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 (Umezawa *et al*. 1977). 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 (Parkin *et al*. 2001: Sankaranarayanan *et al*. 2001 and Thomas *et al*. 2001)

In Thailand, 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 (www.WHO.com). Most cases of cancer were treated 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 (Verweij and Jonge, 2000). 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 (www.oncolink.com). 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 (Witteveen *et al*. 2002: www.biopathic.com/castoverview/htm and www. phamacology. unmc. edu/ cancer). 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, kidney, leukemia and testicular cancer (Wagner *et al*. 2001).

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 that is a major characteristic of drug resistance encountered in cervical cancer (Willemse *et al*. 2002)

The development of chemo-resistance 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 of P-170 glycoprotein (P-glycoprotein) and multidrug resistant associated proteins (MRPs) expressed by a broad range of human tumors at presentation or at relapse. The 170 kDa P-glycoprotein encoded by the MDR1 gene (multiple drug resistance gene), and MRP-1 act as an ATP-driven efflux pump for a wide variety of hydrophobic natural products, chemotherapeutic drugs, and various biological active hydrophobic peptide derivatives. P-glycoprotein and MRPs are found in high level in many chemotherapy-resistant tumors and low level in chemotherapy-responsive tumors. P-glycoprotein and MRPs are expressed in some normal tissue; however, within the context of cancer chemotherapy, P-glycoprotein ``pumps" certain cytotoxic drugs out of tumor cells. It thereby prevents these drugs from reaching toxic levels and destroying the malignant cells. Because P-glycoprotein forces out some cytotoxic drugs, cancers become resistant to certain chemotherapy and renders them unresponsive to this treatment. P-glycoprotein and MRPs -mediated resistance are thus a significant clinical problem that might be mitigated by the development of a drug designed either to prevent P-glycoprotein and MRPs expression in tumor cells or to reverse its effect (Sharom *et al*. 1999). In clinical trial attempting to reverse or modulate MDR in many clinically resistant tumors, new drug developed specifically to inhibit P-glycoprotein and demonstrated the safety and efficacy of this novel MDR reversal agents (MDR-modulator or pump inhibitors or P-glycoprotein 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-glycoprotein, may have the capacity to block the P-glycoprotein pump from expelling cytotoxic drugs from tumor cells and, thus, potentially prevent the malignant cells loss of responsiveness to chemotherapy (Varma *et al*. 2003). Considering of novel biochemical modulators from some foods and beverages, many research groups are interested in the effect of several plant natural products on the activity of P-glycoprotein. Thus researchers have studied the beneficial effects of plant natural products 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, *Stemona tuberosa Lour*. a Thai medicinal herb has been previously shown to moisten the lungs and stop cough, to kill lice and parasites (Lu and Henry, 1991)

Because of the clinical importance of drug efflux mechanism for multidrug resistance and cancer treatment, the modulating properties of *S.tuberosa* on P-glycoprotein and MRP-1 activity was investigated.

Therefore, the effects of *S.tuberosa* root extract on modulation of P-glycoprotein and MRP-1 activities were taken as a mean the modulation of chemotherapeutic drugs sensitivity.

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 has already been present at diagnosis or it can develop after chemotherapy treatment. These two forms of drug resistance are respectively called intrinsic and acquired resistance (Giaccone *et al*. 1996). 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 (Goldstein *et al*. 1996).

Multidrug resistance (MDR) is the protection of a tumor cell population against numerous 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 (Broxterman *et al*. 2003 and Roninson *et al*. 1992). 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-glycoprotein in the cell lines and on the affinity of the substrate for it (Duhem *et al*. 1992).

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 and c) alterations of the genes and their products that involved in the control of apoptosis (especially p53 and Bcl-2). 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.

The human *MDR1* gene product, P-glycoprotein, was the first ATP-dependent system discovered that was implicated in multidrug resistance. The over expression of P-glycoprotein 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-glycoprotein in its capability 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 (Schinkel and Jonker, 2003). 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 1. 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 P-glycoprotein-MDR or topoII-MDR. The terms "apoptosis-MDR" and "clinical MDR" are also used herein. Alterations in apoptosis pathways have been shown to 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.

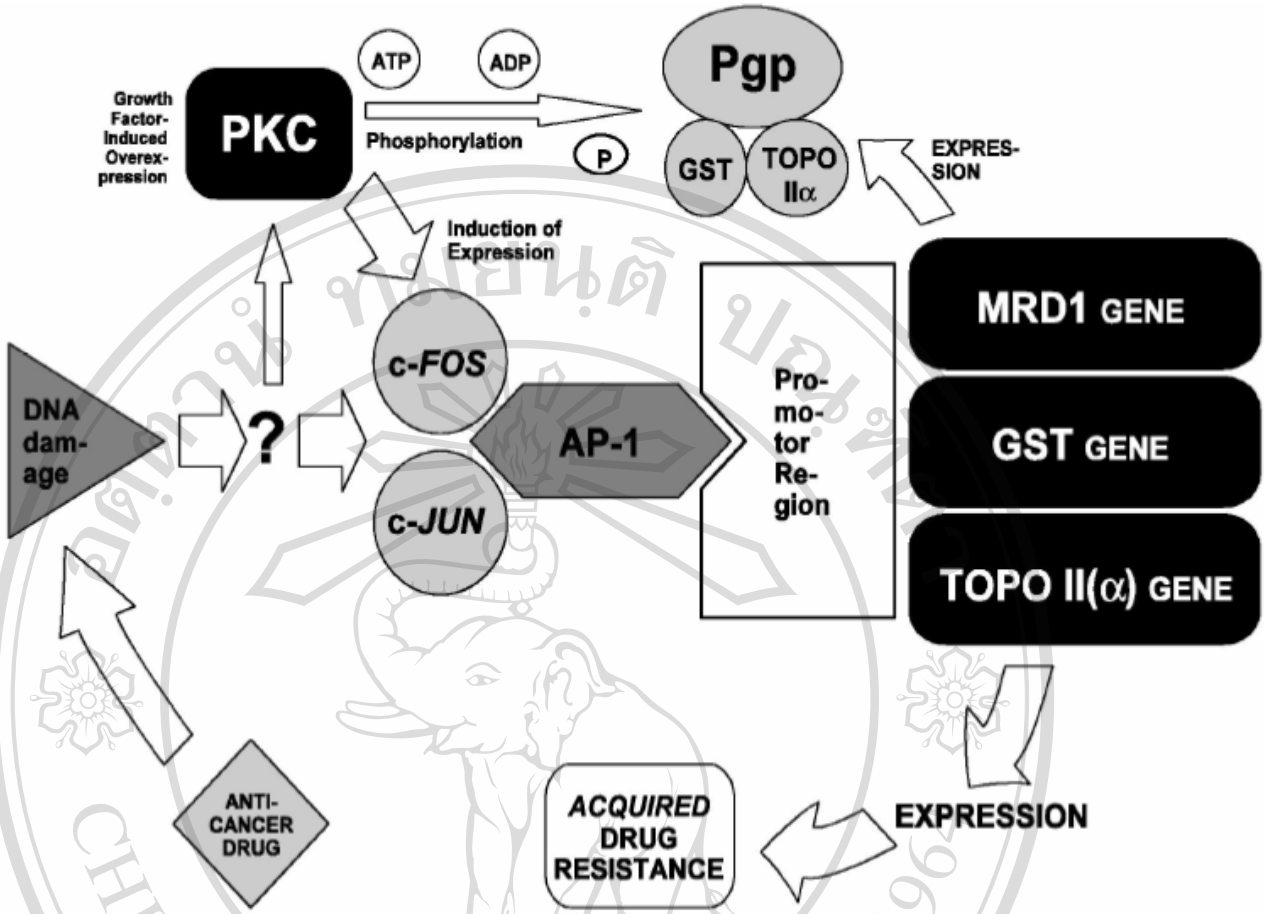

Fig. 1. Model of *acquired* drug resistance and possible explanation for the concomitant expression of different resistance mechanisms in a tumor DNA damage caused by anticancer drugs may induce proto-oncogenic c-*Jun* and c-*Fos* expression leading to the formation of the AP-1 complex. Binding of AP-1 to AP-1 promoter regions affects the transcription rate of various cellular genes. There is evidence that the promoter region of some resistance factor genes, in particular *Mdr* 1 and GST, may contain AP-1 motifs suggesting that these may be modulated by c-*Jun* and c-*Fos*. This may explain cell populations with several resistance mechanisms operating at once may exist in a certain tumor. Expression of these early response genes is thought to be associated with increased protein kinase C (PKC) activity, which is known to be overexpressed in a number of brain tumors, in particular high-gliomas. It becomes increasingly clear that PKC itself is a major regulator of drug resistance and directly affects the expression and phosphorylation of different resistance proteins such as Pgp, Topo II, and GST (Bredel, 2001).

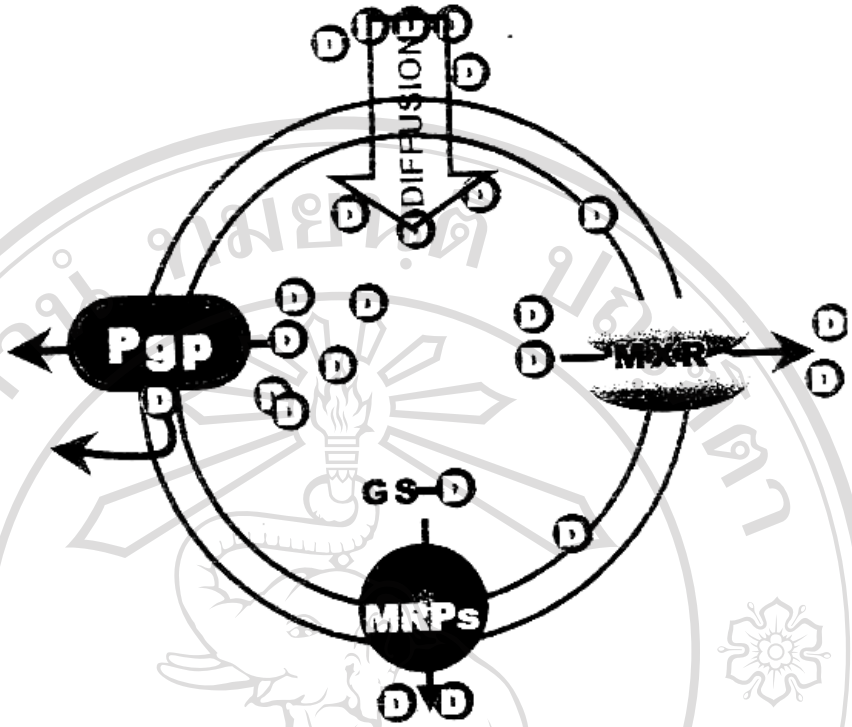

Fig. 2. Role of ABC transporters in the development of the MDR phenotype in cancer cells (Sauna *et al*. 2001). Cancer cells show resistance to cytotoxic agents via one or more of the several mechanisms. Most natural hydrophobic drugs (D) enter the cell by diffusion. These may be pumped out by P-glycoprotein 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 the 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 systems, 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 1. Partial list of MDR mechanisms (Krishna and Mayer, 2001)

Mechanism	Specific enzyme/transporters
1. Decreased therapeutic target levels	(a) Topo I (b) Topo II
2. Altered drug metabolism	(a) GSH and related enzymes
3. Altered cell death (apoptosis)	(a) mutant p53 (b) increased bcl-2, bcl-xl (c) decreased bax, bcl-xs
4. Increased DNA repair enzymes	(a) alkylators
5. Alter drug transport Drug transporters	(a) P-glycoprotein (b) Multidrug resistant protein (MRP-1) (c) CMOAT/MRP-2 (d) Lung resistant protein (e) Breast cancer resistant protein (f) MRP3-6
6. Intracellular/tissue factors	(a) hypoxia (b) altered growth fraction (c) poor blood flow

1.2.2 ABC transporters

Chris Higgins introduced the name ABC transporter in 1992 in a memorable review. The designation ABC was based on the highly conserved ATP-binding cassette, the most characteristic feature of the superfamily. Traffic ATPases and P-glycoproteins are other names used for this family. An inventory of all 48 known and putative human ABC transporters can be found on the Web site of M. Mu¨ller, University of Wageningen, The Netherlands (Borst and Elferink, 2002).

The subunit structure of ABC transporters varies, as shown in Figure 3-5 The basic structure, as found in P-glycoprotein (ABCB1), is thought to consist of 12 transmembrane segments and two ATP-binding sites in a protein of about 1,300 amino acids. This basic structure may be assembled from two equal (ABCG2) or unequal (ABCG5 and 8) halves. Some ABC transporters (e.g., MRP1) have additional domains and are even larger than P-glycoprotein (Borst and Elferink, 2002).

ABC-transporter proteins have to be distinguished from ABC-proteins. Both types of proteins are defined by the presence of a highly conserved approximately 215 amino acids consensus sequence designated as ABC, ABC domain, ABC-ATPase domain, or nucleotide binding domain (NBD). The domain contains two short peptide motifs, a glycine-rich Walker A- and a hydrophobic Walker B motif both involved in ATP binding and commonly present in all nucleotide-binding proteins. A third consensus sequence named ABC signature and is unique in ABC domains. ABC containing proteins couple the phosphate bond energy of ATP hydrolysis to many cellular processes and are not necessarily restricted to transport functions. However, the proper meaning of the term ABC-transporter protein, also designated as traffic ATPase or permease for import systems, is satisfied when the ABC-protein is, in addition, associated with a hydrophobic, membrane embedded transmembrane domain (TMD) usually composed of at least six transmembrane (TM) α-helices. TMDs are also designated as membrane spanning domains (MSD). The TMDs are believed to determine the specificity for the substrate molecules transported by the ABC-transporter protein. The minimal structural requirement for a biological active ABC-transporter seems to be two TMDs and two ABCs [TMD-NBD]2. In 'full-transporters', this structural arrangement may be formed by a single polypeptide chain and in multiprotein complexes by more than one polypeptide chain.

Most of the prokaryotic genes encoding ABC transporters are organized in the operons that contain ABC domains and TMDs as separate subunits requiring assembly as a biologically active transporter. In some ABC-transporter encoding genes the different domains are already fused into higher structural units, so called 'half-transporters' [TMD-NBD] or 'full-transporters' [TMD-NBD]2. The organization of ABC-transporter encoding genes in eukaryotic organisms also shows wide variation. Nevertheless, the different domain combinations are commonly distributed in one or two genes encoding 'half- or full-transporters' (Lage, 2003).

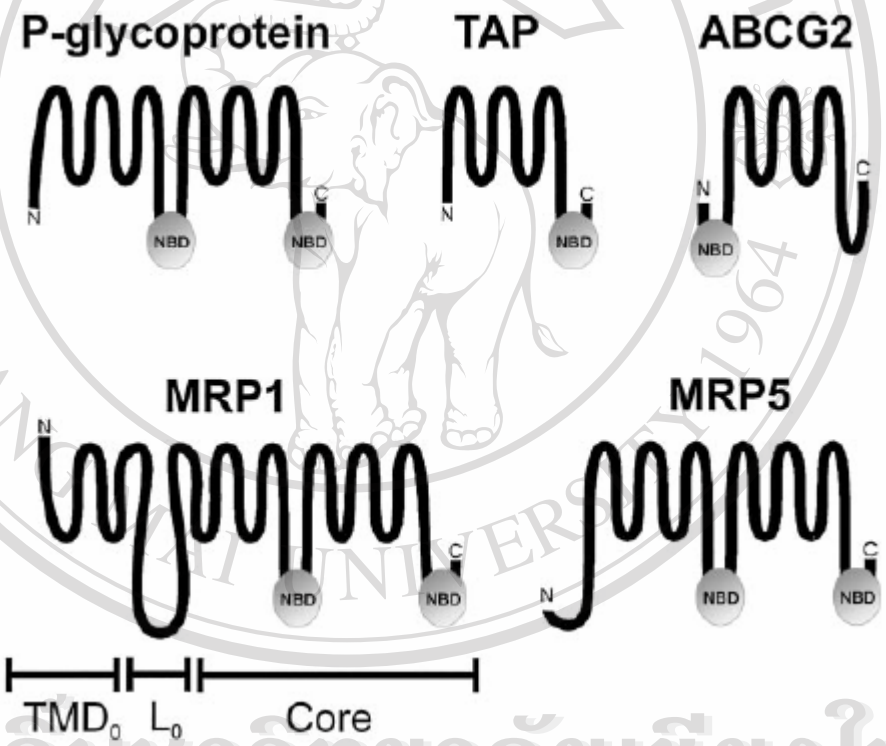

Fig. 3. Predicted topology of the major classes of mammalian ABC transporters. This simplified scheme shows the intracellular nucleotide-binding domains (NBDs) and the transmembrane segments and indicates the N and C termini of the protein. Note that the predicted topology is often based on minimal data, as in the case of ABCG2 (BCRP1/MXR/ABCP) and MRP5 (ABCC5). TAP, the transporter associated with antigen presentation, probably has more than six transmembrane segments. The half-size transporter TAP functions as a heterodimer of TAP1 and TAP2, and ABCG2 functions as a homodimer (Borst and Elferink, 2002).

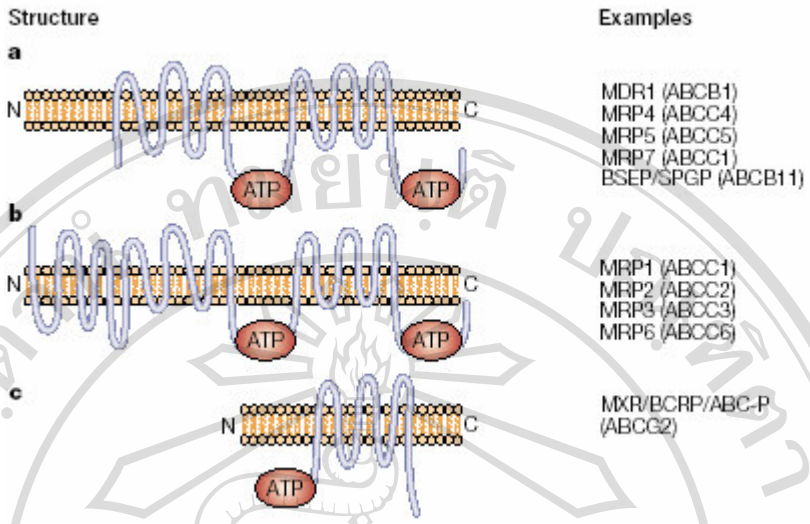

Fig. 4. Structures of ABC transporters known to confer drug resistance**.** The structures of three categories of ABC transporter. **a** | ABC transporters such as multidrug resistance MDR1 and multidrug-resistance-associated protein 4 MRP4 have 12 transmembrane domains and two ATP binding sites. **b** | The structures of MRP1, 2, 3 and 6 are similar in that they possess two ATP binding regions. They also contain an additional domain that is composed of five transmembrane segments at the amino-terminal end, giving them a total of 17 transmembrane domains. **c** | The'half-transporter' ABCG2 contains six transmembrane domains and one ATP-binding region in this case, on the amino-terminal side (N) of the transmembrane domain. In other 'half-transporters', such as the transporter associated with antigen processing (TAP), the ATP-binding cassette is found on the carboxy-terminal (C) side of the transmembrane domain. To function properly, half-transporters may have to be in homodimer or heterodimer form. (Gottesman *et al*. 2002)

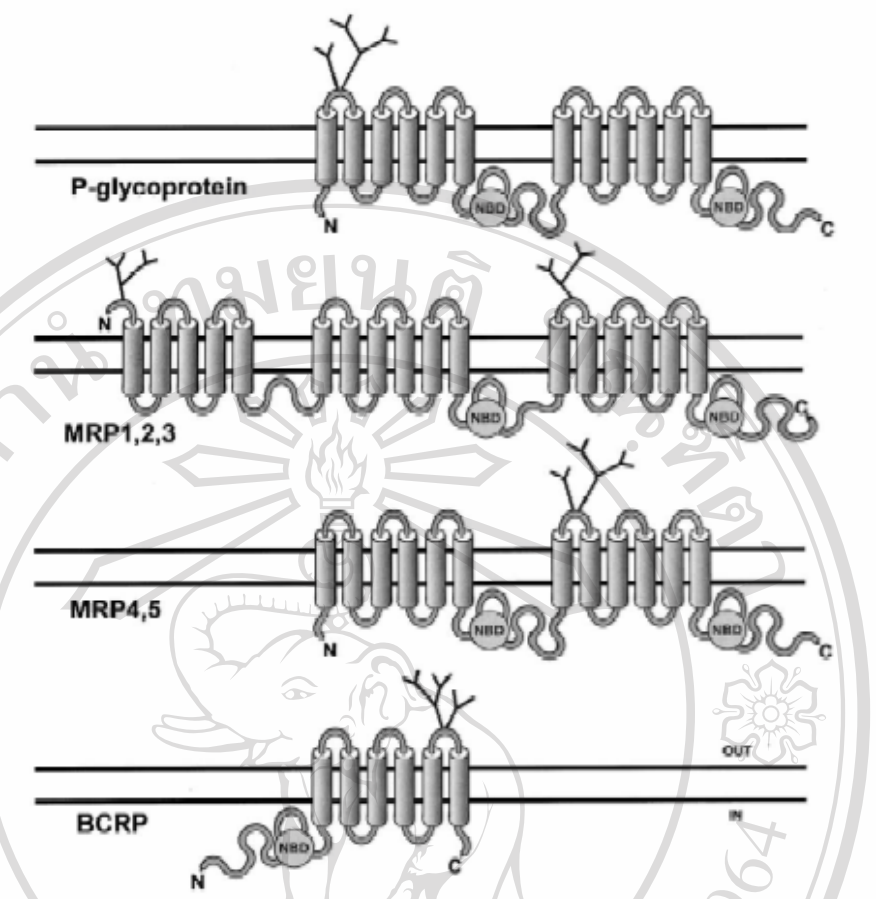

Fig. 5. Predicted secondary structures of drug efflux transporters of the ATP-binding cassette family. Four distinguish classes shown in this figure can be differentiate, based on predicted structure and amino acid sequence homology. P-glycoprotein consists of two transmembrane domains, each containing six transmembrane segments, and two nucleotide binding domains (NBDs). It is *N*-glycosylated (branches) at the first extracellular loop; MRP1, 2 and 3 have an additional aminoterminal extension containing five transmembrane segments and they are *N*-glycosylated near the N-terminus and at the sixth extracellular loop; MRP4 and 5 lack the aminoterminal extension of MRP1–3, and are *N*-glycosylated at the fourth extracellular loop; BCRP is a 'half transporter' consisting of one NBD and six transmembrane segments, and it is most likely *N*-glycosylated at the third extracellular loop. Note that, in contrast to the other transporters, the NBD of BCRP is at the aminoterminal end of the polypeptide. BCRP almost certainly functions as a homodimer. N and C denote amino- and carboxy-terminal ends of the proteins, respectively. Cytoplasmic (IN) and extracellular (OUT) orientation indicated for BCRP applies to all transporters drawn here (Schinkel and Jonker, 2003).

1.2.3 P-glycoprotein

P-glycoprotein expression in normal tissue

P-glycoproteins 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 expressed 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 epithelium, ovarian follicles, and pregnant uterine epithelium (Table 2.).

Expression of P-glycoprotein in these normal tissues was thought to be a protective mechanism against xenobiotics (protection of vital organs against toxic products), excretion and detoxification (Lin, 2003). 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 murine 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 (Fromm, 2002)

In all of these organs, P-glycoprotein 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-glycoprotein is also expressed in 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-glycoprotein, suggesting that P-glycoprotein may be involved in cell-mediated cytotoxicity (Roninson, 1992).

P-glycoprotein expression in human tumors

P-glycoprotein has been expressed in a wide variety of cancers. The expression of P-glycoprotein is usually high and constitutive in tumors that arise from tissues with physiologically expressed pump, such as carcinoma of the colon, kidney, adrenal gland, pancreas, and liver. P-glycoprotein expression has been correlated with the treatment failure and poor prognosis in several types of cancers. There are two

human P-glycoproteins. 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 still 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 leukemia and chronic myelogeneous leukemia) are more frequently positive for P-glycoprotein at relapse than at diagnosis (Chan *et a*l. 1996).

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 3). 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 the tissues of origin highly expressed of the *MDR1* gene, which is conserved in these tumors.

Table 2. Normal tissues, which express P-glycoprotein (Fisher *et al*. 1996)

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 3. Expression of the MDR1 gene in human tumors (Goldstein, 1996)

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 leukemia.

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 membrane protein encoded by the *MDR1* gene on human chromosome 7 and consists of 28 exon. P-glycoproteins 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 (Germann *et al*. 1993 and Hirose, 2002). P-glycoproteins are composed of 43% sequence homology, between the two parts there is a hydrophobic, 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-glycoprotein. This peptide conjugated, commonly called the linker region, is highly charged and contains the *in vivo* sites of phosphorylation (Abraham *et al*. 1993).

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-glycoproteins are necessary for transport of substances out of cells (Stouch and Gudmundsson, 2002).

A general topological model of the membrane associated P-glycoprotein is presented using the human *MDR1* gene product as an example as shown in figure 6. 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-glycoprotein. This twelve TM region model of P-glycoprotein is supported experimentally by cellular epitope localization data obtained from antibodies that specifically recognize the N- or C-terminus of P-glycoprotein, its first and fourth extracellular loop, or the two ATP-binding sites (Germann, 1996).

The two halves of P-glycoprotein are essential for the transporter activity 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-glycoprotein 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 (Ambudkar *et al*. 1999).

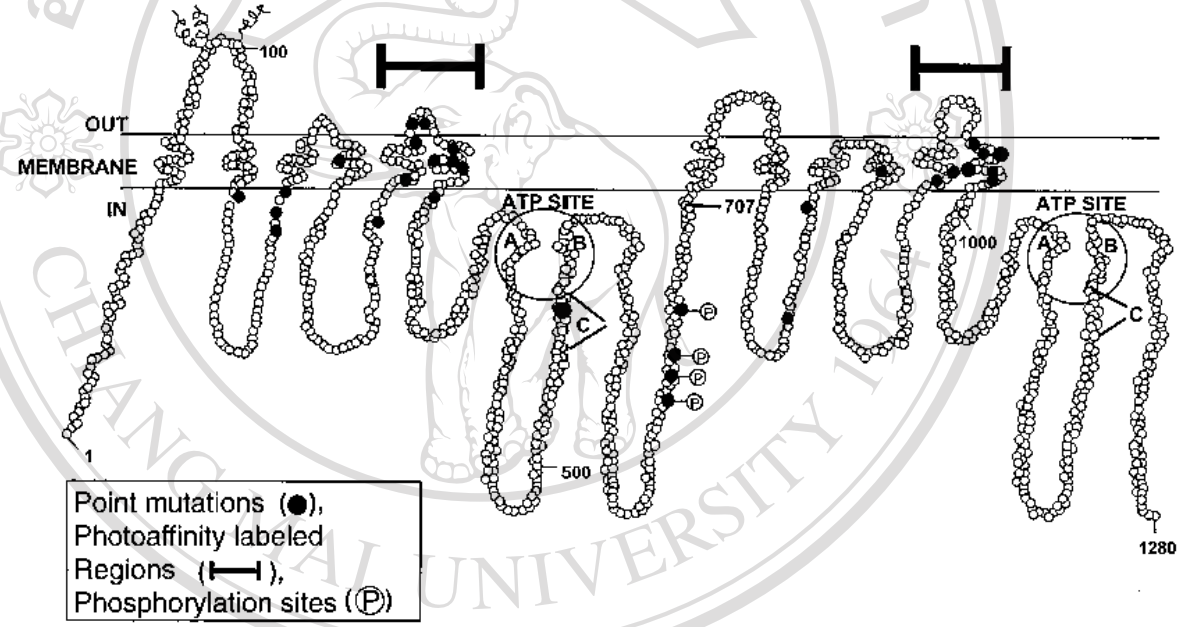

Figure 6. Two-dimensional hypothetical model of human P-glycoprotein structure based on a hydropathy plot analysis of primary amino acid sequence (Hrycyna, 2001). The ATP binding/utilization domains are circled with the Walker A, B, and 'linker dodecapeptide' or 'signature sequence' (LSGGQ) motifs are 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 dark circles with an attached and encircled 'P'. Each circle represents an amino acid residue. The full circles show the positions of mutations that change substrate specificity in human P-glycoprotein.

Physiology and Function of P-glycoprotein

Expression of P-glycoprotein 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 4. Other substrates include calcium channel and calmodulin inhibitors, such as verapamil, trifluoperazine, 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-glycoprotein. Some of these agents are listed in Table 5. 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 (Fardel, 1996).

P-glycoprotein 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 P-glycoprotein. In addition, the pump may serve as an ATP channel and is involved in volume-regulated chloride channel activity (Fardel, 1996).

Several mechanisms have been hypothesized to explain how the anticancer drugs transport via the P-glycoprotein involved in the MDR phenotype. The most widely accepted model is that the P-glycoprotein, a molecular pump, uses the energy from ATP hydrolysis to extrude chemotherapeutic agents from the cell. In this hypothetical model, P-glycoprotein acts as a transmembrane pore-forming protein, when chemotherapeutic agents diffuse into the cell, the drug could be expelled from the bilayer itself, or the drugs could interact with P-glycoprotein in the cytoplasm and be expelled directly into the extracellular medium. According to the “hydrophobic vacumn cleaner” model, P-glycoprotein binds substrates and pumps them out of the cell (Teodori *et al*. 2002 and Loor *et al*. 2002).

In the “Flippase” model, the drugs are transported from the innner leaflet to the outer leaflet of the bilayer. P-glycoprotein 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-glycoprotein 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 (Fardel, 1996 and Ling, 1995)

Table 4. Main group of anticancer drugs (Stavrovskaya, 2000)

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 5. Representative compounds which are substrates for the P-glycoprotein (Fromm, 2002)

Anticancer agents	**Immunosuppressants**	**Hydrophobic peptides**
- actinomycin D	**- cyclosporine A**	**- gramicidin**
- etoposide	- tacrolimus	- valinomycin
- docetaxel	- rapamycin	- N-acetyl-leucyl-leuycl-
- doxorubicin		norleucine
- 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

Pharmacological Modulation of MDR

A primary goal in the investigation of P-glycoprotein-mediated MDR is to discover specific means to reverse or circumvent it. The clinical significance of P-glycoprotein attracts many researchers to find the ways to inhibit P-glycoprotein activity. Understanding of structure and function of P-glycoprotein 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-glycoprotein chemosensitizer (Clynes *et al*. 1998).

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-glycoprotein efflux pump (Tamai and Safa, 1991 and Lo *et al*. 2001) 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 (Ferry, 1996).

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-glycoprotein blockers such as the acridonecarboxamide GF 120918, the diketopiperazine XR9051, the diarylimidazole OC144-093 (Krishna and Mayer, 2002)

A partial list of the wide range of agents with the ability to reverse MDR in preclinical models was shown in Table 6.

Drug substrates are actively transported, into 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 mechanism to antagonise MDR is the direct inhibition of drug efflux mediated by P-glycoprotein 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 7.

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 that leads to 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-glycoprotein, in a specific and saturable manner, and this binding may be inhibited by other chemosensitisers and by chemotherapeutic drug (Cornwell *et al*. 1987 and Naito and Tsuruo, 1989).

Table 6. MDR modulators and levels required toreverse MDR *in vitro* (Ford, 1996)

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.*

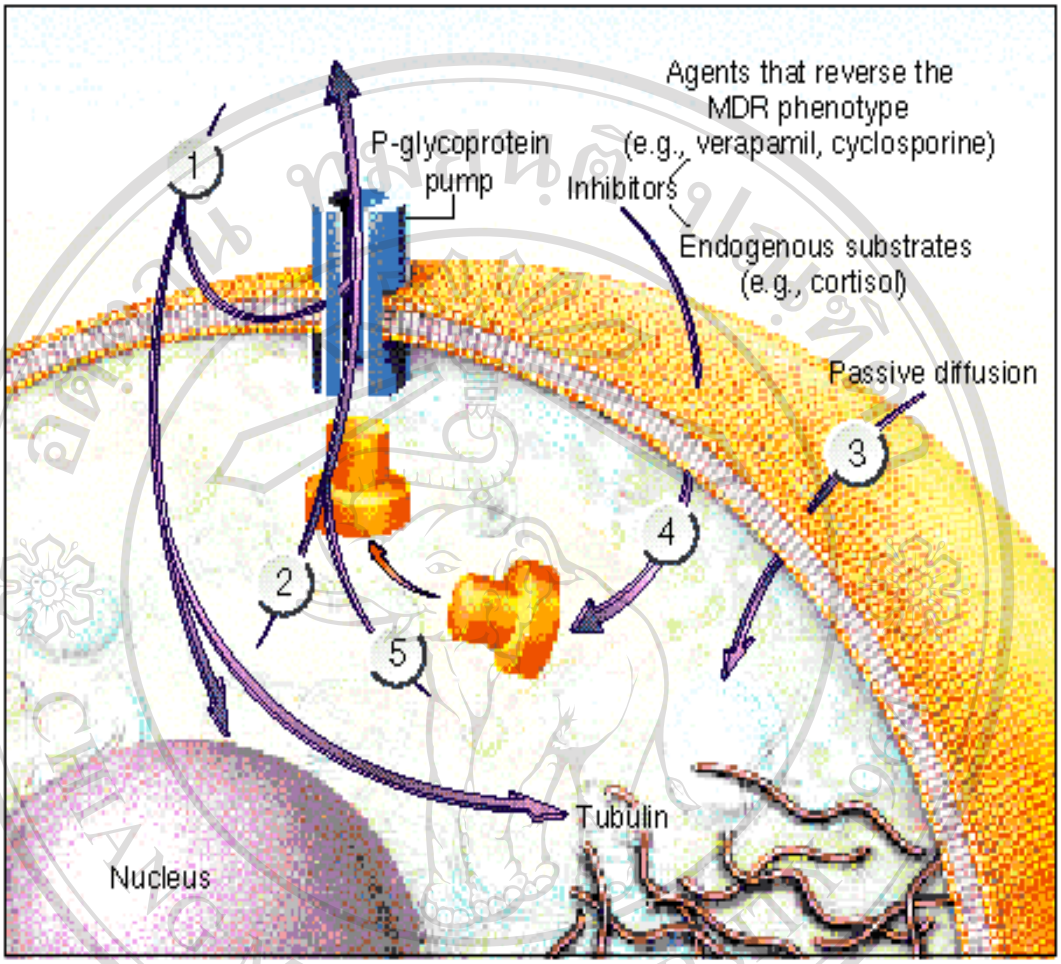

Fig. 7. The P-glycoprotein pump may transport cytotoxic drugs directly from the cell membrane, before such drugs enter the cytoplasm (1), or from the cytoplasm (2), limit the drug concentration of such drugs at the target (DNA or tubulin). Highly lipophilic drugs enter the cell by passive diffusion (3). 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). (Pinedo and Giaccone, 1995)

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

Multidrug resistant cancer cell lines (MDR-cancer cell lines) for biochemical, physiological and genetic study were derived from cancer cell lines bases of alterations that result in the development of multidrug resistance. The highly expressed of P-glycoprotein gene was found in many MDR cancer cell lines. The model tissue culture systems have been developed for studying the mechanism of multidrug resistance. Culture cells selected for resistance to one of the drugs become resistant to other structurally unrelated natural products, such as anthracyclines, vinca alkaloids, and epipodophyllo toxins (Shen *et al*. 1986).

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, are the increasing of P-glycoprotein level and activity in Chinese hamster ovary (CHO) cells and decreasing accumulation of drugs in human multidrug resistant KB-carcinoma cell lines.

Multidrug resistant cell lines have been isolated from tissue cuture by multistep selection. Human multidrug resistant KB-carcinoma cell lines (MDR KB-carcinoma cell) were 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 (Schoenlein *et al*. 1992).

In the preliminary studies, it was impossible to isolate spontaneous mutants of this cell line resistant to colchicine, vinblastine, and adriamycin. The ethylmethanesulfonate (EMS) was used to mutagenized KB cells in the first selection (Figure 8). 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 7.

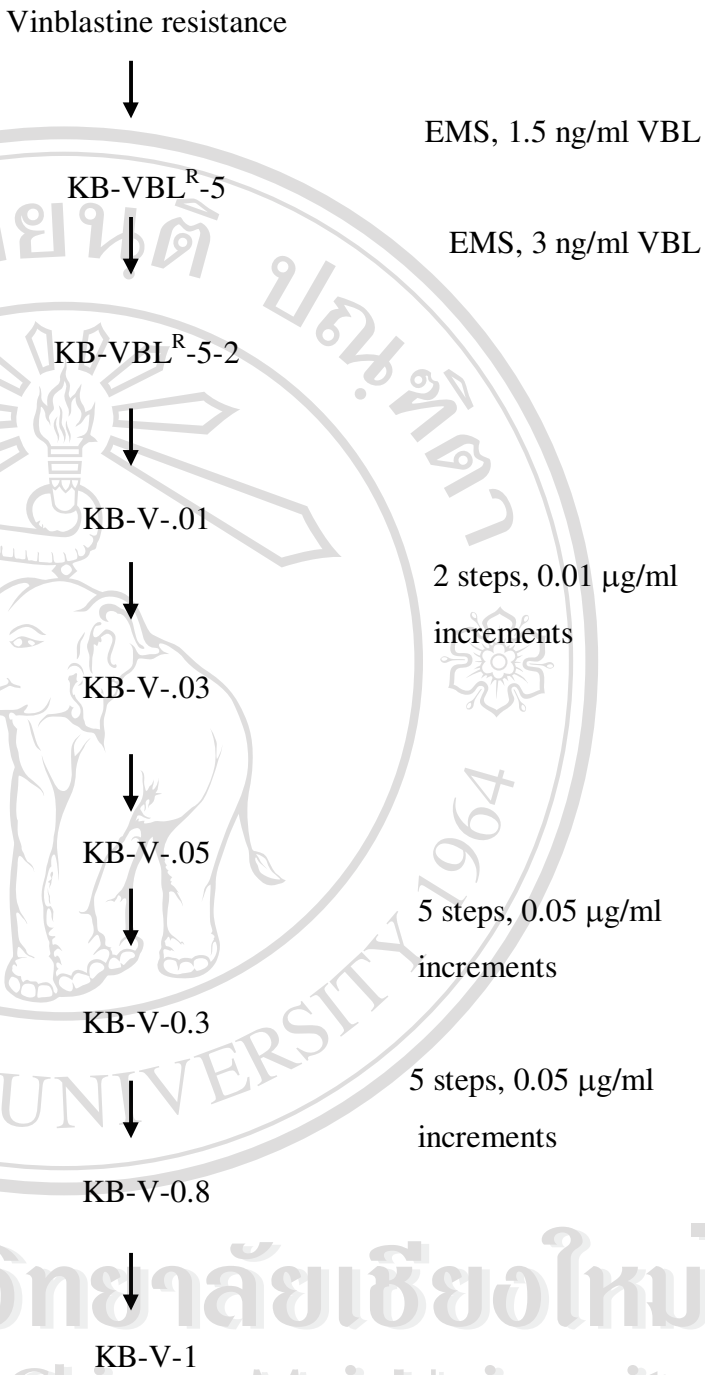

Fig. 8. Flow chart of the steps for increasing vinblastine resistance in the MDR KB-V-1 cell line. The parental cell line was KB subline designated KB-3-1. Abbreviations: VBL^{R}, vinblastine-selected subclones; V, vinblastine-selected populations. In each case, where a letter alone is used, the number following the letter refers to the selecting concentrations of drug in micrograms per milliliter(Shen *et al.* 1986).

Table 7. Properteis of MDR KB cell lines (Shen *et al*. 1986).

	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.

1.2.4 Multidrug resistance-associated protein 1 (MRP-1, ABCC1)

General properties of MRP-1

MRP-1 was first identified in a cell line made highly resistant to a cytotoxic drug (doxorubicin), and subsequent analysis showed that it conferred MDR against a range of anticancer drugs. Substrate anticancer drugs include *Vinca* alkaloids, anthracyclines, epipodophyllotoxins, mitoxantrone and methotrexate. Unlike P-glycoprotein, MRP-1 does not confer high levels of resistance to paclitaxel or bisantrene in cells. In further contrast to P-glycoprotein, MRP-1 functions mainly as a (co-)transporter of amphipathic organic anions. It can transport hydrophobic drugs or other compounds (e.g. the inflammatory mediator leukotriene C_4 (LTC_4) that are conjugated or complexed to the anionic tripeptide glutathione (GSH), to glucuronic acid, or to sulfate. In fact, efficient export of several non-anionic anticancer drugs by MRP-1 is dependent on a normal cellular supply of GSH, and it is likely that MRP-1 exports drugs such as vincristine and etoposide by co-transport with reduced GSH. Inorganic heavy metal oxyanions like arsenite and trivalent antimony are also transported by MRP-1, in all likelihood complexed to GSH. In contrast to P-glycoprotein, MRP-1 localizes to the basolateral membrane of epithelial cell layers,

and its substrates are therefore transported towards the basolateral side of epithelia. Knockout mice lacking MRP-1 are viable and fertile, but they do show deficiencies in LTC_4 -mediated inflammatory reactions, suggesting that secretion of LTC_4 is an important physiological function of MRP-1. (Schinkel and Jonker, 2003)

Pharmacological functions of MRP-1

Even though MRP-1 localizes predominantly to the basolateral membrane of epithelial cells, it still has some important pharmacological and toxicological functions. Wijnholds *et al.* (1997) showed that MRP-1 knockout mice are more sensitive to the toxicity of intravenously administered etoposide in the oropharyngeal mucosal layer and testicular tubules. This can be explained by the fact that these cells are shielded from blood-borne toxins by epithelia that have the basolateral membrane facing the blood circulation. Moreover, MRP-1 in the basolateral membrane of choroid plexus epithelial cells can mediate a substantial clearance of etoposide from the cerebrospinal fluid, indicating that this compartment is also protected by MRP-1. Other studies demonstrated that a combined deficiency of Mdr1a and Mdr1b P-glycoprotein and MRP-1 in knockout mice resulted in a dramatically increased sensitivity not only to intraperitoneally administered vincristine (up to 128-fold), but also to etoposide (3.5-fold), whereas a P-glycoprotein deficiency alone resulted in a 16- and 1.75-fold increased sensitivity to this drug, respectively. In this case, greatly increased vincristine toxicity was observed in bone marrow and the gastrointestinal mucosa, suggesting that these compartments are normally extensively protected by the P-glycoprotein and/or MRP-1 transporters. At the cellular level, the endogenous expression of MRP-1 (and P-glycoprotein) can already contribute substantially to the basal resistance of cell lines to a range of cytotoxic anticancer drugs, as was demonstrated *in vitro* with murine fibroblast and embryonic stem cell lines deficient for murine MRP-1 and/or Mdr1a and Mdr1b. A marked increase in sensitivity to epipodophyllotoxins, *Vinca* alkaloids, anthracyclines, topotecan and SN-38, and arsenite was found in these lines due to MRP-1 deficiency. Such contributions can explain the markedly increased drug sensitivity of bone marrow and intestinal epithelial cells deficient for MRP-1 especially when drug-transporting P-glycoprotein is also absent. For the extrapolation of pharmacological studies in knockout mice and

possibly rats to humans, it is important to be aware that the murine MRP-1 is much less efficient in transporting anthracyclines than human MRP-1 (Schinkel and Jonker, 2003).

Inhibitors of MRP-1

So far, it has been much more difficult to find good and small molecular inhibitors for MRP-1 than for P-glycoprotein, especially the ones that work in intact cells. This probably has to deal with the preference of MRP-1 for anionic compounds substrates and inhibitors: most anionic compounds barely enter cells, so it may be difficult to obtain the intracellular concentrations of the inhibitor for efficient inhibition. A variety of inhibitors of MRP-1 has been described. Some examples are the LTC_4 analogue MK571, LTC_4 itself, S-decylglutathione, sulfinpyrazone, benzbromarone and probenecid. P-glycoprotein inhibitors like cyclosporin A and PSC 833, with reasonable cellular penetration, do inhibit MRP-1, but only with low affinity and (obviously) low specificity. For specific *in vivo* inhibition of MRP-1, the general organic anion transporter inhibitors sulfinpyrazone, benzbromarone and probenecid are not the right one, as they extensively affect organic anion *uptake* systems as well. Moreover, these compounds work only at relatively high concentrations. To be able to improve anticancer chemotherapy by specially inhibit MRP-1 activity, better MRP-1 inhibitor with high specificity but low cytotoxicity need to be developed and penetration (Schinkel and Jonker, 2003).

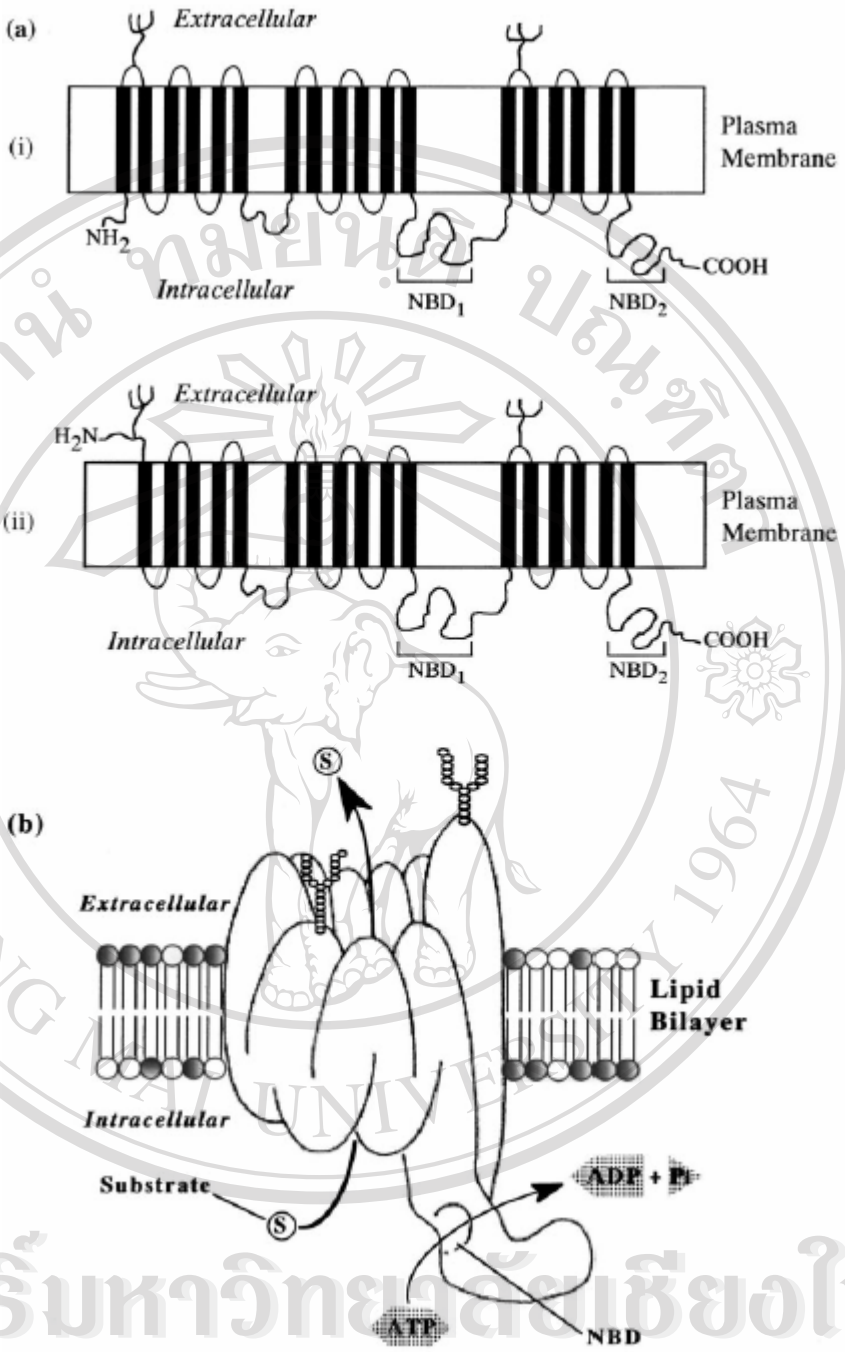

Fig. 9. (a) Models of membrane topology for multidrug resistance-associated protein (MRP) (i) and (ii) according to and, respectively. (b) Schematic illustration for ATP-dependent substrate transport by MRP. NBD, nucleotide binding domain (Quan *et al.* 2000).

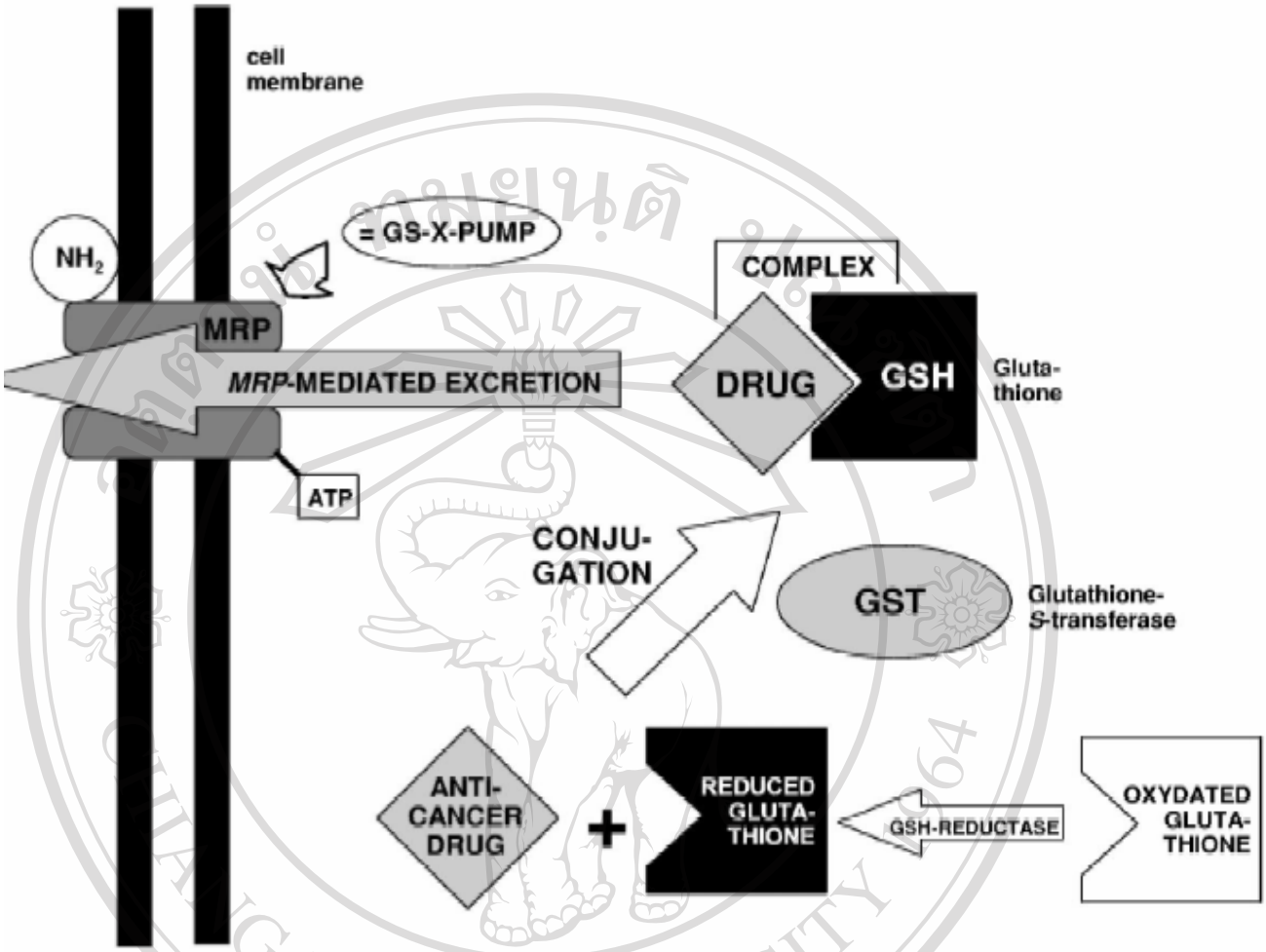

Fig. 10. Model of a putative interaction between *MRP* and the GSH-linked enzyme system in the formation of anticancer drug resistance in human brain tumors: glutathione (GSH)-*S*-transferase (GST) mediates the conjugation of cytotoxic drugs with GSH reductase-derived reduced GSH, which makes them more water soluble, leading to rapid detoxification of these agents. There is some evidence that in addition to its role as a resistance-related chemotherapeutic drug transporter, *MRP* may mediate the excretion of drug/GSH complexes generated by the GSH-linked drug detoxification system, thereby functioning as an active GSH conjugate export pump (GS-X) and, hence, may possess a bifunctional role in the chemoresistance of intracranial neoplasms (Bredel, 2001).

1.2.5 Mitoxantrone resistant protein

Structure, transport properties and possible mechanism of action of ABCG2

The G subfamily of ABC transporters consists of half transporters with a domain arrangement, when the ABC resistance protein; DOX, doxorubicin; MDR1, human multidrug resistance protein (P-glycoprotein, ABCB1); MRP, human multidrug resistance associated protein, ABCC1; MXR, mitoxantrone resistance protein; Sf9 cells, *Spodoptera frugiperda* ovarian cells; TK, tyrosine kinase; TM, transmembrane domain is located towards the N-terminus of the polypeptide chain.The suggested membrane topology of the human ABCG2, based on various prediction models, as presented in Fig. 1.1. The predicted glycosylation sites are also indicated in this model. There has been no detailed study as yet for the experimental verification of this membrane topology, but the double glycosylation of the protein, when expressed in mammalian cells, has already been documented. It has also been shown that the expression or transport function of ABCG2 does not require glycosylation, when the protein was expressed in insect, *Spodoptera frugiperda* ovarian cells (Sf9 cells). Membrane insertion and transport function most probably require the dimerization of ABCG2.

ABCG2 was first cloned from the placenta and from multidrug-resistant tumor cells, not expressing either MDR1 or MRP1. By now it has been established that ABCG2 functions as a high capacity drug transporter with low substrate specificity. This protein can transport large, hydrophobic, both positively or negatively charged molecules, including cytotoxic compounds (mitoxantrone, topotecan, flavopiridol, methotrexate), fluorescent dyes (e.g., Hoechst 33342) and different toxic compounds found in normal food (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, PhIP) or pheophorbide A

ABCG2 mediates the extrusion of the transported compounds towards the extracellular space through a process energized by ATP hydrolysis. This active transport has been directly observed in inside-out membrane vesicles, whenABCG2 was shown to transport methotrexate.

It has been reported that ABCG2 shows a drug-stimulated, vanadate-sensitive ATPase activity, which is Mg^{2+} dependent. This ATPase activity, as in the case of other multidrug transporters, reflects the substrate recognition and is connected to

substrate transport by this protein. However, when the human ABCG2 is expressed in the Sf9 insect cell system, in isolated membranes a large fraction of ABCG2-ATPase seems to be already activated by endogenous substrates, and additional substrates have only a minor effect. In contrast, in certain isolated mammalian cell membranes, ABCG2-ATPase is significantly activated by the transported compounds. Moreover, when human ABCG2 was expressed in a bacterial (lactobacillus) expression system, membrane cholesterol itself was found to activate this protein. Thus, endogenous lipids or lipid derivatives may act as natural transported substrates ofABCG2.

The active, ATP-dependent transport properties of ABCG2 have also been documented by the formation of the ATPase reaction cycle intermediate, trapped ADP, in this protein. This ADP trapping reaction, which is stabilized by the ATPase inhibitor vanadate, is a general feature of all known active transporter ABC proteins. Interestingly, in the case of ABCG2 only the cobalt complex of 8-azido-ATP could be used to obtain a covalent linkage of this trapped intermediate to the protein. ABCG2 has only one ABC and one TM domain, therefore, it requires dimerization to become active. There are several findings indicating that ABCG2 functions as a homodimer. In drug selected cell lines, an exclusive up-regulation of the ABCG2 mRNA and the amplification of the *abcg2* gene alone was enough to circumvent drug-toxicity. In mammalian or insect cells, transfected with the ABCG2 cDNA, ABCG2 was fully functional. Moreover, co-expression of an inactive ABCG2 variant with the wild-type protein resulted in a loss of ABCG2 activity. In this latter study, the formation of ABCG2 homodimer bridged by a disulfide bond, was suggested (Sarkadi *et al*. 2004).

ABCG2 in cancer drug resistance

The overexpression of ABCG2 was observed in certain drug resistant cell lines and tumors, providing a special multidrug resistant phenotype in these cancer cells. Human ABCG2 was shown to confer resistance against various, clinically relevant compounds, e.g., mitoxantrone, methotrexate, topotecan, SN38, and flavopiridol. The first cloning of ABCG2 from either expressed sequence tags or heavily drug selected cell lines yielded sequences coding for different amino acids at position 482. As discussed below, this single amino acid change results in major differences in substrate recognition and transport by ABCG2. Therefore, for some

time, different experimental expressions of ABCG2 yielded different results regarding substrate handling by this protein. Based on overwhelming evidence, in the current review we refer to R482-ABCG2 as the wild-type protein. Therefore, clinical anthracyclin resistance probably cannot be due to ABCG2, while in the case of many new anticancer agents ABCG2 may be a candidate transporter for cancer drug resistance. In fact, polymorphic variants may further modulate substrate recognition or transport activity of ABCG2.

It was reported that the human ABCG2 multidrug transporter interacts with two recently developed specific tyrosine kinase inhibitors (TKIs), Gleevec/Imatinib (STI-571) and Iressa (ZD 1839), with a high affinity. In current antitumor drug research, a large variety of TKIs with increasing specificity and selectivity have been developed. These are highly promising agents for specific inhibition of malignant cell growth and metastasis formation. However, their therapeutic potential also depends on access to their intracellular targets, which may be significantly modulated by ABC membrane transporters. It was indicated that ABCG2 modulation by TKIs may be an important factor in the treatment of cancer patients, moreover, an extrusion of TKIs by ABCG2 may result in tumor cell TKI resistance. Based on the role of ABCG2 in tumor resistance described above, the selective and sensitive detection of the ABCG2 protein has a great impact in cancer diagnostics and treatment. The best known experimental approach in this regard is the analysis of the active extrusion of the fluorescent dye, Hoechst 33342 by ABCG2, by using fluorescence detection, e.g., flow cytometry. Several other fluorescent substrates of ABCG2 (e.g., topotecan, flavopiridol, BODIPY-prazosin or mitoxantrone) have also been applied. However, this dye transport assay is not usually specific for ABCG2, so the combination of ABCG2-specific inhibitors is recommended for these methods. A recent report suggested that the chlorophyll derivative, pheophorbide A, is an ABCG2 selective transported substrate that can be used for flow cytometry analysis of this protein. Unfortunately, a highly sensitive and selective dye transport method *in vivo* sorting of ABCG2 expressing cells is yet to be introduced.

The recent development of a monoclonal antibody, specifically reacting with the human ABCG2 protein on the cell surface, has been a major breakthrough in diagnostic applications. This antibody was prepared by immunizing mice with intact

mouse fibroblasts, expressing the human ABCG2. The antibody, named 5D3, was reported to inhibit the Hoechst 33342 dye transport function of ABCG2 in intact cells. Recently, it was found that detection and inhibition by 5D3 strongly depends on the actual conformation of ABCG2. This conformation-sensitive interaction may be applied for the investigation of the molecular mechanism and the detection of drug interactions of ABCG2 (Sarkadi *et al*. 2004).

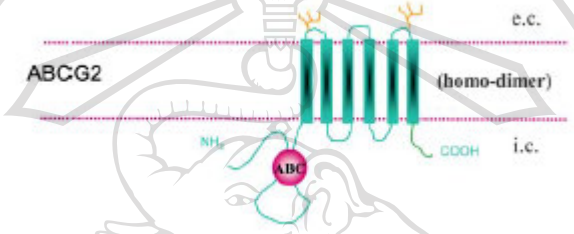

Fig. 11. Proposed membrane topology ABCG2 multidrug transporters (Sarkadi *et al*. 2004)

Table 8. Reported inhibitors of BCRP/Bcrp1 (Allen and Schinkel, 2002)

Compound	Effective concentrarion	Comments
Reserpine	5 μM	Also inhibits P-gp
Cl1033	Low micromolar	HER tyrosine kinase inhibitor
GF120918	EC_{90} about 0.05 μM	Potent P-gp inhibitor, well tolerated *in vivo*
FTC	1-5 μM	Low activity against P-gp and MRP-1, neurotoxic
Ko134	EC_{90} about 0.1 μM	Low activity against P-gp and MRP-1, well tolerated *in vivo*

1.2.6 *Stemona tuberosa Lour*.

Family: Stemonaceae

Synonyms: *Roxburghia gloriosoides* Roxb., *R. viridiflora* Smith, *R. stemona* Steud. (Keys, 1976)

Common names: Pai Pu (Hsu and Hong, 1980)**;** Bai Bu in Chinese (Geng *et al*. 1997), the name translates to "hundred parts" (so named because its roots are over one hundred in number) (Lu and Henry, 1991). Wild Asparagus (English) (Lu and Henry, 1991). Sessile Stemona Root, Japanese Stemona Root, Tuber, Stemona Root.

Habitat: It is an herbaceous plant found in Central China, Indochina, Taiwan, India and Thailand. (Keys, 1976)

Part used: Tuberous roots cropped all year round, especially in autumn. After being well washed and docked at each end, the roots are steam-cooked, then dried in the sun or in ovens at 50-60°C.

Description: The drug occurs as yellowish white, cylindrical tubers, the interior hollow and dark brown. The taste is bittersweet (Keys, 1976).

Nature: Sweet and bitter; slightly cold. It was described also as sweet, bitter and neutral.

Affinity or Meridian: Lungs.

Energy: slightly warm.

Class: 10, herbs to suppress cough and reduce sputum (Lu and Henry, 1991).

Chemical composition: The tubers contain an alkaloid called stemonine ($C_{22}H_{33}NO_4$; white needles, odorless, slightly bitter; soluble in alcohol, ether, acetone, toluene, benzene, chloroform; m.p. 160°), which is mildly toxic (Keys, 1976). In addition, the tuberous roots contain alkaloids: tuberostemonine, isotuberostemonine, stemonidine, sinostemonine; glucides 2.3%, lipids 0.83%, proteins 9%, organic acids (citric, formic, malic, succinic) (WHO 1990). Three bibenzyls were isolated from the roots of *S. tubersosa* (Zhao *et al*. 1995). The root of Stemona plants contains alkaloids. The root of *S. sessilifolia* contains tuberostemonine and oxotuberostemonine. From the root of *S. japonica* protostemonine, stemonamine and isostemonamine were isolated. Tuberostemonine, stenine, oxotuberostemonine, stemonine, stemotinine and isostemotinine were identified in the root of *S. tuberosa* (Zhu and You, 1998). Two new alkaloids, named tuberostemoninol and stemoninoamide, were isolated from the roots of *S. tuberosa* (Lin *et al*. 1994), two new alkaloids, named neotuberostemonine and bisdehydroneotuberostemonine, were also isolated from the roots (Ye *et al*. 1994) and stenine, a new alkaloid (Ueo *et al.* 1976). Also stemonone, stemonal and neotuberostemonine. The full list of alkaloids and all the structures are given as follows: Stenine, Tuberostemonine, Tuberostemonol, Didehydrotuberostemonine, Bisdehydroneotuberostemonine, Neotuberostemonine, Oxotuberostemonine, Stemoamide, Tuberostemoamide (Stemoninoamide), Tuberostemospironine, Stemotinine, Isostemotinine, Tuberostemonone and Tuberostemoninol (Pilli 2000).

Properties: Antitussive; demulcent to lungs; anthelmintic; kills lice (Reid 1993). The herb decoction could inhibit multiple types of bacteria and skin fungi. It was lethal to maggots, mosquitoes, mandarin aphids, cutworms, etc.

Medicinal use: Stemonine calms the respiratory centre; it is strongly effective against *Pediculus capitus, P.corporis* and *Phthirus pubis* without irritation or toxicity (Keys,1976). The insecticidal effect was confirmed, the aqueous and the 70% alcoholic extract of the herb were lethal to *Pediculus capitis* and *P. vestimenti*. It was also able to kill lice ova. The alcoholic extract killed *P. pubis* in a few min after contact. Tuberostemonine produced an inhibitory effect on the motility of *Angiostronglyus cantonensis*, *Dipylidium caninum* and *Fasciola hepatica* at 6.7 x 10^{-6}

– 6.7 x 10^{-5} M *in vitro* (Zhu and You,1998). It is used for killing insects and worms and used externally in pediculosis capitis, pediculosis corporis, oxyuriasis (infestation with pinworms) and pudendal itching (Zhu and You, 1998). Note: The pudendal nerve carries sensations from the external genitals, the lower rectum, and the perineum (between the genitals and the anus). The infusion of the drug can be applied externally to treat head louse, body louse, pubic louse and clothes louse (not effective against louse eggs). When sprayed, the drug can destroy bedbugs. When taken orally, the drug can be used to treat enterobiasis (an infestation with or a resulting infection caused by the pinworm *Enterobius vermicularis*; occurs especially in children); and the disease can also be treated by washing the anus with the decoction of the drug. It can be used to treat coughs; chronic, dry coughs; whooping cough; tapeworm; external application to lice Experiments indicate that Bai Bu is effective for suppression of cough (Zhu and You, 1998); (Tierra and Michael, 2003) and it is also an antituberculotic herb (Lu and Henry, 1991) and recent applications have confirmed the drug to be effective against tuberculosis (Reid 1993), chronic bronchitis (Zhu and You, 1998) and demulcent, antitussive, antifungal (Tierra and Michael, 2003). It has recently been studied for the treatment of pertussis - a disease of the respiratory mucous membrane (also known as whooping cough) (Wang *et al*. 1960). The tuberous roots are well known for their antibacterial, anthelmintic (a medication capable of causing the evacuation of parasitic intestinal worms) (Hsu and Hong, 1980), antiparasitic (Zhu and You, 1998); (Tierra and Michael, 2003) and expectorant properties. The decoction of the herb was also inhibitory against multiple species of bacteria and fungi and the alcoholic extract of the herb at 1:100-1:1600 inhibited and at 1:80 killed *Mycobacterium tuberculosis* var. *hominis* (Zhu and You, 1998). The decoction or extract is applied externally against impetigo and scabies. Can also be used as an insecticide against mosquito larvae (Lee and Chiang, 1994); (WHO 1990), fleas and bugs (WHO 1990). The insecticidal effects have been confirmed recently (Brem *et al*. 2002); (Jiyavorranant *et al*.).

Chinese use: To moisten the lungs and stop cough, to kill lice and parasites (Lu and Henry, 1991); (Geng *et al*. 1997). Also to bring down energy, and destroy worms (Lu and Henry, 1991).

A

B

C

D

Fig. 12. The pictures of each parts of *S.tuberosa* are shown as follow: A; flower, B; seed, C; fruit and D; roots.

1.3 Objectives

1. To study the effect of *S.tuberosa* 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 *S.tuberosa* extracts on drugs sensitivity 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 *S.tuberosa* 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).

4. To study the effect of *S.tuberosa* extracts on cytotoxicity in drug resistant transfected human embryonic kidney cell lines (HEK_{293}/MRP-1) and drug sensitive transfected human embryonic kidney cell lines (HEK_{293}/pcDNA).

5. To study the effect of *S.tuberosa* extracts on etoposide sensitivity in drug resistant transfected human embryonic kidney cell lines (HEK_{293}/MRP-1) and drug sensitive transfected human embryonic kidney cell lines (HEK_{293}/pcDNA).