# CHAPTER IV

# DISCUSSION AND CONCLUSION

Multidrug resistance (MDR) is the resistant phenomenon to broad spectrum chemotherapeutic drugs in cancer cell lines and human tumors. The MDR phenotype is associated with an increase of drugs transport mediated by Pgp. Overexpression of Pgp, encoded by the MDR1 gene, confers resistance to a variety of structurally and functionally unrelated chemotherapeutic drugs. In the past few decades, extensive studies have been performed with the aim of developing effective modulators to overcome the intrinsic and acquired MDR of human cancers. Potent Pgp modulators are being investigated in clinical trial, including calcium channel blockers, verapamil and immunosuppressants, cyclosporinA. However, clinical application has not been attained to date because of the toxicity and undesirable effects.

In a search of MDR modulators from natural sources, many groups have studied the beneficial effect of flavonoids such as quercetin, kaemferol, galangin [101], genistein[102] on the modulation of MDR. We have directed our attention to Japanese green tea flavonoids because many studies have suggested the positive evidence for human health and a variety of green tea products are either being developed or currently sold as dietary supplements and herbal remedies. We determined the antitumor activity of green tea flavonoids and found that catechin and ECG were non-toxic to the drug resistance KB-V1 and drug sensitive KB-3-1 but EC, EGC and EGCG were equally toxic to both cell lines. EGCG is the most potent cytotoxic agent to the human cervical carcinoma cell lines. This result was similar to the study of Shinichi Uerato [103] and colleagues whom suggested that the pyrogallol structure possessing in EGCG and EGC is remarkable inhibitors against human colorectal cell line (HCT116) and human hepatocarcinoma cell line (HepG2). However, the sensitivity of EGCG on growth inhibitory effect was different, depending on cell type and time of incubation. The concentrations caused 50% cell death ($IC_{50}$) of EGCG were 46.7 and 141.6μM in HCT116 and HepG2 respectively, for 3 days incubation. While in human cervical carcinoma cell lines KB-V1 and KB-3-1, the $IC_{50}$ of EGCG were 214.3 and 220.6μM respectively, after 2 days incubation.

**4.1 Effects of green tea flavonoids on Pgp mediated drugs transport in KB-V1 and KB-3-1 cell lines**

In the studies of the effects of green tea flavonoids on Pgp mediated drug transport, the intracellular accumulation and retention of Rh123 and $^{3}$H-vinblastine were measured as direct representative of Pgp transport. Among the catechins present in green tea, EGCG and ECG increased the accumulation and reduced the efflux of Rh123 and $^{3}$H-vinblastine in KB-V1 cells but not in KB-3-1 which lack of Pgp. The inhibition of Rh123 and $^{3}$H-vinblastine transport by EGCG and ECG was found only in Pgp expressing cells supported the hypothesis that EGCG and ECG modulated Pgp activity rather than a modification of the membrane permeability.

However, the exposure to EGCG and ECG in either drugs accumulation or drugs efflux experiments of KB-V1 cells was about 1-2 h, it was unlikely that these compounds acted by reducing the amount of Pgp in the cell membrane. The Pgp level was examined and the results showed that Pgp level in KB-V1 cells was not different by treatment of the cells with EGCG and ECG (100 to 300 μM) after 2 h incubation compared to the vehicle control. In addition, the decrease of $^{3}$H-vinblastine efflux could be detected in only 15 min incubation, after additional of EGCG and ECG. These short periods left no time for gene expression and translation to cause and effect.

Thus, these data demonstrated that EGCG and ECG in the high micromolar range, at a short time incubation, increased the intracellular drugs levels in KB-V1 cells by modulating Pgp activity, not expression. Our findings confirmed the previous studies that EGCG and ECG increased the Rh123 accumulation in the resistant chinese hamster ovary [95], thus the gallate group substituted in EGCG and ECG may be an impotant part that modulated the Pgp activity.

Moreover, it was evident that EGCG modulated Pgp activity, increasing the Rh123 accumulation in MDR1 transfected human fibroblast cell line (NIH-3T3-G185) [104] and P388 leukemia cells (P388/Dox) [105]. Interestingly, in the use of LDS-751 as a fluorescent Pgp substrate, EGCG caused the significant decrease in LDS-751 retention in NIH-3T3-G185 [104]. Taken together, EGCG and ECG are able to modulate Pgp activity *in vitro* and that the interference with the transporter is related not only to the concentration and to the degree of resistance, but more interestingly also to the kind of anticancer drugs used.

**4.2 Effect of green tea flavonoids on Pgp expression (protein level) in KB-V1 cell line**

The other approach for overcoming the MDR is the modulation of the MDR1 gene. Several groups have demonstrated that antisense related to reverse the MDR phenotype in human cancer cells. Different oligodeoxynucleotide sequences were designed to target the MDR1 gene expression. The majority of targets were the sequence located around the mRNA AUG initiation codon [106]. However, this antisense mediated strategies is being under development and not yet in clinical use. Interestingly, some plants derivatives such as berberine, have been evidenced as an inhibitor of the MDR1 gene expression, resulting in the reversal of MDR property [11,12].

In this study, we investigated the effect of green tea catechins for their ability to modulate the MDR1 gene expression after long time incubation (48 h) by determining the level of Pgp at the plasma membrance using Western blot analysis. The results showed that EC significantly decreased the Pgp level but ECG caused a significant increase of this protein in KB-V1, while other catechins had no effect on Pgp level. In KB-3-1 cells, the Pgp band could not be detected by the Western blot analysis compared to the resistant KB-V1 which had a high detectable level of Pgp, which confirmed the different phenomenon of MDR between the resistant and sensitive cell model used in all experiments. This is the first report on the effect of green tea flavonoids on Pgp expression in MDR cancer cells.

**4.3 Effect of green tea flavonoids on cytotoxicity of chemotherapeutic drugs (MDR phenotype) in KB-V1 and KB-3-1 cell lines.**

The co-combination of green tea catechins with the increasing concentration of vinblastine for 48 h were tested on KB-V1 cell proliferation to determine whether catechins potentiate the toxicity of vinblastine. The concentration of each catechin used in this experiment (50 and100 μ M) caused the cell death not more than 20% as show in Section 3.1, leaving the factors that may contribute the cell death. The vinblastine induced cell death was not affected by catechin, EC, ECG and EGC co-incubation, in contrast, co-incubation with EGCG significantly increased the cell viability in a dose dependent manner in KB-V1 cells. The results from the functional test that EGCG caused the increase drugs retention in the cancer cells, which provided the aspect that combination of EGCG with drugs should induced cell death more than the cells that treated with drug alone. However, the concentration of EGCG that caused significant increase of the intracellular vinblastine retention is 250-300 μM, thus higher concentration (50-300 μM) were co-

incubated with the increasing concentration of vinblastine for 24 h. These higher concentration caused the cell death not more than 20% after 24 h incubation and the result showed that EGCG also caused a significant desensitizing effect in a dose dependent manner.

Moreover, EGCG not only caused the desensitizing effect when combined with vinblastine, but also when combined with other drug such as doxorubicin, colchocine and paclitaxel. Taken together, this finding suggested that an agent that can modulated the Pgp function or expression may not directly related with the reversal MDR properties that potentiate the cytotoxicity and sensitize the resistant cells to chemotherapeutic drugs. Other secondary effects of Pgp inhibitors in the MDR cells should be evaluated.

However, pre-inbubation with EGCG alone for 48 h and then treated with the increasing concentration of vinblastine for another 48 h caused no significant effect on vinblastine induced cell death.

In addition, EGCG desensitized chemotherapeutic drugs toxicity not only found in KB-V1 cells but also in KB-3-1, suggested that desensitizing effect did not related with Pgp molecule but the other signaling mechanisms might play a protective role here.

However, other studies showed that EGCG sensitized the toxicity of vinblastine or doxorubicin in multidrug resistant Chinese hamster ovary cell line [95], multidrug resistant human oral epidermoid carcimoma [107] and multidrug resistant human breast cancer when the mixture of green tea polyphenols were co-incubated [108], corresponding to the inhibitory effect of EGCG on Pgp function. In other cell lines such as NIH-3T3-G185 [104] and P388 leukemia cells (P388/Dox) [105], which showed the inhibitory effect of EGCG on Pgp function, had no report about its effect on MDR phenotype. Thus, experimental studies have yielded the clear-cut conclusion concerning the correlation between reversal multidrug resistant property and Pgp molecule inhibiting effects.

**4.4 The possible mechanism of desensitizing effect to chemotherapeutic drug by EGCG in KB-V1 and KB-3-1.**

Because of the complexity of the living cells, the effects of added chemical compounds on overall cellular responses would be difficult to discern. The compound may produce several additional cellular effects that determine the cell destiny. In cancer chemotherapy, anticancer drugs exhibit their lethality by different mechanism inducing apoptosis in tumor cell. Some drugs,

such as vinblastine and paclitaxel interfere with microtubule dynamics leading to G2-M phase arrest and inhibition of cell proliferation [109]. Some drugs, such as etoposide [110] and doxorubicine [111] induce DNA damage by inhibiting topoisomeraseII. In recent years, many evidences have shown that most anticancer drugs alter the activity of different MAPKs in many cancer cell lines [112].

MAPKs, which include extracellutar signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 subfamilies, are important key molecules in survival, proliferation and apoptosis. Molecular modulations of MAPK signals have been shown in many cases to influence the apoptotic response to anticancer drugs. However, the responses caused by MAPKs tend to be depending on cell type, kind of drugs and duration of exposure. For example, ERK activation is commonly response to cisplatin, protecting cell from cisplatin cytotoxicity. Inhibition of ERK sensitized ovarian cancer cells to cisplatin induced apoptosis [113], whereas inhibition of ERK desensitized HeLa cell to cisplatin toxiciy [114]. However, this is not true in other anticancer drugs treatment in HeLa cell. The ERK inhibitor potentiated the cytotoxicity effects of vinblastine and doxorubicin [115].

Taken together, drug induced cytotoxicity is not correlated with any single parameter, but rather a combination of effects on cell survival and apoptosis. In certain combinations of modulation of MAKPs pathway could enhance or decrease drug efficacies.

Zhong Zong Pan and colleague reported that γ-synuclein, the soluble protein that predominantly expressed in neurons and overexpressed in late stage breast and ovarian cancers, protected paclitaxel induced cell death by activation of ERK and down-regulation of JNK [116]. Miroslav Barancik and colleagues [117] reported that the exposure of mouse leukemic resistance cell line (L1210/VCR) to SB203580, the specific inhibitor of P38 resulted in the significant reduction of resistance against vincristine which structure is an analogous to vinblastine. Therefore, the use of traditional anticancer drugs combination with MAPKs modulating agent may provide attractive rational for effective therapy in the future.

EGCG have been reported to activate all three MAPKs in a dose and time dependent manner. Yona L. and colleagues reported that EGCG at low concentration exerted protective activity against 6-OHDA, a neurotoxin induced cell death in neuroblastoma cell (SH SY5Y) by restoring the reduced ERK1/2 and PKC activity induced by 6-OHDA toxicity, whereas at higher

concentration, it was protoxic [118]. Chi Chen also suggested that at 250 μM of EGCG induced ARE-mediated phase II gene expression including glutathione S-transferase and quinone reductase through activation of MAPKs, but at higher concentrations, EGCG activated the caspase pathway leading to apoptosis [119].

In the study of effect of EGCG on MDR phenotype in KB-V1 and KB-3-1, the concentration that used in this experiment were 50and 100 μM, which caused cell death not more than 20%. At this low concentration, EGCG may modulate the signal transduction via MAPKs pathway, especially ERK that plays the protective role from cell death and vinblastine itself also activates ERK activity in HeLa cells as aforementioned. Thus, synergistic effect of EGCG and vinblastine that activated the MAPKs was probably more pronounced than their effects on Pgp activity. Thus, KB-V1 and KB-3-1 cells were desensitized to vinblastine when EGCG was included in the culture medium. More detailed works are required to investigate the MAPK pathway in EGCG treated KB-V1 and KB-3-1 cells.

In conclusion, among of flavonoids found in green tea, EGCG is the most abundant component, which showed the antitumor activity and inhibited the Pgp function. It provided an expectation that EGCG could restore the chemotherapeutic drugs in the cell, leading to cancer cell death. Conversely, the combination of EGCG with the chemotherapeutic drugs such as vinblastine, doxorubicin, colchicine and paclitaxel, showed the desensitizing effect, protecting cancer cell from death. This finding suggested the reversal MDR property and Pgp inhibitor may not completely be related. EGCG may produce several additional cellular signal transductions that more pronounced than its effect on Pgp activity. The rationale for combination of EGCG with chemotherapy should take account of the multifactorial nature of cancer and needs more knowledge on the mechanism-based interaction and efficacy of the intervention.