CHAPTER V

DISCUSSION AND CONCLUSIONS

Tetrahydroisoquinoline compounds have been known for their antitumor and antibiotic activities (Scott and Williams, 2002). Examples of these compounds include saframycin, renieramycin and ecteinascidin. Renieramycin M (RM) is a new tetrahydroisoquinoline compound which can be isolated from *Xestospongia sp.*, marine sponges found in the Gulf of Thailand (Suwanborirux *et al.*, 2003). With tetrahydroisoquinoline core, RM is a promising candidate for anticancer drug development. RM has been reported its anti-tumor activity in several cell culture models including human colon carcinoma (HCT116), human lung carcinoma (QG56), human lung carcinoma (NCI-H460), and human colon carcinoma (DLD1) (Suwanborirux *et al.*, 2003; Saito *et al.*, 2004). In this study, the cytotoxic property of RM was further tested against normal and cancerous cell lines, including dermal fibroblast cells (CC2511), renal epithelial cells (LLC-PK_1), buccal carcinoma cells (KB), lung carcinoma cells (H460) and *MDR1* gene-transfected epithelial cells (LLC-MDR_1) which is a model for P-gp overexpressing cells.

The RM-induced cytotoxicity as measured by MTT reduction assay was concentration dependent. This study revealed that RM is a potent cytotoxic compound which was able to cause cell death at the low concentration of nanomolar range. The data suggested that cancer cell lines (KB and H460) were more sensitive to RM treatment as similar to those described in the previous study (Suwanborirux *et al.*, 2003). In addition, the cells with overexpression of *MDR1* gene (LLC-MDR_1) were more susceptible to RM toxicity than normal LLC-PK_1 cells. The descending order of cell susceptibility toward RM treatment was LLC-MDR_1> > KB > H460 > LLC-PK_1> CC2511. Apparently, this compound elicited its cytotoxic potency against the highly proliferating cells like the cancer cells. This observation was in agreement with the reported doubling times of normal and cancer cell lines. The doubling time of KB and H460 cells were approximately 24 hours (Bergamo *et al.*, 1999; El-Bayoumy *et al.*, 2002) while the doubling time of CC2511 cells were approximately 2-3 days. Interestingly, the doubling time of LLC-MDR_1 cells (*MDR1*-overexpressing cells) and the parental cells (LLC-PK_1) were 16 and 18 hours, respectively. However, the

findings in this study revealed that LLC-MDR_1 cells were 16-fold more sensitive to RM treatment than the LLC-PK_1 cells. Hence, the doubling time was unlikely to be a major factor influencing the differential susceptibility of LLC-MDR_1 and LLC-PK_1 cells.

Multidrug resistance refers to the clinical and laboratory circumstance in which a tumor is no longer susceptible to several groups of anti-cancer drug (Rubin and Hait, 2006). The principle mechanism is the presence of *MDR1* gene products such as P-glycoprotein (P-gp) to pump the drugs out of the cells so that a cytotoxicity concentration is never achieved in the cells. The LLC-MDR_1 cells and its non-MDR counterpart (LLC-PK_1) has been a well-established model to study MDR phenomenon and to screen for anti-MDR compounds (Beumer *et al.*, 2006; Patanasethanont *et al.*, 2006). Generally, it can be postulated that LLC-MDR_1 cells are less sensitive to cytotoxic agent than LLC-PK_1 cells (Tanaka *et al.*, 1997). Surprisingly, LLC-MDR_1 cells were the most sensitive cells to RM treatment. RM clearly exerted more potent anti-proliferative action against LLC-MDR_1 cells than its parental cells (LLC-PK_1).

Although the mechanism of RM-induced cytotoxicity has yet understood, this study demonstrated that RM caused necrotic cell death in LLC-PK_1 cells and LLC-MDR_1 cells, as evidenced by LDH release. LDH is a stable enzyme presented in all cells and rapidly released into the culture medium upon damage of plasma membrane (Al-Ghamdi *et al.*, 2004). Thus, LDH leakage into the supernatant has been accepted as an indicator of necrotic cell death. Although RM induced necrosis in both LLC-PK_1 and LLC-MDR_1 cell lines, the numbers of cells undergone necrosis were significantly higher in LLC-MDR_1 cells than in normal LLC-PK_1 cells.

A number of differences in cellular characteristics between *MDR1*-transfected cells and their non-MDR counterparts have been reported. It has been demonstrated that the levels of intracellular ATP in *MDR1*-transfected cells were higher than those in non-MDR counterparts (Batrakova *et al.*, 2001). Hence, the MDR-overexpressing cells were much more susceptible to metabolic inhibitors, which induced a profound decrease in ATP levels, than its non-MDR counterparts. It is likely that depletion of ATP can lead to cell death and potentiate the cytotoxicity of the drugs in the MDR cells. The presence of ATP inhibitors shifted the IC_{50} value of doxorubicin in LLC-MDR_1 cells 2.5 folds less than those in LLC-PK_1 cells (Batrakova *et al.*, 2001). Furthermore, ATP depletion induced necrosis and apoptosis of cultured mouse

proximal tubular cells (Lieberthal *et al.*, 1998; Stinson *et al.*, 2003). Taken together, RM may disrupt metabolic and energy homeostasis, leading to a more susceptibility of LLC-MDR_1 toward RM treatment.

Interestingly, this study showed that RM caused LDH leakage in LLC-MDR_1 more than in LLC-PK_1 cells. It is possible that the membrane of LLC-PK_1 and LLC-MDR_1 feature dissimilar characteristics, which consequently influence the susceptibility of these two cells toward RM treatment. In this regard, membrane integrity of the LLC-MDR_1 cells is more sensitive to RM than that of the LLC-PK_1 cells. Hence, it can be proposed that RM compromised membrane permeability resulting in an increase in RM entering the cells whereas P-gp could no longer effectively pump RM out of the cells. Subsequently, RM reached equilibrium more rapidly in the LLC-MDR_1 cells, and elicited its cytotoxic action. Moreover, alteration of membrane fluidity may interfere with either the ATPase activity and/or its drug transport capability due to increase membrane resistance to protein conformational change (Wadkins *et al.*, 1993; Drori *et al.*, 1995; Hui *et al.*, 1989).

The interference of P-gp function is of clinical importance leading to drug interaction, drug accumulation and alteration of pharmacokinetic outcomes such as absorption, distribution and elimination. Tumor cells with the MDR phenotype over-express a P-gp efflux pump become resistance to chemotherapy because of the unattained intracellular concentration of cytotoxic agent.

In this study, LLC-PK_1 cells and LLC-MDR_1 cells were used for the drug accumulation assays with P-gp substrates including VBL, puromycin, and Rh123. Similarly to several other studies, the basal P-gp activity in LLC-MDR_1 cells was higher than in LLC-PK_1 cells as evidenced by the less VBL-induced cytotoxicity and Rh123 accumulation in LLC-MDR_1 cells (Quesada *et al.*, 1996). In addition, verapamil, a known P-gp inhibitor could restore the VBL-induced cytotoxicity and increase Rh123 accumulation in LLC-MDR_1 cells (Tanaka *et al.*, 1997; van der Sandt *et al.*, 2000). In this study, the degree of VBL resistance in LLC-MDR_1 cells was 4.1-fold higher than in LLC-PK_1 cells. Generally, tumors with MDR isolated from patients exhibit a 2-10-fold increase in drug resistance (Goldstein *et al.*, 1989).

Based on its molecular structure, RM has high potential to interact with P-gp and to causes drug interaction like other tetrahydroisoquinoline such as Ecteinascidin (ET743) (Beumer *et al.*, 2006) and cepharanthin (Hirai *et al.*, 1995). Kanzaki and co-

workers (2002) found that ET-743 was able to down-regulate *MDR1*/P-gp gene expression after 24-hour treatment and the level of P-gp protein decreased for at least 72 hours. Although this study was not designed to directly determine the expression of P-gp, the degree of P-gp expression can be estimated from its function as drug efflux pump. The decrease in P-gp activity would result in the increase of VBL-induced cytotoxicity. The result of this study revealed that treatment of RM for 24 hours prior to VBL had no effects on VBL-induced toxicity in either LLC-PK_1 or LLC-MDR_1 cells. Hence, RM is unlikely to increase the expression of *MDR1*/P-gp gene in both cell types.

Generally, in addition to increasing the accumulation of P-gp substrates, P-gp inhibitors such as verapamil are able to enhance the cytotoxic response of drug-resistant cells through inhibition of P-gp function. For example, it has been shown that verapamil increased Rh123 accumulation in LLC-PK_1/ADR cells (Decorti *et al.*, 2001), and reversed the resistance to cytotoxic drugs in P-gp over-expressing cells (Quesada *et al.*, 1996; Chen *et al.*, 1999; Qi *et al*, 2004; Wang *et al.*, 2004). Unlike ET-743 or verapamil, RM had no effect on VBL-induced cytotoxicity in LLC-PK_1 and LLC-MDR_1 cells. In addition, RM had no effect on Rh123 accumulation in P-gp over-expressing cells. Hence, it is unlikely that RM is an inhibitor of P-gp. However, the intrinsic inhibitory property of RM toward P-gp function may not be thoroughly explained because of its different effect on the known P-gp substrates in these studies. RM was able to decrease puromycin-induced cytotoxicity in LLC-MDR_1 cells. It appeared that the efflux of puromycin, but not that of VBL and Rh123, was affected by RM. This phenomenon may not be surprising. Certain chemicals such as QB102 which interfered adriamycin-induced activation of p53-resposive reporter *LacZ* gene featured different outcomes in drug accumulation and cytotoxicity depending upon certain type of P-gp substrates in the studies. The action of QB102 may be through modulating P-gp activity, making the pump more or less effective against certain substrates (Kondratov *et al.*, 2001). Thus, it could be hypothesized for further investigation that RM might affect the cellular transport of puromycin either by interfering membrane permeability of puromycin influx or enhancing puromycin efflux.

It is noteworthy that RM elicited differential effects in LLC-PK_1 and LLC-MDR_1 cells which may be attributed to the different characteristics of these two

cell lines (Nagatsuka and Nakazawa, 1982). The results showed that RM enhanced the passive uptake of Rh123 in LLC-PK_1 cells, but not in LLC-MDR_1 cells. In general, LLC-PK_1 cell lines is a proximal tubular cell line derived from normal pig kidney, which has structure and function similar to those of renal proximal tubular cells including P-gp expression (Decorti *et al.*, 2001). However, it is possible that LLC-PK_1 cells may have P-gp and other membrane transporters such as organic cation carrier system (van der Sandt *et al.*, 2000). In this study, co-treatment of RM with verapamil could enhance Rh123 efflux in LLC-PK_1. In addition, RM could enhance Rh123 accumulation in this cell line even when the cells were deprived of energy supply to the pump function. These findings supported that LLC-PK_1 cells had another efflux system for Rh123.

Although RM might not be an inhibitor of P-gp function, it may have intrinsic property of P-gp substrate or modulator. The co-treatment of RM and verapamil revealed that verapamil enhanced RM-induced cytotoxicity in P-gp expressing cells, suggesting that RM may be pumped out of the cells through P-gp. Consequently, the presence of verapamil which is a known P-gp inhibitor caused RM accumulation in the cells reaching its cytotoxic concentration.

In summary, RM has a good potential as an anticancer drugs, featuring highly selective cytotoxicity to *MDR1*-overexpressed and cancer cells. Although RM might not be an inhibitor of P-gp function, it could be a substrate of P-gp and modulate P-gp efflux pump. In addition, RM could increase puromycin resistance in LLC-MDR1 cells. Further studies are needed to elucidate the mechanisms of RM-induced cytotoxicity, especially in *MDR1*-overexpressed cells, and the modulating effects of RM on P-gp function and expression as well as its effects on other transporters.