Herpes simplex virus type 1 propagation in HeLa cells interrupted by *Nelumbo nucifera*

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**Summary**

Inhibitory effects of ethanolic extracts from 10 Chinese herbs on herpes simplex virus type 1 (HSV-1) replication were investigated. By a bioassay-guided fractionation procedure, NN-B-5 was identified from seeds of *N. nucifera*. NN-B-5 significantly blocked HSV-1 multiplication in HeLa cells without apparent cytotoxicity. To elucidate the point in HSV-1 replication where arrest occurred, a set of key regulatory events leading to the viral multiplication was examined, including HSV-1 DNA synthesis and viral immediate early gene expressions. Data from polymerase chain reaction and Southern blotting showed that there were impairments of HSV-1 DNA replication in HeLa cells treated with NN-B-5. Results indicated that the production and mRNA transcription of infected cell protein (ICP) 0 and ICP4 were decreased in NN-B-5 treated HeLa cells. Results of an electrophoretic mobility shift assay demonstrated that NN-B-5 interrupted the formation of α-trans-induction factor/C1/Oct-1/GARAT multiprotein/DNA complexes. The mechanisms of antiviral action of NN-B-5 seem to be mediated, at least in part, through inhibition of immediate early transcripts, such as ICP0 and ICP4 mRNA and then blocking of all downstream viral products accumulation and progeny HSV-1 production.

**Introduction**

Herpes simplex virus type 1 (HSV-1) causes a variety of infections in human [1]. The recipients of organ transplantation and immunocompromised or cancer patients are at high risk for increased severity of HSV-1 infection [2–4]. In addition, HSV-1 and HSV-2 have been shown to be factors for spreading of human immunodeficiency virus [5–7].

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 genes, early (β₁, β₂) genes including DNA polymerase and thymidine kinase genes, and late (γ₁, γ₂) genes containing glycoprotein B (gB), ICP5, and gC, and DNA replication [1, 8]. 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 [9]. Inhibition of any of these stages blocks HSV-1 replication. Nucleoside analogues have been extensively investigated in the search for effective
antiviral activity. Among these, 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. However, it has been observed that the acyclovir-resistant HSV infection has come from immunocompromised patients such as transplant and AIDS patients. 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. Parts of them are used as functional foods because of their immunomodulatory and antitumor functions. However, ethnopharmacology provides scientists with an alternative approach for the discovery of antiviral agents. The polysaccharides, anthraquinones, triterpenes, phloroglucinol, flavonoids, and catechin derivatives isolated from medicinal plants are found to have inhibitory activities against the replication of HSV-1. There has been a promising result of a naturally occurring antiherpetic agent, n-docosanol, which has recently completed extensive clinical evaluation and been approved by the US Food and Drug Administration as a topical treatment for herpes labialis.

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. These findings show that natural products are still potential sources in the search for new antitherpetic agents.

In the present study, ten 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. The herbs were Platycodon grandiflorum (PG), Dioscorea opposita (DO), Lycium chinense (LC), Nelumbo nucifera (NN), Arctium lappa (AL), Ziziphus zativa (ZZ), Momordica charantia (MC), Polygonum multiflorum (PM), Diospyros lotus (DL), and Coix lacryma-jobi (CL). The ethanolic extracts that showed appreciable anti-HSV-1 activity were selected for bioassay-guided fractionation procedure. The effect of active fraction, NN-B-5 isolated from N. nucifera, on HSV-1 DNA replication and gene expression in HeLa cells was evaluated. The mechanisms of antiviral action of NN-B-5 were investigated in vitro.

**Materials and methods**

**Preparation of crude extracts for Chinese herbs**

All ten species of Chinese herbs were purchased from Chinese medicine shops in Taipei. They were identified by Dr. Yun-Lian Lin, resident medicinal-plant expert of The National Research Institute of Chinese Medicine. Each dried Chinese herb (600 gm) was extracted with ethanol (5 l). After removing solvent, the crude extracts were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 100 mg/ml and stored at 4 °C until use.

**NN-B-5 fractionated from seeds of N. nucifera**

The dried seeds of N. nucifera were collected and their ethanolic extracts were evaporated with Rotavapor under vacuum (Büchi, Switzerland) and furthered to partition successively between n-hexane (NN-H) followed by ethyl acetate (EtOAc; NN-E) and butanol (BuOH; NN-B), respectively. The bioactive NN-B fraction was subjected to silica gel chromatography. Extensive gradient elution was then employed using H2O and methanol (MeOH). The like fractions were combined to give nine main fractions (NN-B-1 to NN-B-9) with monitoring by thin-layer chromatography and the solvent was removed under vacuum. All extracted fractions were dissolved by dimethyl sulfoxide (DMSO) to a concentration of 100 mg/ml and stored at 4 °C until use.

**Cell culture and viruses**

HeLa cells were cultured in minimal essential medium (MEM; Gibco, Grand Island, NY) supplement 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% CO2 incubator. The cells were free from mycoplasma contamination, which was checked by Mycotect™ kit (Mycotect™ kit, Life Technology, Gaitherburg, MD). To prepare HSV-1 (KOS strain, VR-1493, ATCC; TK− strain, a gift from Dr. Szu-Hao Kung) stocks, HeLa cells were infected by HSV-1 at a multiplicity of infection (MOI) of 3 plaque forming units (PFU)/cell and harvested at 24 h postinfection (p.i.) and centri-