CHAPTER I

INTRODUCTION

1.1 Statement and significance of the problem

Cancer is one of the leading causes of death in worldwide. The high mortality rates of specific cancers in Thai women are breast, cervical and ovarian cancer, while commonly found cancers in men are liver cancer, lung cancer and leukemia [1]. Presently, cancer treatment can be done by surgery or radiotherapy which have been called local treatment and chemotherapy or immunotherapy which have been called systemic treatment [2]. An advantage of systemic treatment is its ability to treat widespread or metastasis cancer, while local treatment is limited to cancer that is confined to specific areas and diagnosed in early stages.

The best current treatment at early stage cervical cancer is surgery. If the cancer has been spread by metastasis beyond its original site, radiotherapy usually prefers to be used and may be given in combination with chemotherapy.

The efficacy of the continuous treatment by chemotherapy is limited to resistance of cancer cells to a wide spectrum of cytotoxic drugs that do not have a common structure or a common cytotoxic intracellular target. This phenomenon has been called multidrug resistance (MDR) [3]. MDR is manifested by the reduced intracellular drug accumulation resulting from increased drug efflux. Several mechanisms have been proposed, one major type of MDR is attributed to the over expression of a 170 kDa plasma membrane, known as P-glycoprotein (Pgp).

P-glycoprotein is a member of the ATP-binding cassette (ABC) protein family and acts as an energy dependent drug efflux pumps. It is also constitutively expressed in normal organs such as liver, kidney and adrenal cortex which are responsible for the elimination of toxicants from the body, suggesting its role in detoxification and P-glycoprotein is also found to be over expressed in various human cancers.

Because of the likely role of P-glycoprotein in clinical drug resistance, many researchers have focused on the strategies to inhibit the function or expression of this protein. Several classes of drug like calcium channel blocker, e.g. verapamil, nifedipine and azidopine or immunosuppressants, e.g. cyclosporineA reverse the multidrug resistance phenotype by

interfering the transport system responsible for resistance. The agents that reverse the MDR phenotype have been called MDR modulator or chemosensitizer.

However, some MDR modulator applications have been hampered by undesirable side effects because the dose required to reverse MDR phenotype is clinically toxic. For example, the effective dose of verapamil to inhibit Pgp function is 8-10 μM, but the highest steady state plasma level that can be achieved when using continuous infusion was less than 5 μM. Dose limiting toxicity is due to cardiovascular side effect with heart block, congestive heart failure and hypertension [4].

Therefore, much of the current research efforts are directed towards the identification of novel compounds with due attention to dietary agents. An advantage of using dietary agents as a modulator of MDR is that they enhance antitumor activity and exhibit little or virtually no side effects without any further increase in the medication burden on the patient. For this reason, the quality of life of the patient will be improved.

Moreover, many groups have studied the beneficial effects of natural products as anti-tumorigenic, anti-proliferation, anti-inflammation as well as various immunological function, convincing patients to supplement consume diet in combination with conventional medicine.

Several components extracted from plants such as orange juice [5], cruciferous vegetables [6], grapefruit juice [7], rosemary [8] and curcumin [9,10] have been reported as MDR modulator, enhancing antitumor activity of chemotherapeutic drug in multidrug resistance cell. However, one of the major compounds of *Coptis chinensis*, berberine has been found to elevate P-glycoprotein expression, resulting in reduced retention of chemotherapeutic drugs, even though it has been evidenced to have antiproliferative effect on tumor cellls [11,12]. Thus, the rational for combination harbal plants with chemotheraphy in each cancer type must be investigated because some plant derivatives may increase antitumor activity of some chemotherapeutic drug, but some may decrease it.

In this study, I'm interested in green tea which is widely consumed beverage in Japan, China and other Far Eastern countries and become to be popular in Thailand. Epidemiological, experimental and metabolic studies are providing evidence that green tea plays an important role in initiation, promotion and progression of several types of human cancer as reviewed by Chung S. Yang and Zhi-Yuan Wang [13].

Components of the most interest contained in green tea are catechin, epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC) and epigallocatechin gallate (EGCG). These compounds have attracted the attention of many researchers due to their positive effects on human health and becoming popular beverage for both healthy and patients. They were examined in the present study to determine how they interacted with P-glycoprotein function and expression in drug resistant human cervical carcinoma cell (KB-V1) compared with its drug sensitive parent cell (KB-3-1).

1.2 Literature review

1.2.1 The clinical problem and phenotype of multidrug resistance

The pharmacological goal of chemotherapy is to deliver as much active drug as possible to the molecular target in cancer cells in order to cause sufficient molecular target leading cell death. Various factors can reduce the amount of active drug to cancer cells, resulting in cell survival and continuous growth. Clonal expansion of these cell leads to the formation of a tumor resistant to multiple cytotoxic drugs that is so called MDR phenotype (Figure 1).

Factors contributed MDR phenotype can be categorized into three groups as described in the following section (Figure 2).

a.) Factors upstream of the molecular target, which reduce the availability of active drugs.

Most cytotoxic drugs enter cancer cell via passive diffusion through the concentration gradient. The extracellular concentration is the major determinant for how many drugs enter the cells. Moreover, tumors can be poorly vascularized resulting in long distance between capillaries and tumor cells. The interstitial tissue may be rich in solid structures such as collagen, e.g. in particular type of carcinoma or in scar after radiation [14,15].

b.) Factors which reduce the availability of active drug at molecular target.

The first line of defense that chemotherapeutic drugs can face upon entering tumor cells are membrane bound efflux pump, such as Pgp or MRP [16]. Moreover, in the cytoplasm of target cells, drugs are subjected to metabolize by the detoxification system. A large body of evidence supports the role of glutathione-S-transferase (GST) in drug resistance. Many resistant cell lines have a correlative increase in the expression or activity of GST or related enzyme [17]. L-Buthionine-s-sulphoximine (BSO), a γ-glutamylcysteine synthase inhibitor, which depletes cellular GSH level, has been used in many studies to demonstrate that a reduced GSH pool sensitizes cells to drug treatment [18,19,20]. Recent work has also revealed a link between MRP mediated transport and GSH [21].

c.) Factor downstream of the molecular target.

Even if chemotherapeutic drugs reach their molecular target at adequate concentrations, they still may not be able to kill the cancer cells. A mechanism which lead to drug resistance despite adequate drug induced damage is enhancement of cellular repair or alteration of apoptosis. Almost cytotoxic agents appear to kill cancer cells via apoptosis. Alteration of gene involved in apoptosis

pathway such as p53 [22,23] , Bcl2 [24] and others [25,26] showed the influence of apopotosis in effective chemotherapy.

Table 1. shows the terms used to refer to particular MDR mechanism and phenotype. MDR itself is refered to a phenotype of resistance to multiple agents which differ in structure and can be specified by a prefix depend on the mechanism of resistance.

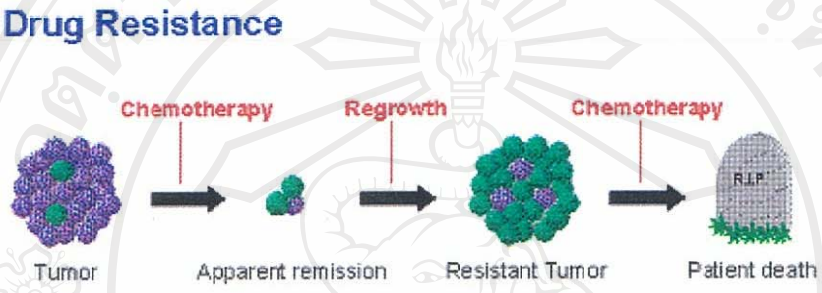

Figure 1. The resistance development during cancer chemotherapy [27]. When chemotherapeutic drug is given, most of sensitive cell will be killed, but the resistant cells will be survived.

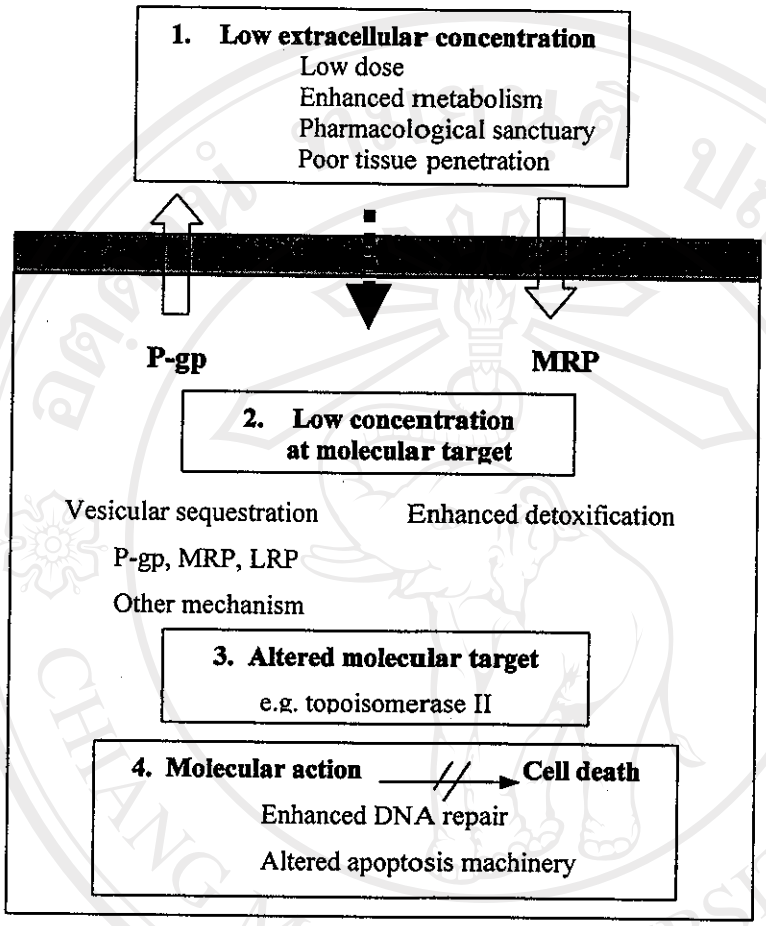

Figure 2. Factors contribute to clinical MDR in patients with cancer [28]

1. Most cytotoxic agents enter cancer cells via passive diffusion through the cell membrane along the concentration gradient between extra- and intracellular compartments. The amount of drug molecules present outside is therefore the major determinant for cellular drug uptake.
2. Various molecular mechanisms can reduce the availability of active drug at the molecular target such as drug efflux pumps and detoxification system.
3. MDR can result from alteration in the amount, structure or activity of the molecular target of cytotoxic agent.
4. Adequate drug induced molecular damage may not translate into cell death if cells are able to repair the damage or are unable to die via apoptosis.

Table 1. The various mechanisms and phenotypes of MDR [28]

Term	Mechanism	Characteistics
Pgp-MDR	Overexpression of *MDR1*/Pgp	- Resistance to natural drugs which differ in structure and function;reduced drug accumulation due to enhanced efflux. - Can be reversed by chemosensitizers such as verapamil or cyclosporins
MRP-MDR	Overexpression of MRP	- Phenotype similar to Pgp-MDR but little resistance to taxanes; changes in cellular pharmacology variable. - Amphipathic cations need to be conjugated prior to transport. - Low activity of typical Pgp-inhibitors.
TopoII-MDR	Diminished content or activity of topoIIα	- Resistance to topoII drugs (i.e. drugs which differ in structure but not in function)
GSH-MDR	Increased content of GSH and/or increased activity of GSH S-transferases	- Resistance to melphalan, BCNU, cyclophosphamide, chlorambucil, thiotepa (and possibly other drugs such as cisplatin and doxorubicin) - Increased phase II metabolism of drugs.
Apoptosis-MDR	Blocked apoptosis; dysfunction of genes involved in apoptosis	- Resistance to most cytotoxic agents.
Clinical MDR	Can be multifactorial; cellular mechanisms possible	- Clinical resistance to multiple cytotoxic drugs which differ in structure (and possibly function)

1.2.2 Multidrug resistance mediated by P-glycoprotein (Pgp)

The primary approach to understand the MDR phenomenon is the isolation step of cultured cells selected for resistance to anticancer drugs by exposing tumor cell line with increasing concentration of drug and analyzing the survival clone for gene, protein expression or morphological alterations. One of these alterations is the increased expression of cell surface, termed P-glycoprotein (Pgp), a 170 kD ATP dependent membrane transporter that acts as a drug efflux pump (Figure 3) [3]

Naturally, MDR mediated by Pgp do not necessary occur only after a single drug treatment. It is connected with the type of cell or the localization of cell in an organism. The cancer cell derived from normal tissues which have high Pgp expression can resist to the toxic effect of chemotherapeutic drugs at the first given, that is called intrinsic MDR. Acquired drug resistance can arise or develop during chemotherapy.

Not only the evidence in support of the rate of Pgp overexpression in MDR *in vitro*, the clinical relevance of MDR1 gene expression has been reported [29,30]. Clinical studies have demonstrated a correlation between MDR1 expression and chemoresistance in many intrinsically resistant tumors and acquire resistance during chemotherapy as shown in Figure 4. Furthermore, several reports have suggested that Pgp positivity is associated with aggressive tumor behavior [31,32]. Thus, the expression of Pgp appears to be prevalent in many type of tumors and be induced rapidly after chemotherapy, indicating its role in multidrug resistance phenotype.

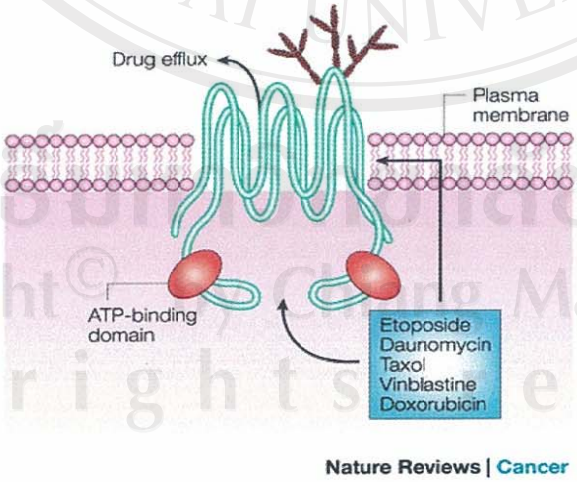

Figure 3. Pgp function in the plasma membrane of a cancer cell during chemotherapy [33]
Activation of the efflux pump by ATP hydrolysis drive the cytotoxic drug out of the cell.

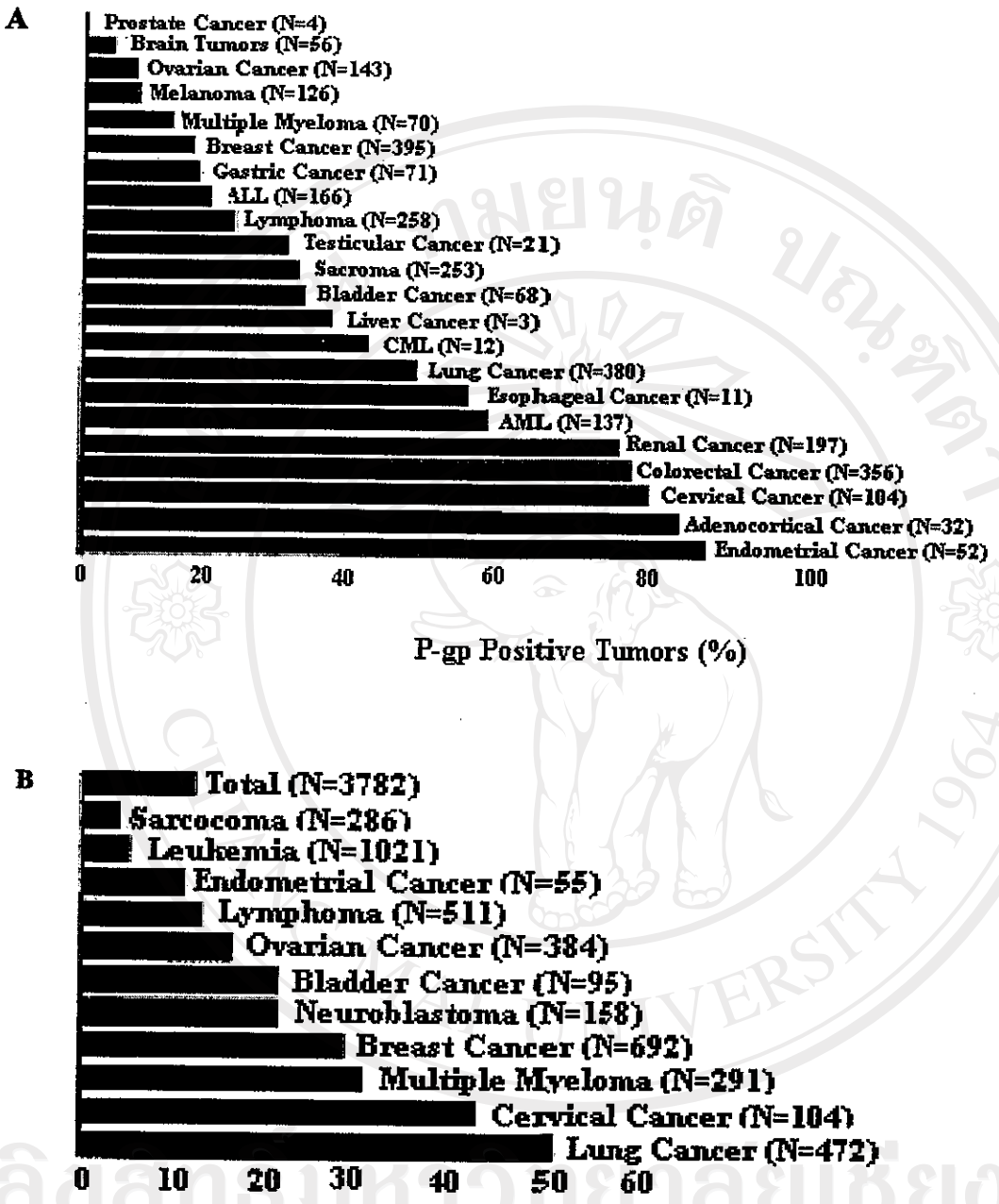

Figure 4. Pgp expression in tumors prior to receiving chemotherapy and after therapy [34]

a. MDR1 expression in tumors obtained from patients prior to receiving chemotherapy.

b. Comparison of MDR1 expression in tumor pre- and post-chemotherapy. This represents in increase of MDR1 expression in treated tumors.

1.2.3 Pgp gene family

The human MDR gene is localized in chromosome7, band q21.1 [35]. Genetic analysis has also revealed the existence of mdr gene in other mammalians such as mouse and hamster. Members of the mdr gene family from different species can be categorized into 3 classed based on sequence similarity of the 3' untranslated regions [36] (Table 2).

ClassI consists of MDR1 gene in human, the genes that correspond to human classI are mdr3 (classI) and mdr2 (classII) in mouse, pgp1 (classI) and pgp3 (classII) in hamster, all encode for multidrug transporter. In human, there is evidence for a second MDR gene, designated MDR3 gene. Smit et al. observed a disruption in the mdr2 gene which is analogous to the human MDR3 gene and found that development of liver disease that was apparently the result of the inability of the liver to secret phospholipid into bile [37], suggesting that mdr2 gene may involved in phospholipid transport.

MDR can occur both due to alteration of MDR1 gene expression and to increase in a gene amplification of the extrachromosomal DNA segments [38,39]. Transcription of MDR1 gene is increased due to very different influences such as anticancer drug, carcinogens, heavy metals, ultraviolet light and heat shock [40].

Table 2. Nomenclature of multidrug resistance genes [40]

	Designation		
	ClassI	ClassII	ClassIII
Human	MDR1	-	MDR3
Mouse	mdr3	mdr1	mdr2
Hamster	Pgp1	Pgp2	Pgp3

1.2.4 Tissue distribution and overexpression of Pgp in cancer cell

Pgp is expressed in different tissues differently from very low level of expression up to high levels. High levels of expression have been found in human adrenal cortical cells, brush border of renal proximal tubule epithelium, the lumenol surface of biliary hepatocytes, small and large intestinal mucosal cells and pancreatic duct [41]. Subsequently, Pgp was also found in capillary endothelial cells of brain [42] and hematopoietic stem cells [43].

The normal tissue specificity of Pgp expression raises a question of its physiological function in the organism. High level expression in the adrenal gland suggests a role in steroid secretion or

protection of the membrane of steroids – secreting cells [44]. In kidney and liver, Pgp is present on the brush border and biliary face, respectively of proximal tubule cells and hepatocytes, consist a role for Pgp in excretion of xenobiotics and endogenous metabolites into the urine and bile. The development of the mdr (-/-) knockout mouse has shown the barrier function, limiting the penetration of toxic substance into the brain [45].

In cancerous cell, Pgp is always expressed in many intrinsically resistance tumor during the course of or after the chemotherapy. Furthermore, rapid activation of mdr gene expression has been observed in metastatic pulmonary carcinoma within an hour of the patient received treatment with doxorubicin [29]. Grogan et al. has demonstrated a correlation in between Pgp expression and prior chemotherapy in multiple myeloma patients. A series of 106 consecutive bone marrow specimens from 104 myeloma patients were detected for P-gp expression. Myeloma patients with prior chemotherapy had a low incidence of P-gp expression (6%), while those received chemotherapy had a significant higher incidence of P-gp expression (43%) [46]. Thus, Pgp expression may represent as two – edged sword due to their elimination of anticancer durgs as well as xenebiotic detoxification. The level of Pgp expression in human tissue is summarized in Table 3.

Table 3. Cellular localization of Pgp in tissues, which are important for drug disposition and effects [47]

Tissue	Localization	Function
Small intestine	Apical (luminal) membrane of epithelial cells	Secretion of drugs into gut lumen
Liver	Canalicular membrane of hepatocytes	Secretion of drugs into bile
Kidney	Apical membrane of epithelial cells of proximal tubules	Secretion of drugs into tubules lumen
CNS	Luminal membrane of endothelial cells forming the blood-brain barrier	Protection of CNS from xenobiotics
Testis	Endothelial cells of capillary blood vessels	Blood-testis barrier
Placenta	Trophoblasts	Protection of fetus from xenobiotics

1.2.5 Structure of Pgp

Mammalian P-glycoprotein is a large transmembrane glycoprotein with approximately 170 kD molecular weight and consist of 1280 amino acid residues, Pgp is composed of two homologous halves, each containing six transmembrane domains and an ATP binding, utilizing domain, separated by a flexible linker region (Figure 4).

The glycosylation site is in the first extracellular loop [48,49,50], but the precise composition of the carbohydrate moiety of Pgp remains unknown. The Pgp lacking N-glycosylation sites yielded drug resistance transfectants with much lower efficiency than the wild type. Based on this observation, it was proposed that the carbohydrate moiety may contributes to the correct folding, proper routing and stabilizing within the plasma membrane [51].

The study of Pgp mutants has been reported to help elucidate the functional unit of the Pgp molecule. The mutation sites in mammalian Pgp that affect substrate specificity are predominantly in the transmembrane domains, mainly 5, 6 and 11, 12. However, they are also found throughout the rest of the molecule [52]. Replacement of nonfunctional regions to the last transmembrane region (TM12) markedly impaired resistance to actinomycin D, vincristine and doxorobicin but not to colchicine. In contrast, replacement of the transmembrane loop between TM11 and TM12 appeared to create a more efficient drug pump for actinomycin, colchicine and doxorubicine but not for vincristine [53]. The mutations of Pgp molecules lie within the ATP binding utilization domains suggested that both nucleotide binding domains are essential for the proper function of Pgp [54,55,56]. Moreover, seperately expression of cDNA encoding the carboxy-terminal half molecules of human MDR, in Sf9 cells revealed that each half molecule had basal ATPase activity but drug stimulated ATPase activity was not present until the wild type human MDR, were expressed. [57]

Taken together, the two halves of human, Pgp molecule was believed to interact to form a single transporter and the major drug binding domain residue in or near transmembrane domains 5, 6 and 11, 12. Both ATP binding sites are necessary for a functional molecule, in fact, interaction between the ATP binding sites and the drug binding domain is essential for drug transport.

This ATP-dependent transporter exports a wide variety of structurally unrelated compounds such as vinka alkaloids, antibiotics, anthracyclines, etoposides and steroids as shown in Table 4.

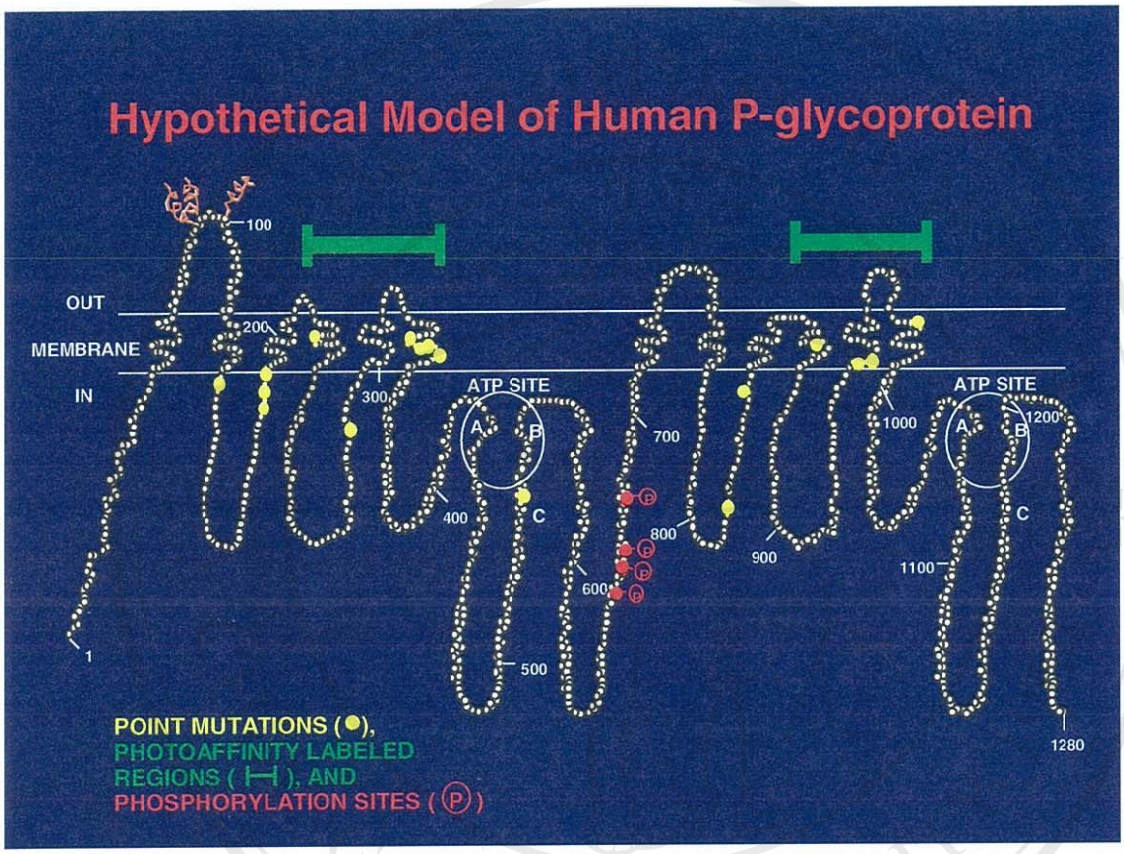

Figure 5. Two-dimensional hypothetical model of human P-glycoprotein structure based on a hydropathy plot analysis of primary amino acid sequence [52]. The ATP binding/utilization domains are circled with the Walker A, B, and 'linker dodecpeptide' or 'signature sequence' (LSGGQ) motifs are designated by the letters 'A', 'B' and 'C'. Putative glycosylation sites are represented by squiggly lines. The regions known to bind photoaffinity drug analogues are designated by heavy dark bars and serine residues that known to be phosphorylated are shown as darkened circles with an attached an encircled 'P'. Each circle represents an amino acid residue. The full circles show many of the positions of mutations that change substrate specificity in human Pgp.

Table 4. Compounds which interact with Pgp [58]

Anticancer drugs	Other cytotoxic agents	MDR reversing agents	Cyclic and linear peptides
Daunorubicin	Colchicine	Verapamil	ActinomycinD
Doxorubicin	Emetine	Nifedipine	GranicidinD
Mitoxantrone	Ethidium bromide	Azidopine	Valinomycin
Etoposide	Pauromycin	Quinidine	Yeast a-factor
Teniposide	Mithramycin	Amiodarone	pheromone
Vinblastine		Reserpine	N-acetyl-leucyl-
Vincristine		Cyclosporine A	norleucine
Mitomycin C		FK 505	
Paclitaxel		Rapamycin	
Actinomycin D		Progesterone	
Topotecan		Forskolin	

1.2.6 The mechanism of action of Pgp

Several mechanisms explaining the increased influx or/and decreased accumulation resulting in reduction of accumulation of drug in the resistance cell have been proposed. The hypothesis of mechanism of Pgp has two major features that are Pgp itself acts as a drug transporter or affects drug accumulation in MDR cell. Another is an indirect mechanism. Consistent with the hypothesis of indirect involvement of Pgp, several studies have indicated that the pH in the cytosol is often increased and the electrical membrane potential is often reduced in MDR cells when compared with drug-sensitive parental cells [59,60,61]. Most of the known Pgp substrates are weakly basic and positively charged at physiological pH, thus an increase of the intracellular pH or a decrease of the negative internal electrical membrane potential would reduce intracellular retention of these cationic compounds [62]. However, conversely studies have indicated that the effect of the reversal of MDR did not correlate with the alteration of the intracellular pH [63].

Additionally, purified Pgp reconstituted into phospholipid vesicles is capable of drug transporting even in the absence of electrochemical gradients [64, 65]

The most widely accepted model of Pgp function that acts as an active transporter using energy provided by ATP hydrolysis for the transmembrane translocation. This model predicts the substrates (cytotoxic drugs) bind to the specific domain which subsequently undergoes an energy dependent conformation change, allowing the substrate to be released [3, 52].

Building upon the Vi – induced trapping and chemical modifications at the ATP site, the catalytic cycle of ATP hydrolysis by Pgp has been proposed [66]. The essential features at the cycle are illustrated in Figure 6. The drug and ATP first bind to Pgp without energetic requirement. The prior binding of ATP is not essential for drug interaction with Pgp. Thus, ATP binding could precede, follow, or accompany the binding of drug. The hydrolysis of ATP is accompanied by a large conformational change that drastically reduces the affinity of both drug and nucleotide. Following hydrolysis, ADP is spontaneously released. The dissociation of ADP is accompanied by a conformational change that allows nucleotide binding but not substrate binding. A second ATP hydrolysis is initiated which is kinetically indistinguishable from the first. The subsequent release of ADP completes one catalytic cycle, bringing the Pgp molecule back to the original state where it can bind both substrate and nuclectide to initiate the next cycle.

Complementary models also have been proposed, suggesting that Pgp directly interact with the drug, flipping drug from the inner leaflet to the outter leaflet, know as flippase model [67].

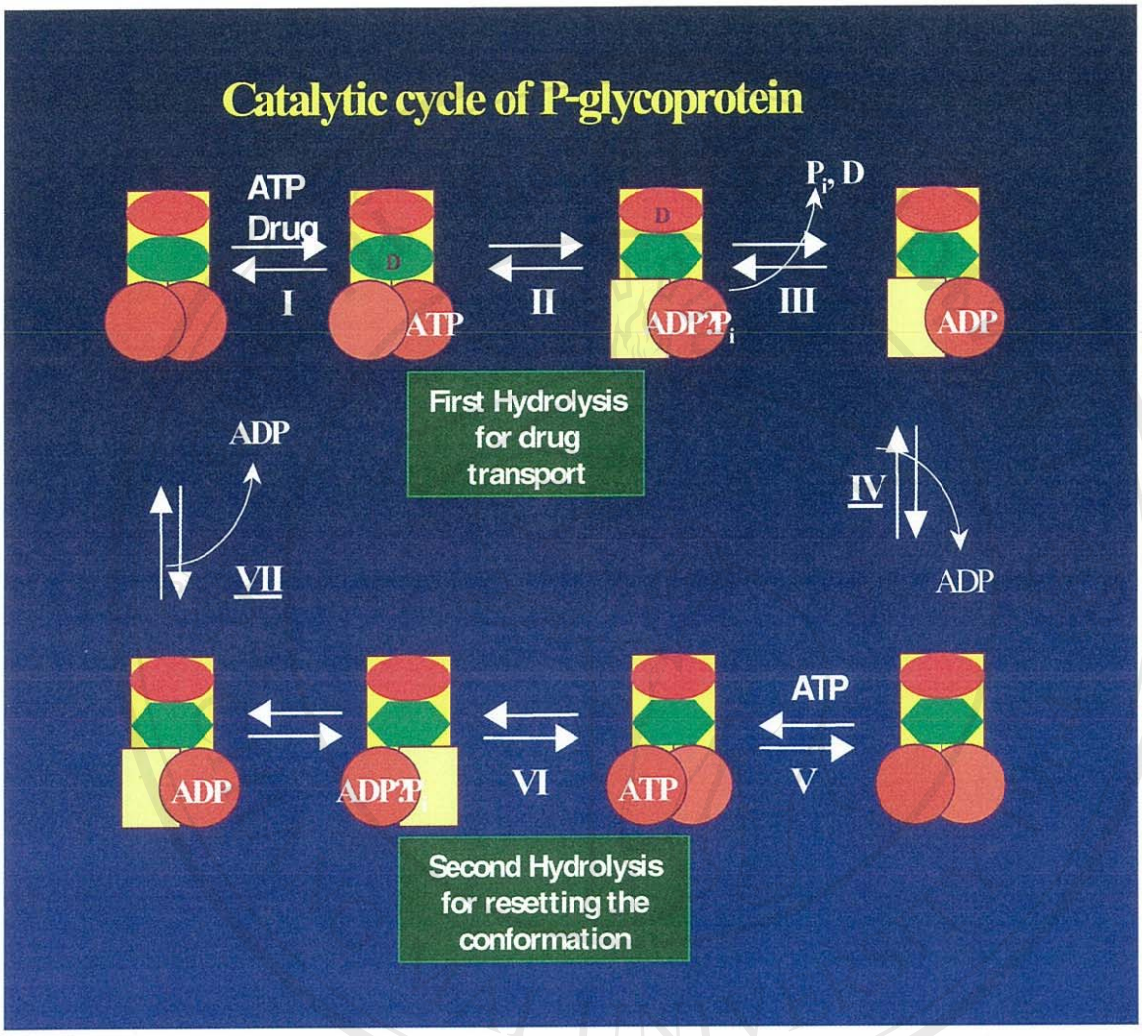

Figure 6. A proposed scheme for the catalytic cycle of ATP hydrolysis by Pgp [66]

The ellipses represent the substrate binding sites, the "ON" and the "OFF" site. The hexagon portrays the "ON" site with reduced affinity for the drug. Two circles represent the ATP site and the circles are shown overlapping to indicate that both sites are required for ATP hydrolysis. The empty square represents the ATP site with reduced affinity for nucleotide.

1.2.7 Reversal of Pgp mediated MDR by chemosensitiser

One of the goals of multidrug resistance studies is to find a means of circumventing drug resistance, which is applicable to the clinical setting. The approach receiving the most attention has been the use of MDR modulator to reverse Pgp mediated drug resistance. Base on the observations by Tsuruo et al. who first reported that the clinically used drugs such as verapamil, were able to reverse vincristine resistance in murine leukemia all line [68, 69], a wide variety of compounds representing several drug classes have been shown to modulate P-glycoprotein – mediated MDR in pre-clinical models as shown in Table 5.

The possible mechanisms of action for the ability of chemosensitisers to inhibit the Pgp is shown in Figure 7. In this scenario, Pgp modulator may serve as a competitive inhibitor by occupying drug – binding site or a non-competitive inhibitors by bind to other binding site which causes allosteric changes resulting in inhibition at cytotoxic drug binding or transport [70].

Now widely knowledge about limitation of many early MDR modulator is that they typically reverse MDR at concentration that results in clinical toxicity. Many at the first chemosensitisers were themselves substrates of Pgp, thus high serum concentration at the chemosensitiser was necessary to serve adequate intracellular concentration, leading to unacceptable toxicity.

To overcome these limitations, several novel analogues of the first generation Pgp modulator were tested and developed. Second generation Pgp modulators have a better pharmacologic profile than the first generation, but they still retain some characteristic that limit their clinical usefulness. In particular, these compounds significantly inhibit the metabolism and excretion of the cytotoxic agents. In response to cytotoxic agent, cytochrome P450 enzymes are often induced [71]. It is thought that the genes of cytochrome P450 enzymes share overlapping regulatory elements with MDR gene [72]. In fact, many of the cytotoxic agents that are substrates for P-gp are also substrates for the cytochrome P450 too. The competition between cytotoxic agents and P-gp modulators to cytochrome P450 activity has resulted in unpredictable pharmacokinetic interactions. Inhibition of P-gp will increase concentration, oral absorption and increase central nervous system penetration, in addition, inhibition of CYP will further enhance oral absorption and decrease hepatic metabolism with consequently further increase high plasma concentration and increase toxicity. For example, the cyclosporine-modulated course of doxorubicin resulted in

the significant alteration of doxorubicin disposition and remarkable toxicity compared with non-combination course [73].

Third generation molecules that specifically and potently inhibit P-gp function have been developed by using structure activity relationships to overcome the limitation of the second generation molecules. These agents do not affect cytochrome P450 at relevant concentration, leading no alteration at the pharmacokinetics at the co- administered cytotoxic agents. The third generation P-gp inhibitors currently in clinical development include the anthranilamide derivatives, tariguidar (XR 9576), the cyclospropyldibenzosuberane zosuguidar (LY 335979), laniquidar (R 101933) and the substituted diarylimidazole ONT-039 [74].

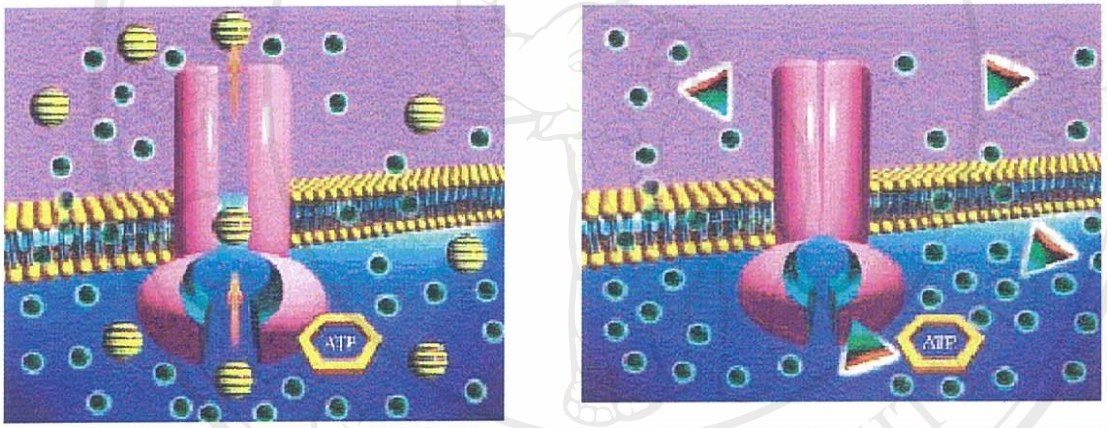

Figure 7. Functional representation of Pgp [74]. The model illustrates a protein, which uses ATP energy to actively efflux drug substrate across the plasma membrane.
a). The MDR modulator competes as a substrate with the drug for transport by the pump. This limits the efflux of the drug, increasing its intracellular concentration.
b). The MDR modulator binds with high affinity to the Pgp but themselves are not substrates. This induces a conformation change in the Pgp, thereby preventing ATP hydrolysis and transport of drugs out off the cell, resulting in an increased intracellular concentration.

Table 5. Selected pharmacological agents with ability to reverse MDR [70]

Calcium channel blockers	**Cyclic peptides**
R-verapamil (5-10 μM)	Cyclosporin A (0.8-2 μM)
Dexniguldpine (0.1-1 μM)	SDZ PSC 833 (0.1-1 μM)
Gallopamil (5 μM)	SDZ 280-446 (0.1-1 μM)
Roll-2933 (2-6 μM)	FK 506 (3 μM)
PAK-200 (5 μM)	Rapamycin (3 μM)
Calmodulin antagonists	**Vinca alkaloid analogues**
Trifluoperazine (3-5 μM)	Vindoline (20-50 μM)
Fluphenazine (3 μM)	Thaliblastine (2 μM)
Trans-Flupenthixol (3 μM)	
Protein kinase C inhibitors	**Miscellaneous compounds**
Calphostin C (250 nM)	GF 120918 (0.02-0.1 μM)
Staurosporine (200 nM)	Tolyporphin (0.1-0.5 μM)
CGP41251 (150nM)	Dipyridamole (5-10 μM)
NPC15437 (60 μM)	BIBW22 (1 μM)
Safingol (20-50 μM)	S9788 (1-3 μM)
Steroidal agents	Terfenadine (3-6 μM)
Progesterone (2 μM)	Reserpine (5 μM)
Tamoxifen (2-10 μM)	Amiodarone (4 μM)
Toremifene (5-10 μM)	Quinidine (10 μM)
Megestrol acetate (5 μM)	Methadone (75 μM)

1.2.8 Green tea

Green tea is one of the most popular and traditional beverages, mainly consumed in Asian countries. Historically, tea has been lauded for various beneficial health effects.

Green tea is produced from the leaves of the plant *Camellia Sinensis.* To make green tea, fresh tealeaves are streamed or dried over the hot pan, which inactivate the enzyme and prevent the oxidation of green tea. Black tea manufacture involves crushing the tea leaves to promote

enzymatic oxidation and subsequent condensation of tea flavonoids in a process known as fermentation, which leads to the formation of theaflavins and thearubigins.

The dried tealeaves contain many functional constitients such as amino acids, flavonoids, purine alkaloids, vitamins and photopigments as reviewed by Horie and Kohata [75]. The major components found in green tea are flavonoids known as catechins which usually account for 30% to 42% of dry weight of solids in 1% green tea water extract [76]. Tea beverage, with 1.25g tea leaves in 100 ml hot water, resulting in tea brew that similar to those consumed by human was measured to estimate consistency of flavonoids in green tea compared with black tea as shown in Table 6.

Flavonoid is formed from the aromatic amino acid, phenylalanine and tyrosine, and malonate [77]. The basic structure is the flavan nucleus, which consists of 15 carbon atoms arranged in three rings (C6-C3-C6), which are labeled A, B and C (Figure 8). The benzene ring A is condensed with a six-member ring (C), which in 2-position carries a phenyl benzene ring (B) are substituent. Ring C can be heterocyclic pyran, which yields flavanols (catechin) and anthocyanidins , or pyrone which yields flavonols [78,79].

Catechins are characterized by di or tri hydroxyl group substitution of the ring B and meta –5,7-dihydroxy substitution of the ring A. The structure of catechin, epicatechin (EC), epicatechin gallate (ECG) , epigallocatechin (EGC) and epigallocatechin gallate (EGCG) are shown in Figure 9.

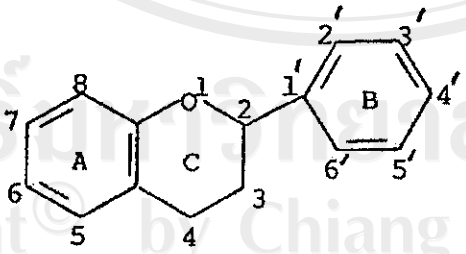

Figure 8. Nuclear structure and numbering system of bioflavonoids [80]

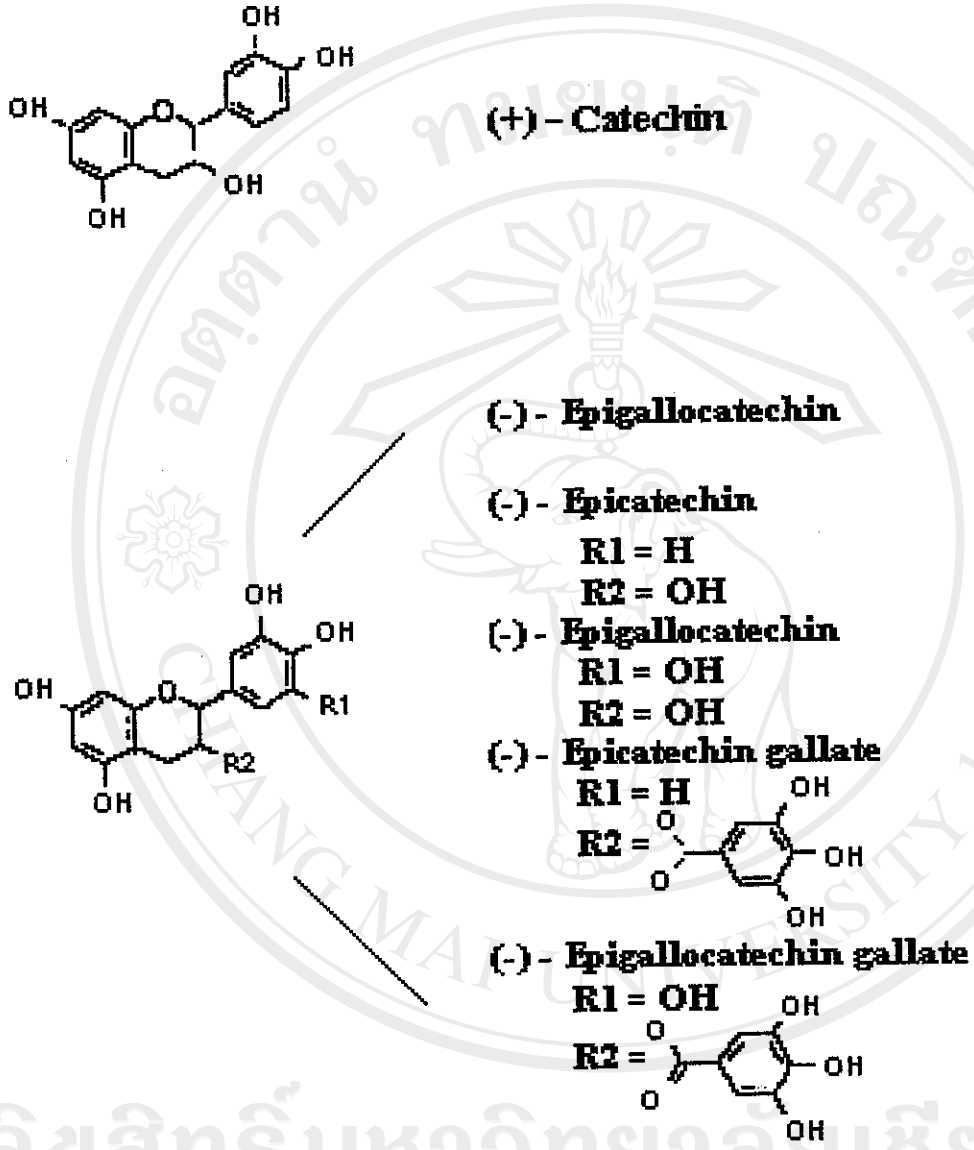

Figure 9. Structure of green tea flavonoids [81]

Table 6. Flavonoids content of 1.25% water extract of green tea and black tea [82]

Tea constituent	Tea (μg/ml)	
	Black tea	green tea
(-)- Epigallocatechin-3-gallate	128	444
(-)-Epicatechin-3-gallate	73	90
(-)-Epigallocatechin	42	411
(-)-Epicatechin	37	98
Catechin	20	21
Total catechins	300	1064
Theaflavin	22	0
Theaflavin-3-gallate	20	0
Theaflavin-3'-gallate	13	0
Theaflavin-3,3'-gallate	9	0
Total theaflavins	64	0
Flavonols and flavonol glycosides	95	101
Undefined polyphenols	1466	589
Total polyphenols	1925	1754

1.2.9 Green tea in cancer

The inhibitory effects of green tea against carcinogenesis and tumor growth have been attributed to the bioactivities of the flavonoids in green tea. The most significant properties of green tea flavonoids that may affect the multistage carcinogenesis, namely tumor initiation, promotion and progression are their antioxidant activities, modulation of carcinogen metabolizing enzyme, trapping of ultimate carcinogens, inhibition of cell proliferation, induction of cell apoptosis and cell cycle arrest [83].

Among the proposed mechanism, the antioxidant theory for cancer prevention by green tea has been widely mentioned. ROS is considered as one of the major biodeterminants in the process of tumor development. ROS arise whenever the cell is involved in oxygen utilization and this

product may be exacerbated by drug, xenobiotics and disease [84]. The concept that green tea flavonoids may inhibit carcinogenesis through their antioxidant property is supported by the finding that oral administration of green tea inhibited the formation of 8-hydroxydeoxyguanosine in mice [85] and EGCG inhibited the formation of 8-hydroxydeoxyguanosine induced by 12-O-tetradecanoyl phorbol-13-acetate in HeLa cell [86].

The chemical structure of green tea flavonoids that contributed to effective antioxidant activity is the dihydroxy or trihydroxy structure, which can chelate metal ions and prevent the generation of free radicals. This structure also allows electron delocalization, conferring high reactivity to quench free radicals [76]. Moreover, the structure of flavanols provides exceptionally strong nucleophillic center at position six and eight. This property enables flavanols to react with electrophillic carcinogen species to form flavanol-carcinogen adduct [83]. Khan WA et al. demonstrated that green tea flavonoids interacted with B(a)P-7,8-diol-9,10-epoxide-2 (BPDE), the ultimate carcinogenic metabolite of B(a)P , supporting that green tea flavonoid inhibited carcinogenesis by trapping the ultimate carcinogen [87].

Another proposed mechanism is that green tea flavonoids inhibit the activation and increase the elimination of carcinogens. Elevation of anti-oxidative enzymes and xenobiotic metabolizing enzymes has been expected to have protective functions against carcinogenesis. Oral administration of green tea flavonoids in mice for 4 weeks has been reported to cause moderate enhancement of glutathione peroxidase, catalase and NADPH-quione oxidoreductase activity in small bowel, lung and liver and glutathione S-transferase activity in small bowel and liver [88]. Thus, the elevation of these anti-oxidative enzymes and xenobiotic metabolizing enzymes may enhance the elimination of carcinogen and reactive oxygen radicals.

Moreover, the inhibition of MAPK and cyclin dependent kinase as well as the suppression of the activation of transcription factor AP-1 and NFKB by EGCG can result in cell cycle arrest, increase in apoptosis as well as inhibition of cell proliferation, cell transformation, tumor invasiveness and angiogenesis [89,90].

Due to its wide range of biological and pharmacological effects, lack of toxicity in animal models, green tea flavonoids were examined in the present study to determine possible interactions with Pgp function and expression.

1.2.10 Isolation and characterization of human MDR KB-V1 cell line [91].

A clonal isolation of KB cells, designated KB-3-1, was used as a parental cell line for drug selection. Ethyl methanesulfonate (EMS) was used to mutagenize KB cells at the initial step of selection (Figure 10). EMS mutagenesis was repeated for the second step of selection with vinblastine. The resistant cells were subcloned at each steps of selection, at the later steps of selection, cell populations were isolated without subcloning. The level of cross-resistance in MDR KB cells increased in parallel with resistance to the selective agent. The cell line selected with vinblastine maintained a relatively uniform level of resistance to vinblastine, doxorubicin and colchicine (Table7).

The MDR KB cells were similar to other MDR cell lines in that they were characterized by decreased accumulation of radiolabeled drugs and increased expression of Pgp, as detected with antibodies C219 and MRK16. The MDR phenotype in KB cell line was dominant, as indicated by the resistance of somatic-cell hybrids formed between MDR KB cells and drug sensitive HeLa D98 cells and by the ability to transfer the resistant phenotype from MDR KB cells to mouse NIH 3T3 cells by transfection with genomic DNA.

Figure 10. Flow diagram showing the steps for increasing vinblastine resistance in the MDR KB-V1 cell line. The parental cell line was KB subline designated KB-3-1. Abbreviations: VBL^R, vinblastine-selected subclones; V, vinblastine-selected populations. In each case, where a letter alone is used, the number following the letter refers to the selecting concentrations of drug in micrograms per milliliter.

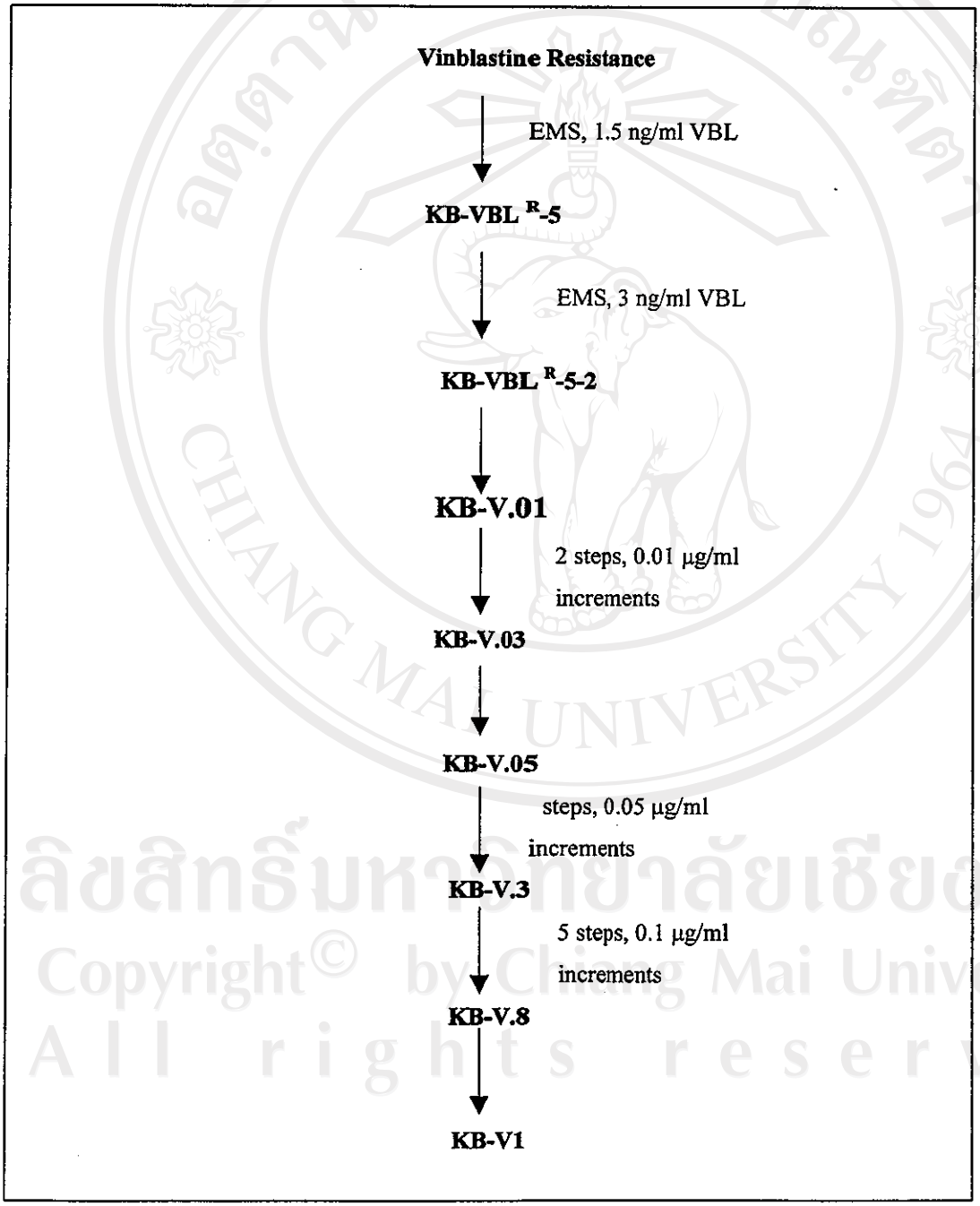

Table 7. Properties of MDR KB cell lines [91]

Cell lines	Relative resistance to		
	Colchicine	Vinblastine	Adriamycin
KB-3-1	1	1	1
KB-VBL ^{R}-5	2	3	2
KB-V1	290	1300	650
KB-V1-R2	1	1	1

Revertant clone, KB-V1-R2, was obtained by culturing resistant lines in the absence of vinblastine.

1.3 Objectives

1. To study the effect of green tea flavonoids on Pgp mediated drug transport in drug resistant human cervical carcinoma cells (KB-V1)

2. To study the effect of green tea flavonoids on Pgp expression in drug resistant human cervical carcinoma cells (KB-V1)

3. To study the effect of green tea flavonoids on the MDR phenotype in drug resistant human cervical carcinoma cells (KB-V1)