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.