ฤทธิ์ต้านไวรัสเฮอร์ปีส์ซิมเพล็กซ์ของกรดไลโปอิก

นางสาวภัทรพร ศศิวิมลพันธุ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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ANTIVIRAL ACTIVITY AGAINST HERPES SIMPLEX VIRUS OF LIPOIC ACID

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A Thesis Submitted in Partial Fulfillment of the Requirements

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ภัทรพร ศศิวิมลพันธุ์ : ฤทธิ์ต้านไวรัสเฮอร์ปีส์ซิมเพล็กซ์ของกรดไลโปอิก. (ANTIVIRAL ACTIVITY AGAINST HERPES SIMPLEX VIRUS OF LIPOIC ACID) อ. ที่ปรึกษา : รศ. ดร.วิมลมาศ ลิปิพันธ์, 119 หน้า.

การศึกษาฤทธิ์ต้านไวรัสเฮอร์ปีส์ซิมเพล็กซ์ทัยป์ 1 สายพันธุ์ KOS และทัยป์ 2 สายพันธุ์ Baylor 186 ของ lipoic acid และ lipoamide ใน Vero cells โดยวิธี inactivation, plaque reduction และ MTT reduction assay พบว่า lipoic acid และ lipoamide มีฤทธิ์ต้าน HSV-1 และ HSV-2 โดยค่าความเข้มข้นของสารที่ยับยั้งการเจริญของไวรัส 50% (IC_{50}) ของ lipoic acid ในการยับยั้ง HSV-1 เท่ากับ 96.34, 115.49 และ 126.85 µg/ml ตามลำดับ และมีค่า SI เท่ากับ 3.04, 2.54 และ 2.31 ค่า IC_{50} ของ lipoic acid ในการยับยั้ง HSV-2 เท่ากับ 111.75, 130.13 และ 135.06 µg/ml ตามลำดับ โดยมีค่า SI เท่ากับ 2.62, 2.25 และ 2.17 ค่า IC_{50} ของ lipoamide เท่ากับ 41.28, 60.47 และ 105.17 µg/ml สำหรับ HSV-1 และ 50.15, 83.49 และ 108.42 µg/ml สำหรับ HSV-2 ตามลำดับ โดยมีค่า SI ในการยับยั้ง HSV-1 เท่ากับ 6.57, 4.48 และ 2.58 ซึ่งสอดคล้องกับค่า SI ในการยับยั้ง HSV-2 ที่มีค่าเท่ากับ 5.41, 3.25 และ 2.50 การทดสอบความเป็นพิษต่อเซลล์ของ lipoic acid และ lipoamide โดยวิธี trypan blue exclusion และ MTT reduction assay พบว่าความเข้มข้นของสารที่เป็นพิษต่อเซลล์เพาะเลี้ยง 50% (CC_{50}) มีค่าเท่ากับ 242.69 และ 292.19 µg/ml สำหรับ lipoic acid และ 239.03 และ 271.10 µg/ml สำหรับ lipoamide ตามลำดับ นอกจากนี้ในการประเมินกลไกการออกฤทธิ์เบื้องต้นของ lipoic acid ในการยับยั้ง HSV โดยใช้วิธี virucidal, post-binding, penetration, prophylactic activity และ virus growth inhibition assay พบว่า lipoic acid ไม่มีฤทธิ์ยับยั้งการจับของ HSV บนผิวเซลล์เพาะเลี้ยง ไม่มีฤทธิ์ป้องกันการเข้าสู่เซลล์ของไวรัสและไม่มีฤทธิ์ทำลายอนุภาคของไวรัสโดยตรง อย่างไรก็ตาม lipoic acid มีผลลดปริมาณ HSV-1 และ HSV-2 ใน Vero cells ได้มากกว่า 70% โดยเฉพาะอย่างยิ่งใน pre-treatment นอกจากนี้ lipoic acid ยังแสดงฤทธิ์ต้าน HSV ในเซลล์เพาะเลี้ยงชนิดอื่น เช่น human cervix epithelium HeLa cells และ normal human dermal fibroblast NHDF CC-2511 เมื่อทำการทดสอบโดยวิธี MTT reduction assay พบว่าค่า IC_{50} ของ lipoic acid มีความแตกต่างกันไปในเซลล์เพาะเลี้ยงแต่ละชนิด โดยค่า IC_{50} ในการยับยั้ง HSV-1 เท่ากับ 126.85, 109.61 และ 58.20 µg/ml และในการยับยั้ง HSV-2 เท่ากับ 135.06, 125.90 และ 92.26 µg/ml ใน Vero, HeLa และ NHDF CC-2511 ตามลำดับ การศึกษานี้แสดงให้เห็นว่า lipoic acid และ lipoamide มีฤทธิ์ต้านไวรัสเฮอร์ปีส์ซิมเพล็กซ์และอาจนำไปพัฒนาเป็นยารักษาโรคที่เกิดจากการติดเชื้อ HSV ได้ในอนาคต

ภาควิชา จุลชีววิทยา ลายมือชื่อนิสิต....ภ.ท.ร.พ.ร.....ศ.ศิ.วิ.ม.ล.พ.น.ธุ์.....

สาขาวิชา จุลชีววิทยา ลายมือชื่ออาจารย์ที่ปรึกษา.....

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PATTARAPORN SASIVIMOLPHAN : ANTIVIRAL ACTIVITY AGAINST HERPES SIMPLEX VIRUS OF LIPOIC ACID. THESIS ADVISOR : ASSOC. PROF. VIMOLMAS LIPIPUN, Ph.D., 119 pp.

Antiviral activity of lipoic acid and lipoamide against herpes simplex viruses type 1 (HSV-) strain KOS and type 2 (HSV-2) strain Baylor 186 on Vero cells using inactivation, plaque reduction, and MTT reduction assay was investigated. Lipoic acid and lipoamide exhibited anti-HSV activity in all assays. The 50% inhibitory concentrations (IC_{50}) of lipoic acid against HSV-1 were 96.34, 115.49, and 126.85 μg/ml, respectively, and the selective index (SI) were 3.04, 2.54, and 2.31. In the inhibition of HSV-2, IC_{50} values of 111.75, 130.13, and 135.06 μg/ml, respectively, and SI of 2.62, 2.25, and 2.17 were observed in lipoic acid treatment. IC_{50} values of lipoamide were 41.28, 60.47, and 105.17 μg/ml for HSV-1 and 50.15, 83.49, and 108.42 μg/ml for HSV-2, respectively. Its SI values against HSV-1 were 6.57, 4.48, and 2.58, correlating with these values of 5.41, 3.25, and 2.50 in the HSV-2 inhibition. In trypan blue exclusion and MTT reduction assays, 50% cytotoxic concentration (CC_{50}) were 242.69 and 292.19 μg/ml for lipoic acid, and 239.03 and 271.10 μg/ml for lipoamide, respectively. In addition, possible mechanism of action of lipoic acid against HSV-1 and HSV-2 was determined using virucidal, post-binding, penetration, prophylactic activity, and virus growth inhibition assays. The results indicated that lipoic acid did not directly inactivated virus particle, or inhibited virus adsorption to cell surface, or penetration into the cells. However, lipoic acid acted mainly by reduction of HSV-1 and HSV-2 titer in Vero cells at more than 70% especially in the pre-treatment. In other cell types, lipoic acid showed inhibitory activity on human cervix epithelium HeLa cells and normal human dermal fibroblast NHDF CC-2511 cells, as determined by MTT reduction assay. However, the IC_{50} of lipoic acid varied among different cell types with the values of 126.85, 109.61, and 58.20 μg/ml for HSV-1 and 135.06, 125.90, and 92.26 μg/ml. for HSV-2 in Vero, HeLa, and NHDF CC-2511 cells, respectively. This study indicated that lipoic acid and lipoamide showed anti-herpes simplex activity and was a the promising anti-herpetic agent.

Department	Microbiology	Student's signature..Pattaraporn....Sasivimolphan
Field of study	Microbiology	Advisor's signature..Vimolmas Lipipun
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LIST OF ABBREVIATIONS

3-OSTs	=	3-*O*-sulfotransferase
ACV	=	acyclovir
AIDS	=	acquired immunodeficiency syndrome
AMP	=	adenosine monophosphate
BVDU	=	brivudin
$^{\circ}C$	=	degree celcius
CC_{50}	=	50% cytotoxic concentration
CDV	=	cidoforvir
CO_2	=	carbondioxide
cm	=	centimeter
CMV	=	cytomegalovirus
CPE	=	cytopathic effect
Cu	=	copper
dATP	=	deoxyadenosine triphosphate
dCTP	=	deoxycytosine triphosphate
dGTP	=	deoxyguanosine 5'- triphosphate
DHLA	=	dihydrolipoic acid
DMEM	=	Dulbecco's modified Eagle Medium
dNTPs	=	deoxynucleoside triphosphate
DMSO	=	dimethylsulfoxide
DNA	=	deoxyribonucleic acid
ED_{50}	=	50% effective dose
EDTA	=	ethylene diamine tetra acetate
ELISA	=	enzyme-linked immunosorbent assay
FBS	=	fetal bovine serum
FCV	=	famcyclovir
GCV	=	ganciclovir
GSH	=	glutathione
h or hr	=	hour

H_2O	=	water
HDL	=	high density lipoprotein
HHV	=	human herpesvirus
HIV	=	human immunodeficiency virus
HPMP	=	3-hydroxy-2-phophonylmethoxypropyl
HPMPA	=	3-hydroxy-2-phophonylmethoxypropyladenine
HPMPC	=	3-hydroxy-2-phophonylmethoxypropylcytosine
HSV	=	herpes simplex virus
HveA	=	herpesvirus entry mediator
HveB	=	nectin-2
HveC	=	nectin-1
IC_{50}	=	50% inhibitory concentration
ICP	=	infected cell polypeptide
ICSP	=	infected cell specific polypeptide
ICTV	=	International Conference for Taxonomy of Viruses
IDU	=	idoxuridine
kg	=	kilogram
l	=	liter
LA	=	lipoic acid
LATs	=	Latency Associated Transcripts
LD_{50}	=	median lethal dose
m	=	meter
MEM	=	Minimum Essential Medium
mg	=	milligram
µg	=	microgram
min	=	minute
ml	=	milliliter
mm	=	millimeter
MOI	=	multiplicity of infection

MTT	=	thaizoyl blue tetrazolium bromide
nm	=	nanometer
PAA	=	phosphonoacetic acid
PBS	=	phosphate buffered saline
PCV	=	penciclovir
PFA	=	phosphonoformic acid (foscarnet)
PFU	=	plaque forming unit
RNA	=	ribonucleic acid
ROS	=	reactive oxygen species
rpm	=	round per minute
SDS-PAGE	=	sodium dodecyl sulfate polyacrylamide gel eletrophoresis
SI	=	selective index
TCID50	=	50% tissue culture infective dose
TK	=	thymidine kinase
U_L	=	long unique sequences
U_S	=	short unique sequences
VACV	=	valacyclovir
VP	=	virion polypeptide
VZV	=	varicella zoster virus

CHAPTER I

INTRODUCTION

Herpes simplex viruses (HSV) are the first of human herpesviruses and remain one of the most common viral infections in humans. Infections of these viruses occur world wide and have been reported in both developed and developing countries. Consequently, the infection has been recognized as a public health concern (Corey *et al*., 1983). In addition, HSV is an intense human pathogen and responsible for causing a widespread spectrum of mild to severe diseases. These include acute primary and recurrent mucocutaneous disorders recognized as herpes labialis, eczema herpeticum, and genital herpes through herpes keratitis, herpes meningitis, and life-threatening herpes encephalitis in the otherwise healthy adult. Moreover, HSV infection in immunocompromised patients and neonates are usually more severe than in the normal host (Whitley, 1995). These individuals, which compose of AIDs patients and immunocompromised patients resulted from pathogenic reasons or receiving immunosuppressive drugs, are also prone to increased frequency of secondary herpes episodes (Greenberg *et al*., 1987), and the severity of herpes infection has been shown to correlate with the degree of immunosuppressive therapy used (Rand *et al*., 1997). In addition, HSV-2 infection may be a risk factor for the transmission of human immunodeficiency virus (HIV) (Hook *et al*., 1992).

The existence of two distinct antigenic types of HSV was revealed in the early 1960s, they are now designated as human herpesviruses 1 (HHV-1 or HSV-1) and human herpesviruses 2 (HHV-2 or HSV-2) by the International Conference for Taxonomy of Viruses (ICTV) (Ginsberg, 1980). HSV-1 and HSV-2 significantly differ in their pathogenic potential. HSV-1 infection is generally limited to the oropharynx and transmitted by direct contact of a susceptible individual with infected secretions. Thus, initial replication of HSV-1 normally occurs in oropharyngeal mucosa, and the trigeminal ganglion becomes colonized and harbors latent virus. Acquisition of HSV-2 infection is usually the consequence of transmission by genital contact. This virus replicates in the genital, perigenital, or anal skin sites with seeding of sacral ganglia for latent infection. However, changes in sexual behaviors have somewhat altered this common pattern: occasionally, HSV-2 viruses are isolated from oral lesions and HSV-1 from genital lesions (Ginsberg, 1980).

Among several populations, between 60% and more than 95% are infected with HSV-1, and between 6% and 50% with HSV-2 (Cunningham and Mikloska, 2001). Recurrences of both oral labial and genital HSV infections in human occur frequently. More than 60% of patients with initial HSV-2 infection develop recurrent infection within 6 months, and patients with recurrent genital disease have a median of 5 recurrences per year. The disease is often painful, sometime debilitating, and causes considerable social and psychological stress. Furthermore, a number of clinical and epidemiological studies have shown a significant correlation between HSV-2 infection and a higher incidence of cervical carcinoma. In Thailand, HSV infections have been frequently found in various populations. Virus Research Institute, Department of Medical Sciences, Ministry of Public Health reported that 10-17% of young people in Bangkok and Chinart provinces ever had symptomatic herpes. In this group, 5% of them were genital herpes and 0.35% had HSV infection in the vagina and cervix which increased in the past years. In Bangkok, almost upward 30-year old people had HSV-1 infections and had a little lower HSV-2 infection incidence. Meanwhile, in Chainart where the population density is lower than Bangkok, there were also lower incidences of HSV infection (ประเสริฐ, 2528). It was also reported that the frequencies of recovery from HSV diseases seemed to be higher in female than in male, particularly during the first episode of infection. Asymptomatic shedding of HSV-2 represented 98.4% of all isolates from female genitalias and the remaining isolates were HSV-1.

There are various antiviral drugs with clinically relevant activity against HSV infection such as idoxuridine, vidarabine, trifluridine, and foscarnet, however, these drugs have some undesirable effects such as they are potentially toxic, mutagenic, and teratogenic to the host (Coen, 1991). Acyclovir (ACV) is most commonly used for the treatment of HSV infections, followed by penciclovir or famciclovir. It has been reported that ACV in topical, oral, or intravenous forms was highly effective especially on the first episode of HSV infection (Leung and Sacks, 2000). However, a serious problem for the use of ACV is the increase of HSV strains that resist to drug treatment (Pottage and Kessler, 1995; Shin *et al*., 2001) particularly in immunocompromised patients. Resistance to ACV and related nucleoside analogues can occur commonly following mutation in HSV thymidine kinase or rarely DNA polymerase (Khan *et al*., 2005). Foscarnet, an antiviral drug inhibiting HSV DNA polymerase, is often used to treat ACV-resistant virus and recommended for only severe infection (Hasegawa and Kaeagushi, 1994). Nevertheless, foscarnet may induce mutation in viral DNA polymerase gene when used upon prolonged period, and

foscarnet-resistant viruses have been isolated (Birch *et al*., 1990; Hwang *et al*., 1992). Consequently, these mutants are often resistant to combination chemotherapy with existing compounds. Therefore, there is a worldwide interest in the development and identification of efficacious new anti-HSV agents with on adverse effects.

α-lipoic acid (LA) is a universal antioxidant that combines free radical scavenging and metal chelating properties with an ability to regenerate the levels of other nonenzymatic and enzymatic antioxidants such as glutathione, ascorbate, α-tocopherol, catalase, and peroxidase (Maitra *et al*., 1995; Packer *et al*., 1995). As a precursor of the lipoamide prosthetic group in α-keto-acid dehydrogenase complexes, LA has been shown to prevent diabetes, hyperglycemia-associated complications such as neuropathy, cataract formation, and radiation injury. It has been reported that LA interrupted HIV replication, while LA supplementation in AIDs patients resulted in increases in CD_4 and CD_4/CD_8 ratios (Lyn, 2000). Moreover, oral LA administration has been shown to be safe with no apparent side effects, no mutagenicity, and no genotoxicity (Cremer *et al*., 2006).

Therefore, the purpose of this study was to investigate the antiviral activities of LA and lipoamide against HSV-1 strain KOS and HSV-2 strain Baylor 186. The antiviral activity was evaluated by inactivation, plaque reduction, and MTT reduction assay. To determine the anti-HSV activities of lipoic acid in different cell cultures, normal human dermal fibroblast cell line and cervix epithelium cell line were used instead of Vero cells and the MTT reduction assay was performed. Furthermore, preliminary mechanism studies of LA were performed through virucidal, post-binding, penetration, and virus growth inhibition assays to find its possible mode of antiviral activity.

The results from this study could provide preliminary information on the *in vitro* anti-HSV-1 and HSV-2 activities of LA and lipoamide. Moreover, LA and lipoamide would be interesting candidates for the anti-HSV-1 and HSV-2 drug development in the future.

CHAPTER II

LITERATURE REVIEW

1. Herpes simplex virus

Herpes simplex viruses (HSV) are the first of the human herpesviruses to be discovered and are among the most intensively investigated of all viruses (Roizman and Knipe, 2001). The investigations into the HSV first began in ancient times, 25-plus centuries ago (Nahmias and Dowdie, 1968). Hippocrates used the term "herpes" to describe the lesions that appeared to creep or crawl along the skin (Beswick, 1962; Wildy, 1973). Herodotus was the first to draw an association between these cutaneous eruptions and fever, the association that has survived to this day. Galen recognized that HSV recurrences develop at the same anatomical site (Roizman and Whitley, 2001). Several observations made in the late 19^{th} and early 20^{th} centuries brought and end to the imprecise descriptive era of HSV infections. Numerous points substantiate this conclusion. First by, histopathologic studies described multinucleated giant cells associated with herpesvirus infections (Unna, 1886). Second by, the unequivocally nature of HSV infections was determined (Lowenstein, 1919).

The advances in major laboratory in the past 25 years have provided a foundation for the recent application of molecular biology technologies of the study of human disease such as restriction enzymes, DNA cloning, and DNA sequencing. One significant advance was the detection of antigenic differences between HSV types. Although suggested by Lipschitz (1921) on clinical grounds more than 60 years ago and by others from laboratory observations (Plummer, 1964), in 1968 Nahmias and Dowdle and in 1971 Schnewels and Nahmias demonstrated biologic and antigenic differences between two types of HSV. These investigators proposed that human herpes simplex virus type 1 (HSV-1) was more frequently associated with oral and nongenital infections; whereas human herpes simplex virus type 2 (HSV-2) was associated with genital tract disease. This observation was pivotal for many of the clinical, serological, immunological, and epidemiological studies. Obviously, other critical advances made over the past decade have contributed to our understanding of the natural history of HSV infections. These include the

following, among others. First came the descriptions of herpes genitalis (De Morbis Veneresis, 1736) and herpes encephalitis (Smith *et al*., 1941), then successful antiviral therapy was established unequivocally for HSV encephalitis by vidarabine therapy (Whitley *et al*., 1977) and, subsequently, for genital HSV infections (Bryson *et al*., 1983) and HSV infections in the immunocompromised host (Meyers *et al*., 1980). In fact, the real therapeutic advance for mucocutaneous and visceral HSV infections was the discovery of acyclovir and the demonstration of its mechanism of action by Elion in 1982 (Whitley and Gnann, 1992). The differences between strains of HSV were demonstrated by restriction endonucluease technology, which has become an important molecular epidemiologic tool (Buchman *et al*., 1978). Type-specific antigens were used for seroepidemiologic studies of HSV infections (Roizman *et al*., 1984). Many studies have focused on the replication of HSV and the resultant gene products. A principal goal of these investigations is to define the biological properties of these gene products that affected the nature and pathology of HSV infection. The engineering of HSV and the expression of specific gene have been expected to provide technology for new vaccines (Roizman *et al*., 1985; Stevens *et al*., 1987), as well as for the use of HSV for gene therapy in cancer (Andreansky *et al*., 1998; Markert *et al*., 2000) and CNS diseases (Roizman and Whitley, 2001). Finally, extensive effort has been spent on the study of HSV latency with incremental advances (Stevens, 1989; Roizman and Sears, 1993).

2. Characteristics of herpes simplex virus

Herpes simplex viruses (HSV) are classified in the genus *Simplexvirus* (White and Fenner, 1994; Taylor *et al*., 2002), in the subfamily *Alphaherpesvirinae*, and in the family *Herpesviridae* by the International Committee on the Taxonomy of Viruses (ICTV) (Roizman *et al*., 1967, 1981, 1992; Van Regenmortel *et al*., 1991). The members of this subfamily are classified on the basis of a variable host range, relatively short reproductive cycle, rapid spread in culture, efficient destruction of infected cells, and capacity to establish latent infections primarily but not exclusively in sensory ganglia (Roizman and Pellet, 2001).

HSV is a large enveloped virus; 150-200 nm in diameter; with a distinct virion structure characteristic of the viruses (Figure 1). A mature HSV particle contains four structural features (Wildy *et al*., 1960): (i) an electron-opaque core containing viral DNA, (ii) an icosahedral capsid surrounding the core, (iii) an amorphous\tegument surrounding the capsid, and (iv) an outer membrane envelope studded with viral glycoprotein spikes on its surface. The core contains the linear double strand DNA (dsDNA) genome wrapped as a toroid or spool structure (Furlong *et al*., 1972; Zou *et al*., 1999). The ends of the genome are probably held together or are in close proximity in as much as a small fraction of the packaged DNA appears to be circular and the bulk of the linear DNA circularizes rapidly in the absence of protein synthesis after it enters the nuclei of infected cells. DNA extracted from HSV virions contains ribonucleotides, nicks, and gaps. The tegument, a term introduced by Roizman an Furlong to describe the structures between the capsid and the envelope (Roizman and Furlong, 1974), has no distinctive features in thin sections, but it may appear to be fibrous on negative staining. The tegument is frequently distributed asymmetrically, and its thickness may vary depending on the location of the virion within the infected cell; when the amount is variable, there is more of it in virions accumulating in cytoplasmic vacuoles than in those accumulating in the perinuclear space. The available evidence suggests the amount of tegument is more likely to be determined by the virus than by the host. The variability in the thickness of tegument results in the variation in size of virions. The capsid is approximately 100 nm in diameter and composed of 162 capsomers arranged in icosahedral symmetry. The hexameric capsomers are 9.5 x 12.5 nm in longitudinal section; a channel 4 nm in diameter runs from the surface along their long axis. Finally, the envelope consists of a lipid bilayer with about 12 different viral glycoproteins called spikes embedded in it. The envelope

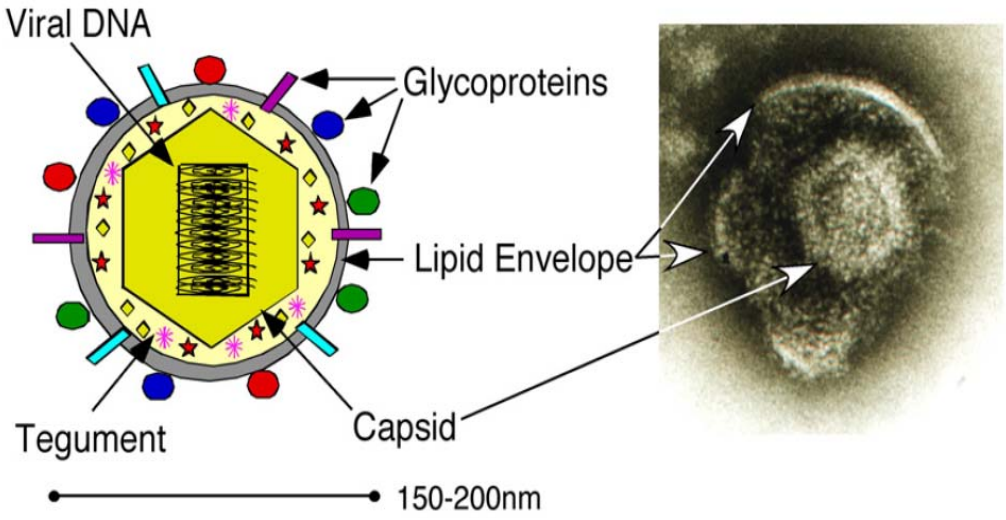


Figure 1. Structure of herpes simplex virus virion (Taylor *et al*., 2002).

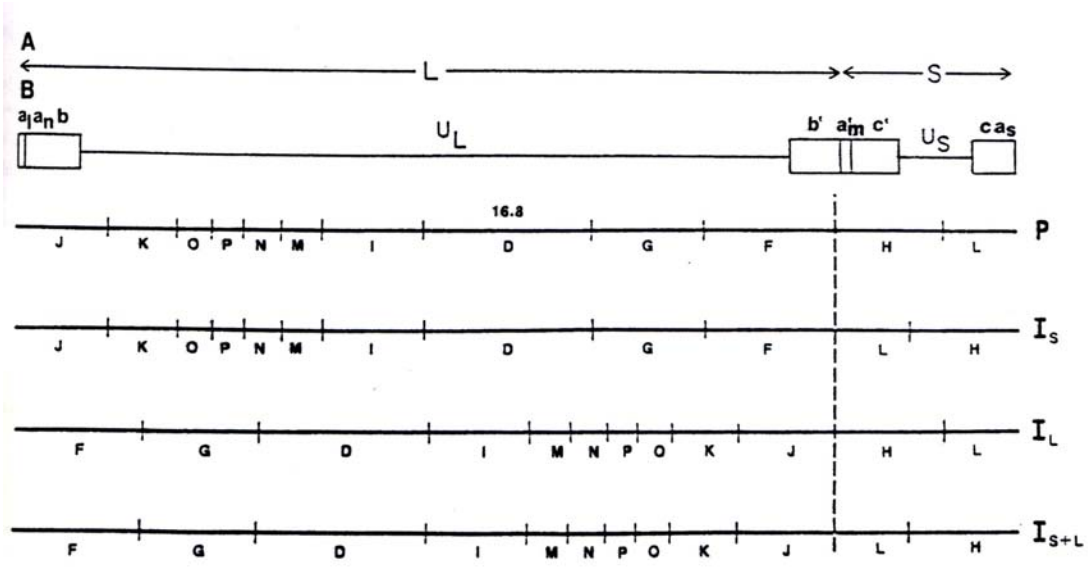


Figure 2. The arrangement of DNA sequences in HSV genome (Riozman and Philip, 2001).

appears to be derived from patches of altered cellular membranes. The presence of lipid was demonstrated by analyzing of virions and by the sensitivity of the virions to lipid solvents and detergents. Early studies on purified HSV virions suggested that they contain more than 30 distinct proteins, which were designated as virion polypeptides (VP). Of the about 30 known and another 10 suspected virion proteins, at least 12 are on the surface of the HSV virion. The viral glycoproteins on envelope surface are gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM, and gN which have many functions in HSV infection (Roizman, 1996; Roizman and Philip, 2001). The gB is required for viral entry and induces neutralizing antibody of host immune response. Glycoprotein C (gC) is involved in cell attachment and plays a role in blocking host immune response to infection. Since it has a C3b complement component receptor, gC can protect infected cells from antibody-dependent cellular cytotoxicity (Friedman *et al*., 2000; Lubinski *et al*., 2002). The gD is required for postattachment entry of virus into cells. The gE has an immune escape function by binding with IgG via Fc receptor in the form of gE-gI complex. This Fc receptor can protect both host cell and viruse against immunologic attack by steric hindrance resulting from the binding of normal IgG or from bipolar bridging of HSV antibody which can attach to gE/gI by its Fc end and simultaneously to another HSV glycoprotein by one Fab arm. The gG plays a role in egress and cell-to-cell spread, whereas gH forms complex with gL. This complex is required for fusion of host membranes, and cell-to-cell spread. The gJ is reported to block apoptosis, and gK is important for efficient viral exocytosis. However, the function of gN is still unknown (Roizman and Knipe, 2001).

The HSV genome consists of 152,000 base pairs, with a G+C content of 68.3% for HSV-1 and 69% for HSV-2 (Roizman, 1992). The homology between the two types of HSV genome is about 50%. The genome is composed of two covalently linked segments, designated as Long (L) and Short (S), based upon their relative length (Figure 2). Each segment consists of unique sequences regions (U_L or U_S) flanked by inverted repeat sequence (Wadsworth *et al*., 1975; Whitley and Roizman, 2001). This large genome allows the virus to encode at least 80 gene products (Taylor *et al*., 2002). Most of the polypeptides specified by one virus type are antigenically related to the polypeptides of the other type (Corey and Spear, 1986). They are designated as either infected cell specific polypeptide (ICSPs) or infected cell polypeptides (ICPs). The three groups of HSV proteins, α, β, and γ, are synthesized in a sequential order. There are six α-proteins namely ICP0, ICP4, ICP22, ICP27, ICP47, and $U_S1.5$. The synthesis of

α-polypeptides occurs very soon after infection, about 2 to 4 hours postinfection, but some α-proteins continue to be produced throughout the period of infection (Honess and Roizman, 1974; Roizman and Knipe, 2001). The β-polypeptides reach peak rate of synthesis about 5 to 7 hours after infection (Honess and Roizman, 1975), and are generally involved in viral DNA replication. These proteins are divided into two groups: β_1 and β_2. The β_1 proteins occur very early after infection, exemplified by polypeptides ICP6, the large component of the viral ribonucleotide reductase (Huszar and Bacchetti, 1981), and ICP8, the major DNA binding protein (Conley *et al*., 1981). They are differentiated from α-proteins by their requirement for functional ICP4 protein for their synthesis (Honess and Roizman, 1975). The β_2 proteins are synthesized later; they include the viral thymidine kinase (TK) and DNA polymerase. Most of the β proteins are responsible for viral nucleic acid metabolism and are the main target of antiviral chemotherapy (Whitley, 2001). The appearance of β gene products signals the onset of viral DNA synthesis. The γ-proteins are primarily structural polypeptides, including viral glycoproteins, capsid, and some tegument components. They are divided into two classes: γ_1 and γ_2. The γ_1 polypeptides are synthesized early in the absence of viral DNA replication, while the γ_2 polypeptides occur after the viral DNA replication has initiated. These γ-proteins also act as a major target for host immune response (Silver and Roizman, 1985; Roizman and Knipe, 2001; Taylor *et al*., 2002). Virulence of HSV that control the course of human HSV infection is composed of invasiveness and neurogrowth. Invasiveness is the capacity of virus to reach a target organ, including nervous system tissues, from the portal of entry. In order to disseminate to the target organ, it is necessary for the virus to multiply at peripheral sites. Neurogrowth is the viral ability to grow in nervous system tissue. Thus, HSV virulence requires at least two distinct sets of viral function. The first comprises viral genes responsible for access and injury of host cells whose destruction is responsible for the disease. The second are viral genes and gene functions that turn off host responses to infection (Whitley and Roizman, 2001).

3. Multiplication of herpes simplex virus

The events of HSV replication (Figure 3) involve several major steps, including entry, viral gene expression, viral DNA synthesis, virion assembly, and egress of progeny virions. The entry of HSV into cell is affected in three stages (Figure 4). The first involves the attachment of the virion to the cell surface. The second step involves the interaction of gD with one of the several cellular receptors. In the last stage, the viral envelope and the plasma membrane fuse to release the capsid-tegument structure into cytoplasm.

The initial virus attachment depends on the interaction of viral envelope gC, and, to a lesser extent gB, with the glycosaminoglycan moieties of cell surface heparin sulfate (WuDunn and Spear, 1989; Herold *et al*., 1991; Shieh *et al*., 1992). In addition, gB also binds to cell surface independently of heparin sulfate and allows viral entry into cell (Bender *et al*., 2005). While this initial step enhances infection (Banfield *et al*., 1995), it is not an absolute requirement for either entry or viral replication, and cells that do not express heparin sulfate remain permissive of HSV entry at low levels. After the initial step, virion attachment is stabilized by binding to a coreceptor. This sequential step involves the interaction of gD with one of the several cellular molecules that belong to three structurally unrelated molecular families (Spear *et al*., 2000, 2003, 2006). The first of these coreceptors is a member of the tumor necrosis factor (TNF) receptor family originally called herpesvirus entry mediator (HVEM) but renamed HveA (Montgomery *et al*., 1996; Whitbeck *et al*., 1997). HveA is present primarily in lymphoid cells, but also found in other cell types such as lung, liver, and kidney (Kwon *et al*., 1997). It serves as a receptor for entry of HSV-1 and HSV-2, but not for the related alphaherpes virus (Spear *et al*., 2006). The second family of HSV coreceptors belongs to the immunoglobulin (Ig) superfamily, including nectin-1 or HveC (Geraghty *et al*., 1998) and nectin-2 or HveB (Warner *et al*., 1998). These cellular proteins were shown to act as intercellular adhesion molecules and to be localized at adhesion junctions (Aoki *et al*., 1997; Lopez *et al*., 1998; Takahashi *et al*., 1999; Satoh-Horikawa *et al*., 2000). Nectin-1 is broadly expressed in cells of epithelial, fibroblast, neural, and hematopoietic cells. It mediates entry of all HSV-1 strains, HSV-2 and also mediates cell-to-cell spread of HSV (Cocchi *et al*., 2000). Nectin-2 is expressed in gall bladder, kidney, and testis. It acts as entry receptor for HSV-2 selectively, but not for wild-type HSV-1 (Lopez *et al*., 2000). The third coreceptor is specific sites in heparin sulfate generated by certain isoforms of 3-*O*-sulfotransferases (3-OSTs)

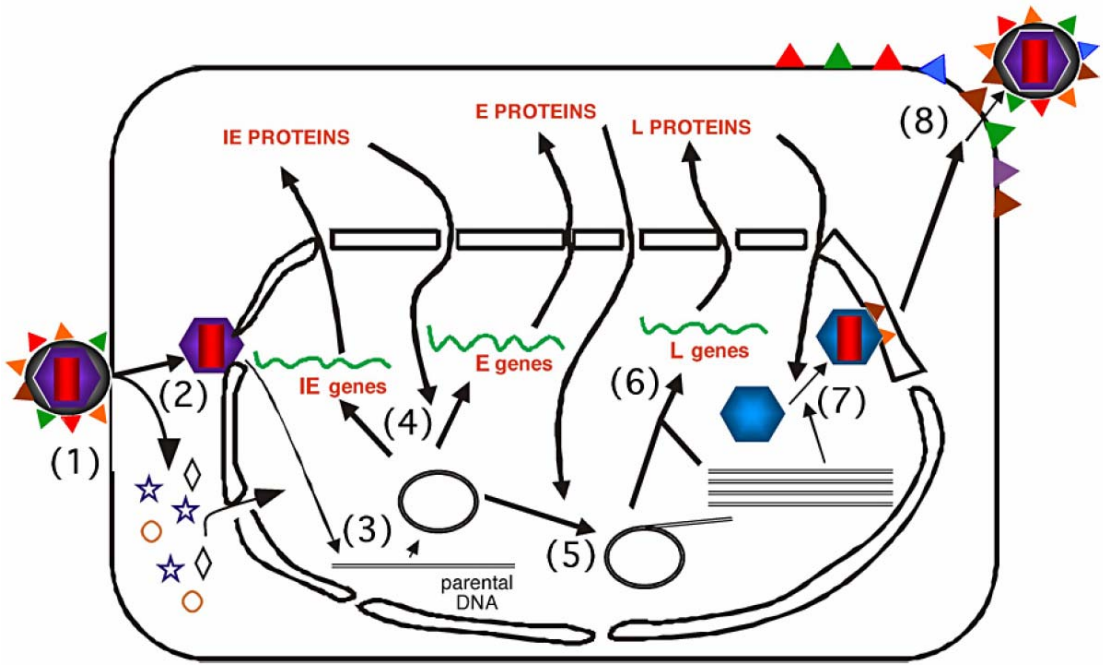


Figure 3. The cycle of productive HSV replication in a cell (Taylor *et al.*, 2002).

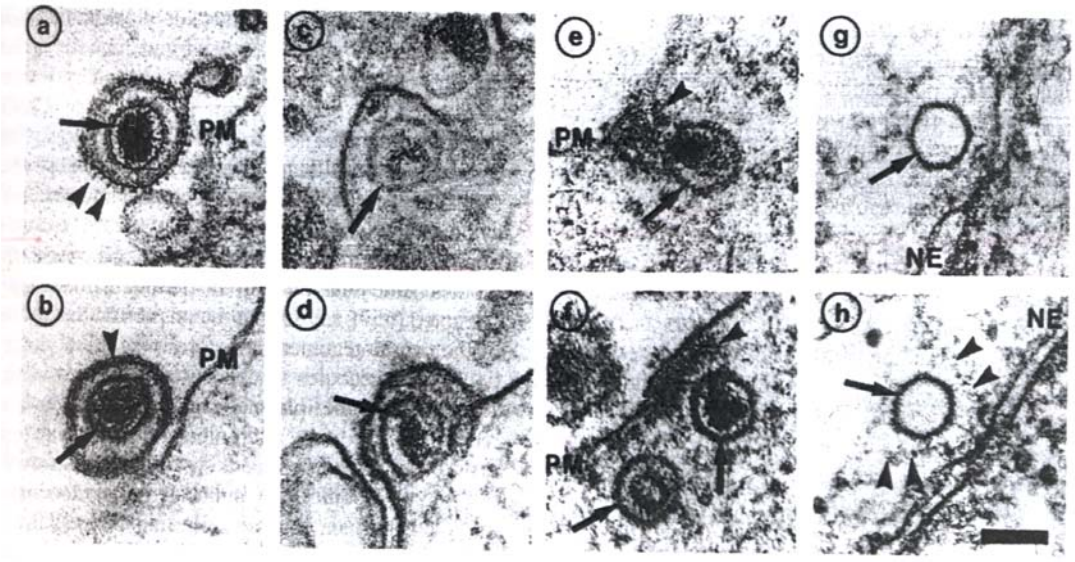


Figure 4. The entry and uncoating of HSV in infected cell. These figure show the stages of viral binding (a and b), fusion (c and d), release of the capsid into the cytosol (e and f), and binding of capsid to the nuclear pore (g and h) (Roizman and Philip, 2001).

called 3-*O*-sulfated heparin sulfates. The 3-*O*-sulfated heparin sulfates generated by 3-OST isoforms 2, 3A, 3B, 4, 5, and 6 can efficiently mediate HSV-1 entry but not HSV-2 entry. These coreceptors are broadly distributed on human tissue, primarily in heart, brain, lung, kidney, liver, skeletal muscle, and placenta (Shukla *et al*., 1998, 1999; Liu *et al*., 1999; Shworak *et al*., 1999; Xia *et al*., 2002; Tiwari *et al*., 2004, 2005; Xu *et al*., 2005; O'donnell, 2006). Moreover, recent work has identified a novel type II cell surface membrane protein, designated B5, which can serve as a receptor for HSV entry into the cells. However, it has not yet been shown that B5 interacts directly with HSV virions or with gD (Perez *et al*., 2005). The last step in viral entry is the fusion of the HSV envelope with the plasma membrane of the host cell by an undetermined mechanism. Current evidence indicates that virus-cell fusion requires gD (Ligas and Johnson, 1988), gB (Sarmiento, 1979), and gH-gL heterodimer (Forrester *et al*., 1992; Perez-Romero *et al*., 2005). Nevertheless, intact virions may also enter via endocytic pathway into some cell types in certain conditions (Nicola and Straus, 2004; Milne *et al.*, 2005; Nicola *et al*., 2005).

Following the fusion, viral nucleocapsid and tegument proteins are released into cytoplasm of the host cell. The nucleocapsid and some tegument proteins, such as VP16 and VP1-2, are transported through the microtubules network to the nuclear pore (Sodeik *et al*., 1997) while other tegument proteins remain in the cytoplasm. At the pore, the nucleocapsid releases its DNA into the nucleus, leaving an empty capsid at the cytoplasmic side of the complex.

The bulk of incoming viral DNA circularizes rapidly after infection and in the absence of viral protein synthesis (Garber *et al*., 1993), and then the transcription of HSV gene begins. Host RNA polymerase II is responsible for synthesis of all viral mRNAs (Alwine *et al*., 1974; Costanzo *et al*., 1977). While cellular proteins are sufficient for the synthesis of viral transcripts, viral proteins are sufficient for the synthesis of viral transcripts, viral proteins are necessary for the initiation and enhancement of transcription of certain genes. These proteins act in concert with an abundance of cellular proteins to produce the full range of viral gene products needed for productive viral infection and replication. The first gene transcribed during viral infection are the immediate early (IE) or α genes. Initiation of transcription of these genes proceeds by recruitment of cellular transcriptional machinery to IE gene promoters that contain numerous host regulatory sequences. IE gene expression does not require prior HSV protein synthesis; however, an HSV protein brought in with the virion tegument, VP16, plays an important role in enhancing the expression of the α-proteins (Batterson and Roizman, 1983). The α proteins include several multifunctional proteins that play essential roles in the regulation of later viral gene expression as well as in the control of host cell. Expression of the next set of HSV genes, the early (E) or β genes, requires at least 3 IE proteins, ICP0, ICP4, and ICP27, but this expression is not associated with the onset of viral DNA synthesis (Watson and Clements, 1980; Stow and Stow, 1986; Uprichard and Knipe, 1996). The β-proteins are generally involved in viral DNA replication. These proteins block further synthesis of α proteins and lead to transcription of the third set of viral RNAs (γ genes). Prior to DNA replication, α proteins initiate the transcription of not only the E genes, but also a subset of the late (L) or γ genes, called early-late, leaky-late, or γ_1 genes. The synthesis of these proteins later in the infection cycle is not strictly dependent on viral DNA replication. However, their levels are significantly enhanced upon initiation the initiation of DNA replication. A second subset of late genes, the true late or γ_2 genes, is transcribed only after the initiation of viral DNA replication.

Viral DNA replication occurs in nucleus initiated at the origins of replication within the HSV genome and is believed to proceed initially via theta replication mechanism (Figure 5). Once DNA synthesis begins, it is likely that a rolling-circle replication mechanism takes over to produce concatemeric molecules. Thus, most of the viral progeny DNA molecules that accumulated in the infected cell nucleus are head-to-tail concatemers (Jacob *et al*., 1979). Seven herpes genes were identified as essential for viral DNA replication (Challberg, 1986).

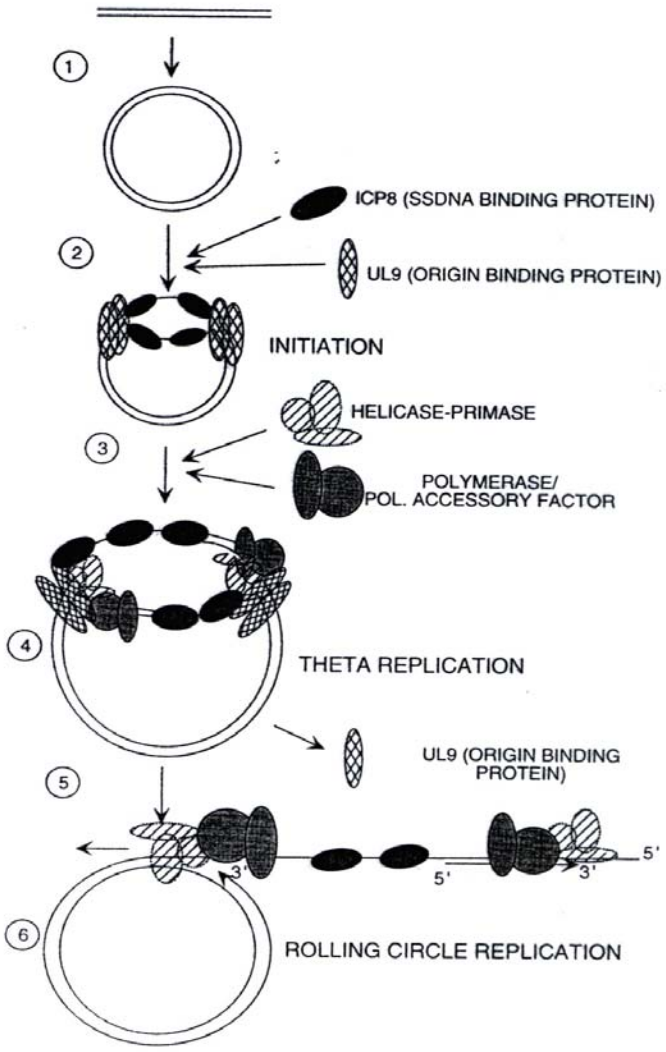


Figure 5. A model of HSV DNA replication (Riozman and Philip, 2001).

These seven genes encode protein products that function as an origin binding protein (U_L9) (Elias *et al*., 1986), a DNA binding protein ICP8 (U_L29) (Conley *et al*., 1981), a helicase-primase complex (U_L5, U_L8, U_L52), and a DNA polymerase (U_L30, U_L42) (Purifoy *et al*., 1977). In addition, HSV expresses several other early viral gene products, such as thymidine kinase (Kit and Dubbs, 1965), ribonucleotide reductase (Bacchetti *et al*., 1986), and uracil N-glycosylase (Caradonna *et al*., 1987). These proteins are considered nonessential for viral replication, but are required for nucleotide metabolism and viral DNA synthesis and repair in resting cells, such as neurons.

Assembly of viral capsid requires synthesis of numerous late proteins, and occurs within the nucleus. These capsid proteins, such as VP5, VP19c, VP21, VP22a, VP24, and VP26, are synthesized in cytoplasm and transported into the nucleus. Empty capsid shells are loaded with viral DNA by a process that simultaneously cleaves of HSV progeny DNA concatemers and packs genome-length monomers within the capsid (Ladin *et al*., 1980, 1982). The mechanism of DNA cleavage and packaging is not well understood; however, it is known to require site-specific breaks to the concatemers at specific distances from the packaging signal (Varmuza and Smiley, 1985; Deiss *et al*., 1986; Smiley *et al*., 1990).

After encapsidation of full-length viral genomic DNA molecules, the nucleocapsids are capable of budding through the inner nuclear membrane (Vlazny *et al*., 1982). Interactions between capsid and tegument proteins and between tegument proteins and viral glycoproteins promote this budding process. It is clear that nucleocapsids acquire some tegument proteins and a glycoprotein studded envelope upon budding through the inner nuclear membrane. From this point, the route for egress of the virion particle from the space between the inner and outer nuclear membranes to the exterior of the infected cell remains controversial. Two general pathways have been hypothesized for virion egress. A re-envelopment pathway suggested that enveloped particles fuse with the outer nuclear membrane; thereby de-envelop the nucleocapsids and release free nucleocapsids into the cytoplasm. These nucleocapsids are re-enveloped by budding into the golgi compartment and the re-enveloped particles are secreted from the cell through secretory vesicles (Siminoff and Menefee, 1966; Stackpole, 1969). A luminal pathway has been proposed in which the enveloped particles traffic from the inner nuclear space through the cytoplasm in the lumen of the endoplasmic reticulum or in vesicles to the golgi where final maturation of the virion glycoprotein occurs. These mature virions are released from the cell by a

normal secretory route (Enquist *et al*., 1999). However, recent evidences lend considerable support to the re-envelopment model as the major route of virion egress (Granzow *et al*., 2001; Skepper *et al*., 2001).

The eclipse period is 5 to 6 hours in monolayer cell cultures, and virus increases exponentially until approximately 17 hours after infection. Each cell has then made 10^4 to 10^5 physical particles, of which about 100 are infectious (Roizman and Knipe, 2001; Taylor *et al*., 2002).

4. The fate of the infected cells

HSVs require living cell for their replication. The longer the infected cells remain alive, the more progeny viruses are made, and ultimately the more the virus spreads. Cells productively infected with herpesviruses do not survive. Cell death is the result of not only irreparable injury caused by viral replication but also cellular responses to infection. Almost from the beginning of the reproductive cycle, the infected cells undergo major structural and biochemical alterations that ultimately result in their destruction.

The structural alterations in infected cells occur in many ways. First, the nucleolus becomes enlarged and finally disaggregates. Concurrently, host chromosomes become marginated, and later in infection, the nucleus becomes distorted and multilobed (Roizman and Furlong, 1974). The viruses then induce changes in appearance of cellular membranes. The duplication and folding of intracellular membranes are characteristic of cells in late HSV infection (Morgan *et al*., 1959; Epstein, 1962; Leestma *et al*., 1969). HSV also causes a cell-type-dependent fragmentation and dispersal of golgi vesicles throughout the cytoplasm (Campadelli-Fiume *et al*., 1993). In addition, viral proteins especially glycoproteins are inserted into cellular membranes (Spear and Kellejmroian, 1970; Roizman and Spear, 1971) resulting in alterations of cellular membrane structure and antigenicity (Roizman and Roane, 1961; Roane and Roizman, 1964; Roizman and Spring, 1967). Moreover, polykaryocytosis is another character of HSV infected cells. Both HSV-1 and HSV-2 cause cells to round up and adhere to each other or fuse into polykaryocytes (Hoggan and Roizman, 1959; Ejereito *et al*., 1968; Ruyechan *et al*., 1979). Rearrangements of the microtubular network are apparent very early in infection. Thus, the microtubules at the junction of the network with the plasma membrane appear to be disrupted in infected cells (Ward *et al*., 1998). Finally, the accumulation of viral replication proteins, progeny viral DNA, and nucleocapsid components within nucleus (De Bruyn Kops and Knipe, 1988) may cause the formation of intranuclear inclusion bodies in herpesvirus-infected cells (Schwartz and Roizman, 1969; Smith and Sutherland, 1986). These large eosinophilic intranuclear inclusion bodies are one of the characters of HSV infections that can usually be found both in herpesvirus-infected tissues and in appropriately stained cell cultures (White and Fenner, 1994).

It has been known for many years that HSV shuts down host cell RNA, DNA, and protein synthesis (Roizman and Tognon, 1983) because the virus uses numerous cellular proteins for its own protein synthesis (Taylor *et al*., 2002). Thus, host DNA synthesis is shut off (Roizman and Roane, 1964), host protein synthesis declines rapidly (Roizman and Borman, 1965; Sydiskis and Roizman, 1966), and glycosylation of host proteins ceases (Smiley *et al*., 1992). Furthermore, host macromolecular metabolism is altered in HSV infected cells in at least three ways. First, the virion virion-host shut-off (vhs) protein causes the degradation of mRNA present in infected cells early in infection (Kwong *et al*., 1988; Zelus *et al*., 1996; Karr and Read, 1999). Next, multiple viral genes are involved to impede cellular transcription (Preston and Newton, 1976) and translation (Wagner and Roizman, 1969) to facilitate the cellular to viral gene transcription and translation. In one example, ICP27 inhibits RNA maturation by redistributing host splicing factors (Sandri-Glodin *et al*., 1995; Sandri-Goldin and Hibbard, 1996). Thus, HSV regulatory protein ICP24 inhibits host RNA splicing and contributes to the decrease in cellular mRNA levels during infection (Hardwice and Sandri-Goldin, 1994). This effect has little or no effect on viral RNA synthesis because very few viral transcripts are spliced. Another viral protein, ICP22, has been shown to be required for modification of host RNA polymerase II following infection (Spencer *et al*., 1997), perhaps altering the ability of this complex to transcribe from the genome. Finally, the virus selectively destabilizes (Advani *et al*., 2000) and degrades (Everett *et al*., 1994) a variety of cellular proteins, especially those involved in regulation of host cell cycle (Advani *et al*., 2000; Roizman and Knipe, 2001).

5. Latent infection

One of the hallmarks of all herpesvirus infections is the ability of the virus to establish a latent infection that can last the lifetime of the host. The major site of HSV latent infection is sensory neurons in ganglion tissue, either trigeminal ganglia for HSV-1 or sacral ganglia for HSV-2. After the initial primary infection, generally at an oral or genital mucosal surface, the virus enters nerve ending and is transported retrograde along axon to the neuronal cell body (Cook and Stevens, 1973; Hill, 1981, 1985). During latency, the HSV genome remains in the nucleus of the sensory neuron as circular, extra-chromosomal DNA (Rock and Fraser, 1985;

Mellerick and Fraser, 1987). In neurons latently infected with HSV, no viral progeny is produced and only very limited gene transcription is detected. This viral mRNA, called LATs or Latency Associated Transcripts, may limit viral gene expression (Garber *et al*., 1997) or protect neurons from apoptosis (Perng *et al*., 2000). Clearly, HSV in latent infections is not cause lytic infection, as occurs in mucous membranes and still promote neuronal survival during its latency. The virus remains in this state for the lifetime of host, or until the proper signal periodically reactivate the virus and new progeny viruses are generated. Infection virus travels anterogradely to peripheral tissues by axonal transport (Colberg-Poley *et al*., 1981), usually to cells at or near the site of initial infection (Carton and Kilbourne, 1952; Roizman, 1966). The signal and mechanisms involved in this process are poorly understood, but it appears that host immune status (Roizman and Knipe, 2001) and certain physical stresses, such as illness or exposure to ultraviolet light, increase the chance of reactivation (Taylor *et al*., 2002).

6. Pathology of herpes simplex virus infection

The pathologic changes induced by the replication of HSV are similar for both primary and recurrent infection but vary in the quantitative extent of cytopathology. These changes represent a combination of virally mediated cellular death and associated inflammatory response because HSV causes cytolytic infections. Lesion induced in the skin and mucous membranes by HSV-1 and HSV-2 are the same and resemble those of varicella zoster virus.

HSV productive infections are lytic, as a result of virus-induced shutdown of host protein and nucleic acid synthesis, and obvious when microscopically detected. The histopathologic changes induced by viral infection include ballooning of infected cells and the appearance of condensed chromatin within the nuclei of cells, followed by subsequent degeneration of the cellular nuclei of cells, followed by subsequent degeneration of the cellular nuclei, generally within parabasal and intermediate cells of the epithelium. Cell fusion which appears as multinucleated giant cells provides an efficient method for cell-to-cell spread for HSV, even in the presence of neutralizing antibody (Brooks *et al*., 2001).

With cell lyses, clear fluid called vesicular fluid containing large quantities of virus appears between the epidermis and dermal layer. The vesicular fluid contains cell debris, inflammatory cells, and multinucleated giant cells. In dermal substructures, there is an intense inflammatory response, usually in corium of the skin, more so with primary infection than with recurrent infection. With healing, the vesicular fluid becomes pustule with the recruitment of inflammatory cells, and then it scabs. Scarring is uncommon but has been found in some patients with frequently recurrent lesions. Vascular changes in the area of infection include perivascular cuffing and areas of hemorrhagic necrosis. These histopathologic findings become particularly prominent when the infection occurs in organs of the body other than skin, for example HSV encephalitis or disseminated neonatal HSV infection.

HSV is transmitted by contact of a susceptible person with an individual excreting virus. The virus must come in contact with mucosal surfaces or abraded skin for infection to be initiated. The site of primary infection depends on the way in which the patient acquires the virus. It is often noted that HSV-1 usually causes infection at oropharyngeal tract, and is spread by respiratory droplets or by direct contact with infected saliva. HSV-2 is usually sexually transmitted and replicates in the genital, perigenital, or anal skin sites. This reflects the mode of transmission rather than any intrinsic property of virus (Sack *et al*., 2004).

Once epithelial cells are infected, there is replication of the virus around the lesion and entry into the innervating neuron. Because of infection with HSV-1 generally to oropharynx, initial replication of virus occurs in the oropharyngeal mucosa. The trigeminal ganglion becomes colonized and harbors latent virus. Acquisition of HSV-2 infection is usually the consequence of transmission by genital contact. Virus replicates in genital mucosa with seeding of the sacral ganglion (Bastein *et al*., 1972; Stevens and Cook, 1974; Stevens, 1975). After the establishment of latency as described above a recurrence of HSV infection is known as reactivated infection or recurrent infection. This form of infection leads to recurrent vesicular lesions of the skin such as herpes labialis or recurrent genital herpes (Baringer and Swoveland, 1973). Reinfection with a different strain of HSV can occur but extremely uncommon in the normal host. It is called exogenous reinfection (Brook *et al*., 2001).

Primary HSV infections are usually mild. In fact, most are asymptomatic. Only rarely virus can spread beyond the dorsal root ganglia, thereby becoming systemic. Such circumstances include multiorgan disease of pregnancy, disseminated neonatal HSV infection, and disseminated HSV infection in immunocompromised host. It is likely that the widespread organ involvement is a result of viremia in the host incapable of limiting viral replication to mucosal surfaces.

The natural history of HSV infections is influenced by both nonspecific and specific host defense mechanisms (Lopez *et al*., 1993). It has been reported that host genetic background (Lopez, 1975; Lopez *et al*., 1981), macropharges (Starr *et al*., 1976; Schneweis *et al*., 1982), natural killer cells, specific T-lymphocyte subsets (Kohl *et al*., 1989), specific antibodies (Eberle and Courtney, 1981; Bernstein *et al*., 1985; Kahlon *et al*., 1987), and lymphokine responses (Sheridan *et al*., 1982; Cunningham and Merigan, 1983) are the important host defenses against HSV infection.

During primary infection, IgM antibodies directed against envelope glycoprotein gB and gD appear transiently and are followed by IgG (Kurtz, 1974) and IgA (Friedman and Kimmel, 1982) that persist over time. The more severe the primary infection or the more frequent the recurrences, the greater the level of antibody response. However, the pattern of antibody response does not correlate with the frequency of recurrent disease. Cell-mediated immunity and nonspecific host defense mechanisms are also important in controlling both primary and recurrent infections of HSV. All these responses may well influence on the acquisition of disease, the severity of infection, and the host resistance to subsequent HSV reactivation. However, spontaneous reactivations can occur in spite of the presence of both host's HSV-specific humoral and cellular immunity. This immunity helps in limiting local viral replication, so that recurrent infections are less extensive and less severe. Accordingly, many recurrences are asymptomatic and detected only by viral shedding in secretions. When symptomatic recurrent infections occur, episodes of recurrent HSV-1 infection are usually manifested as cold sores or fever blisters near the lip. More that 80% of the human population harbors HSV-1 in a latent form, but only a small portion experience recurrences.

Passively transferred maternal antibodies are acquired in many newborns. These antibodies are lost during the first 6 months of life, and the period of greatest susceptibility to primary herpes infection occurs between 6 months and 2 years. Clearly, humoral immunity does

not prevent either recurrences or exogenous reinfection. Thus, transplacentally acquired antibodies from the mother are not totally protective against infection of newborns (Sullender *et al*., 1987, 1988; Kahlon and Whitley, 1988). HSV-1 antibodies begin to appear in early childhood and are present in most persons by adolescence. Antibodies to HSV-2 rise during the age of sexual activity and adolescence.

7. Epidemiology of herpes simplex virus infection

HSV-1 and HSV-2 occur worldwide, have no seasonal variation, and naturally only infect human beings. HSV spreads principally by close person-to-person contact with lesions or mucosal secretions. Most human beings have been infected and harbor latent virus that can be reactivated; therefore there is a vast HSV reservoir for transmission to susceptible individuals. Although HSV-1 and HSV-2 are usually transmitted by different routes and involve different areas of the body, there is a great deal of overlap between the epidemiology and clinical manifestations of infection. HSV-1 is probably constantly present in humans than any other viruses. Primary HSV-1 infections usually occur in the young child, less than 5 years of age, and most often asymptomatic. With clinical illness, the mouth and lips are the most common sites of this virus infection. Gingivostomatitis usually is the clinical manifestation in young children while pharyngitis is associated with HSV-1 primary infection in young adults.

Many demographic factors influence the acquisition of HSV-1 infection including geographic location, socioeconomic status, and age. In developing countries, seroconversion happens early in life (Bader *et al*., 1978). By 5 years of age, approximately one third of children seroconverted and this frequency increased to 70% to 80% by early adolescence (Nahmias *et al*., 1970). In comparison, middle-class and upper-class individuals in more developed countries become infected later. Seroconversion occurs in about 20% of children younger than 5 years; then no substantial rise in incidence happens until an increase to 40% to 60% at age 20-40 years (Wentworth and Alexander 1971). In the United States, race also affects acquisition of HSV-1. By 5 years of age, over 35% of African-American versus 18% of white children is infected with HSV-1. Incidence of infection among university students is about 5% to 10% annually (Nahmias *et al*., 1990). These studies suggested that the frequency of direct person-to-person contact,

indicative of crowding encountered with lower socioeconomic status, appears to be the major mediator of HSV-1 infection. The largest reservoir of HSV-1 infections in the community is recurrent herpes labialis. The frequency of recurrent HSV-1 infection is approximately 33% in several studies (Friedman *et al*., 1977; Ship *et al*., 1977). Recurrent infections may occur in the absence of clinical symptoms, but still have viral shedding. At any given time approximately 1% to 5% of normal adults will be excreting HSV (Sheridan and Hermann, 1971; Hatherley *et al*., 1980).

Usually, HSV-2 is transmitted by sexual intercourse and results in genital HSV infection, so antibodies to this virus are seldom found before puberty. Although most genital HSV infections are caused by HSV-2, an increasing proportion is attributable to HSV-1 (Wolontis and Jeansson, 1977; Corey *et al*., 1983). Genital HSV-1 infections are usually less severe and less prone to recur than those caused by HSV-2 (Reeves *et al*., 1981). HSV-2 seroprevalence rises from about 20% to 30% at age 15-29 years to 35% to 60% at age 60 years (Fleming *et al*., 1997). Factors that affect the acquisition of HSV-2 infection include sex, race, marital status, number of sexual partners, and place of residence. HSV-2 infection is more frequent in women than men (Rawls *et al*., 1971; Poste and Howkin, 1972) and in African-Americans than whites. This infection prevalence is higher in cities than in suburbs. Importantly, the number of sexual partners greatly influences the acquisition of HSV infection (Rawls and Gardner, 1972; Rawls *et al*., 1976) according to the highest prevalence of antibodies against HSV-2 among female prostitutes (75%). As with HSV-1 infection of the mouth, primary and recurrent HSV-2 infection may be symptomatic or asymptomatic. Either situation also provides a reservoir of virus for transmission to susceptible persons. HSV-2 infection tends to recur more often than HSV-1 infection (Whitley and Roizman, 2001).

Maternal infections of genital HSV pose risks to both mother and fetus. Rarely, pregnant women may develop widely disseminate maternal disease after HSV infection (Anderson and Nicholls, 1972; Peacock and Sarubbi, 1983) that led to life-threatening disease, such as hepatitis, thrombocytopenia, leucopenia, and encephalitis. The mortality rate among these pregnant women is greater that 50%. The major risk to the fetus is maternal primary or initial genital HSV infection (Kulhanjian *et al*., 1992). Primary infection before 20 weeks of gestation has been associated with spontaneous abortion; however, recurrent infection is the most common form of HSV infection during gestation. Neonate infection can occur in uterus (about 5% of infections),

intrapartum (around 80%), or postnatally infection (Whitley, 2001). Transmission of infection to the fetus is most frequently related to the actual shedding of virus from infected maternal secretions in the mother's birth canal at the time of delivery. Estimates of the frequency of cervical shedding of virus among pregnant women vary widely. Nevertheless, the majority of infants (70%) who develop neonatal disease are born to women who do not have a history of genital herpes and are asymptomatic at the time of delivery (Brooks *et al*., 2001). The rate of occurrences of neonatal HSV infections is approximately 1 in 3,000 to 1 in 5,000 deliveries per year (Nahmias *et al*., 1989). Neonatal HSV infection is almost symptomatic and frequently lethal from disseminated infection especially CNS infection. Mortality in the absence of therapy exceeds 80% and all of few survivors have neurologic impairment (Whitley *et al*., 1988).

The clinical manifestations of primary HSV-1 infection range from totally asymptomatic to gingivostomatitis in young child and pharyngitis or tonsillitis in adult (Glezen *et al*., 1975). Following recovery from primary oropharyngeal infection, the individual retains a chance of suffering from recurrent attacks of herpes labialis, otherwise known as fever blisters or cold sores. Recurrent oralabial lesions are preceded by a prodome of pain, burning, tingling, or itching which generally lasts for 6 hours. Vesicles, normally three to five, appear most commonly on the vermilion border of the lip. These lesions are completely healed after 8 to 10 days. The frequency of recurrence varies among individuals. Other skin HSV-1 infections can occur, such as eczema herpeticum in atopic dermatitis patients, herpes gladiatorium in wrestlers, and herpes whitlow in dentists and nurses.

Primary genital herpes appears as macules and papules followed by vesicles, pustules, and ulcers (Corey *et al*., 1983) on vulva in female, penis in male, and perianal region in male homosexuals. Systemic complications in men are rare; however, aseptic meningitis and urinary retention are common in women. Recurrent genital herpes appears as three to five vesicles on genital and is characterized by a prodrome and localized irritation. Genital HSV infection is rarely transferred from mother to fetus during pregnancy and causes neonatal herpes. This disease can result in, skin, eye, or mouth infections, encephalitis with or without skin infection, or disseminated disease involving multiple organs (Whitley *et al*., 1981).

Herpetic keratoconjunctivitis is HSV-1 infection of eye beyond the newborn age (Binder, 1977). Primary infection is associated with either unilateral or bilateral conjunctivitis, while

recurrent infection is usually unilateral. The clinical symptom includes photophobia, tearing, and eyelid edema accompanied by dendritic lesions. Geographic ulcers of the cornea develop with advanced disease and lead to blindness from repeat HSV infection.

Herpes encephalitis is a rare manifestation of HSV, usually type 1, infection; nevertheless, the virus is thought to be the most common cause of sporadic fatal encephalitis. The temporal lobes are principally involved and mortality rate in untreated cases is over 70%. HSV can also affect almost all areas of the nervous system, causing significantly meningitis, myelitis, and radiculitis (Whitley and Roizman, 2001). Moreover, patients compromised by immunotherapy, underlying disease, or malnutrition are at increased risk for potentially lethal disseminated HSV infection. These patients can develop progressive disease involving respiratory tract, commonly esophagus, or gastrointestinal tract.

8. *In vitro* methods for antiviral test

The methodology used in the determination of the antiviral activity as well as the interpretation of the results have been virtually specific to each laboratory and are consequently not comparable to one another, so simple procedures and guidelines for evaluation of antiviral or virucidal activity of compounds are urgently needed. This is a series of experimental assays through which the anti-HSV activity of candidate antiviral compound may be evaluated *in vitro*, the potency of the compound can be assessed, and the antiviral mechanism of the candidate substance may be investigated.

HSV grows well in a wide variety of cell types to yield high titers of virus stocks. Cell lines routinely used to grow HSV and test for the anti-HSV activity of compounds include BHK (baby hamster kidney cell line), RK13 (rabbit kidney cell line), Vero (monkey kidney cell line), and CV1 (monkey kidney cell line) (Harland and Brown, 1997). Antiviral compounds may block HSV replication cycle at different stages (Figure 6), thus virus replication in cell cultures may also be monitored by the detection of viral products, for example viral DNA, RNA, or glycoproteins.

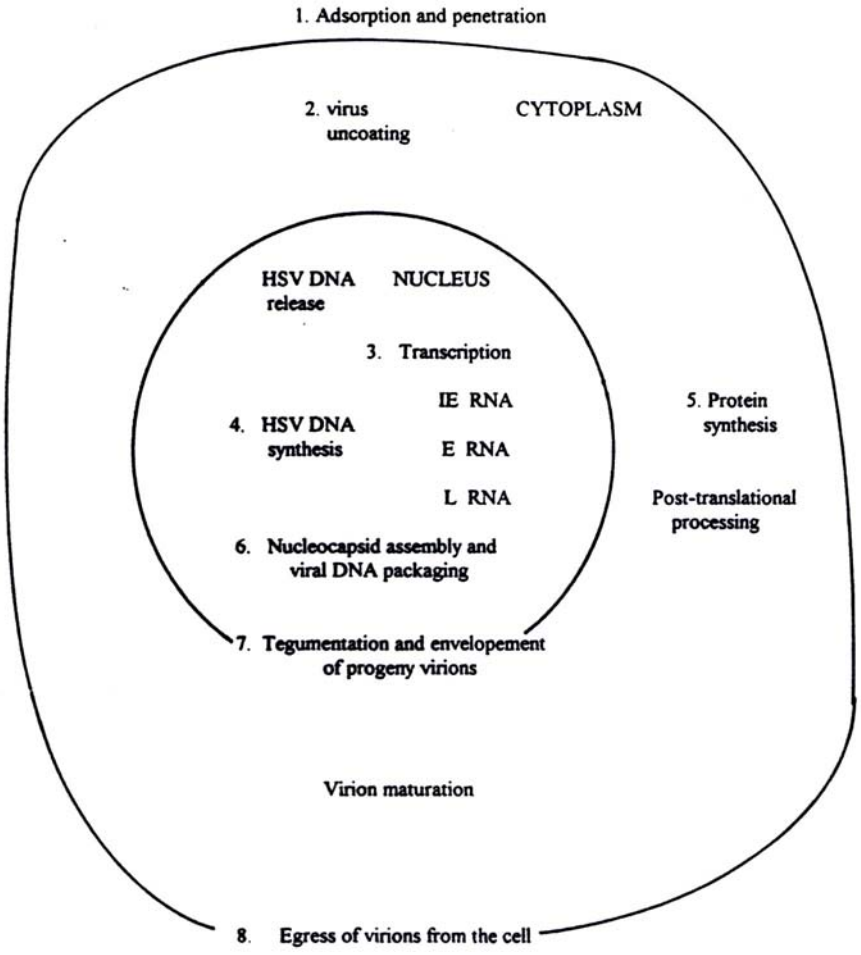


Figure 6. Stages in the HSV replication cycle that might be blocked by antiviral agents (Dargan, 1998).

In order to study the antiviral activity of a new drug, it is important to determine whether antiviral activity can be uncoupled from the effect of cellular toxicity. Cytotoxicity tests define the upper limit concentration of candidate compounds which can be used in subsequent antiviral tests. The simplest cytotoxicity tests *in vitro* are the vital-staining techniques. These methods are easy to perform, need little equipment, and give reliable results (Galt *et al*., 1990; Sydiskis *et al*., 1991). In these tests, cells are treated with trypan blue or neutral red dyes. Trypan blue is excluded by live cells, but stains dead cells blue (Anderson *et al*., 2003; Washman *et al*., 2003). In contrast, neutral red is taken up by live cells, staining them a brownish-red color, whereas dead cells remain colorless. In addition to vital-staining test, cell viability can be confirmed by additional experiment, such as measuring incorporation of ^{3}H thymidine into cellular DNA, and measuring cell ability to cleave tetrazolium salt by mitochondrial enzymes to give a color product (Konsula and Bariel, 2005). Absence of cytotoxicity in *in vitro* test does not necessarily mean that test compound does not have toxicity *in vivo*. Similarly, a moderate level of cytotoxicity in *in vitro* test may not necessarily exclude *in vivo* use of the compound.

The method most commonly used for evaluation of *in vitro* antiviral activities is based on the different abilities of viruses to replicate in culture cells in which test substances appeared and determine ED_{50}. The ED_{50} is the effective concentration or dose of test compounds that eliminates 50% of the viral infectivity. As previously mentioned, HSV can cause cytopathic effects (CPE) or form plaques in cell cultures, thus ED_{50} is the dose or concentration of the test compound which inhibited CPE, reduced or inhibited plaque formation, and reduced virus yield or other viral functions by 50% (Vlietinck and Berghe, 1991).

Generally, plaque reduction or inhibition assay is considered as a reference or standard for antiviral assay (Ellis *et al*., 1987). This assay uses a constant number of viral particles and varying the non-toxic concentrations of test substance (Abou-Karam and Shier, 1990; Liu *et al*., 2004). Typically, a monolayer of cultured cells is allowed to bind virus and then overlaid with a layer of semisolid medium which prevents spreading of virus from the area of originally infected cells. The test substance can be added into cell monolayer before or after virus adsorption is accomplished. The infected cultures are incubated further for an appropriate period of time, then they are fixed, stained with dye, and plaques (the areas of infected cells) are counted. By

reference to the number of plaques observed in virus control or untreated culture, ED_{50} is calculated and expressed.

Histochemical staining for plaque assay using a cell line, Vero ICP6LacZ#7, that expresses β-galactosidase activity was established (Tebas *et al*., 1995). Antiviral compound was added into HSV-infected cell monolayers, followed by pooled human immunoglobulin which limited the spread of virus to the surrounding cells. Two days later, the cell monolayers were stained for β-galactosidase activity. The plaques appear blue against a clear background of unstained, uninfected cells. The procedure of the CPE inhibition assay is similar to the plaque inhibition assay except the semi-solid substance is not included in the culture medium. The infectivity of virus could be examined by microscopic observation of characteristics of viral CPE (Yip *et al*., 1991) or by the dye uptake assay (Marchetti *et al*., 1996). The therapeutic index (TI) can be computed by the ratio of the maximum drug concentration at which 50% of the growth of normal cells is inhibited to the minimum drug concentration at which 50% of virus is inhibited.

Another method in measuring antiviral activity is virus yield inhibition assay (Yoosook *et al*., 1999; Tenser *et al*., 2001; Washman *et al*., 2003). In this method cell monolayer is infected with virus, and increasing concentrations of test compound are added after virus adsorption. Following a cycle of viral replication, the harvested cell cultures are disrupted by three cycles of freeze-thaw, supernatants are kept, and virus yields are determined by plaque assay. Plotting the diminishing infectious virus yields provides drug dose-response curves that yield more information than can be obtained from EC_{50} value alone.

The major goal in the investigation of the antiviral mechanism is to identify the virus gene product that is the target of test compound. According to the concept of this assay, it its first necessary to identify and isolate drug resistant virus mutants from treated cultures by various methods, including single round selection, mapping the drug resistance gene, or DNA sequencing. However, if no drug-resistant variant can be isolated, it will be important to investigate the point in the virus replication at which drug-sensitive function operates.

Virucidal assay is the method to examine the elimination of infectivity when virus particles are mixed with increasing concentration of test compound in solution. Viral titers are obtained from the titration of this mixture solution in cell monolayer. Virucidal activity may be caused by disintegration of the entire virus particle, solubilization of the herpersvirus envelope, or

the chemical modification, degradation, or masking of some essential envelope proteins. HSV particles treated with a virucidal agent are blocked at adsorption and/or penetration, the earliest stage of the virus replication cycle.

To investigate the stage of HSV adsorption to target cells, two techniques have been used. The first technique indirectly measures virus adsorption by quantifying the rate of decrease in infectivity of the inoculum applied to cell monolayers (Hayashi *et al*., 1992). The second technique quantified virus particles bound to cell surfaces by measuring accumulation rate of the radioactivity associated with ^{35}S-methionine labeled virus particles. Both techniques should be performed at 4^{o}C, since at this temperature HSV particles can bind to the receptors at cell surfaces but cannot penetrate into target cells.

Penetration assay is the test to investigate the effect of test compound on the rate of HSV entry into target cells (Rosenthal *et al*., 1984). This assay depends on the observation that HSV binds to cells at 4^{o}C but does not penetrate until the temperature is raised. After virus attachment at 4^{o}C, the medium containing test compound is added to cell monolayer and then temperature is shift to 37^{o}C to allow virus penetration. Virus particles that have not yet penetrated the cells are inactivated, at various times after temperature upshift, by treating the infected cell cultures with low pH solution. The infectivity that becomes resistant to low pH inactivation with time is the rate of virus penetration.

The assays to examine the stage of HSV replication cycle inhibited by test compound at a point subsequent to virion entry include electron microscope studies, which show morphological changes during viral replication, or single step HSV growth experiments in which a single antiviral concentration of test substance is added to infected cell cultures at progressively later times throughout the virus replication cycle. If the target protein of antiviral agents has function in early stage of infection, late addition of test compound will have no inhibitory effect on infectious virus yield. On the other hand, if test compound inhibits protein function that is required in late infection, or throughout the replication cycle, the viral infectivity will decrease whenever the drug is present.

Viral DNA synthesis detection is the assay that determines the effect of test compound on HSV-DNA synthesis. In the past, HSV-DNA synthesis has been studied by separation of ^{3}H-thymidine labeled infected-cell DNA into viral and cellular fractions by cesium chloride gradient

centrifugation (Dargen and Subak-Sharpe, 1986). More recently, this technique has been substituted by a dot-blot, Southern blotting method (Kuo *et al*., 2001; Evers *et al*., 2004). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is another technique that can be used to investigate the effect of antiviral compound on viral protein synthesis and post-translational processing. The inhibition in HSV replication is usually reflected in changes in the quantity or the apparent molecular weights of HSV specific polypeptide bands on SDS-PAGE gels (Wachsman *et al*., 2003). These reductions may resulte from test compounds that inhibit viral gene transcription, viral mRNA translation, or reduce viral mRNA or protein products stability (Dargan, 1997). In addition, other antiviral assays based on measurement of specialized function and viral products have been studied using recently developed methods such as flow cytometric analysis (Pavic *et al*., 1997), nucleic acid hybridization (Lin *et al*., 2000), and enzyme-linked immunosorbent assay (ELISA) (Anderson *et al*., 2003).

9. Antiherpes virus agents

There are three categories of anti-herpesvirus drugs in current clinical use (Andrei *et al*., 1995). The first category is pyrophosphate analogues such as phosphoacetic acid (PAA) and phosphonoformic acid (PFA), known as foscarnet or trisodium phosphonoformate. These drugs act as a direct inhibitor of viral DNA polymerase by reversibly blocking the pyrophosphate binding site of viral polymerases (Oberg, 1989; Chrisp and Clissold, 1991). This binding site involves in releasing the pyrophosphate product of DNA synthesis during DNA polymerization process. PFA inhibits this cleavage of pyrophosphate groups from the deoxynucleoside triphosphates (dNTPs), a crucial step in DNA chain elongation, thus PFA inactivates virus by interfering with the elongation of the viral DNA chain. According to very poor absorption after oral administration, foscarnet used in the form of cream has been reported to accelerate healing of recurrent facial and genital herpes lesion, and given systematically it can halt the progression of HSV, varicella zoster virus (VZV) and cytomegalovirus (CMV) infection. However, the most common side effect of foscarnet is renal toxicity, associated with tubular intestinal lesions (Snoeck, 2000).

The second category is the nucleoside analogues that can be divided into two groups. One is a variety of purine nucleoside analogues including acyclovir (ACV), valacyclovir (VACV), penciclovir (PCV), and famcyclovir (FCV). Antiviral activity and selectivity of drugs in this group are based on their specific activation by herpesvirus-encoded thymidine kinase, which converts these drugs intracellularly to their monophosphate metabolites. Viral thymidine kinase has broader specificity than cellular thymidine kinase which unable to phosphorylate these compounds, therefore these drugs are non-toxic to uninfected cells. After initial conversion of the purine nucleoside analogues to their monophosphate, then further activation to diphosphate and triphosphate metabolites are catalysed by cellular enzymes, such as kinases, involved in nucleotide metabolism. These drugs in triphosphate forms inhibit the DNA polymerase reaction (Naesens and Clercp, 2000). Valacyclovir is the L-valyl ester of acyclovir that was developed as an oral prodrug of ACV to increase oral bioavailability and provide higher plasma ACV levels (Beutner *et al*., 1995). VACV is rapidly and extensively metabolized to ACV and L-valine after oral administration. Penciclovir is also a guanine analogue that is used topically as a cream to treat cold sores. Famciclovir is the diacetyl ester of 6-deoxy-penciclovir, an oral prodrug of PCV.

FCV was synthesized to improve oral absorption of PCV because FCV is rapidly and extensively adsorbed in the upper intestine. After adsorption, FCV undergoes substantial first pass metabolism via deacetylation and oxidation in liver to yield PCV (Vere Hodge, 1993; Perry and Wagstaff, 1995). Another group comprises pyrimidine nucleoside analogues, for instances idoxuridine (IDU), trifluridine (TFT), and brivudin (BVDU). Similar to purine nucleoside analogues, drugs in this group require specific phosphorylation to their monophosphte forms by viral enzyme; however, the monophosphate is converted to the diphosphate form by HSV or VZV encoded thymidine kinase (Clercq, 2004). Upon further phosphorylation by cellular kinases, nucleoside triphosphate can then interact with viral DNA polymerase, either as competitive inhibitor with natural substrate or as an alternative substrate incorporated into growing viral DNA chain and affect both the stability and functioning of the DNA. Idoxuridine and trifluridine in the form of eye drops or ophthalmic cream have been used to treat HSV keratitis (Clercq, 2005), while both oral and topical administration of brivudin can be used as a therapeutic agent in herpes labialis, herpes keratitis, and herpes zoster (Clercq, 2004).

The third class consists of those drugs that are independent from viral thymidine kinase for their activation. These drugs include the acyclic nucleoside phophonates, such as cidoforvir (HPMPC; 3-hydroxy-3-phosphonylmethoxypropyl-cytosine). Cidoforvir (CDV) has a potent and broad spectrum anti-DNA virus activity that includes all human herpesviruses (Naesens *et al*., 1997). CDV targets the viral DNA polymerase. After intracellular phosphorylation by cellular kinase to their monophosphate and diphosphate derivatives, acyclic nucleoside analogues incorporate at the 3'-end of the viral DNA chain and act as chain terminators or competitive inhibitors of further DNA chain elongation (Neyts *et al*., 1994).

Acyclovir (ACV), 9-(2-hydroxyethoxymethyl) guanine or acycloguanosine, has been a drug of choice for the prophylaxis and treatment of HSV infections for many years. This acyclic guanosine analogue is a selective inhibitor of HSV-1, HSV-2, and VZV DNA replication with low host cell toxicity. Following uptake of ACV by virus-infected cells, the first phosphorylation of the drug to ACV-monophosphate occurs via HSV-encoded thymidine kinase. Subsequent conversion to ACV-diphosphate and ACV-triphosphate is catalysed by host cell enzymes. ACV-triphosphate is a potent inhibitor of HSV DNA polymerase and is a competitive inhibitor of cellular deoxyguanosine triphosphate resulting in viral enzyme inactivation. The inactivation of viral DNA polymerase occurs due to lack of the 3'-hydroxy group required for subsequent 5' to

3’phosphodiester linkages to elongate viral DNA chain; thereby, DNA chain termination appears once the drug enters the viral DNA (Figure 7).

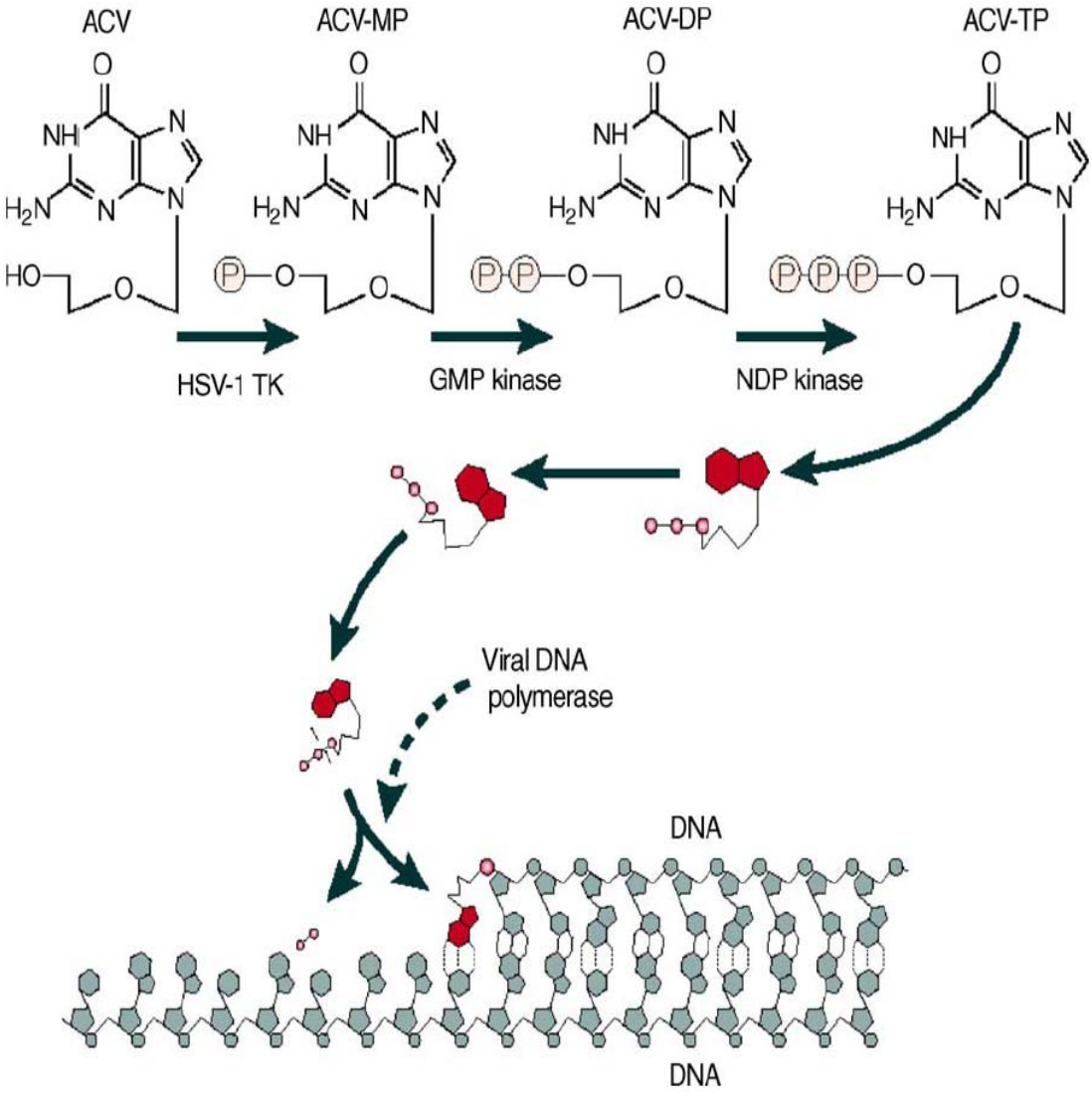


Figure 7. Mchanism of antitviral action of acyclovir (ACV) (Clercq, 2004).

ACV is clinically used in the treatment of mucosal, cutaneous, and systemic HSV-1 and HSV-2 infection and VZV infection, and may be administered orally, topically as an aqueous cream, or by slow intravenous infusion. Nevertheless, this widespread use of ACV has led to the emergence of HSV strains resistant to ACV. ACV resistant mutants of HSV can be recovered from both *in vivo* and in cell cultures. The first case of clinical viral resistant strains was published in 1982 (Crumpacker *et al*., 1982; Silbrack *et al*., 1982) and the incidence had continued increasing mainly among immunocompromised patients and particularly allogenic bone marrow transplant patients of which the prevalence reaches 5% and 30%, respectively (Morfin and Thouvenot, 2003). The mutation is most frequently located in the viral gene encoding thymidine kinase, but more rarely occurs in viral DNA polymerase gene. There are three kinds of ACV-resistant mutants. Firstly, thymidine kinase deficient virus is the mutants that lose thymidine kinase activity or fail to produce this enzyme, and 95% of ACV-resistant isolates from patients represent a thymidine kinase deficient phenotype (Hill *et al*., 1991). Secondly, the group of mutant that can produce the enzyme but has altered thymidine kinase substrate specificity by point mutation is called thymidine kinase altered virus. Thirdly, some viruses mutate by alteration of DNA polymerase activity (Larder *et al*., 1983). These mutations leading to resistance occur spontaneously during viral replication and resistant viruses are then selected by antiviral treatment. Management of drug-resistant HSV infections depends on the mechanism of resistance of mutant strains. Foscarnet and cidoforvir that directly act on viral DNA polymerase without requiring activation by viral thymidine kinase are still active against viruses resistant to ACV because of a mutation in their thymidine kinase gene; however, they may be associated with a significant level of toxicity in clinical practice (Morfin and Thouvenot, 2003).

Vaccination remains the ideal method for prevention of HSV infection; nevertheless, prevention of HSV infections has unique problems because of its recurrences even in the presence of humoral immunity. However, protection from life-threatening infection can be achieved in animal models with avirulent, inactivated, or subunit glycoprotein vaccines. The first vaccine developed was killed whole-virus HSV-1 and HSV-2 vaccines which were licensed in Germany (Allen and Rapp, 1982). Nevertheless, the results from many clinical studies in the efficacy of these vaccines differ widely. Inactivated virus vaccines may benefit some patients with recurrent infection, but long-term benefit could not be established, since there are potential risks of inactivated vaccine especially as a cofactor in the development of cervical carcinoma. These were

followed by a partially purified HSV-1 vaccine, glycoprotein HSV-1 and HSV-2 subunit vaccines, and recombinant glycoprotein vaccines. Some recombinant glycoprotein vaccines appear highly immunogenic and now a few of them are being tested in clinical trails, particularly recombinant HSV-2 vaccine. One of these is a recombinant HSV-2 glycoprotein D vaccine (Straus *et al*., 1993) and another is glycoprotein B and glycoprotein D recombinant vaccine (Stanberry *et al*., 1987). However, to demonstrate high antibody titer and protective effects against HSV-2 recurrent infection, these two vaccines require efficient adjuvant, such as complete Freund's adjuvant, lipophilic muramyl tripeptide (Bernstein *et al*., 2005), or immune enhancer (Quenelle *et al*., 2006). In addition, these vaccines did not provide 100% protection in all patient groups (Corey *et al*., 1999), especially in men (Spruance, 2000), even in the presence of adjuvant. Other two promising HSV vaccines are genetically engineered, live-attenuated HSV-deletion mutant vaccine and glycoprotein H deletion mutant vaccine. The former was made by carefully selected gene deletions to remove putative neurovirulence sequences in HSV gene. The latter, known as the HSV-DISC vaccine, is a vaccine deleted of an essential glycoprotein H, thus allowing only a single cycle of infectious HSV replication, and has been studied in phase I clinical trail in the United Kingdom (Markert *et al*., 2000).

10. Antiherpes virus activity of natural substances

There are a number of natural options available for the prevention and treatment of herpes simplex virus infection. Natural remedies that show promises either for prophylaxis or treatment include lysine, vitamin C, vitamin E, zinc, adenosine monophosphate, glutathione, copper, and medicinal plants. These substances have different antiviral mechanisms, and some of them also have biological functions in human such as amino acid, vitamin, trace element, and antioxidant.

Various kinds of essential oils from medicinal plants have a long history of use. They have been used widely in both developing and developed countries for the treatment of various human diseases such as respiratory infection, asthma, atopic dermatitis, allergic rhinitis, and gastrointestinal disease (Buckle, 1997). These uses of medicinal plants are gaining popularity because of several advantages, including often fewer side effects, better patient tolerance, local

availability, relative acceptance due to long-time use, and less prone to the emergence of drug resistance strains. It has been reported that essential oils show not only anti-bacterial and anti-fungal activities but also anti-viral activity. Sandalwood oil, the essential oil of *Santalum album*, has antiviral activity against HSV-1 and HSV-2 virions, thus preventing adsorption of virions to host cells and inhibition of cell-to-cell virus spread *in vitro* (De Logu *et al*., 2000). Other essential oils from medicinal herbs that completely inhibited HSV replication include *Cupressus sempervirens* (cypress), *Juniper communis* (juniper), *Melaleuca alternifolia* (tea tree), *Eucalyptus globulus* (eucalyptus) (Schnitzler *et al*., 2001), *Ocimum basilicum album* (tropical basil), *Mentha piperita* (peppermint), *Origanum majorana* (majoram), *Ravensara aromatica* (ravensara), *Lavandula latifolia* (Lavender), *Citrus limon* (lemon), *Rosmarinus officinalis* (rosemary), and *Cymbopogon citratus* (lemongrass). These essential oils, especially lemongrass oil, possessed strong activity according to its complete inhibition of HSV-1 growth at a concentration of 0.1% *in vitro*, inactivation against viral particles by interaction with the virions, and binding to viral envelopes or glycoproteins (Minami *et al*., 2003). In clinical use, there are two medicinal plant extract preparations used as topical treatment for HSV infection. First, the extract of the leaves of *Melissa officinalis* or lemon balm is used as a lemon balm cream containing 1% extract of the leaves. Lemon balm cream can reduce the symptoms (Koytchev *et al*., 1999) and promote rapid lesion healing (Wolbling and Leonhardt, 1994). Second, leaf extract of *Clinacantus nutans* Lindau displayed anti-HSV-1 anti-HSV-2, and anti-inflammatory activity (Lipipun *et al*., 2003). The cream containing 40 mg of the extract of *Clinacantus nutans* Lindau commercially produced by Government Pharmaceutical Organization (GPO) could effectively treat HSV lesions in herpes labialis patients (เกศริน บุษรานนท์, 2550).

Lysine, an essential amino acid, has been shown to inhibit normal replication of HSV (Betsy *et al*., 2005), block the binding of HSV-1 by interfering with cellular receptor function (Langeland *et al*., 1988), and antagonize the growth-promoting action of arginine on HSV replication in tissue cultures (Griffith *et al*., 1981). The proteins synthesized by HSV normally contain more arginine than those synthesized by host cells (Kagan, 1974), and arginine is required for HSV replication. Lysine appears to antagonize arginine by several mechanisms. For examples, lysine acts as an antimetabolite of arginine, increases arginine excretion by competing with arginine reabsorption at renal tubule, competes with arginine absorption in the intestine, induces arginine degradation via enzyme arginase acrivation, and competes with arginine transportation

into cells (Miller and Foulke, 1984). Treatment with oral and topical lysine preparations in patients with orofacial or genital herpes could shorten the course and duration of the disease, reduce the severity of lesions, and reduce the frequency of recurrences.

Zinc ions at concentration of 0.1 mM have been reported to almost completely inhibit the replication of HSV-1 and HSV-2 *in vitro* (Gupta and Rapp, 1976). The inhibition appeared to result from the selective inhibition of viral DNA polymerase (Gordon *et al*., 1975; Fridlender *et al*., 1978). Topical application of various zinc preparations has been shown to be effective in the treatment of cutaneous human HSV infections. Most of the studies using zinc sulfate (Brody, 1981; Finnerty, 1986) or zinc monoglycerolate (Apisariyakulm *et al*., 1990) indicated that topically applied zinc could shorten the duration of HSV skin infection and possibly prevent both spontaneous and sunlight-induced recurrent infections with a few adverse effects such as irritation, unpleasant dryness, or nausea (Godfrey *et al*., 2001). Besides topical zinc treatment, oral zinc supplement also could reduce the duration and severity of HSV infection (Jones, 1979) and might prophylaxis recurrences induced by sun exposure (Fitzherbert, 1979); however, long-term zinc supplementation should be accompanied by a copper supplement in order to prevent zinc-induced copper deficiency (Fosmire, 1990).

Copper or cupric ions have been shown to inactivate several types of viruses, including members of *Herpesvirus* (Sagripanti, 1992) and completely inhibit HSV plaque formation when combined with reducing agents such as ascorbic acid (Sagripanti *et al*., 1997). The killing of HSV by copper followed a pattern in which Cu (II) ions bound with high affinity to viral DNA favoring guanosine residues (Sagripanti and Kraemer, 1989; Sagripanti, 1991). This binding produced oxidative base damage and gave the products including single and double DNA strand breakages as well as base modifications, mainly 8-OH-deoxyguanosine, and free radicals (Toyokuni and Sagripanti, 1996).

Vitamin C or ascorbic acid is an important antioxidant and has been shown to inactivate a wide range of viruses both *in vitro* and *in vivo* including HSV (White *et al*., 1986; Betanzos-Cabrera *et al*., 2004). There are numerous studies that reported the effect of oral or topical ascorbic acid treatment on HSV infection in both healthy and immunocompromised patients. These results suggested that vitamin C accelerated the healing of HSV lesions (Klenner, 1940), reduced the mean time until remission of symptoms (Terezhalmy *et al*, 1978) and severity of

symptom, and significally reduced HSV yield after the first day of treatment (Hovi *et al*., 1995). The vitamin C treatment was most effective when initiated drug during the prodromal period and the antiviral effect of ascorbic acid was more pronounced at higher dose especially in treatment of an acute infection (Betsy, 2006).

Vitamin E or α-tocopherol, a lipid soluble antioxidant, protects cell membranes from oxygen free radical causing damage. Like other antioxidants, vitamin E has been reported to enhance the healing of wounds (Slater and Block, 1991; Martin, 1996). In various *in vivo* (Martin, 1995) and clinical studies, topical application of vitamin E oil (Starasoler and Haber *et al*., 1978) or capsule (Fink and Fink., 1980) relieved pain, aided in the rapidly healing of oral herpetic lesions, reduced lesion development and severity of symptoms when compared to control (Sheridan *et al*, 1997)

Resveratrol (3, 5, 4'-trihydroxystilbene) is a non-flavonoid phenol compound produced naturally by some spermatophytes, such as grapes, in response to injury or fungal attack. This compound has antioxidant (Stivala *et al*., 2001), anti-cancer (Jang *et al*., 1997), antimicrobial (Jeandet *et al*., 1995), and anti-HSV activities. Resveratrol effectively inhibits HSV *in vitro* by targeting immediate early events in HSV replication (Docherty *et al*., 1999). The precise mechanism of action of resveratrol is unknown, but it has been reported that resveratrol disrupted the cell cycle by inhibition cell cycle factors (Schang *et al*., 1998) or phosphorylation (Stewart *et al*., 1999). Because HSV requires cellular function associated with cell cycle progression in order to replicate (Hossain *et al*., 1997), this affect might contribute to the inhibitory effects of this compound on HSV replication. Topical application of resveratrol cream effectively suppressed the development of HSV wide type and ACV resistant strain induced cutaneous lesion without dermal toxicity *in vivo* (Docherty *et al*., 2004).

Glutathione (γ-glutamyl-cysteinyl-glycine or GSH) an SH group containing tripeptide, is the most prominent intracellular low-molecular weight thiol found in eukaryotic cells. GSH serves as an important intracellular water-soluble antioxidant and detoxifying agent (Droge *et al*., 1994). Many viral infections, including hepatitis C virus (Boya *et al*., 1999), human immunodeficiency virus (HIV) (Kalebic *et al*., 1991), parainfluenza-1, sendai virus, and HSV (Vogel *et al*., 2005) have been shown to associate with marked depletion of extra-cellular and intracellular GSH levels. Furthermore, it has been reported that exogenous GSH was able to

induce a strong concentration-dependent inhibition of HIV, sendai virus, and HSV replication *in vitro*. In clinical use, GSH has been suggested for use as a dietary supplement in HIV patients (Lyn, 2000). Although the mechanism of antiviral activity of GSH needs to be fully elucidated, all data indicated that GSH inhibit HSV replication by interfering with very late stages of HSV life cycle (Palamara *et al*., 1995).

11. α-Lipoic acid and derivatives

α-lipoic acid (LA) or thioctic acid (chemical name: 1,2-dithiolane-3-pentanoic acid) (Figure 8) is present in all plant and animal species primarily in mitochondria. In human being, this compound acts as coenzyme in the α-keto acid dehydrogenase complex that takes part in energy formation (Lyn, 2000). As a cofactor, LA linkes to lysine residues of the 2-oxo acid dehydrogenase multienzyme complexes by binding with acyl groups and transfering them from one part of the enzyme complex to another (Morris *et al*., 1995; Fujiwara *et al*., 1996). In this process LA is reduced to dihydrolipoic acid (DHLA). LA contains a five-membered ring which contains two sulfur atoms and a carboxylic acid group. It is insoluble in water, but soluble in organic solvents such as methanol and ethyl ether. LA is not only found in vegetable such as potato, spinach, and tomato, but also synthesized in mammalian cells (Glantzounis *et al*., 2006). Food derived from tissue with a high metabolic activity has a high LA content (Herbert and Guest, 1975), thus this indicated that most LA in diet originates from the multienzyme complex. Therefore, it has been suggested that LA from mammalian diet is absorbed in the form of lipoyllysine because proteolytic enzymes do not effectively cleave the peptide bond between LA and lysine. In addition LA can be obtained by *de novo* biosynthesis in mitochondria from fatty acids and cysteine which required enzyme lipoic acid synthase (Cakatay, 2006). Because it is a low molecular weight substance (molecular weight: 206), LA is readily absorbed from gut and passes through the blood-brain barrier (Packer *et al*., 1995). Exogenous LA supplement enters the cells and converts easily to its reduced from (DHLA) by cytosolic enzymes including GSH reductase, thioredoxin reductase, and also mitochondrial enzyme E3.

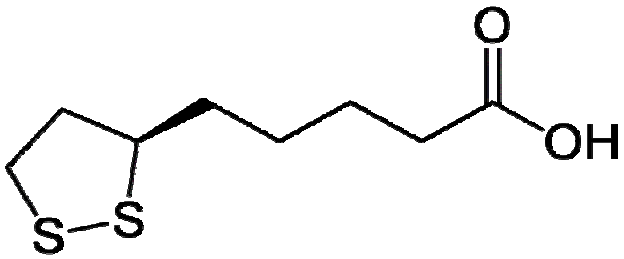


Figure 8. Structure of α-lipoic acid.

LA is a potent antioxidant in both fat-soluble and water-soluble environments. Furthermore, its antioxidant activity extends to both its oxidized and reduced forms. Both LA and DHLA can act as antioxidants against reactive oxygen species, including superoxide radicals, hydroxyl radicals, hypochlorus acid, peroxyl radicals, and singlet oxygen. However, the mechanism of the free radical scavenging process performed by these compounds still remains controversial, and there is also evidence indicating that LA and DHLA may exert prooxidant activity (the activity of antioxidant in a situation which it produces more oxidative stress) both *in vitro* (Maini *et al*., 2002) and *in vivo* (Cakatay *et al*., 2005). For example, DHLA can easily reduce Fe^{3+} to Fe^{2+} which promotes lipid peroxidation (Bast and Haenen, 1988). Another important property of LA is its ability to regenerate other antioxidants, such as vitamin C, glutathione, and vitamin E. LA, after reduction to DHLA, is able to contribute to the nonenzymatic regeneration of GSH and vitamin C (Rose and Bode, 1995) and regenerate vitamin E in a cascade of regenerating reactions (Bast and Haenen, 1988). These interactions of LA with other antioxidants result in membrane protection from lipid peroxidation at lipid bilayer. Many researches have also reported that LA can increase coenzyme Q10 levels (Kagan *et al*.,

1990). LA appears capable of chelating certain metals. It binds and forms stable complexes with copper, manganese, and zinc (Sigel *et al*., 1978). In animal models, it has been found to protect against arsenic poisoning (Grunert, 1960), and reduce cadmium-induced hepatotoxicity in both animal and *in vitro* studies (Muller and Menzel, 1990). In addition, LA chelated mercury from renal tissue *in vitro* (Keith *et al*., 1997). LA and DHLA can reduce the pathology of several diseases of which it is assumed that reactive oxygen species (ROS) or oxidative stress accompanies their etiology in animal models. For example, LA administration minimizes oxidant generation and macromolecular damage in skeletal muscle of aged rats. It also dose-dependently prevented the development of clinical signs in a rat model for multiple sclerosis and acute allergic encephalomyelitis resulted from ROS (Schreibelt *et al*., 2006). Both LA and DHLA could provide protection from the harmful effects of free radicals including ischemia and reperfusion injury in heart (Serbinova *et al*., 1992) and brain tissues (Prehn *et al*., 1992). DHLA was able to inhibit the peroxidation of linoleic acid and of the non-HDL fraction catalyzed by rabbit reticulocyte 15-lipoxygenase (Lapenna *et al*., 2003). LA enhanced glucose utilization in isolated rat diaphragm (Haugaard and Haugaard, 1970), heart (Singh and Bowman, 1970), and myotubes (Bashan *et al*., 1993); furthermore, it protected rat pancreatic islet cells from destruction by ROS (Heller *et al*., 1997).

LA administration has been used as therapeutic agent for numerous clinical indications. In diabetes, LA has potential applications for many aspects of diabetic pathology. These include the improvement of insulin sensitivity in type 2 diabetes (Jacob *et al*., 1999), slowing the development of cataractogenesis (Maitra *et al*., 1995; Ou *et al*., 1996), treatment of diabetic neuropathy (Reljanovic *et al*., 1999; Ametov *et al*., 2003), and prevention diabetic nephropathy (Melham *et al*., 2001). Exogenous LA administration has been reported to improve the biochemical parameters of glaucoma and visual function in open-angle glaucoma, and be beneficial in the treatment of mushroom poisoning (Packer *et al*., 1995). Moreover, LA may have a positive effect on patients with Alzheimer's disease and other types of memory dysfunction by decreasing and protecting oxidative damage in the central nervous system. LA administration, alone or together with vitamin E, is an effective treatment for radiation injury by lessening indices of oxidative damage and normalizing organ function (Ramakrishnan *et al*., 1992; Korkina *et al*., 1993).

Another therapeutic use of LA and DHLA is protection of HIV viral activation in AIDS patient. Oxidative stress has been reported to occur in several different viral infections, including parainfluenza, HSV, and HIV (Ciriolo *et al*., 1997). In HIV infection, oxidative stress induces both HIV replication and DNA damage, leading to immunosuppression (Marmos *et al*., 1997), and the role of antioxidants as antiviral in the treatment of HIV has been supported in multiple studies (Kalebic *et al*., 1991; Israel *et al*., 1992). Oral LA interrupted HIV replication by completely blocking the activation of nuclear factor kappa-B (Susuki *et al*., 1992; Packer and Susuki, 1993), a protein that functions as a nuclear factor and appears to play a role in inflammation, and inhibition of reverse transcriptase (Lyn, 2000). LA treatment resulted in increases in CD_4 T-helper cells and CD_4 T-helper cell/CD_8 T-suppressor cell ratio (Packer *et al*., 1995).

LA appears to be safe in dosages generally prescribed clinically. It was no acute toxicity and did not show any adverse effects in rat. LD_{50} was 400 to 500 mg/kg after an oral dosage in dogs (Parker *et al*., 1995). There was no evidence of mutagenic activity and genotoxic activity, but there have not been sufficient studies to guarantee the safety of LA supplement in pregnant women (Cremer *et al*., 2006). Although few adverse effects were noted, LA at 20 mg/kg given intraperitoneally to severely thiamine-deficient rats proved fatal. This effect was prevented by administration of thiamine prior to LA administration (Gal, 1965). In human, allergic skin reactions are among the few reported side effects of LA supplementation (Parker *et al*., 1995). It is estimated that humans can tolerate several grams of LA administered orally (Biewenga *et al*., 1997); however, therapeutic dosages of LA range from 600 to 1800 mg daily. In addition, it is critical to select the appropriate pharmacological doses of LA for use in oxygen-related disease according to balance between its prooxidant and antioxidant activity of thiol compound (Cakatay, 2006).

Lipoamide (6, 8-dithiooctanoic amide or thioctic acid amide) (Figure 9) is a functional form of lipoic acid which linked to lysine side chain of multienzyme complexes that catalyzed oxidative decarboxylation of keto acid. Similar to LA, lipoamide is a small substance with molecular weight of 205, is found in the inner membrane of mitochondria, and is a powerful antioxidant. It has been reported that lipoamide, which is the analogue that more closely resemble the cellular protein-bound lipoyllysine than free LA, could effectively prevent oxidant-mediated apoptosis of lysosomes (Persson *et al*., 2001), and inhibit nitric oxide production in macrophages better than free LA (Guo *et al*., 2001).

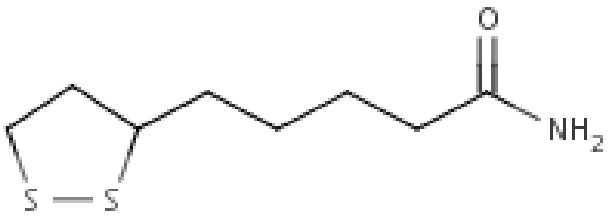


Figure 9. Structure of lipoamide.

CHAPTER III

MATERIALS AND METHODS

1. Cell culture

1.1 Vero cells

A continuous cell line of African green monkey (*Cercopithecus aethiops*) kidney, called Vero cell CCL-81, purchased from ATCC, was propagated in an MEM growth medium supplemented with 10% FBS and 1% antibiotic-antimycotic agents.

1.2 HeLa cells

HeLa cell is a cervix epithelium cell line derived from cervical cancer cells taken from a young African-American woman, Henrietta Lacks, in 1951. This cell culture was kindly provided by Professor Dr. Garnpimol C. Ritthidej, Department of Industrial Pharmacy, Faculty of Pharmaceutical Sciences, Chulalongkorn University and also by Professor Dr. Pornthep Tiensiwakul, Department of Clinical Microscopy, Faculty of Allied Health Sciences, Chulalongkorn University. HeLa cells were grown in DMEM medium supplemented with 10% FBS and 1% antibiotic-antimycotic agents.

1.3 NHDF CC-2511 cells

NHDF CC-2511, an adult normal human dermal fibroblast cell line, was kindly provided by Associate Professor Dr. Ubonthip Nimmannit, Department of Pharmacy, Faculty of Pharmaceutical Sciences, Chulalongkorn University. This cell was propagated in DMEM growth medium supplemented with 15% FBS and 1% antibiotic-antimycotic agents.

All of the cell lines were washed once with phosphate buffer saline solution (PBS) and 1 ml of trypsin-EDTA was added for 2-3 minutes. When the cells were detached, trypsin-EDTA was discarded, and 2 ml of growth medium was added. The cells were tapped lightly and mixed thoroughly by a pipette. The viable cells were counted by trypan blue staining. The cell

suspension was diluted in complete growth medium to an appropriate concentration and distributed into a new 25 cm^2 or 75 cm^2 tissue culture flask. Then, the cells were incubated at 37°C in a humidified-5% CO_2 incubator until the cell monolayer was confluent.

2. Herpes simplex virus

HSV-1, strain KOS, and HSV-2, strain Baylor 186, were kindly provided by the Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University. A virus stock was prepared from Vero cell monolayer infected with the virus at multiplicity of infection (M.O.I.) of about 0.1 plaque forming unit per cell (PFU/cell) (Lipipun *et al*., 2000). After one hour of viral adsorption at 37°C, unabsorbed viruses were washed with PBS and replaced with maintenance medium. The infected Vero cells were incubated in a humidified-5% CO_2 incubator at 37°C until the cell population showed CPE more than 80%. Then, the cells were disrupted by being repeatedly freeze-thawed three times at -70°C. The disrupted cell suspension was pelleted by centrifugation at 3,000 rpm for 10 minutes. The supernatant was distributed into small aliquots into microtubes and stored at -70°C as virus stock.

3. Lipoic acid

(±)-α-lipoic acid (Sigma, Lot. No. 055K1352) was prepared into a stock solution at a concentration of 1 mg/ml in distilled water with 2% DMSO. The lipoic acid stock solution was distributed into small aliquots and stored at -20°C until used.

4. Lipoamide

(±)-α-lipoamide (Sigma, Lot. No. 020K-0652) was prepared into a stock solution at a concentration of 800 µg/ml in distilled water with 2% DMSO. The lipoamide stock solution was distributed into small aliquots and stored at -20°C until used.

5. Acyclovir

Acyclovir (Sigma, Lot. No. 117F0756) was prepared as a stock solution at a concentration of 500 µg/ml in distilled water with 2% DMSO. The ACV stock solution was distributed into small aliquots and stored at -20°C until used.

6. Determination of viral titer

Titration of virus was performed by the following plaque assay. Serial ten-fold dilutions of virus stock in MEM were added onto Vero cell monolayer in a 96-well tissue culture plate (Nunc, Denmark) in amount of 25 µl/well in quadruplicate. The virus was allowed to be adsorbed for 1 hour in a humidified-5% CO_2 incubator at 37°C. Then, 75 µl of overlay medium (see appendix) were added to each well and the plate was incubated for 2 to 3 days in a humidified-5% CO_2 incubator at 37°C. After the incubation period, the medium was discarded and the infected cells were fixed with 12% formalin in normal saline solution and stained with 0.05% methylene blue solution for 1 hour. The number of plaques was counted under an inverted microscope and the virus titer was calculated as plaque forming unit per milliliter (PFU/ml).

7. Cytotoxicity test

In this study, cytotoxicity test was performed to determine the effects of lipoic acid and lipoamide on cell proliferation and viability by using trypan blue exclusion method and MTT reduction assay.

7.1 Trypan blue exclusion method

Cytotoxicity was determined by staining uninfected cells with trypan blue. Trypan blue exclusion test was modified from Hayashi *et al*., (1996) and Liu *et al*., (2004). Various concentrations of lipoic acid and lipoamide in growth medium were added to Vero cell monolayer quadruplicately (100 µl/well) in 24 well-tissue culture plates. The cells were incubated in a humidified-5% CO_2 incubator at 37°C for 5 days and then were trypsinized. The number of cells in collected suspensions was determined in a hemocytometer after staining the cells with an equal

volume of trypan blue solution. Trypan blue is excluded by live cells, but stains dead cells blue. Mean value of the cells number was calculated. Results were expressed as the ratio between the number of viable cells in treated cultures and viable cells in the untreated control cultures. The curve between percent of cell viability and drug concentration was plotted. The 50% cytotoxicity concentration (CC_{50}) was defined as the concentration which caused 50% reduction in the number of viable cells.

7.2 MTT (Thiazolyl blue tetrazolium bromide) reduction assay

For cytotoxicity assay, MTT reduction method modified from Watchmans *et al*., (2003) and Liu *et al*., (2004) was used. Vero cells were seeded in 96-well tissue culture plate at a cell concentration of $2x10^5$ cells per well in 100 µl of growth medium. After incubation of the cells in a humidified-5% CO_2 incubator at 37°C for 24 hours, various concentrations of lipoic acid and lipoamide were added to the plate, and the incubation was continued for 72 hours. Cell viability was examined based on the ability of the cells to cleave the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]; Sigma, Lot. No. 085K5304, U.S.A.) by the mitochondrial enzyme succinate dehydrogenase to give a blue formazan crystal. MTT was dissolved in PBS at a concentration of 5 mg/ml and sterilized by filtration to remove a small amount of insoluble residue present in some batches of MTT. At the time indicated above, the MTT solution was added to each well (25µl/well) and the plates were incubated again in a humidified-5% CO_2 incubator at 37°C for 4 hours. Then, 100 µl of acid-isopropanol were added to all wells and mixed thoroughly on shaking plate at 150 rpm to dissolve the dark blue crystals. After 20 minutes at room temperature to ensure that all crystals were dissolved, the plates were read on Bio Rad microplate reader (Model 3550), using a test wavelength of 595 nm and a reference wavelength of 655 nm. Optical density should be directly correlated with cell quantity. The 50% cytotoxic concentration (CC_{50}) was defined as the concentration which caused 50% reduction in optical density compared with that of untreated control cultures.

CC_{50} values for both trypan blue exclusion method and MTT reduction method were calculated by regression analysis, $Y = aX + b$ or $Y = a\log X + b$, whereas Y is the amount of cells (% of control); X is the concentration of the test substances; and a and b are constant values.

8. Anti-HSV-1 and HSV-2 activity of lipoic acid and lipoamide

The antiherpes simplex virus activities were investigated by using inactivation and plaque reduction assays.

8.1 Inactivation assay (Hayashi *et al*., 1996)

To determine neutralizing activity of lipoic acid, lipoamide, or ACV (as positive control) to herpes simplex virus, inactivation assay was performed by the following method. Twenty five microliters of virus were incubated with 25 µl of sample dilutions in a humidified-5% CO_2 incubator at 37°C for 1 hour. This mixture was then added onto Vero cell monolayer in quadruplicated wells and incubated in humidified-5% CO_2 incubator at 37°C for another 1 hour. The overlay medium containing different concentration of test substances in appropriate concentration was added to the cultures. The cultures were incubated in a humidified-5% CO_2 incubator at 37°C for 48 hours. The number of plaques was counted and confirmed by staining with methylene blue as previously mentioned.

8.2 Plaque reduction assay

To determine the activity of lipoic acid, lipoamide, or ACV (as control) against intracellular viral replication, plaque reduction or post-treatment assay was performed by the following method modified form Hayashi *et al*., (1996) and Gong *et al*., (2004). Twenty-five microliters of virus were added onto Vero cell monolayer in 96-well tissue culture plates. After 1 hour of viral adsorption in a humidified-5% CO_2 incubator at 37°C, 50 µl of various concentrations of test substances were added. The cultures were incubated in a humidified-5% CO_2 incubator at 37°C for 1 more hour and then the overlay medium containing appropriate dilution of test substances was added to each well. After further incubation in a humidified-5% CO_2 incubator at 37°C for 48 hours, the infected cells were fixed with formalin, stained with methylene blue solution as previously mentioned, and the number of plaques was counted.

The antiviral activity of lipoic acid and lipoamide from both assays was tested and compared with that of ACV in term of 50% inhibitory concentration (IC_{50}). The IC_{50} was determined by

equation Y = aX + b, or Y = alogX + b, whereas Y is the number of plaques (% of control); X is the concentration of the test substances; and a and b are constant values.

8.3 MTT reduction assay (Serkedjieva and Ivancheva, 1999; Liu *et al*., 2004)

Determine of the activity of lipoic acid, lipoamide, or ACV (as positive control) in the inhibition of cytopathic effect (CPE), MTT reduction assay was performed by the following method. Semi-confluent Vero cells in 96 well-tissue culture plates were infected with virus. The cells were incubated in a humidified-5% CO_2 incubator at 37°C for 1 hour and then various concentrations of test substances were added. After further 48 hours incubation in, the cell cultures were examined for evidence of cytopathic effect by using MTT reduction assay as previously mentioned in cytotoxicity test.

IC_{50} value was calculated based on the regression equation of the percentage ratio of viral inhibition of test sample groups to virus control group determined as follows;

$$\% \text{ of HSV inhibition} = \frac{[\,(OD_t)_v - (OD_c)_v\,]}{[\,(OD_c)_{mock} - (OD_c)_v\,]} \times 100$$

Where $(OD_t)_v$ is the optical density (OD) of the cells, treated with herpes simplex virus and lipoic acid, lipoamide, or ACV; $(OD_c)_v$ is the OD of the cells, treated with virus alone as a virus control; and $(OD_c)_{mock}$ is the OD of the mock infected cells only as a cell control.

9. Virucidal assay

The direct effect of lipoic acid on HSV-1 and HSV-2 infectivity was evaluated by the method described by Minami *et al*., (2003) and Yang *et al*., (2005). Briefly, different concentrations of lipoic acid were mixed thoroughly with 10^5-10^6 PFU/ml of virus. The mixture was then incubated in a humidified-5% CO_2 incubator at 37°C for 1 hour. After incubation, serial 10-fold dilutions of mixture were added on confluent Vero cell monolayer. The cells were incubated in a humidified-5% CO_2 incubator at 37°C for another 1 hour for viral adsorption and then unadsorbed viruses were removed. The residual virus infectivity was determined by plaque assay as previously mentioned.

10. Preliminary tests for the mechanism of action of lipoic acid

Preliminary tests for anti-HSV-1 and HSV-2 activities of lipoic acid were performed by the following methods.

10.1 Post-binding assay (Piret *et al*., 2002)

The effect of lipoic acid on HSV attached to Vero cells was examined. Confluent Vero cells seeded in 24-well tissue culture plates were maintained at 4°C for few minutes. The cells were first incubated with 50-100 PFU of viruses at 4°C for 2-3 hours to allow stable attachment of the viruses without fusion with cell membrane. After incubation period, unbound viruses were removed, and cells were then washed with cold PBS (as control), low pH citrate buffer (as positive control), or PBS containing increasing concentrations of lipoic acid at 4°C for 1 minute. Cells were washed once with PBS and overlaid with overlay medium. After being incubated in humidified-5% CO_2 atmosphere at 37°C for 2 days, cells were fixed, washed, and stained as described previously. The amount of viruses which had attached and penetrated into cells after temperature shift to 37°C was evaluated according to the number of PFU.

10.2 Penetration assay (Piret *et al*., 2002)

The effects of lipoic acid on the rate of penetration of HSV into Vero cells were investigated. Confluent Vero cells seeded in 24-well tissue culture plates were incubated with viruses 50-100 PFU of viruses at 4°C for 2 hours of viral attachment. After removal of unbound viruses, the incubation temperature was shifted to 37°C to allow penetration of bounded virus into cells. At selected times after the temperature shift (0, 15, 30, and 60 minutes), the cells were treated with PBS (as control) or PBS containing increasing concentrations of lipoic acid for 1 minute. Then, the cells were overlaid with overlay medium and incubated in a humidified-5% CO_2 atmosphere at 37°C for 2 days. The cells were then fixed, washed, and stained. The amount of viruses which had penetrated into the cells was evaluated following the determination of numbers of PFU.

Another penetration assays were performed using modified method of De Logu *et al*. (2000). After incubation with viruses at 4°C for 2 hours of viral attachment and removal of unbounded viruses, the temperature was then abruptly increased to 37°C to get maximun penetration of bounded viruses. Penetration proceeded for various time periods (0, 15, 30, and 60 minutes) in the absence or presence of various concentrations of lipoic acid. The Vero cell monolayers were then treated with citrate buffer pH 4.0 for 1 minute to neutralize any remaining attached virus and after several washes with PBS the cells were added with overlay medium and incubated in a humidified-5% CO_2 atmosphere at 37°C for 2 days. The cells were then fixed, washed, and stained as above and the surviving virus was quantitated versus time of lipoic acid exposure.

10.3 Virus growth inhibition assay

The antiviral activity of lipoic acid was evaluated in time-of-addition experiments to determine the effect of this substance on the growth of HSV-1 and HSV-2 by virus yield inhibition assay. This assay was modified from Kurokawa *et al*., (1995) and Yang *et al*., (2005). Briefly, confluent Vero cells monolayer in 25 cm^3 tissue culture flasks were infected with HSV-1 or HSV-2 at 0.01 PFU/cell and allowed the viruses to be absorbed for 1 hour in a humidified-5% CO_2 incubator at 37°C. Unadsorbed viruses were discarded and the infected cells were washed three times with MEM. The growth medium (as control) or MEM containing lipoic acid at concentration greater than the IC_{50} value was then added onto the cultures at various time points either 1 hour before viral infection or after viral infection periods (1 and 3 hour post infection). After carefully washing by MEM in every step, the infected cells were then maintained in MEM alone or MEM containing lipoic acid for 3 and/or 9 hours at 37°C in a humidified-5% CO_2 incubator. Then the media were discarded, each infected cell monolayer was washed three times with MEM and supplied with fresh growth medium. The cultures were freeze-thawed three times, then the suspension was pooled and centrifuged at 3000 rpm for 15 minutes to release cell-associated viruses into supernatants. The supernatants were kept at -70°C until use in viral assay. Virus titers in the supernatants were determined by the plaque assay which serial 10-fold dilutions of each supernatant were added onto confluent Vero cell monolayer in 96-well tissue culture plates, as previously mentioned.

Total infectivity was quantified by PFU and expressed as percent of virus yield as compared to control.

10.4 Prophylactic activity assay (Hayashi *et al*., 1996)

To determine an antiviral activity of lipoic acid against viral adsorption, viral penetration, or other events before virus entering into the cells, prophylactic activity assay or pre-treatment assay was performed by the following method. Fifty microliters of lipoic acid were added onto Vero cell monolayer in triplicated wells and incubated in a humidified-5% CO_2 incubator at 37°C for 1 hour. After each lipoic acid dilution was discarded, the cells were infected with 25 µl of HSV-1 or HSV-2 and incubated in a humidified-5% CO_2 incubator at 37°C for another 1 hour. During this period, the growth medium (as control) or MEM containing lipoic acid was added to the cells together with the virus. The overlay medium with or without lipoic acid was added to the cultures after unadsorbed virus was discarded. The cell cultures were incubated in a humidified-5% CO_2 incubator at 37°C for 48 hours. The infected cells were fixed with formalin and stained with methylene blue solution as previously mentioned. The number of plaques was counted as PFU.

11. Anti-HSV-1 and anti-HSV-2 activities of lipoic acid in various cell types

The anti-HSV-1 and anti-HSV-2 activities of test substance in cultured cells originated from different anatomical sources were evaluated by using MTT reduction method as previously described in section 8.3. Normal human dermal fibroblast (NHDF CC-2511) cell culture and cervix epithelium (HeLa) cell culture were used in this study instead of Vero cell culture. IC_{50} value of each cell type was calculated and compared with the result obtained from Vero cells in the same method.

CHAPTER IV

RESULTS

1. Cytotoxicity of lipoic acid, lipoamide, and ACV on Vero cells

In this study, cellular toxicity of lipoic acid, lipoamide, and ACV was determined as CC_{50}. All CC_{50} values in this study were calculated by regression analysis (see appendix). The maximal concentration of DMSO that did not affect the cytotoxicity to Vero cells was 2% (Lipipun *et al*., 1999). In addition, the result of cytotoxicity of DMSO on Vero cells determined with MTT reduction assay was shown in Figure 10. The CC_{50} value of DMSO was 4.712%. Therefore, the final concentration of DMSO in each drug stock solution was not more than 2%, and the concentration would be further diluted by growth medium before used in this study. Finally, the maximum concentration of DMSO used in all antiviral tests was 0.25% which did not show any effect to cell growth.

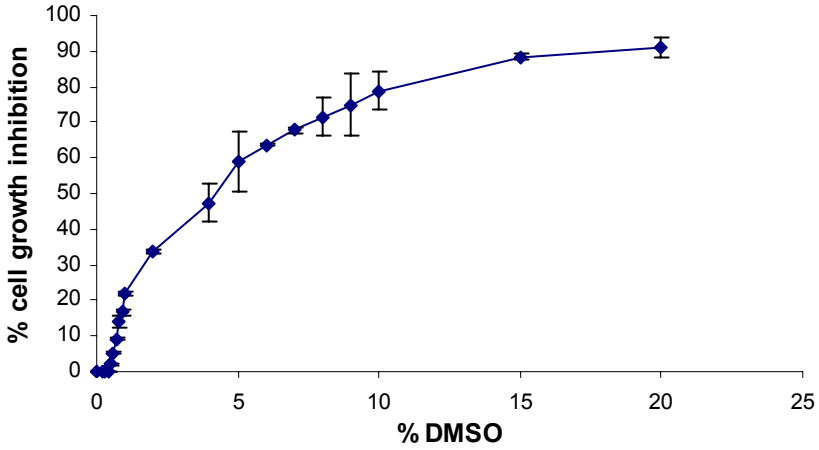


Figure 10. Cytotoxicity of DMSO on Vero cells determined by MTT reduction assay. Each bar of point represents the percentage of cell growth inhibition compared with the controls. Data were reported as the mean ± S.D. from three independent experiments.

Cytotoxicity of lipoic acid, lipoamide, and ACV was examined by trypan blue exclusion test and MTT reduction test. Trypan blue exclusion method, in which dead cells were stained blue while living cells remained clear, showed the total viable cell numbers in treated Vero cells as compared with untreated control Vero cells. The results were shown in Figure 11. Lipoic acid,

lipoamide, and ACV exhibited CC_{50} values of 242.69, 239.03, and 1,650.59 µg/ml, respectively.

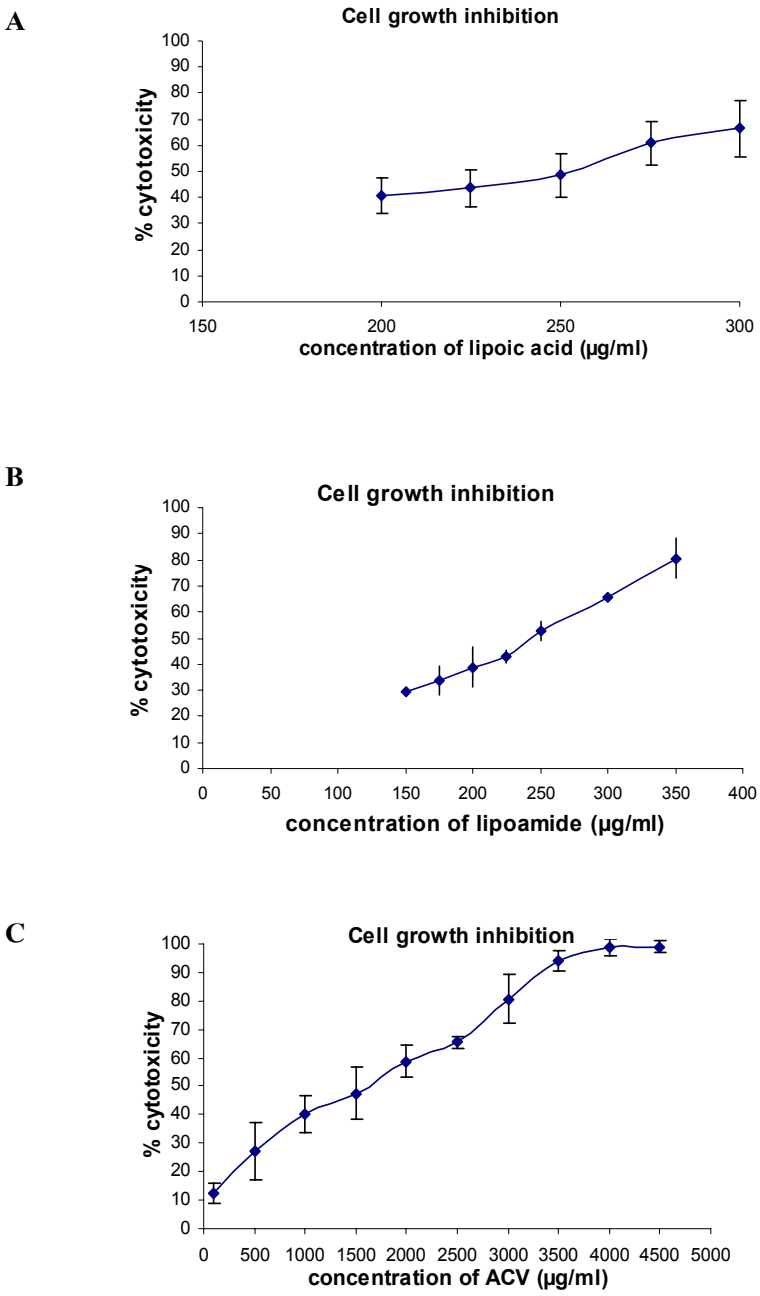


Figure 11. Cytotoxicity of lipoic acid (A), lipoamide (B) and ACV (C) determined by trypan blue exclusion method. Vero cells were incubated with various concentrations of test substances for 5 days, then the cells were counted after staining with trypan blue. The data were reported as mean ± S.D. from at least three experiments.

In the second method, MTT reduction assay, which measured cellular enzymes activity that correlated with cell viability, showed the similar results. Cytotoxicity of lipoic acid, lipoamide, and ACV on Vero cells performed with this method was shown in Figure 12. The CC_{50} values of lipoic acid, lipoamide, and ACV were 292.19, 271.10, and 1,602.81 µg/ml, respectively.

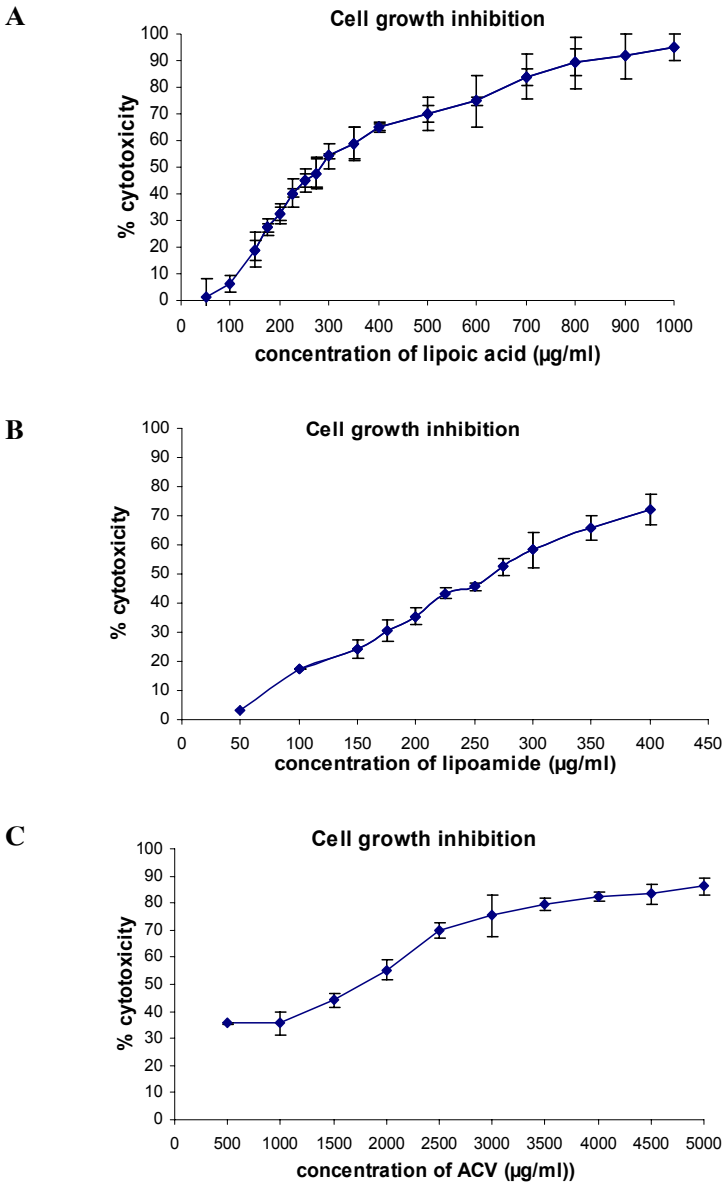


Figure 12. Cytotoxicity of lipoic acid (A), lipoamide (B), and ACV (C) determined by MTT reduction assay. Vero cells were incubated with various concentrations of test compounds in media for 72 hours. The media were replaced with MTT solution. After incubation at 37°C for 4 hours, acid-alcohol was added and optical density was read at 595 nm. The data were reported as mean ± S.D. from at least four independent experiments

The CC_{50} of all test substances on Vero cells performed by both methods were summarized in Table 1. The CC_{50} values of lipoic acid, lipoamide, and ACV determined by the trypan blue exclusion test were similar to those obtained by the MTT test.

Table 1 Cytotoxicity of lipoic acid, lipoamide, and ACV on Vero cells.

	CC_{50} (μg/ml)	
Antiherpetic substances	Trypan blue exclusion method	MTT reduction assay
Lipoic acid	242.69	292.19
Lipoamide	239.03	271.10
Acyclovir	1650.59	1602.81

50% cytotoxic concentration (CC_{50}) values represent concentrations of the compounds required to reduce the viability of the cells by 50%. The reported values were derived from at least three independent assays of both methods.

2. Anti-HSV-1 and HSV-2 activity of lipoic acid and lipoamide

Antiviral activities of lipoic acid, lipoamide, and ACV (as a positive control) were determined as 50% inhibitory concentration (IC_{50}) (see appendix).

2.1 Effect on viral inactivation

Inactivation assay was used to investigate the inhibitory activity of lipoic acid and lipoamide to HSV-1 and HSV-2 on Vero cells, and ACV was used as a positive control. The anti-HSV-1 and anti-HSV-2 activities of each substance were shown in Figure 13. The IC_{50} values of lipoic acid, lipoamide, and ACV against HSV-1 were 96.34, 41.28, and 0.26 μg/ml, respectively. For anti-HSV-2 activity, the IC_{50} values were 111.75, 50.15, and 0.32 μg/ml for lipoic acid, lipoamide, and ACV, respectively. The IC_{50} values of each substance required for inactivation of HSV-2 was higher than the value required for inactivation of HSV-1. When the concentration of both lipoic acid and lipoamide was increased to 200 μg/ml, the inhibitory activities of these substances were almost 100%.

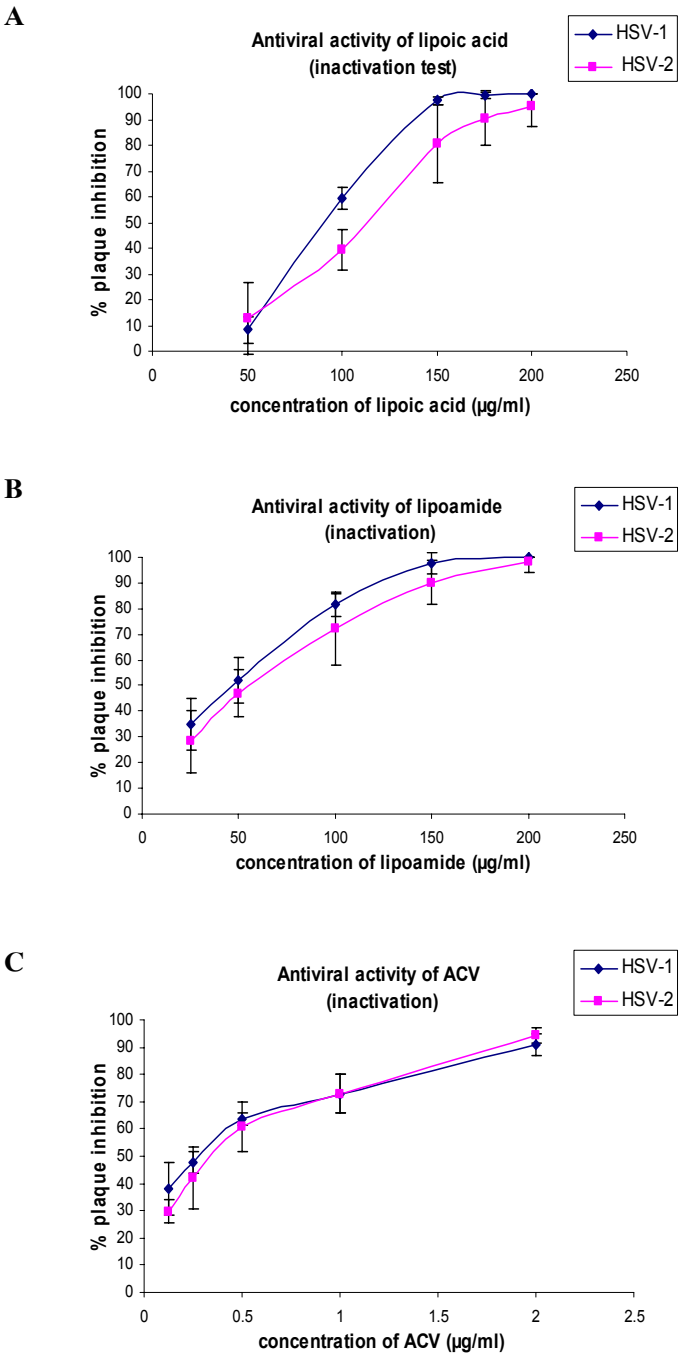


Figure 13. Anti-HSV activity of lipoic acid (A), lipoamide (B), and ACV (C) as determined by inactivation assay. Percentages of plaque inhibition were determined using infected cells as control. The data were reported as mean ± S.D. derived from at least four independent experiments. Percent of plaque inhibition between treated and untreated cells after infection was significantly different ($P<0.05$).

2.2 Effect on plaque formation

The antiviral activity of lipoic acid, lipoamide, and ACV (as positive control) obtained from plaque reduction assay or post-treatment was shown in Figure 14. The IC_{50} values of lipoic acid against HSV-1 and HSV-2 were 115.49 and 130.13 µg/ml, respectively. The IC_{50} of lipoamide were 60.47 µg/ml for HSV-1 inhibition, and 83.49 µg/ml for inhibition of HSV-2. ACV inhibited HSV-1 and HSV-2 plaque formation on Vero cells with IC_{50} of 0.34 and 0.60 µg/ml, respectively. The results showed that at the same concentration the efficiency of plaque inhibition of all test substances to HSV-1 infected cells is higher than to HSV-2 infected cells. When the concentration is as high as 200 µg/ml, lipoic acid and lipoamide almost completely inhibited the HSV-1 and HSV-2 plaque formation.

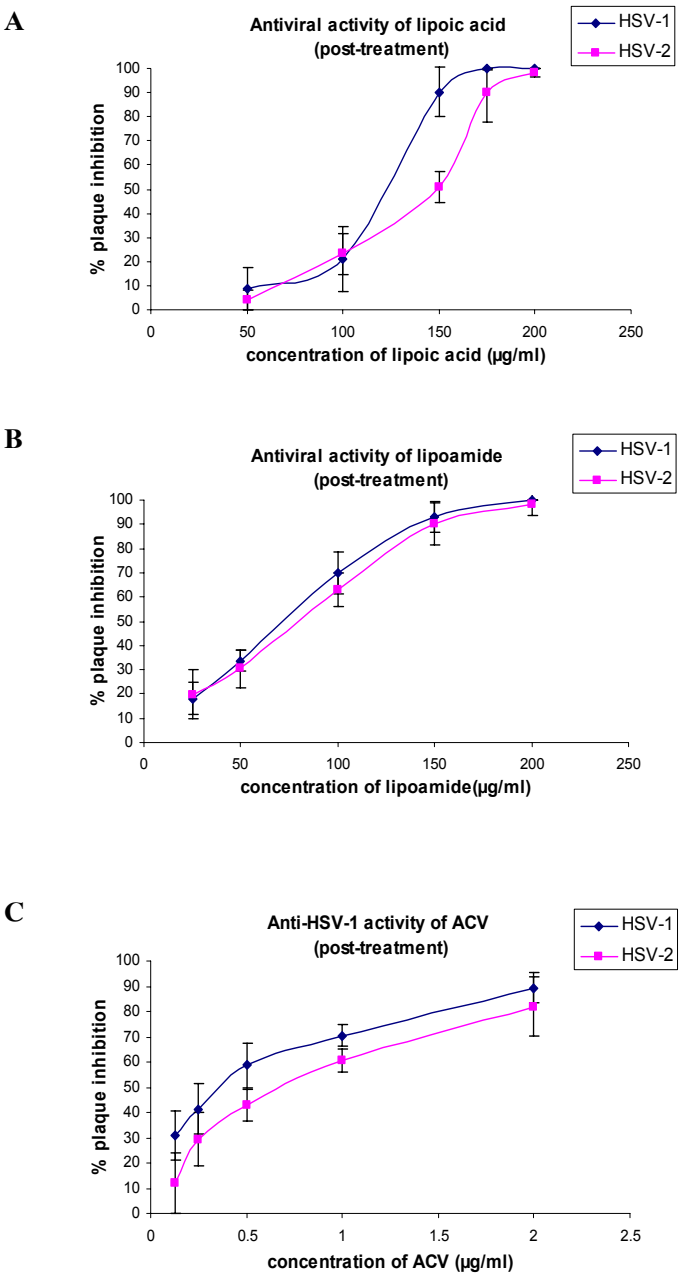


Figure 14. Anti-HSV activity of lipoic acid (A), lipoamide (B), and ACV (C) as determined by plaque reduction assay. The data were reported as mean ± S.D. from at least four independent experiments. Percent of plaque inhibition between treated and untreated cells after infection was significantly different ($P<0.05$).

2.3 Inhibition of HSV cytopathic effect (CPE)

The inhibition of the cytopathic effect was measured by MTT reduction assay. The results of viral inhibition were shown in Figure 15. In the inhibiton HSV-1 effect on Vero cells, the IC_{50} values of lipoic acid, lipoamide, and ACV were 126.85, 104.90, and 0.46 µg/ml, respectively. For HSV-2 effect on Vero cells, the IC_{50} values of lipoic acid, lipoamide, and ACV were 135.06, 107.48, and 0.59 µg/ml, respectively. The inhibition of HSV-2 effect by all three substances was less than that obtained with HSV-1.

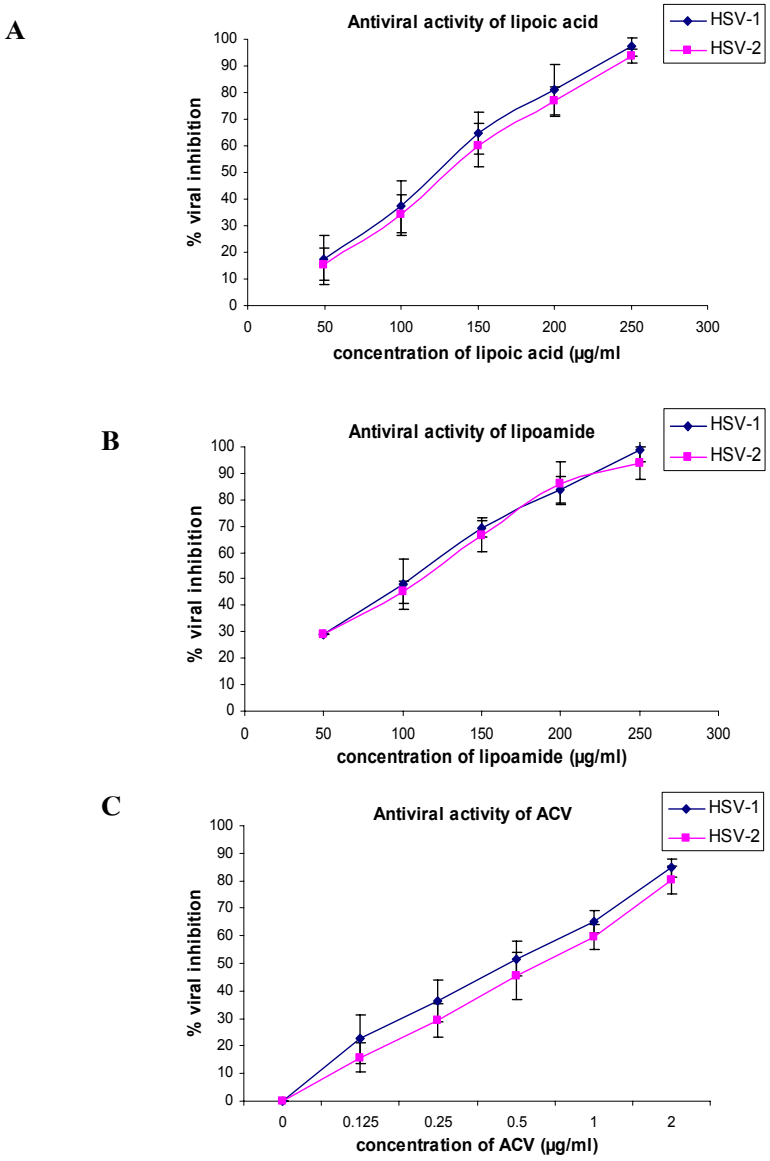


Figure 15. Anti-HSV activity of lipoic acid (A), lipoamide (B), and ACV (C) determined by MTT reduction assay. The data were reported as mean ± S.D. from at least four independent experiments. Percent of virus inhibition between treated and untreated cells after infection was significantly different ($P<0.05$).

The IC_{50} and SI (selective index) values against both HSV-1 and HSV-2 of lipoic acid, lipoamide, and ACV obtained form inactivation, plaque reduction, and MTT reduction assays on Vero cells were summarized in Table 2 and Table 3. In all cases, SI were more than 2 according to the CC_{50} of each drug higher than its IC_{50}. There were observed differences in the degree of viral inhibition depending on the assay used. The IC_{50} obtained from inactivation test was less than the IC_{50} obtained from the other two methods.

Table 2 Antiviral activity of lipoic acid, lipoamide, and ACV on HSV-1.

		HSV-1					
Antiherpetic substances	Host cells	IC_{50} (μg/ml) [a]	SI[d]	IC_{50} (μg/ml) [b]	SI[d]	IC_{50} (μg/ml) [c]	SI[d]
Lipoic acid	Vero	96.34	3.04	115.49	2.54	126.85	2.31
Lipoamide	Vero	41.28	6.57	60.47	4.48	105.17	2.58
Acyclovir	Vero	0.26	>100	0.34	>100	0.46	>100

a: obtained by inactivation assay

b: obtained by plaque reduction assay

c: obtained by MTT reduction assay

d: selective index of each drug calculated from CC_{50} / IC_{50} in each treatment and the results were determined from at least four independent experiments.

Table 3 Antiviral activity of lipoic acid, lipoamide, and ACV on HSV-2.

Antiherpetic substances	Host cells	HSV-2					
		IC_{50} (μg/ml) [a]	SI[d]	IC_{50} (μg/ml) [b]	SI[d]	IC_{50} (μg/ml) [c]	SI.[d]
Lipoic acid	Vero	111.75	2.62	130.13	2.25	135.06	2.17
Lipoamide	Vero	50.15	5.41	83.49	3.25	108.42	2.50
Acyclovir	Vero	0.32	>100	0.60	>100	0.59	>100

a: obtained by inactivation assay;

b: obtained by plaque reduction assay;

c: obtained by MTT reduction assay;

d: selective index of each drug calculated from CC_{50} / IC_{50} in each treatment and the results were determined from at least four independent experiments.

3. Effect of lipoic acid on HSV infectivity

The virucidal activity of lipoic acid against HSV-1 and HSV-2 was displayed in Table 4. The HSV-1 and HSV-2 titers after cell were treated with increasing concentration of lipoic acid slightly decreased compared with untreated control cells in condition of 37°C, 1 hour incubation. However, this difference on viral titer was not significant ($P>0.05$) even when treated with higher concentration of lipoic acid (500 μg/ml). It could be concluded that lipoic acid did not have direct effect on HSV-1 and HSV-2 infectivity.

Table 4 Effect of lipoic acid on HSV infectivity[a].

concentration of lipoic acid (μg/mL)	HSV-1 titer (x 10^5 PFU/mL)	HSV-2 titer (x 10^6 PFU/mL)
control	5.76 ± 0.68	7.65 ± 0.64
50	5.43 ± 0.57	5.73 ± 0.88
100	4.50 ± 0.60	7.60 ± 0.60
150	4.68 ± 0.54	5.65 ± 0.62
200	4.36 ± 0.61	7.64 ± 0.99
250	4.39 ± 0.29	6.31 ± 0.49
300	4.03 ± 0.22	6.74 ± 0.67
350	5.57 ± 0.34	5.66 ± 0.47
400	4.99 ± 0.35	5.77 ± 0.56
450	4.48 ± 0.30	6.36 ± 0.29
500	3.84 ± 0.14	7.76 ± 0.60

[a]Virucidal assay was performed by incubation of virus and lipoic acid at 37°C for 1 hour. The residual virus infectivity was titrated by plaque assay and reported as mean ± S.D. of three independent experiments.

4. Mode of anti-HSV activity of lipoic acid

Preliminary tests for possible mechanisms of action of lipoic acid in anti-HSV-1 and HSV-2 infection were performed using post-binding assay, penetration assay, virus growth inhibition assay, and pre-treatment assay.

4.1 Effect on viral adsorption

The effect of lipoic acid on HSV-1 and HSV-2 attachment to Vero cells was analyzed by post-binding assay. The results were shown in Figure 16. Citrate buffer pH 4 was used as a positive control and no plaque was detected. No statistically significant difference ($P>0.05$) in the amount of adsorbed HSV-1 and HSV-2 was observed between cells treated with lipoic acid and untreated control cells. The data indicated that HSV-1 and HSV-2 attachment was not affected by lipoic acid.

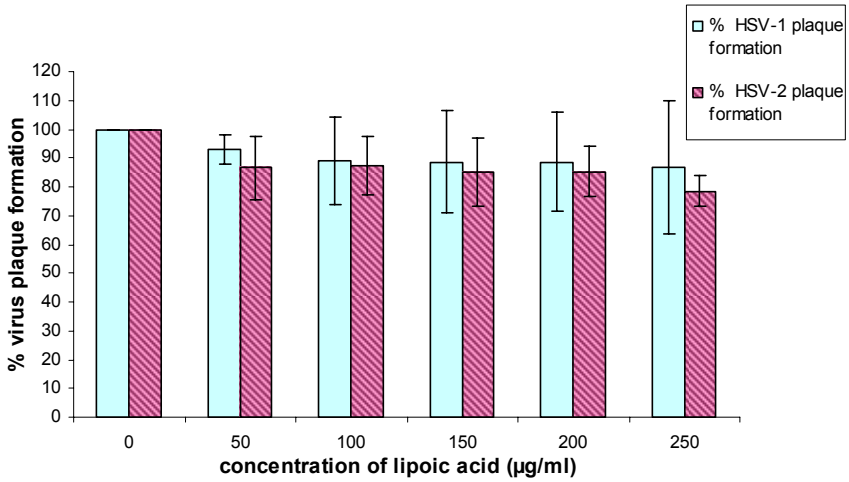
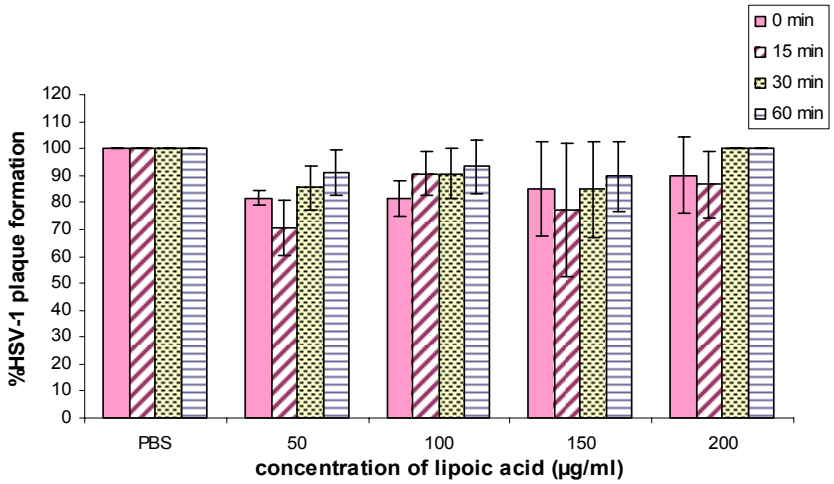


Figure 16. Effect of lipoic acid on HSV-1 and HSV-2 adsorption as determined by post-binding assay. HSV-1 and HSV-2 were attached to Vero cells and then incubated with lipoic acid at 4°C. The number of plaque was reported as mean ± S.D. from four independent experiments. Low pH citrate buffer was used as a positive control and no plaque was detected.

4.2 Effect on viral penetration

In penetration assay modified from Piret *et al*. (2002), no viral inhibition was observed when Vero cells were infected with HSV-1 or HSV-2 at 4°C. The penetration of viruses was prevented by increasing the temperature to 37°C and treating the infected cells with lipoic acid for different period of time. The result was shown in Figure 17. The number of both HSV-1 and HSV-2 plaque formation between treated and untreated infected control cells was not significantly different ($P>0.05$). Another method modified from De Logu *et al.* (2000) that increase the incubation time of lipoic acid with the infected cells after temperature shift showed the similar results (Figure 18). The quantities of HSV-1 and HSV-2 in treated cells at any time point were not significantly different ($P>0.05$) from those observed from untreated infected control cells. Altogether, these results suggested that lipoic acid did not interfere with HSV penetration into Vero cells.

A



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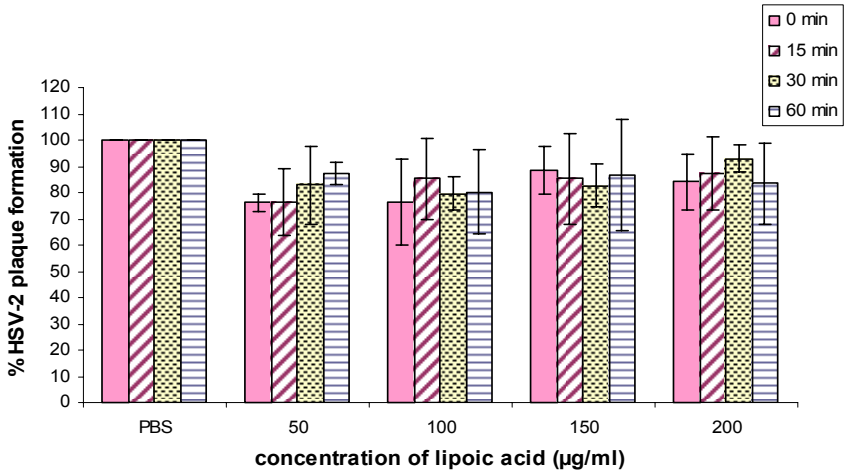

Figure 17. Effect of lipoic acid on HSV-1 (A) and HSV-2 (B) penetration determined by the assay derived from Piret *et al.* (2002). Penetration assay was performed on Vero cells. After viral adsorption at 4°C, the temperature was shifted to 37°C for 0, 15, 30, and 60 minutes to allow penetration of bounded virus into treated and untreated control cells. The number of plaques was reported as mean ± S.D. from four independent experiments.

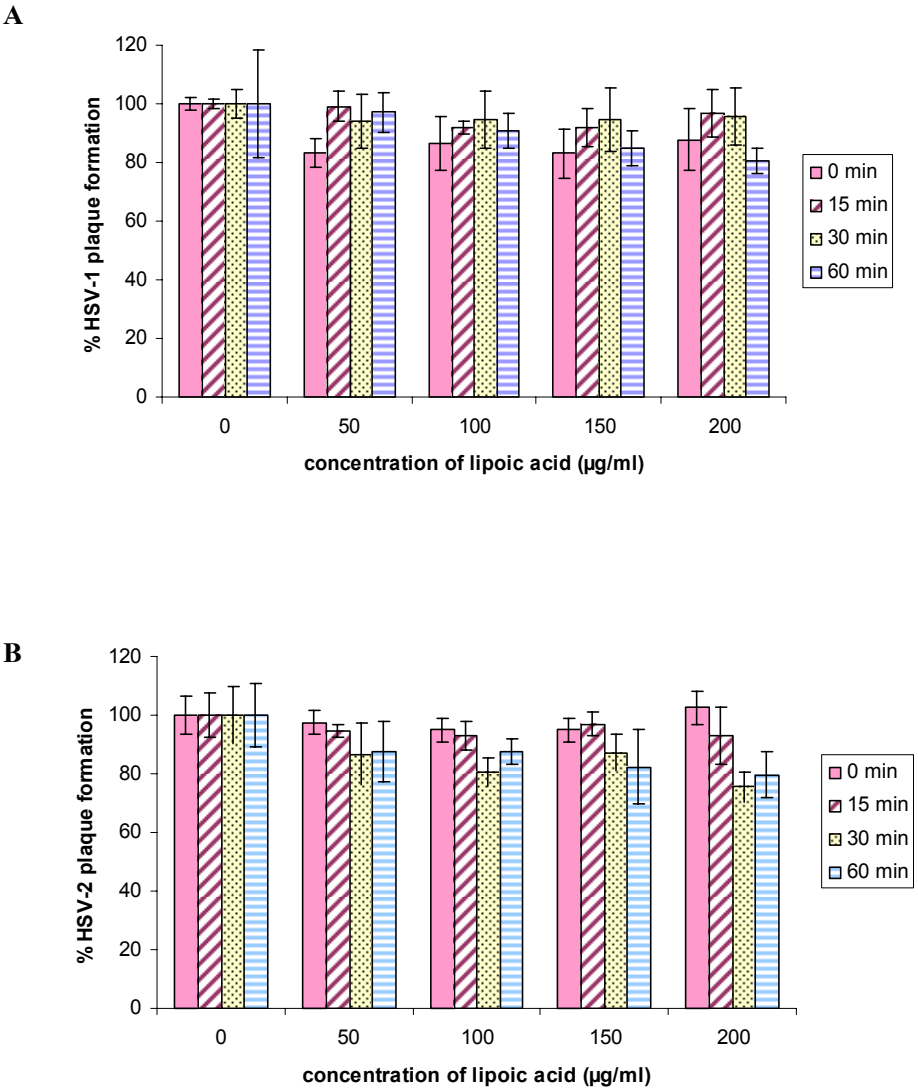

Figure 18. Effect of lipoic acid on HSV-1 (A) and HSV-2 (B) penetration determined by the assay derived from De Logu *et al.* (2000). Penetration assay was performed on Vero cells. After viral adsorption at 4°C, the temperature was shifted to 37°C for 0, 15, 30, and 60 minutes to allow penetration of bounded virus into treated and untreated control cells. The number of plaques was reported as mean ± S.D. from four independent experiments.

4.3 Effect on virus growth

To study the inhibitory effect of lipoic acid on the stages of HSV-1 and HSV-2 infection, a time of addition experiment was performed. Lipoic acid was added to Vero cells at various time points including before and after virus infection. The results shown that lipoic acid treatment both before and after virus inoculation potentially suppressed HSV-1 and HSV-2 infection as displayed in Figure 18. When lipoic acid was added to the cells as 1 hour pre-treatment at 37°C and then washed out before viral infection, the reduction of virus yield was more than 80% for both HSV-1 and HSV-2 as compared with untreated control cells. Moreover, the addition of lipoic acid to pretreated cells at 1 and 3 hour post infection exhibited HSV inhibitory activity higher than 98% for HSV-1 and 94% for HSV-2. The extent of inhibition of HSV-1 and HSV-2 production was observed when the treatment with lipoic acid was started either 1 or 3 hour after virus inoculation and removed at 3 and 9 hour post infection, respectively. Indeed, the inhibition of HSV-1 and HSV-2 replication was greater than when lipoic acid was given to the cell at 1 hour after virus infection, and the compound was maintained in the culture until the end of the experiment. However, all three conditions in which lipoic acid was added to the cells after virus challenge resulted in reduction of HSV-1 and HSV-2 infectivity by more than 50% as compared to untreated infected cells. The result indicated that lipoic acid affected all steps of HSV replication. In addition, it probably affected the susceptibility of the target cells to infection by HSV.

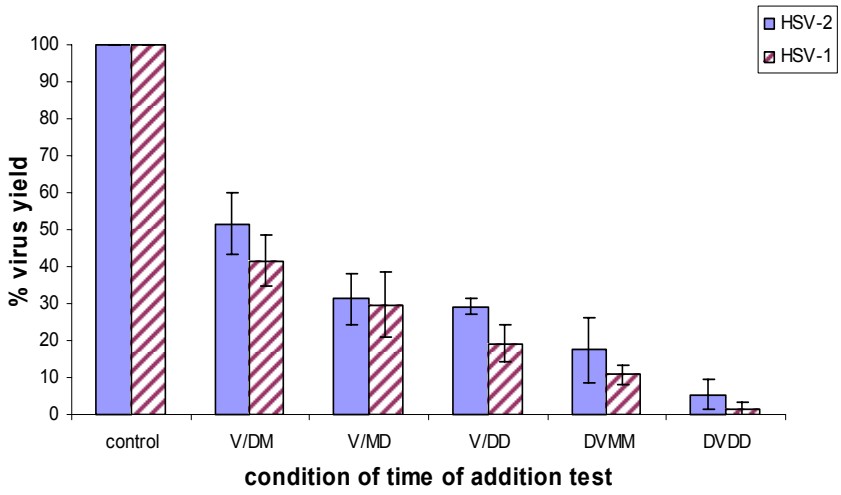

Figure 18. Inhibition of HSV growth by lipoic acid as determined by time of addition assay. Control: untreated infected control cells; V/DM: lipoic acid added at 1 hour after viral adsorption and then withdrawn and replaced by growth medium at 3 hour post infection; V/MD: lipoic acid added at 3 hour after viral adsorption and then withdrawn and replaced by growth medium at 9 hour post infection (the end of experiments)**;** V/DD: lipoic acid added at 1 hour after viral adsorption and then withdrawn and replaced by growth medium at 9 hour post infection (the end of experiments)**;** DVMM: lipoic acid given to uninfected cells for 1 hour and removed before viral infection; DVDD: lipoic acid given to uninfected cells for 1 hour and removed before viral infection and then added again at 1 hour after viral adsorption and then withdrawn and replaced by growth medium at 9 hour post infection (the end of experiments). The time of addition experiments were performed on Vero cells as described in chapter III. Each bar represented the percentage of virus yield (PFU/ml) compared with controls. Data were reported as the mean $\pm$ S.D. from three independent experiments. The difference in virus yield of all cases was statistically significant ($P<0.05$).

4.4 Prophylactic activity of lipoic acid

Prophylactic activity assay or pretreatment assay was used to determine the activity of lipoic acid in preventing HSV-1 and HSV-2 infection on Vero cells. Pretreatment with lipoic acid before virus adsorption showed dose-dependent inhibitory activity on plaque formation as shown in Figure 19. HSV-1 and HSV-2 plaque formation was almost completely inhibited by the added lipoic acid at all time periods including before, during, and after virus infection. For each condition, only when uninfected cells were pretreated with lipoic acid both HSV-1 and HSV-2 plaque formation was reduced by more than 80%. Antiviral activity was higher when lipoic acid was added to the cells again either during or after virus adsorption. The addition of lipoic acid to pretreated cells during 1 hour of virus adsorption produced less antiviral activity in the inhibition of plaque formation than the addition after viral infection.

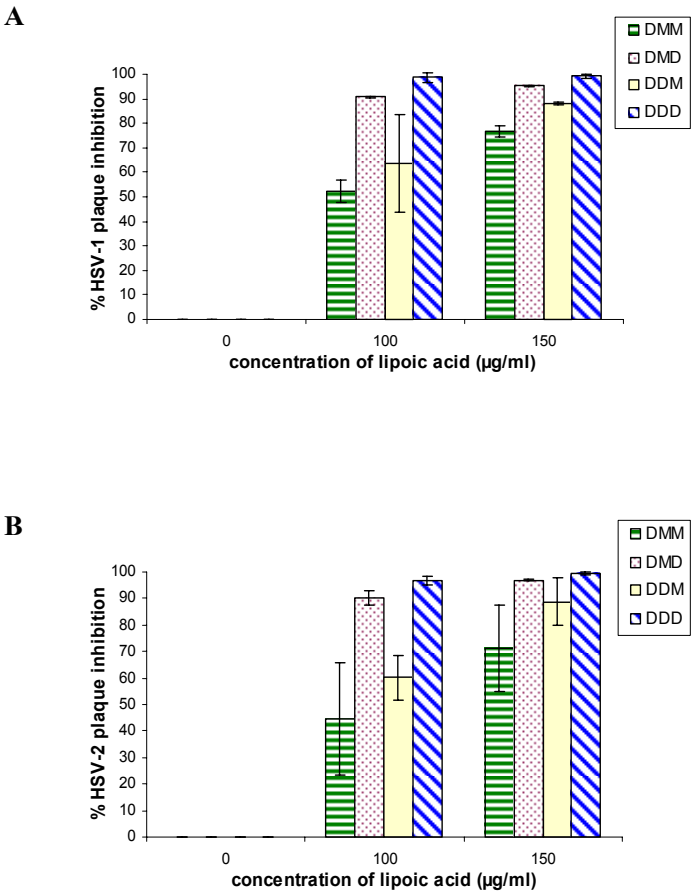

Figure 19. Anti-HSV activity of lipoic acid against HSV-1 (A) and HSV-2 (B) determined by pre-treatment assay. DMM: lipoic acid given to uninfected cells for 1 hour and removed before viral infection; DMD: lipoic acid given to uninfected cells for 1 hour and removed before viral infection and the test substance added again at 1 hour post infection and maintain during 48 hours of incubation; DDM: lipoic acid given to uninfected cells for 1 hour and maintained during 1 hour of viral adsorption; DDD: lipoic acid given to uninfected cells for 1 hour and maintained until the end of experiments (48 hour). Each bar represented the percent inhibition of plaque formation with respect to the untreated infected control cells. Data were reported as the mean $\pm$ S.D. from four separate experiments. The difference in inhibition of plaque formation of all cases was statistically significant ($P<0.05$).

5. Anti-HSV-1 and anti-HSV-2 activities of lipoic acid in various cell types

Effect of lipoic acid against HSV-1 and HSV-2 infection on cervix epithelium HeLa cell culture and Normal human dermal fibroblast NHDF CC-2511 cell culture were determined.

5.1 Effect of lipoic acid on viability of cells

Cytotoxicity of lipoic acid was examined by means of MTT reduction assay. The results were presented in Figure 20. Similar to Vero cells, lipoic acid had an effect on the proliferation of HeLa cells, and the CC_{50} value of this substance in this cell line was 259.05µg/ml as shown in Table 5. On the contrary, lipoic acid affected NHDF CC-2511 cell proliferation less than that on Vero cells and HeLa cells. The CC_{50} of lipoic acid in NHDF CC-2511 was more than 500 µg/ml. The data suggested that variation in the toxicity of lipoic acid depended on cell types used in this study.

Table 5 Cytotoxicity of lipoic acid on different cell types

Host cells	CC_{50} (µg/ml)[a]
Vero	292.19
HeLa	259.10
NHDF CC-2511	569.14

[a]50% cytotoxicity concentration (CC_{50}) values represent concentration of lipoic acid that shows 50% cytotoxicity in MTT reduction test. The data were determined from at least four independent experiments.

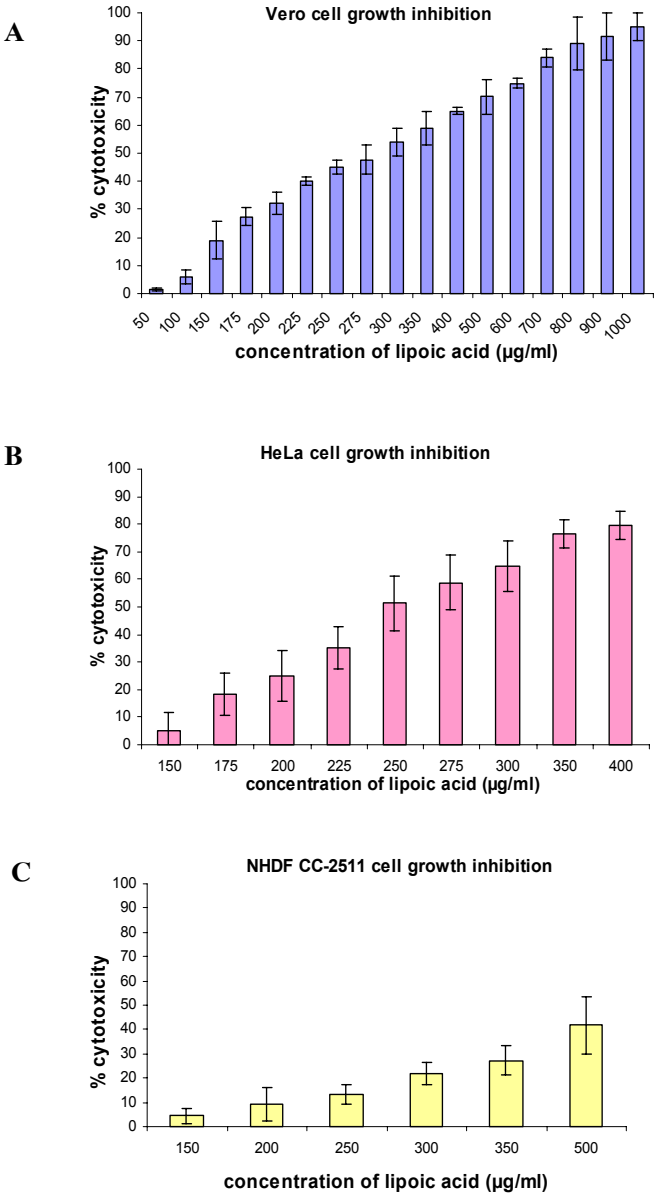

Figure 20. Cytotoxicity of lipoic acid on Vero cells (A), HeLa cells (B), and NHDF CC-2511 (C) as determined by MTT reduction method. Cells were incubated with various concentrations of test compounds in media for 72 hours. Cell viability was determined by MTT assay. The data were reported as mean ± S.D. from at least four independent experiments and the difference in % cytotoxicity between treated cells and control in each cell type was statistically significant ($P<0.05$).

5.2 Inhibition of HSV-1 and HSV-2 infection by lipoic acid

MTT reduction assay was used to determine antiviral activity of lipoic acid in HeLa cells and NHDF CC-2511 cells. The IC_{50} values of lipoic acid against both HSV-1 and HSV-2 on Vero cells, HeLa cells, and NHDF CC-2511 cells were summarized (Table 6). In HSV-1 inhibition, the IC_{50} values were 109.61 and 58.20 μg/ml for HeLa cells and NHDF CC-2511 cells, respectively. The IC_{50} values of lipoic acid against HSV-2 infection in HeLa cells and NHDF CC-2511 cells were 125.90 and 92.26 μg/ml, respectively. These IC_{50} values were lower than the IC_{50} obtained from Vero cells. These IC_{50} and SI values were shown in Figure 21.

Table 6 Anti-HSV activity of lipoic acid on different cell types.

	HSV-1		HSV-2	
Host cells	IC_{50} (μg/ml)[a]	SI	IC_{50} (μg/ml)	SI[b]
Vero	126.85	2.30	135.06	2.16
HeLa	109.61	2.36	125.90	2.06
NHDF CC-2511	58.20	9.78	92.26	5.80

[a]50% inhibitory concentration (IC_{50}) was the concentration of lipoic acid required to inhibit 50% of virus-induced CPE.

[b]SI: selective index of each drug calculated from CC_{50} / IC_{50}. of at least four independent experiments.

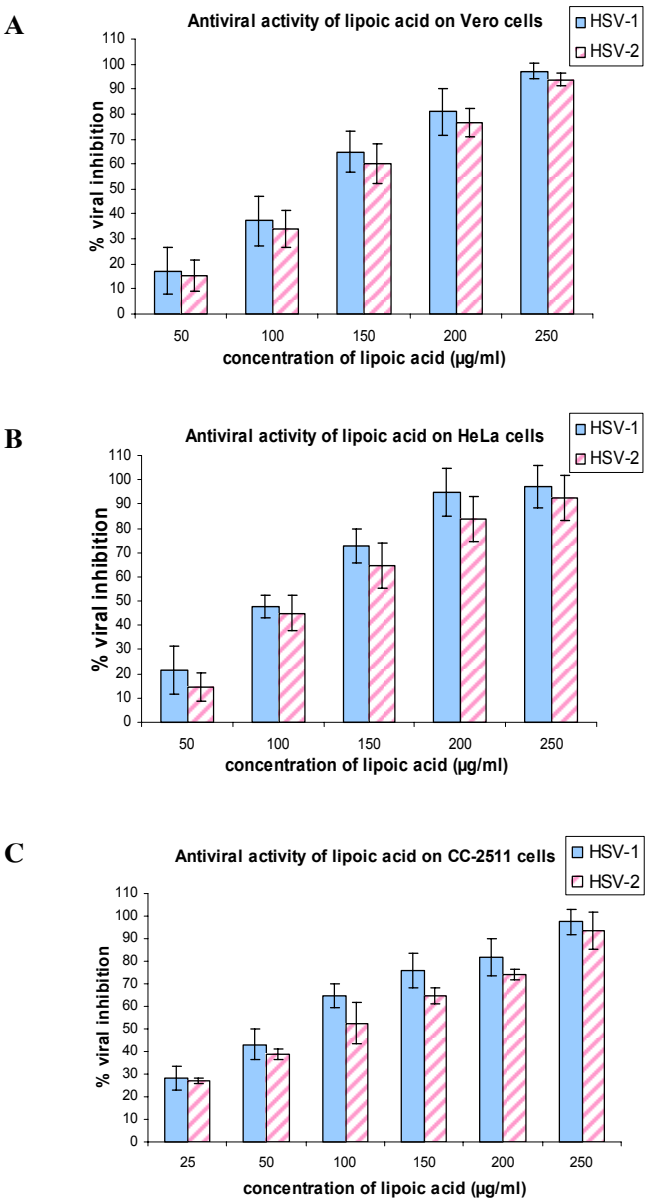

Figure 21. Anti-HSV activity of lipoic acid on different cell types. A: Vero cells; B: HeLa cells; C: NHDF CC-2511. Virus inhibition was examined by MTT reduction assay. The data were reported as means ± S.D. from at least four independent experiments. The viral inhibition on each cell type was significantly different ($P<0.05$).

CHAPTER V

DISCUSSION AND CONCLUSION

Current chemotherapeutic antiviral drugs have been characterized as having in many cases limited clinical efficacy, suboptimal pharmacokinetics, and toxic side effects (Patrick and Potts, 1998). In response to this, it is necessary to identify and develop new antiviral agents with different targets from the standard therapy. Lipoic acid, a potent antioxidant, has been used as a supplement therapy in several diseases associated with oxidative stress such as diabetic neuropathy (Ametov *et al*., 2003) and HIV infection (Packer and Susuki, 1993). In addition, many antioxidants including glutathione, ascorbate, and tocopherol have shown anti-HSV activity (Starasoler and Habers, 1987; Palamara *et al*., 1995; Betanzos-Cabrera *et al*., 1994). Thus, lipoic acid and its derivative, lipoamide, were investigated for their antiviral activity against HSV-1 and HSV-2 infection *in vitro*.

An effective antiviral agent should be non-toxic to the cell culture at the antiviral concentration. Therefore, cytotoxicity of lipoic acid, lipoamide, and ACV was primarily investigated. Dimethylsulfoxide (DMSO) was used as solvent and diluent to all test substances in this study. The maximum final concentration of DMSO in test solutions used in all antiviral assays was 0.25%, which did not show any toxicity to cell cultures. Hence, no effect of DMSO was interfering in the antiviral activity assays. The concentrations of all test substances in determining antiviral activity were lower than their CC_{50} and the incubated cultures did not show the cytopathic effect.

Acyclovir triphosphate, an active form of acyclovir, has significantly high affinity to HSV DNA polymerase than to intracellular α-DNA polymerase. For this reason, ACV exhibited high CC_{50} (1,602.81 and 1,650.59 μg/ml) in this study and has been proven to be safe in cell cultures, animal models, and humans (Kurokawa *et al*., 1995; Liu *et al*., 2004) with high selectivity. As a current standard treatment for HSV-1 and HSV-2 infection, ACV was used as a positive control in this study.

Cytotoxicity of lipoic acid and lipoamide on Vero cells showed that both lipoic acid and lipoamide affected Vero cell proliferation in some way and resulted in reduction of cell viability. Trypan blue exclusion method and MTT reduction method exhibited similar CC_{50} for both substances; however, several recent studies proved that lipoic acid did not show any serious side effect in either animals or humans (Cremer *et al*., 2006). In addition, high doses of lipoic acid approved in Germany for diabetic polyneuropathy in humans showed only few allergic skin reactions (Packer *et al*., 1995). Moreover, the human doses of lipoic acid ranged from 200-1800 mg/day were shown to be safe and without side effects (Wollin and Jones, 2003).

Lipoic acid and lipoamide showed antiviral activities against HSV-1 and HSV-2 on Vero cells. Like ACV, lipoic acid and lipoamide inhibited HSV-1 plaque formation with more efficiency than HSV-2 when treated at the same concentration. In inactivation assay, the IC_{50} values of all three antiherpetic agents were lower than the IC_{50} values obtained from plaque reduction assay. The difference in IC_{50} value between the two methods might be related to the prolonged contact time of the test substances with the cells, since Vero cell monolayers were incubated with lipoic acid, lipoamide, or ACV only after viral adsorption period in plaque reduction assay, while, in inactivation assay, the cell cultures were incubated with the test substances both during and after viral adsorption period as the result of mixing the virus and each test substance together before infection to the cells. The IC_{50} values for HSV-1 and HSV-2 of lipoic acid, lipoamide, and ACV measured with the MTT reduction assay were higher than the values measured with both inactivation and plaque reduction assays, due to the fact that the amount of viruses used in the MTT reduction test (70-700 PFU) is higher than in the inactivation and plaque reduction assays (30 PFU). It was therefore concluded that lipoamide was more effective against both HSV-1 and HSV-2 than lipoic acid, according to the lower IC_{50} and similar CC_{50} values. In addition, lipoic acid also exhibited anti-HSV-1 and anti-HSV-2 activities in other cell lines when measured with MTT reduction assay, which was chosen in the case of some cell lines that did not grow to confluent monolayer as required in plaque reduction assay. The results showed that cellular toxicity and antiviral activity of lipoic acid varied in different cell types. In monkey kidney Vero cells, human cervix epithelium HeLa cells, and normal human dermal fibroblast NHDF CC-2511 cells, lipoic acid exhibited different effect on these cell proliferation according to its CC_{50} values. In addition, the IC_{50} and SI values of lipoic acid against HSV-1 and HSV-2 infection in Vero cells were higher than those in HeLa and NHDF CC-2511 cells. Except

for lipoic acid activity, these differences may be due to variation in proliferation rate and/or susceptibility of each cell type to viral infection.

In an attempt to determine how lipoic acid and lipoamide inhibited HSV infection in Vero cells, lipoic acid was selected as the representative of both substances. The compound is changed to lipoamide inside the cells. Overall data from virucidal, post-binding, and penetration assays suggested that lipoic acid did not directly inactivated virus by itself, or inhibited virus adsorption to the cells, or significantly inhibited virus penetration into the cells. This concluded that lipoic acid did not have directly interact with HSV-1 and HSV-2 virions and bind to viral envelopes, glycoproteins, or other viral structures required for virus entering into the cells. In virucidal assay, lipoic acid was used at higher concentration than its CC_{50} values without producing cellular toxicity because mixture of virus and lipoic acid was diluted with media before adding to the cells. Possible mechanisms of action of lipoic acid were confirmed by virus growth inhibition and pre-treatment testing. Lipoic acid at concentration higher than its IC_{50} (150 μg/ml) was used in virus growth inhibition assay (Kurokawa *et al*., 1995). When pre-incubation of the Vero cells with lipoic acid and then washing the substance out before virus challenge, significant substantial inhibition of HSV-1 and HSV-2 production was observed in virus growth inhibitory assay and prophylactic activity assay. Moreover, the addition of lipoic acid after viral adsorption period without pretreatment of the cells still had potential to reduce HSV-1 and HSV-2 infection. The inhibition of viral production when lipoic acid was added after viral challenge was less than when lipoic acid was added before viral challenge, corresponding with the antiviral activity obtained from inactivation and plaque reduction assay. In addition, addition of lipoic acid at 3 hour post infection inhibited virus production to a higher extent than the addition at 1 hour post infection. All together, these results demonstrated that lipoic acid acted mainly by reducing the susceptibility of Vero cells to HSV-1 and HSV-2 infection and inhibiting HSV replication after the viruses had entered into the cells

The mechanisms by which lipoic acid inhibited HSV-1 and HSV-2 infection is unclear. Nevertheless, the fact that change in intracellular redox status occurs both *in vivo* and *in vitro* in different kinds of viral infections such as influenza, parainfluenza, and HSV-1 (Hennet *et al*., 1992; Ciriolo *et al*., 1997) suggests that the impairment of redox status inside host cells is essential for the initiation and maintenance of virus replication. Therefore, lipoic acid, a potent thiol antioxidant, might prevent this impairment which is a primary event produced by viral

infection. Moreover, the decrease in both extracellular and intracellular levels of total glutathione, a cellular thiol antioxidant, which occurred at early time points after HSV-1 infection of Vero cells has been reported as a major reason in the impairment of intracellular redox status (Palamara *et al*., 1995; Vogel *et al*., 2005). Previous studies demonstrated that supplementation with exogenous glutathione could inhibit HSV-1 replication and replenish intracellular glutathione level (Palamara *et al*., 1995; Nucci *et al*., 2000). Exogenous lipoic acid administration to cellular medium has been proven to cause a rapid increase of intracellular glutathione in a human T-lymphocyte Jurkat cell line (Han *et al*., 1995). This was believed to result from facilitation of cysteine delivery, a limiting factor in glutathione synthesis, to the cell by lipoic acid (Sen, 1998). Thus, it was possible that lipoic acid might act as anti-HSV-1 and HSV-2 agent by elevating cellular glutathione level.

However, the effect of lipoic acid on HSV DNA synthesis, transcription, translation, and post-translation level in infected cells should not be excluded. With regard to the effect of lipoic acid upon HSV protein synthesis and HSV mutation, the amount of viral glycoprotein should be evaluated. Therefore, further researches are required to better characterize the exact mechanisms of action of lipoic acid.

In summary, these results suggested that lipoic acid and lipoamide act as antiviral agent against HSV-1 and HSV-2 infection by reducing the susceptibility of target cells to virus infection and inhibiting virus production inside the cells after infection. Lipoic acid did not inhibit virus by directly inactivating virus, or inhibiting virus attachment to cell receptors, or inhibiting virus penetration into the target cells. Cytotoxicity and anti-HSV-1 and anti-HSV-2 activities of lipoic acid varied among different cell types. In all cell lines used, lipoic acid showed antiviral activity at concentrations below its CC_{50}. Therefore, based on these favorable profiles, lipoic acid may have a potential use in preventive therapy and treatment therapy for HSV-1 and HSV-2 related disease.

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APPENDICES

APPENDIX A

Chemical agents and instruments

1. Chemical agents

Absolute methanol GR (E. Merck, Darmstadt, Germany)

Citric acid (E. Merck, Darmstadt, Germany)

Chloroform (Sigma, MO, U.S.A.)

Crystal violet (Fluka, Switzerland)

Dimethyl sulfoxide (DMSO) (Sigma, MO, U.S.A.)

Formaldehyde 38% w/v AR (Carlo Erba, Milano, Italy)

Hydrochloric acid (E. Merck, Darmstadt, Germany)

Methylcellulose (Sigma, MO, U.S.A.)

Potassium chloride (KCl) (May & Bayer, England)

Potassium dihydrogen phosphate (KH_2PO_4) (E. Merck, Lot. NO. 547A17873, Darmstadt, Germany)

Dipotassium hydrogen phosphate (K_2HPO_4) (E. Merck, Darmstadt, Germany)

Propan-2-ol (Fisher Scientific, U.K.)

Sodium bicarbonate ($NaHCO_3$) (E. Merck, Darmstadt, Germany)

Sodium chloride (NaCl) (E. Merck, Lot. No. K27736104021, Darmstadt, Germany)

Disodium hydrogen phosphate (Na_2HPO_4) (May & Bayer, Lot. No. 50028, England)

Thiazolyl blue tetrazolium bromide (MTT) (Sigma, Lot No. 085K5304, U.S.A.)

Tragacanth (Pharmaceutical chemicals, Denmark)

Trypsin-EDTA (1X) (Gibco, Canada)

2. Instruments

Analytical balance (Satorius, Germany)

Automatic pipet (Drummond Scientific, U.S.A.)

Automatic pipet P2-20/P20-200/P100-1000 (Socorex, Switzerland)

Centrifuge (Sigma, Germany)

Hemocytometer (Boeco, Germany)

Laminar air flow (Holten, U.S.A.)

Microplate reader model 3550 (Biorad, U.S.A.)

Multichannel automatic pipette, 8 channel (Socorex, Switzerland)

Orbital Shaker SO3 (Stuart scientific, Redhill, UK)

pH meter (Beckman, U.S.A.)

Refrigerator 4˚C (Sharp, Thailand)

Refrigerator 20˚C (Ariston, U.S.A.)

Refrigerator -80˚C (Forma Scientific, U.S.A.)

Ultrasonicator

Vortex mixer (Scientific, NY, U.S.A.)

Water bath (Thelco, U.S.A.)

3. Laboratory supplies

Cryotubes (Nunc, Denmark)

Glasswares (Pyrex, U.S.A.)

Microcentrifuge tubes

Millipore filters 0.2 μm, Acrocap filter unit (Gelman Laboratory, U.S.A.)

Pipette tips (Nunc, Denmark)

Syringe filters, Acrodisc (Pall Corporation, U.S.A.)

Tissue culture flasks (Nunc, Denmark)

Tissue culture plates (Nunc, Denmark)

APPENDIX B

Medium and Reagents

1. Growth medium

Vero cells CCL-81 were grown and maintained in Eagle's minimum essential medium (MEM). Minimum essential medium with Eagle's salts and Glutamine (Gibco, U.S.A.) without sodium bicarbonate powder 9.5 g was dissolved in deionized distilled water. 2.2g/L of sodium bicarbonate (Sigma, U.S.A.) were added to the solution. This medium solution was mixed well and adjusted pH to 7.2-7.4 with 6N HCl. Then, the solution was adjusted volume to 1,000 ml by deionized distilled water. This solution was sterilized by filtration with 0.22 μm millipore filter membrane. Before use, this MEM solution was supplemented with 10% fetal bovine serum (FBS, Gibco, U.S.A.) and 1% antibiotic-antimycotic agents (Gibco, U.S.A) which contained 10,000 units/ml of penicillin G sodium, 10,000 μg/ml of streptomycin sulfate, and 25 μg/ml of amphotericin B as fungizone in 0.85% saline.

For HeLa cells, Dulbecco's modified Eagle medium (DMEM) with high glucose, L-glutamine, pyridoxine hydrochloride and 170 mg/L sodium pyruvate (Gibco, U.S.A.), and without sodium bicarbonate was used instead of MEM in Vero cell. These DMEM powder was dissolved in deionized distilled water and 3.7 g/L of sodium bicarbonate (Sigma, U.S.A.) was added into the solution. After mixed well, the solution was adjusted pH to 7.2-7.4 with 6N HCl and adjusted volume to 1,000 ml. This solution was sterilized by filtration with 0.22 μm millipore filter membrane. 10% fetal bovine serum and 1% antibiotic-antimycotic agents were added to the medium solution before use as mentioned in MEM.

For NHDF CC-2511, growth and maintenance medium were prepared by DMEM as the same as previously mentioned in HeLa cell except for increasing fetal bovine serum supplementation to 15%.

2. 0.05% Methylene blue in distilled water

Methylene blue	5 g
Distilled water	100 ml

3. 12% Formaline in normal saline solution

38% Formaldehyde	320 ml
0.85% Normal saline (NaCl) solution	680 ml

4. Phosphate Buffer Saline Solution (PBS)

NaCl	8.00 g
KCl	0.20 g
KH_2PO_4	0.20 g
Na_2HPO_4	1.15 g
Deionized distilled water to	1,000 ml

This solution is sterilized by autoclaving for 15 minutes at 121˚C, 15 lb/in^2

5. Low-pH Citrated buffer,pH 3.0

Sodium citrate	40 mM
Potassium chloride	10 mM
Sodium chloride	135 mM
Deionized distilled water to	1,000 ml

pH of this solution is adjusted to 3.0 and then the buffer is sterilized by autoclaving for 15 minutes at 121˚C, 15 lb/in^2

6. Plaque overlay medium

Solution A

2x MEM with 20% FBS and 2% antibiotic-antimycotic agent

This solution was sterilized by filtration through 0.22 μm Millipore filter membrane

Solution B

Methyl Cellulose 4000 (Sigma, U.S.A.)	1.6 g
Deionized distilled water	100 ml

This solution was sterilized by autoclaving for 15 minutes at 121˚C, 15 lb/in^2

The solution A and B were mixed well at a ratio of 1:1 before use

7. MTT solution

Thiazolyl blue tetrazolium bromide (MTT)	500 mg
PBS	100 ml

This solution was sterilized by filtration to remove a small amount of insoluble residues present in some batches of MTT

8. Acid-isopropanol

HCl	1.23 ml
Isopropanol	1,000 ml

Calculation of median cytotoxic concentration (CC_{50}) and effective concentration (EC_{50}) by regression formula.

CC_{50} is the concentration of lipoic acid, lipoamide, or acyclovir which exhibit 50% cytotoxicity. CC_{50} is calculated from the regression formula Y = aX + b or Y = alogX + b, when Y is optical density or number of viable cells, X is the concentration of lipoic acid, lipoamide, or ACV in µg/ml, a is slope, and b is an intercept or the distance between X axis and the point where the regression line come across Y axis

EC_{50} is the concentration of lipoic acid, lipoamide, or ACV which can inhibit 50% of plaque formation or optical density reduction. EC_{50} is calculated from the regression formula Y = aX + b or Y = alogX + b, when Y is number of plaque or optical density, X is the concentration of lipoic acid, lipoamide, or ACV in µg/ml, a is slope, and b is an intercept or the distance between X axis and the point where the regression line come across Y axis

Determination of correlation coefficient

Correlation coefficient between concentration and percent inhibition or incubation time and percent inhibition was determined using SPSS program as following example

Concentration (µg/ml)	%inhibition	Correlation coefficient
50	10.58	R = 0.95
100	27.05	
200	48.77	
400	71.01	
800	92.92	

BIOGRAPHY

Miss Pattaraporn Sasivimolphan was born on August 17, 1981 in Bangkok, Thailand. She received her Bachelor Degree of Science in Pharmacy (1st Class Honours with gold medal) in 2003 from the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand.