CHAPTER III

MATERIALS AND METHODS

1. Cell cultures

LLC-PK_1 cells (porcine renal epithelial cell line) [ATTC no. CL-101] and LLC-MDR_1 cells (*MDR1* gene-transfected epithelial cells) were kindly provided by Dr. A. H. Schinkel (Netherlands Cancer Institute, Amsterdam, the Netherlands). They were cultured in medium 199 with 100 unit/ml penicillin-streptomycin and 10% (v/v) fetal bovine serum. KB cells (buccal carcinoma cell line) were cultured in MEM medium with 1% (v/v) MEM non essential amino acid (100X), 1% (v/v) of 100 mM MEM sodium pyruvate (100X), 100 unit/ml penicillin-streptomycin, and 10% (v/v) fetal bovine serum. H460 cells (lung carcinoma cell line) were cultured in RPMI medium with 1% (v/v) L-glutamine, 100 unit/ml penicillin-streptomycin, and 5% (v/v) fetal bovine serum. CC2511 cells (human normal dermal fibroblast) were cultured in DMEM medium with 100 unit/ml penicillin-streptomycin and 10% (v/v) fetal bovine serum. All cell lines were incubated at 37 °C, 95% O_2, 5% CO_2 in humidified carbon dioxide incubator. All other tissue culture reagents were obtained from Gibco Life Technologies, Inc. (Grand Island, NY, USA) and fetal bovine serum was purchased from Hyclone Laboratories (UT).

2. Chemicals

Reneiramycin M (RM) was kindly provided by Dr. Khanit Suwanborilux, Department of Pharmacognosy, Faculty of the Pharmaceutical Sciences, Chulalongkorn University. The compound was kept in DMSO at the concentration of 10 mg/ml and stored at -20 °C. On the day of experiment, RM was freshly diluted with sterile ultra-pure water with the final concentration of DMSO as 10% (v/v). Other principle chemicals, including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), *In vitro* toxicology assay kit, Lactate dehydrogenase - based, Hoechst 33342, rhodamine 123, verapamil, Bovine serum albumin (BSA), puromycin, vinblastine, and propidium iodide (PI) were purchased from Sigma Chemical Co. (St Louis, MO, USA).

3. Cytotoxicity studies

Cellular damage induced by RM was evaluated by the MTT assay (Kanzaki *et al.*, 2002; Mueller *et al.*, 2004; Holst and Oredsson, 2005) and by monitoring the release of lactate dehydrogenase (LDH) in the culture medium (Wispriyono *et al.*, 1998; Ryan *et al.*, 2005).

3.1 MTT assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay is one of the established methods to assess cell viability (Chiba *et al.*, 1998). For MTT assay (Carmichael *et al.*, 1987), 190 µl aliquot of cell suspension, LLC-PK_1, LLC-MDR_1, H460, and KB cells were plated at 500 cells/well and CC2511 cells were plated at $1 x 10^3$ cells/ well in 96 wells plate. After 24 hours of incubation (37 °C, 95% O_2, 5% CO_2), 10 µl of various concentrations of RM or 10% DMSO (vehicle control) were added and plates were incubated for 72 hours. At the end of the incubation, culture mediums were removed and 100 µl of MTT (0.4 mg/ml in culture medium without supplement) were added, and the plates were incubated for 4 hours. MTT were taken up via endocytosis by living cells and reduced to blue formazan crystal inside the viable cells via mitochondrial nicotinamide-adenine dinucleotide phosphate (NADH)-dependent dehydrogenase. The amount of formazan generated is assumed to be directly proportional to the viable cell number when using homogenous cell populations. Based on this principle, the damaged cells decrease its ability to cause the reduction of MTT to formazan (Mueller *et al.*, 2004). After removal of the MTT containing medium, the formazan crystals were dissolved in 100 µl DMSO. Plates were shaken mechanically for 10 sec and read immediately at 570 nm with reference wavelength 620 nm by using a microplate reader (Anthos Labtec HT2 version 1.21E, Australia).

3.2 LDH release assay

The principle of LDH release assay is to determine LDH activity via measuring the reduction of NAD to NADH (Petrik *et al.*, 2005). Then, NADH is ultilized in the stoichiometric conversion of tetrazolium dye which can be measured spectrophotometrically. For LDH release assay, LLC-PK_1 and LLC-MDR_1 cells were plated at 5×10^4 cells per well in 24 wells-plate and cultured for 48 hours. At the end of incubation, culture mediums were removed and RM was added in Medium 199 without phenol red and supplement. After incubation time of 24 hours, the activity of LDH in medium (LDH release) was determined using an *in vitro* toxicology assay kit (Lactate dehydrogenase-based). In addition, cells were treated with 0.5% Triton–X 100 for determination of total LDH. The developed chromophore was measured at a wavelength of 490 nm with reference wavelength 690 nm by using a microplate reader (Anthos LabtecHT2 version 1.21E, Australia).

4. P-gp interaction studies

In this study, the *in vitro* experiments to determine whether RM could interact with P-gp and modulate P-gp functions were the growth inhibition assay and the cell-based drug accumulation assay.

4.1 Growth inhibition assay

MTT assay was carried out to measure drug-induced cytotoxicity in the conditions specified for evaluation of RM as substrate or inhibitor of P-gp function. The inhibitory effect of RM against P-gp function was determined by measuring an accumulation of P-gp substrate, vinblastine (VBL) and puromycin in the presence of RM (Kanzaki *et al.*, 2002; Mueller *et al.*, 2004; Holst and Oredsson, 2005). In addition, the potential of RM as P-gp substrates was determined by measuring the accumulation of RM in the presence of verapamil, a P-gp inhibitor.

In these experiments, the VBL-induced cytotoxicity was determined in two conditions, which were the 24 hours pre-treatment and the co-treatment with RM 72 hours in LLC-PK_1 and LLC-MDR_1 cells. In some experiments, puromycin or

verapamil were used instead of VBL to further investigate the inhibitory action of RM against P-gp function in LLC-MDR_1 cells.

In the co-treatment experiments, cells were plated at $5x10^2$ cells in 180 μl of culture medium. After 24 hours of incubation, 10 μl of various concentrations of RM together with VBL, puromycin, verapamil or 10% DMSO (vehicle control) were added and incubated for another 72 hours prior to an MTT assay.

In the pre-treatment experiment, cells were plated at $5x10^2$ cells in 190 μl of culture medium and 10 μl of various concentration of RM were added. After 24 hours of incubation, RM was removed and various concentrations of VBL were added and incubated for another 72 hours prior to an MTT assay.

4.2 Accumulation of Rhodamine123

Accumulation of rhodamine 123, a known substrate of P-gp, was one of the methods used to determine the P-gp function. LLC-PK_1 and LLC-MDR_1 cells were plated at $2x10^5$ cells/ ml and $1.5x10^5$ cells/ml in a 24 wells-plate. Culture medium was replaced every 2 days until cells reached confluence. On the experimental day, cells were pre-incubated with RM for 30 min at 37 °C in CO_2 incubator under various condition, including in energy-free medium (15 mM NaN_3 in PBS), energy-supply medium (10 mM glucose in PBS), and PBS with 10% FBS. Then, rhodamine 123 (10 μM) was added into the culture and incubated at 37 °C for another 2 hours. In certain experiment, RM and rhodamine 123 were added to the culture simultaneously. At the end of incubation, the cells were chilled on ice and washed with ice-cold PBS three times to eliminate the extracellular drug. Cells were then lysed in 0.5 ml of 0.2 M NaOH 30 min. The amount of extracted rhodamine 123 was determined by spectrofluoremeter (Multilabel microplate reader (Perkin Elmer VICTOR3 wallac 1420, Germany) with excitation at 504 nm and emission at 540 nm. An aliquot of cell lysates was used in parallel to determine the cellular protein concentration by Bradford method (Bradford, 1976). The intracellular rhodamine 123 level was expressed in nmole/mg of protein. In these experiments, verapamil, a known P-gp inhibitor was used as positive control.

5. Statistical analysis

Cytotoxicity measuring by MTT assay was calculated based on percent inhibition of cell viability.

$$\text{Percent inhibition} = 100 - \left[\frac{\text{Mean treatment OD x 100}}{\text{Mean control OD}}\right]$$

Cytotoxicity curve was plotted as percent inhibition versus log RM concentrations. The IC_{50} values were determined at 50% inhibition by Microcal origin 5.0.

For LDH assays, percentage of LDH release was determined according published protocols by the formula.

$$\text{Percent LDH release} = \frac{\text{Mean LDH release OD x 100}}{\text{Mean LDH release OD + Mean total LDH OD}}$$

All experiments were repeated at least three times. In each experiment, all data were done in triplicate and presented as means ± S.E. (standard error). A significant difference between the control and the modulator data was determined by Student's *t*-test. P value of < 0.05 was considered to be statistical significant.