CHAPTER I

INTRODUCTION

1.1 Statement and significance of the problem

Cancer is a group of diseases that are all caused by a disturbance in growth metabolism. The origin is, according to general consensus in the field, a combination of exogeneous and endogeneous factors, which step by step lead normal cells along the path of transformation to cancer cells [1, 2]. Cervical cancer is an important public health problem for women in developing countries in South and Central America, South and Southeast Asia, where it is the most or the second most common cancer among women. The majority of cervical cancer cases are caused by infection with certain subtypes of human papilloma virus (HPV), a sexually transmitted virus that infects cells and may result in precancerous lesions and invasive cancer [3-5].

In Thailand, The World Health Organization (WHO) reported that the first most common cancer in females in the year 2000 was cervival cancer (20.12%), before chest cancer (15.48%) and breast cancer (13.78%), respectively [6]. In the cases of cancer treated diagnosed by surgery, radiotherapy and chemotherapeutic drugs. While the surgery and radiotherapy are the conventional method for cancer treatment and result in a cure for some 40% of all cancer patients, the remaining 60% die as a result of metastatic disease [7]. A major advantage of chemotherapy is its ability to treat widespread or metastatic cancer, whereas surgery and radiation therapies are limited to treating cancer that are confined to specific areas [8]. Chemotherapy is useful mostly for disseminated cancers and is the tool of choice to slow down the evolution of several cancers, such as cervical cancer, ovarian cancer, leukemia cancer and testicular cancer. Successful chemotherapy will track and kill all cancer cells while avoiding attacking the healthy cells of one's organism [9-11]. Different natural chemotherapeutic drugs such as anthracyclines (doxorubicin, daunorubicin), vinca alkaloids (vinblastine, vincristine), epipodophyllotoxins (etoposide) and taxanes (paclitaxel) are used for many types of cancer such as cervical, ovarian, liver, kiidney, leukemia and testicular cancer [12].

Cancer cell resistance is considered to be one of the major reasons for failure of chemotherapy for the majority of cancer patients. Some tumors are intrinsically resistant to treatment whereas others acquire resistance with exposure to structurally unrelated drugs. Although chemotherapy is a common choice for treatment of late state of cervical, some tumors develop resistance mechanisms against the cytotoxic effects of anticancer drugs. Several different mechanisms have been proposed to account for multidrug resistance which is a major characteristic of drug resistance encountered in cervical cancer [13].

The development of chemoresistance is a major serious problem in medicinal oncology, limiting the success of chemotherapy in human cancers. In the clinic, one major cause of multidrug resistance (MDR) is the overexpression in tumour cells of P-170 glycoprotein (P-gp) glycoprotein expressed by a broad range of human tumors at presentation or at relapse. The 170 kDa plasma membrane protein encoded by the MDR1 gene (multiple drug resistance gene), acts as an ATP-driven efflux pump for a wide variety of hydrophobic natural products, chemotherapeutic drugs, and various biological active hydrophobic peptides derivative, including proteinase inhibitors (pepstatin), chemoattractants, ionophores (valinomycin, granicidin) and enkephalins [14].

P-gp is found in high level in many chemotherapy-resistant tumors and in low level in chemotherapy-responsive tumors. P-gp is expressed in some normal tissue; however, within the context of cancer chemotherapy, P-gp ``pumps'' certain cytotoxic drugs out of tumor cells. It thereby prevents these drugs from reaching toxic levels and destroying the malignant cells. Because P-gp forces out some cytotoxic drugs, cancers become resistant to certain chemotherapy and renders them unresponsive to this treatment. P-gp-mediated resistance is thus a significant clinical problem that might be mitigated by the development of a drug designed either to prevent P-gp expression in tumor cells or to reverse its effect [15].

In clinical trial attempting to reverse or modulate MDR in many clinically resistant tumors, new drug developed specifically to inhibit P-gp and demonstrated the safety and efficacy of this novel MDR reversal agents (MDR- modulator or pump inhibitors or P-gp inhibitors) that can enhance the efficacy of anticancer agents and have been interested in cancer chemotherapy. Findings from preclinical studies suggest that PSC 833, the specific inhibitor of P-gp, may have the capacity to block the P-gp pump from expelling cytotoxic drugs from tumor cells and, thus, potentially prevent the malignant cells loss of responsiveness to chemotherapy [16].

Considering of novel biochemical modulators from some foods and beverages, many research groups are interested in the effect of several natural products, plant-derived chemical on the activity of P-gp. Many groups have studied the beneficial effects of natural products, plant-derived chemicals in cancer prevention or treatment because plants have been an exemplary source of drugs and many of the currently avaible drugs have been derived directly or indirectly from them. For example, bitter melon (*Momordica Charantia*), a commonly used herb have been previously shown to have potent antitumor activity [17, 18], and shown other biological activities that are potentially useful for clinical uses, such as antioxidant activities [19], anti-viral (HIV-inhibitor) [20], anti-diabetes [21, 22], trypsin and elastase inhibitors [23] and immunomodulating properties [24].

Recently, it has been reported that bitter melon ingestion in cervical cancer patients with radiotherapy did not affect natural killer (NK) cell, B lymphocytes and NKT cell level. However it was effective on the maintenance of T lymphocyte level. Bitter melon affects the decrease of P-gp level on NK cell, B cell and lymphocyte membrane. Taken together, bitter melon intake may not benefitial to cervical cancer patients who were treated with radiation. It may be good for coordinating with chemotherapy and should be investigated before used [25].

Because of the clinical importance of drug efflux mechanism for multidrug resistance and cancer treatment, the modulating properties of bitter melon on P-gp activity was investigated. Due to multidrug resistance phenotype, KB-V-1 human cervical carcinoma cells have been shown to expressed only P-gp at high level on their plasma membrane, but P-gp was not expressed in the drug sensitive KB-3-1 cells

Therefore, the effects of extracts from various parts of bitter melon on vinblastine accumulation and efflux across P-gp in P-gp expressive KB-V-1 cells and non P-gp expressive KB-3-1 cells were taken as a measure for the modulation of the P-gp efflux activity.

1.2 Literature reviews

1.2.1 Drug resistance

The presence or development of resistance to anticancer drugs is the main cause of failure of chemotherapy in the majority of the most common forms of cancer (e.g. lung, colon, breast and cervix). Resistance to chemotherapeutic drugs can be already present at diagnosis or it can develope after chemotherapy treatment. These two forms of drug resistance are respectively called intrinsic and acquired resistance [26]. The development of drug resistance cells is shown in Figure 1.

Intrinsic resistance or de novo resistance of cancer cells can be present before chemotherapy resulting in initial treatment failure such as Hodgkin's disease, testicular cancer and acute childhood leukemia but acquired resistance can develop in response to chemotherapeutic intervention eventually leading to early disease progression despite and initial treatment response for example, lymphoma and breast cancer [27].

Multidrug resistance (MDR) is the protection of a tumor cell population against numberous drugs with different chemical structure and mechanisms of action. Multidrug resistance cell lines can be derived by in vitro selection with a single lipophilic cytotoxic drug such cells show cross-resistance to many other compounds with different mechanisms of cytotoxicity including anthracyclines (doxorubicin, daunorubicin), vinca alkaloids (vincristine, vinblastine), epipodophyllotoxins (etoposide) taxanes (paclitaxel), colchicine, and some other drugs [28, 29]. All of the MDR cell lines displayed a similar pattern of resistance, suggesting a common underlying mechanism , a decreased intracellular accumulation of the cytotoxic agent. The cytotoxic agent resulted from the action of an adenosine triphosphate (ATP) dependent efflux pump, P-170. The degree to which the drug is pumped out of the cell depends upon the level of expression of P-gp in the cell lines and on the affinity of the substrate for it [30].

The mechanisms with clinical significance are a) action of transmembrane proteins effluxing different chemical substances from the cells; b) action of the glutathione detoxification system; c) alterations of the genes and their products that involved in the control of apoptosis (especially p53 and Bcl-2). The main steps of cell injury from the drug uptake by program cell death (apoptosis) was shown in table 1. Most chemotherapeutic drugs induced apoptosis. The simplified list of cell alterations resulting in cell death is indicative of the ability of cells to disrupt the pathway of injury at any step. This list demonstrates the diversity of the mechanisms of cellular resistance to drugs.

Table 1. Main mechanisms of drug resistance of tumor cells [31]

No.	Steps in the cytostatic action of drugs	Alterations resulting in drug resistance	Mechanism of drug resistance (example)
1	drug uptake by the cell ↓	decrease of drug accumulation by the cell	activation of transporter protein (P-gp, MRP, MXR, etc.)
2	activation or preservation of activity of a drug in the cell ↓	detoxification of the drug or inability of drug-activating systems	activation of the enzymes of the glutathione system, sequestration of the drug in intracellular vesicles
3	damage to drug target ↓	alterations of drug target, increased repair of the damaged target	mutations of the genes coding for topoisomerases, enhancement of DNA repair
4	arrest of cell cycle and/or death of the cell *	abrogation of apoptosis or cell cycle arrest: alterations of the genes controlling apoptosis	mutations of p53 gene, activation of Bcl-2 gene

The human *MDR1* gene product, P-gp, was the first ATP-dependent system discovered that was implicated in multidrug resistance. The over expression of P-gp is not the only cause of MDR. Another member of the ATP-binding cassette (ABC) super family, which is involved in MDR, is the 190 KDa multidrug resistance associated protein (MRP1), encoded by the MRP1 gene. MRP1 is simlar to P-gp in that it is capable of decreasing intracellular levels of drugs, and is ATP-dependent. The most recently discovered ABC drug efflux transporter is BCRP (breast cancer resistance protein or ABCP). BCRP, 655-amino acid protein that constitutes a half-transporters, consists of only a single N-terminal intracellular ATP binding site, followed by 6 putative transmembrane segments [32]. The transport proteins that may involved in mdr were shown in Figure 2.

The terms used in this article refering to particular MDR mechanisms and phenotypes was shown in Table 2. The term "MDR" as used herein refers to a phenotype of simultaneous resistance to multiple agents with different structure (but not necessarily function), without implying any particular mechanism. If applicable, these are specified by prefix, as in Pgp-MDR or topoII-MDR. The terms "apoptosis-MDR" and "clinical MDR" are also used herein. Alterations in apoptosis pathways have been shown involve in resistance to a variety of cytotoxic agents. Thus, it seems appropriate to refer to apoptosis-related chemotherapy resistance as a type of MDR.

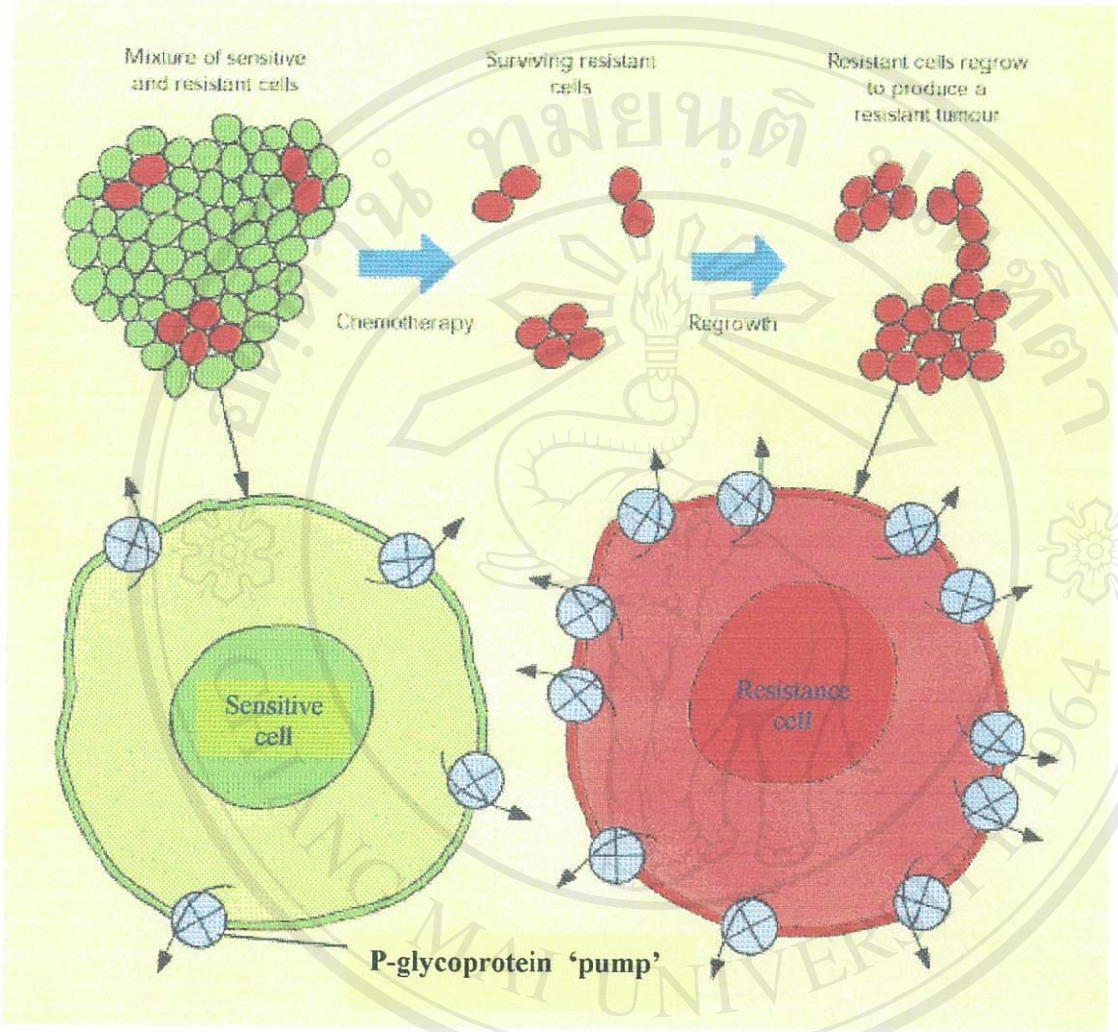


Figure 1. The resistance development during cancer chemotherapy [33]. When cancer chemotherapeutic drug is given, most of the sensitive cells will be killed, but the resistant ones that carry the resistance genes will survive. At the end of the treatment, these resistant cells will begin to grow again to develop into a tumor that is mostly composed of resistant cells and therefore less likely to respond to chemotherapy.

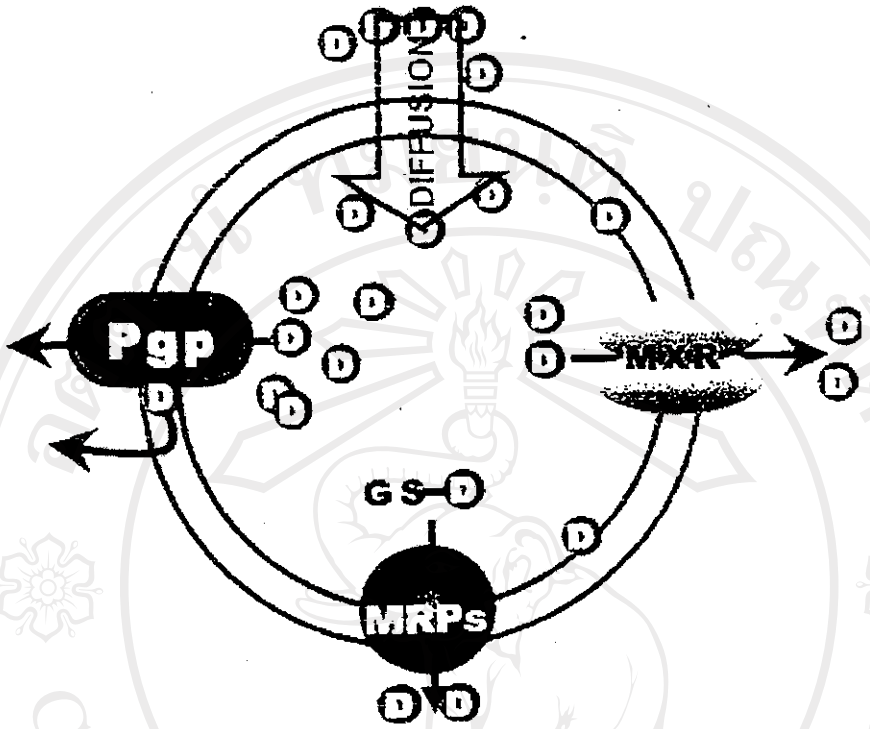


Fig. 2. Role of ABC transporters in the development of the MDR phenotype in cancer cells [34]. Cancer cells show resistance to cytotoxic agents via one or more of the several mechanisms. Most natural hydrophobic drugs (D) enter the cell by diffusion. These may be pumped out by P-gp using the energy of ATP hydrolysis, entering from the cytoplasm or from the membrane phase itself before reaching the cytoplasm. Drug complexed with glutathione (GSH) may also be transported out of cell by MRPs (MRP1-4). The half-transporter MXR (or ABCP or BCRP) also efflux drugs in an energy-dependent manner, with the dimer possibly being the functional unit. Besides these ATP-dependent transport system, the cells may also acquire resistance by a number of intracellular mechanisms such as intracellular compartmentalization, metabolic degradation, altered cell cycle, and increased DNA repair.

Table 2. The various mechanisms and phenotypes of MDR [35]

Term	**Mechanism**	**Characteristics**
Pgp-MDR	Overexpression of *MDR1*/Pgp	- Resistance to natural products of structuraal and functional difference reduced drug accumulation due to enhanced drug efflux. - Can be reversed by chemosensitizers such as verapamil or cyclosporins.
MRP-MDR	Overexpression Of MRP	- Phenotype similar to Pgp-MDR but less resistance to taxanes; changes in cellular pharmacology variable.
Topo II-MDR	Diminished content or activity of topoisomerase IIα	- Resistance to topoisomerase II drugs (i.e. drugs with different structure but not function).
GSH-MDR	Increased content of GSH and/or increased activity of GSH S-transferase	- Resistance to melphalan, BCNU, cyclophosphamide, chlorambucil, thiotepa (and possible other drugs such as cisplatin and doxorubicin). - Increase 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 with structural and possibly functional difference.

1.2.2 P-glycoprotein gene family

The mammalian P-gps are highly conserved group of ABC transporters found as multigene families. P-gps genes from hamster, mouse, rat, and human have been cloned and sequenced, and P-gp homologues have been identified in several other species. Two highly homologous members have been identified in human, three in mouse, three in hamster, and three in rat. The *mdr* gene families are localized in a cluster on chromosome 7q21.1 in human, on chromosome 5 in mouse, and chromosome lq26 in hamster [36].

P-gps are encoded by a small multigene family (*mdr* class I, II ,and III). All three isoforms are present in rodents (mouse, rat and hamster), while humans express only the class I and II. Transfection studies have demonstrated that the class I and II isoforms can confer *mdr*, while the class III isoform is a PC (phosphatidylcholine) translocase, or flippase, responsible for export of this phospholipid into the bile [37].

Class I P-gps are located in the plasma membrane at the cell surface consistent with their function as drug-transpoters(or multidrug transporters) which include the human *mdr1*, the mouse *mdr1a* (or *mdr3*) and *mdr1* (or *mdr1b*), and the rat *pgp1* and *pgp2* (or *mdr1b*) gene products. Class II P-gps (sometimes also referred to as class III) are present in plasma membranes of various specialized cells in normal tissues, includes the non-drug-transporting P-gps, such as the human *mdr3/2*, the mouse *mdr2*, the hamster *pgp3*, and the rat *mdr3/mdr2* gene products (Table 3).

The *mdr1a* and *mdr1b* knockout mice are an extremely powerful tool to explore other putative functions of P-gp. For example, the high level expression of P-gp on the mucosal surface of small and large intestinal epithelial cells suggests that it may block absorption of substrate xenobiotics given orally and, indeed, most of the anticancer drugs which are substrates for P-gp must be given intravenously as they are not absorbed after oral dosing [38].

Studies in *mdr1a* (-/-)knockout mice have demonstrated increased sensitivity to as well as increased concentration of xenobiotics such as invermectin, vinblastine, cyclosporin A and digoxin. Indeed, twenty to fifty fold higher brain concentrations of cyclosporin A and digoxinare were found in *mdr1a* (-/-) knockout mice. Gene products of other multidrug resistance gene family member do not play a role in the drug resistance phenotype. These include human mdr3 (mdr2 in rodent), which

encodes for a phospholipid transporter and SPGP (the sister gene of P-glycoprotein),which encodes for a hepatic bile salt transporter [39].

1.2.3 P-glycoprotein expression in normal tissue

P-gps are encoded by a small family of closely related genes with two members in humans (*MDR1* and *MDR2*) and three in rodents (*mdr1*, *mdr2*, and *mdr3*) which are expressed in a tissue-specific manner.The human *MDR1* gene has been evidenced in several human normal tissues, including the liver, proximal tubules of the kidney, the biliary canaliculi, pancreatic ducts, small and large intestine, colonic epithelium, bronchial mucosa, prostatic epithelia, ovarian follicles, and pregnant uterine epithelium (Table 4).

Expression of P-gp in these normal tissues is believed to be a protective mechanism against xenobiotics (protection of vital organs against toxic products), excretion and detoxification [40]. In mice, *mdr1*(Class I) is expressed mostly in the adrenal cortex, kidney, placenta, and gravid uterus, *mdr2* (Class II) in the liver, spleen and kidney, as well as the marine adrenal gland and heart and *mdr3* (Class III) in the intestine and brain. The level of *mdr* gene expression can be modulated by a number of treatments, including exposure to carcinogens, anticancer drugs and differentiating agents [41].

In all of these organs, P-gp is localized at the luminal surface of epithelial cells, suggesting that the pump may have a physiological role in elimination of xenobiotics or some endogenous metabolites. P-gp is also expressed by the endothelial cells at blood-tissue barrier sites, such as the blood brain barrier and may protect the brain from circulating xenobiotics, including anticancer drugs. Some peripheral blood mononuclear cells, such as cytotoxic T lymphocytes and natural killer cells, also express P-gp, suggesting that P-gp may be involved in cell-mediated cytotoxicity [29].

1.2.4 P-glycoprotein expression in human tumors

P-gp has been evidenced in a wide variety of cancers. P-gp expression is usually high and constitutive in tumors that arise from tissues known to physiologically express the pump, such as carcinoma of the colon, kidney, adrenal gland, pancreas, and liver. P-gp expression has been correlated with treatment failure and poor prognosis in several types of cancers. There are two human P-gps. The class I protein encoded by the overexpressed, rather than by the amplified, *MDR1* gene confers multidrug resistance in human cancers. The class II protein is rare except in certain B-cell malignancies in which its role is unknown. Some cancers (renal and colon) are constitutively highly positive for P-glycoprotein. Other cancers (lung, myeloma, breast, ovary, lymphoma, acute myeloblastic leukaemia, acute lymphoblastic leukaemia and chronic myelogeneous leukaemia) are more frequently positive for P-glycoprotein at relapse than at diagnosis [42].

After the *MDR1* gene was cloned and found to be expressed in specific normal tissues, several investigators analysed human tumors for *MDR1* gene expression. Fojo *et al.* found that untreated adenocarcinomas from tissues that normally expressed the *MDR1* gene overexpressed *MDR1* RNA. Other untreated malignancies including colon cancer, renal cell carcinoma, hepatoma, adrenocortical carcinoma, phaeochromocytoma, islet cell tumors of the pancreas, and carcinoid tumors frequently express high levels of the *MDR1* transcript (Table 5). Clinically, these tumors are resistant to chemotherapy and many are derived from tumors that normally express the *MDR1* gene. A plausible explanation for the intrinsic resistance of these tumors is that their tissues of origin are highly expressive of the *MDR1* gene, which is conserved in these tumors.

Table 3. P-glycoprotein gene nomenclature [43, 44]

Class	Human	Hamster	Mouse	
			Scheme A	Scheme B
I	*MDR1*	*Pgp1*	*mdr3*	*mdr1a*
II		*Pgp2*	*mndr1*	*mdr1b*
III	*MDR2/3*	*Pgp3*	*mdr2*	*mdr2*

Table 4. Normal tissues which express P-glycoprotein [45]

Liver: lumenal surface of bile canaliculi
Kidney: lumenal surface of proximal tubules
Colon and small intestine: mucosa lining lumen
Adrenal cortex
Pancreas: ducts
Capillary endothelium of:
- CNS (blood-brain barrier)
- Testis (blood-testicular barrier)
- Placenta

Reactive mesothelial of malignant pleural and peritoneal effusions
Haematopoietic cells
Macrophages
Activated T-cells
Pluripotent "stem cells" (CD34+)

Table 5. Expression of the MDR1 gene in human tumors [27]

A. High expression of the *MDR1* gene in untreated tumors	
Colon	Renal
Hepatoma	Adrenocortical carcinoma
Pheochromocytoma	Pancreatic carcinoma
NSCLC-NE	Carcinoid
Multiple myeloma	CML-Blast Crisis
B. Occasionally high expression of the *MDR1* gene in untreated tumors	
ALL (adult)	AML (adult)
Non-Hodgkin's lymphoma	Neuroblastoma
Astrocytoma	CLL
C. Low or no expression of the *MDR1* gene in untreated tumors	
Breast	Mesothelioma
NSCLC	Ovarian
Bladder	Prostate
CML-Chronic phase	Sarcoma
Oesophageal	SCLC
Gastric	Thymoma
Head and neck	Thyroid
Melanoma	Wilms'
D. High MDR1 gene expression in tumors relapsing after treatment	
Non-Hodgkin's lymphoma	Neuroblastoma
CML-Blast Crisis	ALL (adult)
ANLL (adult)	Multiple myeloma
Breast	ALL (childhood)
Cervix	Ovarian
Phaeochromocytoma	CLL

CML, chronic myelocytic leukemia; SCLC, small cell lung cancer; NSCLC-NE, non-small cell lung cancer with neuroendocrine properties; ALL, acute lymphoblastic leukemia; ANLL, acute non-lymphocytic leukemia; AML, acute myeloblastic leukemia; CLL, chronic lymphocytic lekemia.

1.2.5 The structure of P-glycoprotein

P-glycoprotein (P for drug Permeability) is a member of the highly conserved super family of ATP-binding cassette (ABC) transporter proteins. It is a 170 kDa plasma membrane protein encoded by the *MDR1* gene in human, located on chromosome 7 and consist of 28 exon. P-gps are single polypeptide chain consisting of 1280 amino acid residues span the plasma membrane 12 times and has been shown to consist of two homologous halves [36, 46]. P-gps are composed of 43% sequence homology, between the two parts there is a hydrophobic, membrane associated domain (approximately 250 amino acid residues) followed by hydrophilic nucleotide binding fold domain (approximately 300 amino acid residues). These two parts are connected by a linked peptide of approximately 75 amino acids defined as amino acids 633-709 in human P-gp. This peptide conjugated, commonly called the linker region, is highly charged and contains the *in vivo* sites of phosphorylation [47].

The C-terminus of each half contains the sequence for a nucleotide-binding site, these two putative nucleotide-binding regions are located intracellularly which is consistent with the postulated ATP-dependent transport activity responsible for ATP-binding and hydrolysis. Both nucleotide binding sites of P-gps are necessary for transport of substances out of cells [48].

A general topological model of the membrane associated P-gp is presented in using the human *MDR1* gene product as sample was presented in figure 3. According to this model, both the N-terminal membrane associated domains and the C-terminal membrane associated domains harbour six predicted transmembrane (TM) regions. The N- and C-terminus, as well as the nucleotide binding folds domain, are located intracellularly. The first extracellular loop is glycosylated and displays 3 putative glycosylation sites in a region that appears to lie in the first extracellular loop of the protein; it seems unlikely that glycosylation affects the function of P-gp. This twelve TM region model of P-gp is supported experimentally by cellular epitope localization data obtained from antibodies that specifically recognize the N- or C-terminus of P-gp, its first and fourth extracellular loop, or the two ATP-binding sites [49].

The two halves of P-gp are essential for activity of the transporter as measured by its ability to confer drug resistance or drug-stimulated ATPase activity. Both TM domains 5, 6 and 11, 12 and the extracellular loops connecting them were determined by photoaffinity labeling with P-gp substrate analogs to be a major sites of drug interaction. These TM domains are important determinants of drug binding site(s), but do not offer any insight as to whether these sites are autonomous or interdependent [50].

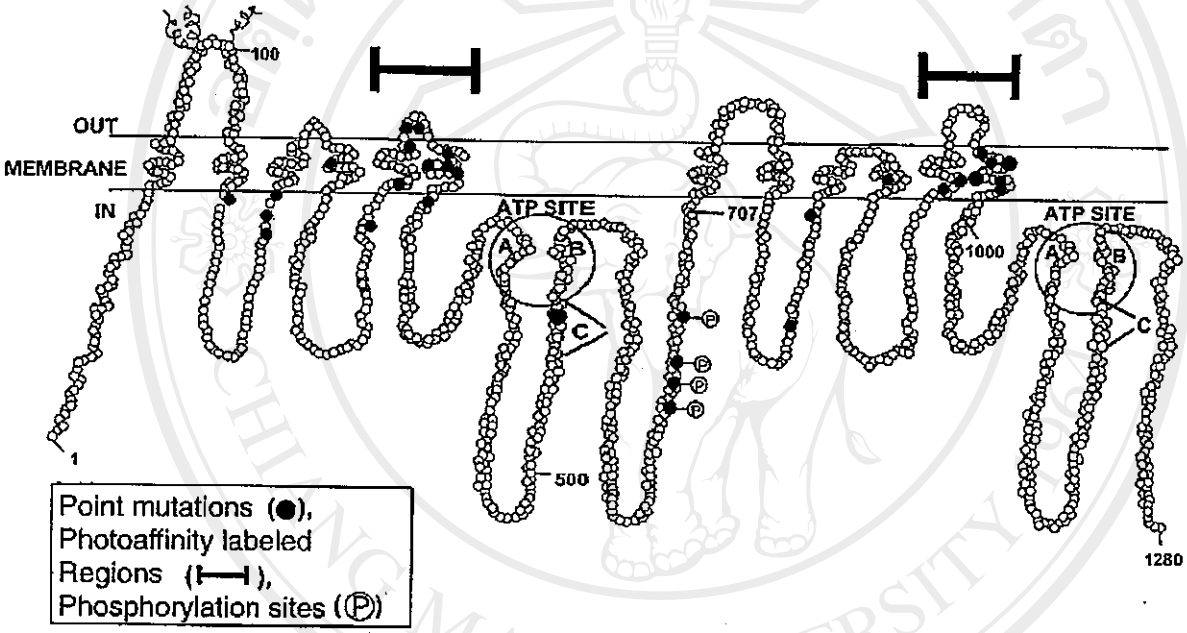


Figure 3. Two-dimensional hypothetical model of human P-glycoprotein structure based on a hydropathy plot analysis of primary amino acid sequence [51]. The ATP binding/utilization domains are circled with the Walker A, B, and 'linker dodecapeptide' or 'signature sequence' (LSGGQ) motifs are designated 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 with the heavy dark bars and the serine residues that are known to be phosphorylated are shown as darkened circles with an attached and 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 P-gp.

1.2.6 Physiology and Function of P-glycoprotein

Expression of P-gp confers drug resistance to numerous antitumor agents, including doxorubicin, daunorubicin, actinomycin D, etoposide, teniposide, colchicine, taxol, vincristine, and vinblastine. Examples of some drugs and their targets are shown in Table 6. Other substrates include calcium channel and calmodulin inhibitors, such as verapamil, trifluoperazine, and quinine, and various biologically active hydrophobic peptide derivatives, including proteinase inhibitors (pepstatin), chemoattractants, ionophores (valinomycin, gramicidin), enkephalins, and immunosuppressants (cyclosporin A, PSC 833). Both synthetic and natural opiates also interact with the P-gp. Some of these agents are listed in Table 7. These compounds are chemically diverse; some carry positive charge at physiological pH and since most of them are relatively hydrophobic, permeate the cell membrane by passive diffusion [52].

P-gp also mediates the transport of various structurally unrelated compounds, such as toxin peptides, including gramidicin D, valinomycin, and N-acetyl-leucyl-leucyl-norleucinal (ALLN), fluorescent dyes such as rhodamine 123 and polycyclic aromatic hydrocarbons such as benzo(a)pyrene. Endogeneous compounds, such as some steroid hormones, have also been found to be substrates for Pgp. In addition, the pump may serve as an ATP channel and is involved in volume-regulated chloride channel activity [52].

Several mechanisms have been hypothetized to explain how the anticancer drugs transport via the P-gp involved in the MDR phenotype. The most widely accepted model is that the P-gp, a molecular pump, uses the energy from ATP hydrolysis to extrude chemotherapeutic agents from the cell. In this hypothethical model, P-gp acts as a transmembrane pore-forming protein, when chemotherapeutic agents diffuse down a concentration gradient into the cell, the drug could be expelled from the bilayer itself, or the drugs could interact with P-gp in the cytoplasm and be expelled directly into the extracellular medium. According to the "hydrophobic vacumn cleaner" model, P-gp binds substrates and pumps them out of the cell [53,54].

In the "Flippase" model, the drugs are transported from the innner leaflet to the outer leaflet of the bilayer. P-gp encounters drugs in the inner leaflet of the plasma membrane and flips them to the outer leaflet from which they diffuse into the extracellular medium. An alternative mechanism suggested that P-gp affects intracellular pH and/or the plasma membrane electrical potential of the cell by acting as a proton pump or a chloride channel, thereby indirectly reduces intracellular accumulation of weakly basic, cationic lipophilic anticancer drug. However, the Flippase model was not supported by the direct drug transport [52, 55].

Table 6. Main group of anticancer drugs [31]

Families of anticancer drugs	**Examples of drugs**	**Some mechanisms of action**	**Examples of drugs targets**
Alkylating agents	melphalan, cyclophosphamide, chlorambucil, cisplatin	binding with DNA, breaks and inappropriate links between DNA strands	DNA molecule
Anticancer antibiotics	dactinomycin, daunomycin, doxorubicin	topoisomerase inhibition	topoisomerase II and I
Drug derived from plants	vinca alkaloids (vinblastine) podophyllotoxins (etoposide), taxanes	depolymerization of microtubules, damage to mitotic spindle	cytoplasmic microtubules, mitotic spindle
Antimetabolites	Methotrexate, fluorouracil, 5-azacytosine, 6-mercaptopurine, gemcitabine	inhibition of enzymes participating in DNA and RNA synthesis	Dihydrofolate reductase, thymidylate synthetase, etc.

Table 7. Representative compounds which are substrates for the P-gp [56]

Anticancer agents	**Immunosuppressants**	**Hydrophobic peptides**
- actinomycin D	- cyclosporine A	- gramicidin
- etoposide	- tacrolimus	- valinomycin
- docetaxel	- rapamycin	- N-acetyl-leucyl-leuycl-norleucine
- doxorubicin		
- daunorubicin	**Antiemetic drugs**	**Lipid lower agents**
- irinotecan	- domperidon	- atorvastatine
- mitomicin C	- ondansetron	- lovastatine
- mitoxantrone		
- paclitaxel	**Antidiarrheal agents**	**Antifungal**
- teniposide	- loperamide	- itraconazole
- topotecan		
- vinblastine	**Antibiotics**	**Micellaneous**
- vincristine	- erythromycin	- rhodamine 123
- colchicine	- levofloxacin	- Hoechst 33342
- **Cardiac drugs**	**Hormone**	**calcium channel blocker**
- β-acetyldigoxin	- hydrocortisone	**and metabolites**
- α-methyldigoxin	- progesterone	- verapamil
- digitoxin	- testosterone	- diltiazem
- digoxin	- dexamethasone	- mibefradil
- quinidine	- estradiol	- D-617 and D-620
HIV protease inhibitors	**Detergents**	**β-adrenoceptor**
- indinavir	- triton x-100	**antagonist**
- nelfinavir	- tween 80	- bunitrolol
- saquinavir	- solutol HS-15	- talinolol

1.2.7 Pharmacological Modulation of MDR

A primary goal in the investigation of P-gp-mediated MDR is to discover specific means to reverse or circumvent it. The clinical significance of P-gp attracts many researchers to find the ways to inhibit P-gp activity. Understanding of structural and functiona of P-gp related to the inhibition of the MDR transporter, will lead to the discovery of new agents for potential use in clinical trials. In general, agents used to antagonise MDR alter the drug accumulation defect present in MDR cells, but have little or no effect on drug-sensitive cells. Many pharmacologic agents from diverse structural classes have been identified as P-gp chemosensitizer [57].

Unfortunately, clinical studies with the so called first-generation MDR modulators indicated dose-limiting side effects of these chemosensitizers with a low therapeutic index. Most of these modulators (e.g. verapamil, cyclosporin A (CsA), quinidine and tamoxifen) modulated MDR at very high concentrations ranging from 5 to 50 μM and they are also substrates for the P-gp efflux pump [58, 59]. Less toxic, second-generation MDR reversing drugs, e.g., the less cardiotoxic d-verapamil and less immunosuppressive, nephrotoxic PSC 833 cyclosporine analog, are currently being clinically evaluated in clinical studies. Some of these compounds which are effective at concentrations ranging from 1 to 20 μM [60].

The third generation modulators work at low dosage to achieve effective reversing concentration *in vivo*. These agents exhibit effective reversing concentrations in the nanomolar range (20–100 nM), thus requiring low doses to achieve effective reversing concentrations *in vivo*. Examples include specific P-gp blockers such as the acridonecarboxamide GF 120918 , the diketopiperazine XR9051, the diarylimidazole OC144-093 [61]. A partial list of the wide range of agents with the ability to reverse MDR in preclinical models was shown in Table.

Drug substrates are actively transported, in to the cell, generate a concentration gradient, and MDR cells display resistance to be destroyed by these compounds. Combined with the drugs, chemosensitizer reversed drug resistance and lead to the destruction of intact MDR cells. The primary mechaism to antagonise MDR is the direct inhibition of drug efflux mediated by P-gp which resulted in the restoration of cytotoxic drug accumulation in MDR cells. A simplified model for a

potential mechanism of action of chemosensitisers to inhibit the MDR efflux pump is shown in Figure 4.

Chemosensitisers may block cytotoxic drug efflux by acting as competitive or non-competitive inhibitors, perhaps by binding to similar drug substrate binding sites, or to other chemosensitiser binding sites which cause allosteric changes resulting in inhibition of cytotoxic drug binding or transport. Evidence support that this model come from many studies which reported that certain chemosensitisers may bind directly to cellular membranes enriched for P-gp, in a specific and saturable manner, and this binding may be inhibited by other chemosensitisers and by chemotherapeutic drug [62, 63]

Table 8. MDR modulators and levels required to reverse MDR in vitro [64]

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)
Ro11-2933 (2-6 μM)	FK506 (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)	GF120918 (0.02-0.1 μM)
Staurosporine (200 nM)	Tolyporphin (0.1-0.5 μM)
CGP41251 (150 nM)	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)

Concentrations in parentheses are those shown to have effect in reversing MDR *in vitro*

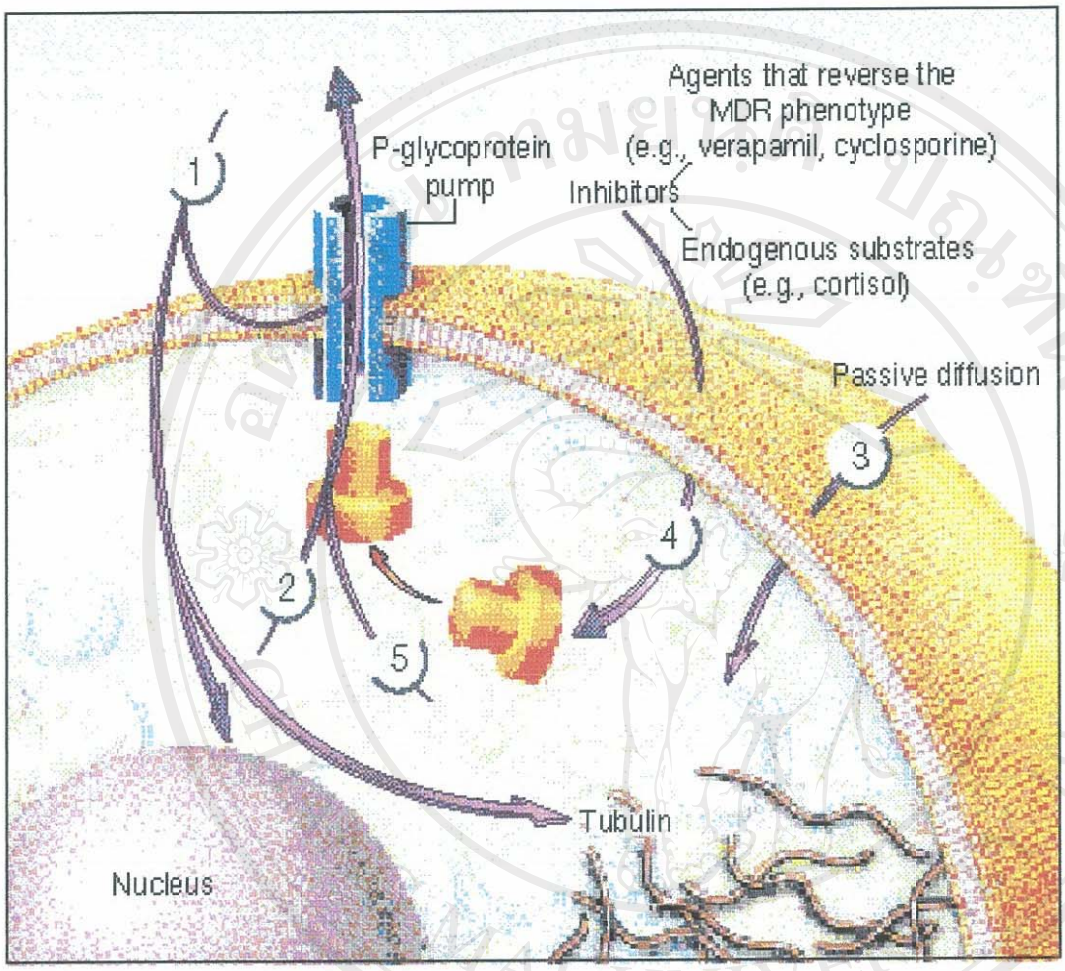


Figure 4. The P-gp pump [65]. P-glycoprotein may transport cytotoxic drugs directly from the cell membrane, before such drugs enter the cytoplasm (1), or from the cytoplasm (2), limit the drug concentration of such drugs at the target (DNA or tubulin). Highly lipophilic drugs enter the cell by passive diffusion (3). Inhibitors of P-glycoprotein–mediated transport may be carried through the blood supply (e.g., steroid hormones and agents that reverse the multidrug-resistance [MDR] phenotype) (4), or hypothetical natural substrates may be produced in the cell (5).

1.2.8 The development of the human MDR KB-V-1 cell lines

Multidrug resistant cancer cell lines (MDR-cancer cell lines) were derived from cancer cell lines to study the biochemical, physiological, and genetic bases of alterations that result in the development of multidrug resistance. The highly expressed of P-gp gene was found in many MDR cancer cell lines. The model tissue culture systems have been developed for studing the mechanism of multudrug resistance. Culture cells that are selected for resistance to one of the drugs become resistant to many other structurally unrelated natural products, such as anthracyclines, vinca alkaloids, and epipodophyllo toxins [66].

Multidrug-resistance cell lines have been developed using hamster, rodent, and human cells. The physiological characteristic of MDR cell lines which accumulated drugs much less than parental drug sensitive cell lines, includs the increase of P-gp level and activity in Chinese hamster ovary (CHO) cells and decreased accumulation of drugs in human multidrug resistant KB-carcinoma cell lines.

Multidrug resistant cell lines have been isolated in tissue cuture by multistep selection. Human multidrug resistant KB-carcinoma cell lines (MDR KB-carcinoma cell) was isolated from human KB-cell lines which is originally derived from a carcinoma of cervix cell. KB-cell line was chosen as the experimental model because of its drug sensitivity, high cloning, efficiency, rapid growth and stable Karyotype [67].

In the preliminary studies, its was impossible to isolate apontaneous mutants of this cell line resistant to either colchicine, vinblastine, and adriamycin. The ethylmethanesulfonate (EMS) was used to mutagenized KB cells in the first selection (Figure 5). EMS mutagenesis was repeated for the second step of selection with vinblastine. The resistant cells were immediately subcloned at each step of selection; but at the later stages of selection, cell populations were isolated without subcloning.

The vinblastine selected cell lines maintained a relatively uniform level of resistance to all three drugs(colchicine, vinblastine, and adriamycin) as shown in Table 9.

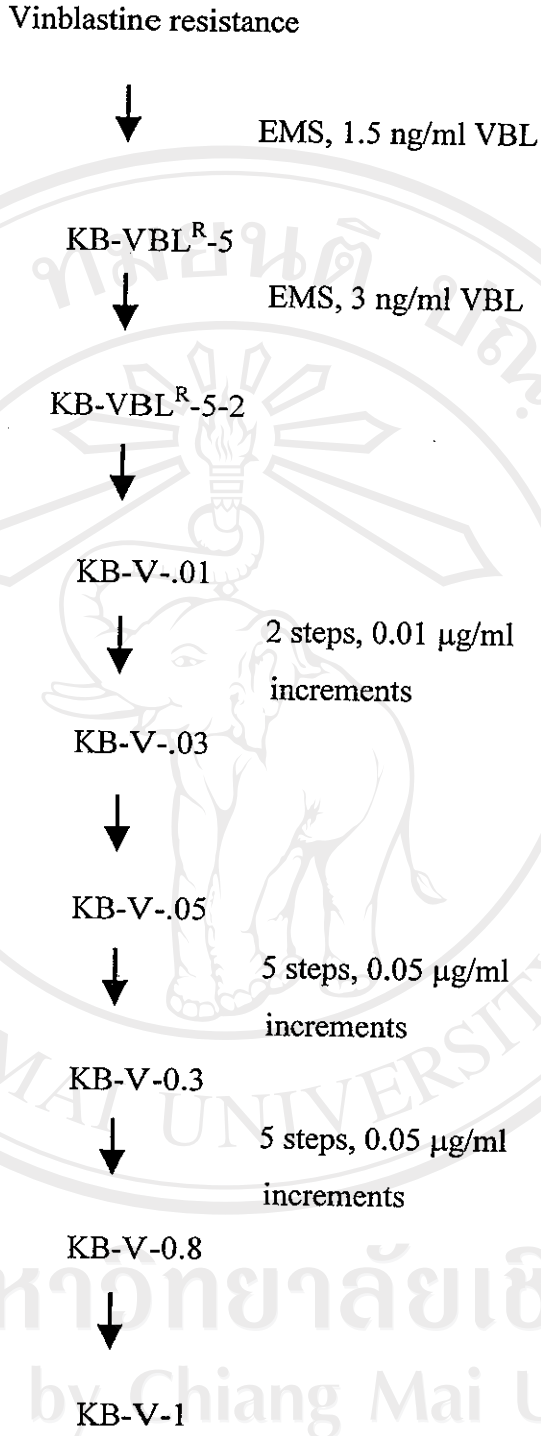


Figure 5. Flow chart of the step for increase vinblastine resistance in the MDR KB-V-1 cell line [68]. The parental cell line was KB subline designated KB-3-1. Abbreviations: VBL^{R}, vinblastine-selected subclones; V, vinblastine-selected populations. In each case, where a letter alone is used, the number following the letter refers to the selecting concentrations of drug in micrograms per milliliter.

Table 9. Properteis of MDR KB cell lines [68]

	Relative Resistance of MDR Cell Lines		
	Colchicine	**Vinblastine**	**Adriamycin**
KB-3-1	1	1	1
Vinblastine-selected			
KB-Vbl-5	2	3	2
KB-V-1	290	1300	650
KB-V1-R2	1	1	1

Revertant clone, KB-V-1-R2, were obtained by culturing resistant lines in the absence of vinblastine

1.2.9 Bitter melon

Momordica charantia (family, Curcubitaceae), common name known as bitter melon or karela, is a plant widely-cultivated plant in many tropical and subtropical regions of the world. It was frequently used in South Asia and the Orient as a foodstuff, tonic and medicinal plant. Bitter melon is a slender, climbing annual vine with long-stalked leaves and yellow, solitary male and female flowers born in the leaf axils. The fruit appears as a warty gourd, usually oblong and resembling a small cucumber. The young fruit is emerald green, turning to orange-yellow when ripe. At maturity the fruit splits into three irregular valves that curl backwards and release numerous brown or white seeds encased in scarlet arils. Although the seeds, leaves, and branch of bitter have all been used, the fruit is the safest and most prevalent part of the plant used medicinally (Figure 6). Taxonomy of bitter melon and other characteristics are shown in table 10.

Table 10. The taxonomy and other characteristics of bitter melon [69-71]

Family : Cucurbitaceae **Genus** : *Momordica* **Species** : charantia **Synonyms:** *Momordica chinensis, M. elegans, M. indica, M. operculata, M. sinensis, Sicyos fauriei*
Common Names: Bitter melon, papailla, melao de sao caetano, bittergourd, sorosi, a'jayib al maasi, assorossie, balsam apple, balsam pear, chin li chih, ejinrin gule khandan, fu-kua, karela, k'u kua kurela, kor-kuey, ku gua, lai p'u t'ao, pava-aki, salsamino, sorci, sorossi, sorossie, sorossies, pare, peria laut, peria, mara-kee-nok
Part Used: Whole plant, fruit, seed and leave
Properties/ Actions: Anthelmintic, antibacterial, antibiotic, antidiabetic, anti-inflammatory, antileukemic, antimicrobial, antimutagenic, antimycobacterial, antioxidant, antitumor, antiulcer, antiviral, aperitive, aphrodisiac, astringent, carminative, cytostatic, cytotoxic, depurative, hormonal, hypocholesterolemic, hypotensive, hypotriglyceridemic, hypoglycemic, immunostimulant, insecticidal, lactagogue, laxative, purgative, refrigerant, stomachic, styptic, tonic, vermifuge .

Phytochemicals:

Leaves:

Octacosane, 1-triacontanol, 7-stigmasten-3β-ol, 7,25-stigmastadien-3β-ol, 5,25-stigmastadien-3β-ol glucoside, phytosphingosine, momordicine I, II, III.

Tendrils:

Benzyl alcohol, myrtenol, *cis*-3-hexenol, *trans*-2-hexenal, 1-penten-3-ol, *cis*-2-penten-1-ol

Unripe fruits:

Momordicosides G, F1, characterized as 3-O-β-D-allopyranoside and 3-O-β-D-glucopyranoside, respectively, of 5,19-epoxy-25-methoxy-5β-cucurbita-6,23-dien-3β-ol, momordicosides F2, I characterized as 3-O-β-D-allopyranoside and 3-O-β-D-glucopyranoside of 5,19-epoxy-5β-cucurbita-6,23-dien-3β,25-diol, 3-O-[6'-O-palmitoyl-β-D- glucosyl]-stigmasta-5,25(27)-diene and stearil derivative

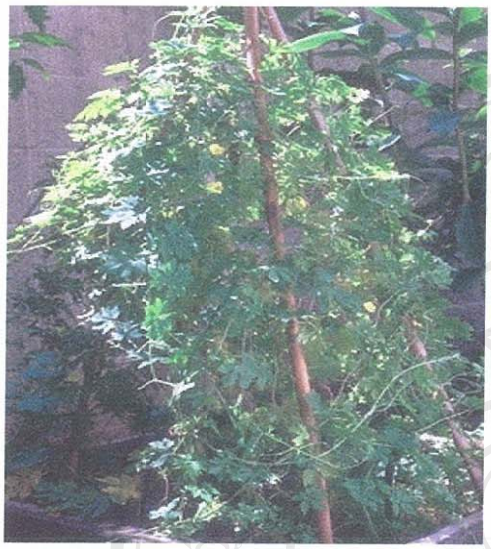
Fruits:

Momordicoside K, L, two acylglycosylsterols-3-O-[6'-O-palmitoyl-β-D -glucosyl] stigmast-5,25(27)-diene and 3-O-[6'-O-stearyl-β-D -glucosyl] stigmasta-5,25(27)-diene, benzyl alcohol, myrtenol, *cis*-3-hexenol, *trans*-2-hexenal, 1-penten-3-ol, *cis*-2-penten-1-ol, charantin, stigmast-5,25-diene-3β-O-glucoside

Seeds

β-sitosterol-β-D-glucoside, stearic acid, two lectins, triterpene glycosides – momordicosides A,B,C, D and E, two cytokinins – zeatin and zeatin riboside –; two proteins α and β momorcharins, p-cymene, hexadecanol, menthol, nerolidol, pentadecanol, and squalene, 10α-cucurbit-5,24-dien-3β-ol, 24-methylencycloartanol, taraxerol, β-amyrin, campesterol, cycloeucalenol, 24β-ethyl-5α-cholesta-7-trans-22-dien-3-ol, 24-ethyl-5-cholesta-7-trans-22,25(27)-trien-3β-ol, lophenol, 4α-methylzymosterol, obtusifoliol, spinasterol, stigmasterol, stigmasta-7,25-dienol and stigmasta-7,22,25-trienol, momordica anti-protein (MAP 30) , ribosome-inactivating-proteins (RIPs).

A



B

C

Figure 6. The pictures of each parts of bitter melon are shown as follow: A; whole plants of bitter melon, B; fruits and leaves, C; tendril, leaves and flowers [72-74].

1.2.10 Pharmacological properties of bitter melon

Bitter melon has been suggested for many uses, based on tradition or on scientific theories. However, these uses have not been thoroughly studied in humans, and there is limited scientific evidence about the safety or effectiveness. Some of these uses are for conditions that are potentially very serious and even life-threatening, so that we should consult a health care provider before taking bitter melon for any unproven use. Many studies found that the methanol extract of bitter melon have clinically preventioned hypoglycemia and hyperinsulinemia properties in fructose fed rats [75], or the other actions against type-2 diabetes [76].

At least three different groups of constituents in bitter melon have been reported to have hypoglycemic (blood sugar lowering) actions of potential benefit in diabetes mellitus. These include a mixture of steroidal saponins known as charantin, insulin like peptides, and alkaloids. It is still unclear whether only one of the components act solely or they have to work together which of these is most effective, or if all three work together. Multiple controlled clinical studies have confirmed the benefit of bitter melon diabetes patients [77].

A novel phytochemical from the fruit of bitter melon has contained a compound that can act as trypsin inhibitor. This inhibitor is thought to be linked to the cancer chemopreventive agent and can inhibit two-state carcinogenesis and breast cancer in cell culture and animal stuides [78]. Other phytochemicals that have been investigated with cytotoxic activity are a group of ribosomal-incativating protein, α and β momorcharin, which inhibit the AIDS virus *in vitro* ; however this research has only been conducted *in vitro* and not *in vivo* [79, 80]. The phytochemical momordin I has cytotoxic activity against murine colon cancer *in vivo*, and *in vitro* studies have demonstrated cytostatic and AP-1 inhibitotry effect inhibiit AP-1 function and also cell proliferation. The mechanism of momordin I comes from its inhibitory effect on the protein-DNA interaction [81].

Another clinical study showed that Map-30 (Momordica anti-human immunodeficiency virus [HIV] protein; molecular weight 30 kd), an antiviral protein from bitter melon could inhibit the proliferation of primary effusion lymphoma cell lines derived from AIDS patients [82, 83]. The use of MAP-30 in combination with low pharmacological doses of dexamethasone and indomethacin may improve the

efficacy of anti-HIV therapy in MT-4 lymphocytes [84]. Recently it has also been reported that anti-HIV plant protein MAP-30 is capable of inhibiting the infection and replication of herpes viruses as well as ACV-resistant strains [85].

1.3 Objectives

1. To study the effect of bitter melon extracts on cytotoxicity in drug resistant human cervical carcinoma cell lines (KB-V-1) and drug sensitive human cervical carcinoma cell lines (KB-3-1).

2. To study the effect of bitter melon extracts on the MDR phenotype in drug resistant human cervical carcinoma cell lines (KB-V-1) and drug sensitive human cervical carcinoma cell lines (KB-3-1).

3. To study the effect of bitter melon extracts on P-glycoprotein-mediated drug transport in drug resistant human cervical carcinoma cell lines (KB-V-1) and drug sensitive human cervical carcinoma cell lines (KB-3-1).