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.