CHAPTER IV
DISCUSSION AND CONCLUSION

The development of multidrug resistant (MDR) of the tumor cells is a major obstacle to successful cancer chemotherapy. Multidrug resistance (MDR) P-glycoprotein and multidrug resistant associated protein – 1 are members of the ATP-binding cassette (ABC) super family of membrane transporters. These proteins are thought to function as energy dependent efflux pumps for a variety of structurally diverse chemotherapeutic agents thereby decreasing their intracellular drug accumulation. As a result, tumor cells evade cytotoxic effects of drugs.

A number of substances have been shown to modulate or inhibit the transport function of P-glycoprotein, thus enhancing or restoring chemosensitivity of MDR cells to cytotoxic agents. These include verapamil, cyclosporin A, tacrolimus (FK506) and yohimbine. Unfortunately, attempts to use these agents clinically have met the limited success.

The ideal modulator should effect the resistant mechanism at a low and readily physiologically achievable concentration. Therefore, the effect of many natural products on P-glycoprotein was investigated. A major group of these products were the powerful antioxidants, others were phenolic in nature, and the remainder includes reactive groups that confer protective properties. Many reports have reported the effect of natural products or other phytochemicals from plants which inhibit P-glycoprotein function and reverse MDR in cancer prevention and treatment.

This work reports an attemption in searching for MDR modulator from *S. tuberosa.* This experiment is the first attempt to study the effect of *S.tuberosa* extract. The screening of the P-glycoprotein based on cytotoxic assay and transport assay comfirmed the high potential of the plant extract as MDR modulator.

Experimental models for P-glycoprotein and MRP-1 study

In this study human cervical carcinoma cell lines were used. All of the cell lines were provided by Dr. Michael Gottesman (National Cancer Institute, Bethesda, MD., USA). These cell lines had two types: drug sensitive cervical carcinoma cell lines (KB-3-1) and multidrug resistant cervical carcinoma cell lines (KB-V-1). Both

cell lines were cultured in complete DMEM, but vinblastine was added to KB-V-1 culture medium. Khantamat *et al*. (2004) investigated the expression in both cell lines by Western blot analysis. It was found that a 170 kDa plasma membrane P-glycoprotein highly expressed in KB-V-1 cells maintained in vinblastine containing medium, but not in KB-3-1. So in this study both KB-3-1 and KB-V-1 were the excellent tools for P-glycoprotein studying.

In this study, MRP-1 was investigated by using the transfected human embryonic kidney cells. These cell lines were generous gifts from Dr. Suresh V. Ambudkar. Cell lines were classified into two types as follow: drug sensitive transfected human embryonic kidney cells (HEK293/pcDNA) and multidrug resistant transfected human embryonic kidney cells (HEK293/MRP-1). Only HEK293/MRP-1 cell lines were maintained in etoposide (VP-16) containing culture medium. Muller *et al*. (2002) were the first researchers to study the MRP-1 in both cell lines. They examined the expression by Western blot analysis and MRP-1 was found in HEK293/MRP-1 but not found in HEK293/pcDNA. Thus the experimental model for studying the MRP-1, HEK293/pcDNA and HEK293/MRP-1 were used in this research.

Phytochemical group of *S. tuberosa* extract

S. tuberosa extract was prepared from dried root of *S. tuberosa* and the phytochemical groups were tested as described in section 2.16. Alkaloid group was the only phytochemical group found. According to Gotz *et al*.1961, the main phytochemical group in *S. tuberosa* was alkaloid, tuberostemonine. This compound with the empirical formular $C_{22}H_{33}NO_4$, was found in stemonaceae plants such as S. *tuberosa* and *S. sessifolia.* Xu *et al*. (1982) have isolated three alkaloids from the 95 % EtOH extract of *S. tuberosa* roots and had the structure elucidated. They found that three alkaloids were stemotinine, isostemotinine and stemonidine. Ye *et al*. (1994) discovered two new alkaloids from *S. tuberosa* extract including, neotuberostemonine and bisdehydroneostemonine. Moreover, Lin *et al*. (1994) found the other two alkaloids tuberostemoninol and stemoninoamide. Neotuberostemonol and neotuberostemoninol were reported in *S. tuberosa* by Jiang *et al*. 2002. In

conclusion, alkaloid was the major component in *S. tuberosa* extract and may act as the P-glycoprotein modulator.

Antiproliferative effect of *S. tuberosa* extract on human cervical carcinoma cells (KB cells)

Antiproliferative effect of plant extract was evaluated by using MTT assay after incubating the plant extract with both cell lines, and then the survival cells were detected. Our results showed that the plant extract did not inhibit cell growth. The IC_{50} values of the plant extract on antiproliferative effect of KB-3-1 and KB-V-1 were > 400 μg/ml. *S. tuberosa* extract was not toxic to both cells, and non-toxic doses selected were 50 μg/ml and 150 μg/ml because they can inhibit growth in both cells lines less than 20 %. P-glycoprotein modulator property of plant extract was determined. In this study the antiprolifertive effect of verapamil, a standard P-glycoprotein inhibitor on both cells, was studied to select the non-toxic concentration. The condition for examining the non-toxic concentration of verapamil was similar to plant extract. It was found that verapamil inhibited both cells growth. The IC_{50} values of verapamil on antiproliferative effect of KB-3-1 and KB-V-1 were 37 μM and 41 μM, respectively. Verapamil was toxic to both cells, then 20 μM verapamil was used in the next study. Mei *et al*. (2003), explained the term resistant factor value (RF), it is equal to IC_{50} value of drug resistant cells versus IC_{50} value of drug sensitive cells. In this study the RF value of *S. tuberosa* extract was not investigated, but it could be estimated from the IC_{20} value of both cells because both of the growth curves were identical; the estimated RF value of plant extract was 1.25. The RF value of verapamil was 1.1. According to Mei *et al*. 2003, the putative P-glycoprotein substrates, including doxorubicin, colchicine and vinblastine, showed the RF values 44.6, 115.9 and 68.6, respectively, but tea polyphenol and EGCG from green tea had RF values 1.0 and 1.2. It was concluded that verapamil was not P-glycoprotein substrate compared to anticancer drugs.

Increasing of anticancer drugs sensitivities by *S. tuberosa* extract in P-glycoprotein overexpressing cells

The effect of *S. tuberosa* extract on MDR phenotype was determined by MTT assay. A cytotoxic IC_{50} end point was a frequently used method to evaluate P-glycoprotein inhibitors. Most often the IC_{50} for several concentration of a cytotoxic drug was examined in the presence and absence of a non-toxic concentration of a P-glycoprotein modifier. In this assay the structurally diverse chemotherapeutic agents were tested by co-incubating with 50 μg/ml and 150 μg/ml of plant extract. The results showed that vinblastine, paclitaxel and colchicine sensitivities were significantly stimulated by decreasing the % survival in KB-V-1 cells. In KB-3-1 cells, the vinblastine and paclitaxel sensitivities did not change but colchicines sensitivity increased when treated with plant extract. Verapamil (positive control agent) could significantly activated vinblastine, paclitaxel and colchicines sensitivities in KB-V-1 cells as much as the plant extract. Interestingly, verapamil had not effect on vinblastine and paclitaxel sensitivities but not colchicines sensitivity. Shapiro *et al*. (1997) reported that P-glycoprotein possessed multiple drug-binding sites. The binding of several P-glycoprotein substrates could be classified into four categories: (i) binding to the Hoechst-binding site (such as colchicines), (ii) binding to the rhodamine-binding site (such as daunorubicin), (iii) binding to both sites (such as vinblastine) and (iv) binding to neither site (such as progesterone). From the results, it was found that *S. tuberosa* extract may interfered the binding of P-glycoprotein substrates at the Hoechst-binding site or Hoechst-binding site and rhodamine-binding site. Verapamil, a phenylalkylamine, first developed as a useful dependent blocker of voltage-dependent L-type calcium channels. Verapamil was the first compound recognized as a chemosensitizer of P-glycoprotein mediated multidrug resistant. Many groups reported that verapamil inhibited the transport function of P-glycoprotein, as it increased the accumulation of several drugs, such as the anthracyclines and vinca alkaloids, in the cells with P-glycoprotein overexpression (Willingham *et al*. 1986). In this study verapamil increased vinblastine, paclitaxel and colchicines sensitivities of KB-V-1 cells by inhibiting P-glycoprotein activities.

The colchicine sensitivity in KB-3-1 cells, which lack P-glycoprotein expression, was surprisingly increased by the plant extract at concentration of

150 μg/ml or 20 μM verapamil. Fu *et al*. (2002) evaluated the effect of alkaloid tetradrine (Tet) on MDR reversal properties on multidrug resistant MCF-7/adr cells and counterpart MCF-7. They found that Tet inhibited the P-glycoprotein mediated efflux of doxorubicin and Fura-2. Interestingly, Tet increased the fluorescence polarization and decreased the cell membrane fluidity in both MCF-7/adr and MCF-7 cells. In this study the plant extract may interact with lipid membrane of the cells thus perturbing the membrane environment or modifying the drug-membrane interaction. Kerr *et al*. (1986) reported that verapamil prolonged the terminal half-life and increased volume of distribution at steady state of doxorubicin. Now, no evidence show how verapamil increase colchicines sensitivity in cancer cells with no P-glycoprotein expression. In previous study, it was suggested that verapamil may disturb the lipid membrane of KB-3-1 cells; moreover, it may prolonged the half-life of colchicines similar to doxorubicin.

Inhibition of P-glycoprotein activities by *S. tuberosa* extract

To verify the increasing of anticancer drugs sensitivity by *S. tuberosa* extract, P-glycoprotein activities were examined in terms of drug uptake assay and drug retention assay. *S. tuberosa* extract significantly increased the intracellular ^{3}[H]-vinblastine accumulation in KB-V-1 cells in a concentration dependent manner (200-500 μg/ml). Moreover, the positive control verapamil also caused the increase in the amount of intracellular ^{3}[H]-vinblastine accumulation in KB-V-1 cells. On the other hand the plant extract and verapamil did not influence the accumulation of the intracellular ^{3}[H]-vinblastine accumulation in KB-3-1 cells.

P-glycoprotein has been shown to function as a drug efflux pump to remove substrates out of tumor cells through an ATP dependent mechanism in a unidirectional manner. ^{3}[H]-vinblastine retention was measured after exposing KB-V-1 cells to the plant extract and verapamil for 30 min in an efflux period. The data showed that plant extract (200-500 μg/ml) and verapamil significantly increased the amount of ^{3}[H]-vinblastine retention compared to KB-3-1. It was due to differences of P-glycoprotein in both cell lines. KB-V-1 cells showed the overexpression of MDR-1 gene, which is known as a main reason in changing the intracellular drug concentration.

Fu *et al*. (2001) found that naturally occurring bisbenzylisoalkaloids (BBI) could reverse multidrug resistance by increasing the intracellular drug accumulation through inhibiting the activity of P-glycoprotein. Alkaloid was found as the major component in *S. tuberosa* extract and may have played a role as P-glycoprotein modulator. This could solve the problem of multidrug resistance faced by conventional cancer chemotherapy which has been linked to overexpression of membrane associated with P-glycoprotein that acts as an energy-dependent drug efflux pump.

Antiproliferative effect of *S. tuberosa* extract on transfected human embryonic kidney cells (transfected HEK 293 cells)

Using the MTT, antiproliferative effect of plant extract on MRP-1 overexpressing cell lines, HEK293/MRP-1 and wild type, HEK293/pcDNA were investigated by incubating the plant extract with both cell lines for 96 h. The IC_{50} values of plant extract of HEK293/MRP-1 and wild type were 83 μg/ml and 98 μg/ml. The selected non-toxic concentrations were 25 μg/ml and 50 μg/ml. Indomethacin, an MRP-1 inhibitor, was studied in comparison to the plant extract. It was found that indomethacin could not inhibit cells growth. The RF value of plant extract was 0.85 but RF value of indomethacin was not evaluated because both growth curves were parallel to the X-axis. According to Muller *et al*. 2002, they found that the IC_{50} value of VP-16 (MRP-1 substrate) in HEK293/MRP-1 and wild type were 38.2 μM and 0.36 μM. Thus the RF value of VP-16 was 106.1. From these results, indomethacin was not MRP-1 substrate.

Unchanging of VP-16 sensitivity by *S. tuberosa* extract on MRP-1 overexpressing cell lines

MRP-1 modulating property of *S. tuberosa* extract was determined by MTT assay. VP-16 was used as a MRP-1 substrate. After co-incubating VP-16 with 25 μg/ml and 50 μg/ml of plant extract for 96 h the survival cells were checked. *S. tuberosa* extract did not change the sensitivity of VP-16 in both cell lines compared with vehicle control. Interestingly, in this study indomethacin at the concentration of

40 μM and 70 μM significantly increased cytotoxicity of VP-16 in HEK293/MRP-1 ($P < 0.05$). No change in MDR phenotype mediated by MRP-1 was found in wild type, HEK293/pcDNA cells, which lack MRP-1 expression. Courtois *et al*. (1999) studied the cellular accumulation and efflux of the fluorescent dye carboxy-2',7'-dichlorofluorescein (CF) in rat liver SDVI cells. These cell lines were found to express MRP-1 mRNAs at relatively high levels, similar to those observed in lung, a tissue known to physiologically display high expression of MRP-1. They reported that indomethacin treated cell demonstrated strong CF retention increase compared to the control. Moreover, Duffy *et al*. (1998) used the DLKP (a human lung squamous cell line) and A549 (a human lung adenocarcinoma cell line) to test the effect of non-steroidal anti-inflammatory drugs (NSIAD) on enhancing chemotherapeutic drugs toxicity. It was shown that indomethacin at 2.5 μg/ml enhanced the toxicity of doxorubicin, vincristine and VP-16 in A549 cells and VP-16 in DLKP cells. Two cell lines had MRP-1 expression observed by RT-PCR technique. In an anthracycline resistant COR L23R, 2.5 μg/ml indomethacin also activated doxorubicin, vincristine and VP-16 cytotoxicity (Touhey *et al*. 2002). From these studies indomethacin was the good inhibitor and can be used as a positive control in MRP-1 work.

Possible mechanisms of P-glycoprotein modulation by *S. tuberosa*

The mechanism for P-glycoprotein modulation may be different among different classes of compounds. The three main mechanisms of modulators are: (i) direct interaction with one or more of the drug binding sites on P-glycoprotein, thus inhibiting transport by acting as competitive or non-competitive blocker; (ii) inhibition of ATP binding, ATP hydrolysis or coupling of ATP hydrolysis to the translocation of the P-glycoprotein substrates, and (iii) interaction with lipid membrane of the cells thus perturbing the membrane environment or modifying the drug-membrane interaction. (Ambudkar *et al*. 1999)

Data from our studies showed that the major component found in the extract of *S. tuberosa* was alkaloid. This extract strongly increased vinblastine, paclitaxel and colchicines sensitivities in KB-V-1 cells. The radiolabeled drug transport assays indicated that the plant extract cause the increase in radiolabeled drug accumulation

and retention in KB-V-1. The extract had no effect on MRP-1 as observed in HEK293/MRP-1 cells.

Previously alkaloids that act as P-glycoprotein modulators were investigated. (2R)-anti-5-(3-(4-(10,11-difluromethanodibenzo-suber-5-yl)piperazin-1-yl)-2-hydroxy-propoxy) quinoline trihydrochloride (LY335979), (Starling *et al.* 1997) was a second generation modulator of P-glycoprotein. It binds to P-glycoprotein with high affinity and specificity. The reversal of P-glycoprotein mediated resistant to vinblastine, doxorubicin, VP-16 and Taxol in multidrug resistant cell line CEM/VLB_{100} was observed as treating with LY335979. LY335979 also blocked ^{3}[H]-azidopine photoaffinity labeling of P-glycoprotein in CEM/VLB_{100} plasma membrane and competitively inhibited equilibrium binding of ^{3}[H]-vinblastine. But LY335979 did not appear to affect P-glycoprotein ATPase activity at concentrations of the modulator that were sufficient for full reversal of P-glycoprotein induced drug resistant.

Nanaumi *et al.* (1997) studied VA-033, a novel derivative of apovincominic acid ester and found that it enhanced the sensitivity of vincristine-resistant P388 (P388/VCR) and adriamycin-resistant P388 (P388/ADM) to vincristine. Adriamycin sensitivity was also found to increase in adriamycin-resistant K-562 (K562/ADM) in the presence of the agent. The intracellular accumulation of ^{3}[H]-vincristine in P388/VCR cells also increased. Finally, they found that it inhibited ^{3}[H]-azidopine photolabeling with P-glycoprotein.

Tropane alkaloid aromatic esters were isolated from the roots of *Erythroxylum pervillei* by Silva (Silva *et al.* 2002). Four new alkaloids, including pervilleine B-F and pervilleine G, were evaluated for their efficacy as potential modulators of P-glycoprotein. This assay assessed the ability of these compounds to increase the cytotoxic substrate of P-glycoprotein (4,9-dihydro-3-isobutyl-2-methyl-1-(p-nitrophenacyl)-4,9-dioxo-1 H-naphth (2,3-d) imidazolium bromide (DINIB), compared to an agent which is not a P-glycoprotein substrate 5-fluorouracil (5-FU) was used as a negative control. The data from the alkaloids tested indicated that they are excellent modulator of the multidrug resistant phenotype as exemplified by their ability, at non-toxic doses, to significantly increase the toxicity of the DINIB.

Moreover, Mi *et al*. (2002) isolated two new alkaloid aromatic esters including pervilleines B and C (PB and PC), from the roots of *Erythroxylum pervillei* and examined the ability of MDR modulator. They found that cell growth was not significantly inhibited when PB or PC were administered as single agent, but when used in combination with vinblastine, the growth inhibition occurred in multidrug resistant oral epidermoid carcinoma KB-V1 compared to wild type KB-3[a].

Next, tetradine (Tet), one of the bisbenzylisoquinoline alkaloids had been used as an antifibrotic drug to treat the lesions of silicosis in China since 1960s. Fu *et al*. (2002) found that it was relatively non-toxic to humans. It was found that when treated with Tet, the IC_{50} of doxorubicin for multidrug resistant MCF-7/adr cells and wild type MCF-7 to be 16.13 and 0.33 μmol/l, respectively. Tet, at the concentrations of 25,1.25 and 0.65 μmol/l, were barely cytotoxic (> 90 % cell survival) to the MCF-7/adr cells and lowered the IC_{50} of doxorubicin to 0.79, 1.37 and 2.98 μmol/l in the MCF-7/adr cells. They also reported that Tet inhibited the P-glycoprotein mediated efflux of doxorubicin and Fura-2. Interestingly, Tet increased the fluorescence polarization and decreased the cell membrane fluidity in both MCF-7/adr and MCF-7 cells.

Again six tropane alkaloid esters were isolated from stems of *Erythroxylum rotundifolium*. They reversed MDR phenotype in multidrug resistant oral epidermoid carcinoma KB-V1 cells incubated in the presence of vinblastine. (Chavez *et al*. 2002)

The alkaloid thaliblastine (TBL) was obtained from *Thalictrum* spp. including *T. dasycarpum* and *T. minus*. It was a dimeric aporphine benzylisoquinoline alkaloid. It stimulated the doxorubicin cytotoxicity in doxorubicin selected resistant subclone HL-60/DOX. (Horvath *et al*. 2004)

Kam *et al*. (2004) screened the indole alkaloids, laundurines A-D from Kopsia tenuis. They found that laundurines A-D displayed appreciable *in vitro* cytotoxicity of vincristine in vincristine-resistant human oral epidermoid carcinoma cell line KB/VJ300.

Indole-3-carbinol (I3C) was a naturally occurring component of cruciferous vegetables. Non-toxic doses (10×10^{-3} M) of I3C enhanced the cytotoxic effect of vinblastine in vinblastine-resistant human leukemia (K562/R10). Besides, similar

dose of I3C also showed complete restoration of doxorubicin accumulation in K562/R10 cells (Arora *et al*. 2005)

From table 35 it was concluded that all of the alkaloids (included *S. tuberosa* extract) could modify the P-glycoprotein by interaction with drug binding site(s) thus inhibiting the transport by themselves. As previously described in this chapter ten alkaloids found in *S. tuberosa* extract were tuberostemonine, stemotinine, isostemotinine, stemonidine, neotuberostemonine, bisdehydroneostemonine, tuberostemoninol, stemoninoamide, neotuberostemonol, and neotuberostemoninol. However, none of the alkaloids have been reported as the MDR reversing agent. Therefore, the result from this study presents the first evidence that the alkaloids from *S. tuberosa* may be an important role as MDR modulator(s). The purified form of these alkaloids present in *S. tuberosa* extract should be studied in more details in order to explain the molecular mechanisms involved in P-glycoprotein modulation. Additionally, *S. tuberosa* extract is needs more investigation to confirm the effectiveness and safety in P-glycoprotein modulating properties for utilizing in animal model.

Table 35. The summary of modulator classified as alkaloid to modulate the P-glycoprotein in different mechanism

Alkaloids	Mechanisms		
	(i) interaction drug binding site(s) and blocking transport	(ii) interfering ATP hydrolysis systems	(iii) interaction lipid membrane
1.LY335979	-Drug sensitivity assay (+) -^{3}[H]-azidopine lebeling (+)	-ATPase activity assay (-)	?
2.VA-033	- Drug sensitivity assay (+) -^{3}[H]-vincristine uptake (+) -^{3}[H]-azidopine lebeling (+)	?	?
3.Pervilleine B-F, G from *Erythroxylum pervillei*	- Drug sensitivity assay (+)	?	?
4.Pervilleine B and C from *Erythroxylum pervillei*	- Drug sensitivity assay (+)	?	
5.Tetradine	- Drug sensitivity assay (+) - doxorubicin uptake assay (+) - doxorubicin retention assay (+) - Fura-2 uptake assay (+)	?	- cell membrane fluidity assay (+)
6.Tropane alkaloids esters from *Erythroxylum rotundifolium*	- Drug sensitivity assay (+)	?	?
7.Thaliblastine	- Drug sensitivity assay (+)	?	?
8.Laundurines A-D	- Drug sensitivity assay (+)	?	?
9. Indole-3-cacbinol	- Drug sensitivity assay (+) - doxorubicin uptake assay (+)	?	?
10. *S. tuberosa* extract	- Drug sensitivity assay (+) -^{3}[H]-vinbastine uptake (+) -^{3}[H]-vinbastine retention (+)	?	?

+ positive result in the assay

- negative result in the assay

? assay not investigation