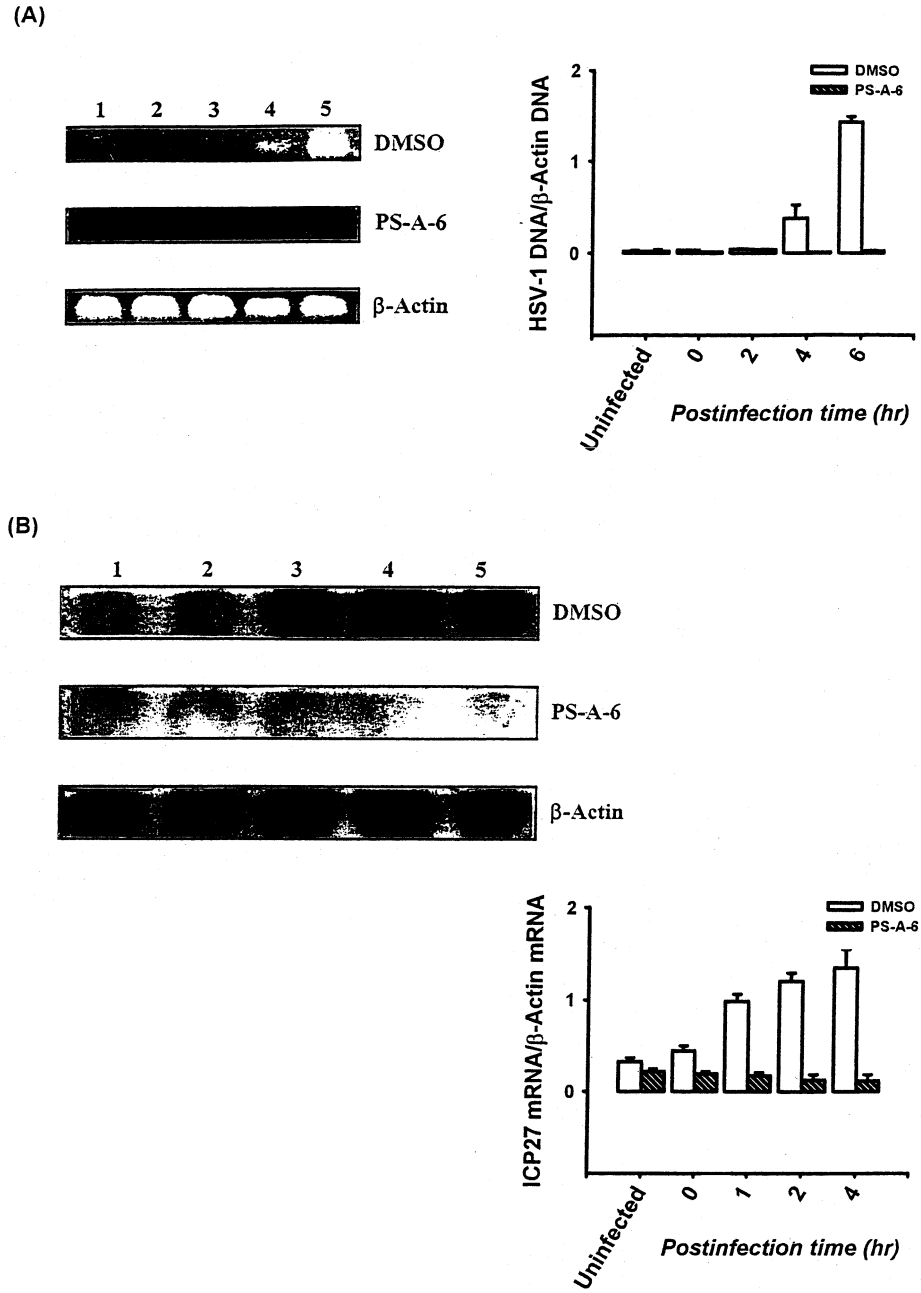

Fig. 8. Effects of PS-A-6 on viral DNA synthesis and ICP27 mRNA expression in Vero cells infected with 3 m.o.i. of HSV-1. Vero cells (5×10^6) were infected with or without 3 m.o.i. of HSV-1 in the presence or absence of 20 μg/ml PS-A-6. (A) The total cellular DNA was isolated from uninfected cells at 6 h p.i. (Lane 1) and infected cells at 0 (Lane 2), 2 (Lane 3), 4 (Lane 4), and 6 (Lane 5) h p.i., respectively. The PCR was done as described in Section 2.11. Following the reaction, the amplified product was run on a 2% agarose gel. Each band was quantitated by densitometer and the ratio of HSV-1 DNA to b-actin DNA was calculated. Each bar represents the mean of three independent experiments. (B) The total cellular RNA was isolated from uninfected cells at 4 h p.i. (Lane 1) and infected cells at 0 (Lane 2), 1 (Lane 3), 2 (Lane 4), and 4 (Lane 5) h p.i., respectively. The dot blot analysis was done as described in the Section 2.9. The filter paper was hybridized with ^{32}P-labeled ICP27 cDNA or β-actin cDNA. Each exposed band was quantitated by densitometer and the ratio of ICP27 mRNA to β-actin mRNA was calculated. Each bar represents the mean of three independent experiments.

gene such as ICP27 and tk mRNAs. In addition, results indicated that the HSV-1 gB mRNA was detected at 0–24 h p.i. and PS-A-6 significantly blocked the levels of gB mRNA in Vero cells at 0–24 h p.i.. The data showed that inhibitory functions of PS-A-6 were maintained to 48 h p.i.. Thus, we predict that PS-A-6 does not lose its activity in the second round of infection.

In the host cells, HSV-1 replication is believed to proceed, as follows: (1) α-TIF, a late (γ) protein packaged in the virion, turns on immediate-early (α) genes (e.g. ICP4 and ICP27) to be transcribed; (2) expression of a genes regulates early (β) genes (e.g. tk) to be expressed; (3) both α and β genes expression initiate HSV-1 DNA replication; (4) γ genes such as gB and gC proteins begin to be synthesized, then virions are assembled; and (5) HSV-1 was enveloped as it buds through the nuclear membrane (Jones and Roizman, 1979; Roizman and Sears, 1996). Thus, we predict that impairments of HSV-1 multiplication in PS-A-6 treated Vero cells, at least in part, were related to: (1) PS-A-6 decreased the level of HSV-1 ICP27 and tk mRNAs in the cells; (2) the viral DNA synthesis was inhibited; (3) owing to blocking of gB mRNA synthesis, gB proteins synthesis was blocked; and (4) the HSV-1 plaque formed in Vero cells was not observed. Although the RT-PCR and PCR are not so strong in quantitative determination, and they are quick methods to detect viral mRNA and DNA. In the present study, the β-actin was used as an internal control, then the ratios of viral mRNA or DNA to β-actin mRNA or DNA were statistically analyzed. Unlike dextran sulfate, which inhibits HSV-1 replication at adsorption step (Hoshino et al., 1998), PS-A-6 blocked HSV-1 replication at an early step of DNA synthesis. As we know, the ICP27 gene is an essential gene for HSV-1 replication (Honess and Roizman, 1974). It has been reported to perform several functions including selection of transcriptional termination sites, inhibition of RNA splicing, stimulation of DNA synthesis, and posttranscriptional destabilization of α mRNA (Honess and Roizman, 1975; Mackem and Roizman, 1982). We are currently attempting to further elucidate suppressory mechanisms of PS-A-6 on ICP 27 mRNA expression in virus-infected cells.

The PS-A, PS-A-3 and PS-A-4 fractions were crude extracts and both viral replication enhancers and inhibitors may be included in the mixtures. The ratios of inhibitor contents to stimulator contents in the PS-A fraction may be higher than those in the PS-A-3 and PS-A-4 subfractions. Thus, the results indicated that PS-A completely inhibited HSV-1 replication, but PS-A-3 and PS-A-4 separated from PS-A stimulated HSV-1 replication. In our experiences, this phenomenon is often observed in studies with Chinese herbs. However, plans are underway for the isolation of pure bioactive principles from PS-A-6 by a bioassay-guided fractionation procedure. The preliminary results indicated that the band corresponding to R_f 0.1 developed by thin-layer chromatography plate (chloroform/methanol = 5:1; v/v) was active. Comparison with standards showed that the active components may be related to polyphenol compounds (data not shown). Many polyphenols are known for their antiherpes virus activities (Vanden Berghe et al., 1986). Future experiments with treatment of HSV-1 infected animals with PS-A-6 or pure components will be necessary to define whether *P. serpens* can reduce experimental viral infection injury. Moreover, this study not only demonstrates that Chinese herbs are potential therapeutic drugs for the viral infection but also support a model for future protocol design in preclinical studies.

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