CHAPTER II

LITERATURE REVIEW

1. Cancer Chemotherapy

Cancer is basically a disease of cells characterized by a shift in the control mechanisms that govern cell proliferation and differentiation. The neoplastic transformation usually induced expression of cell surface antigens that differ from normal mature cells. In addition, the neoplastic cells may exhibit qualitative or quantitative chromosomal abnormalities, including various translocations and appearance of amplified gene sequences. The excessive proliferation can compress or invade adjacent normal structures. Moreover, certain cancerous cells migrate to distant organs in the body in the process called metastasis. The invasive and metastatic processes as well as a series of metabolic abnormalities cause illness and eventual death of the cancer patients unless the neoplasm can be eradicated with treatment (Chu and Sartorelli, 2004; Rubin and Hait, 2006).

Under current treatment algorithm, one third of patients has not developed the metastasis and can be effectively cured with local modalities (surgery or radiation therapy). However, the remaining cases have early micrometastasis and require a systemic approach in treatment such as chemotherapy (Chu and Sartorelli, 2004).

The anticancer drugs have been classified into 5 groups based on their sites of action along the synthetic pathway of cellular macromolecules such as DNA, RNA and protein. These drugs are alkylating agents, antimetobolites, cytostatic antibiotics, alkaloids, and lignans (Table 1). They are sharing some structural and functional similarities, such as small size, biplanar molecules, particle hydrophobicity or presence of nitrogen atoms in their aromatic rings. These compounds enter a cell by passive diffusion, accumulate in the cell and exert its cytotoxic effect (Sawicka *et al.*, 2004).

Table 1 Structural basis of anticancer drugs (Sawicka *et al*., 2004).

Groups	Chemical structure	Example drugs
Alkylating agents	Structurally different classes of chemical compounds, small size	Busalfan, Carmustine, Cyclophosphamide, Lomustine, Mechlorethamine, Mephalan, Thiotepa
Antimetabolites	Structure analogues of natural metabolites or coenzymes occurring in cellular biological systems	Capecitabine, Cladribine Cytarabine, Fludarabine, Fluorouracil, Gemcitabine, Mercaptopurine, Metrotrexate
Cytostatic antibiotic	One common feature of their chemical structures is the presence of a bond between the glycoside ring and the daunosamine aminosugar	Anthracycline: Epirubicin, Idarubicin Mitroxantrone Daunorubicin, Doxorubicin
Alkaloids	*Vinca* rosea indole alkaloids the complex structure is almost identical	Vinblastine, Vincristine, Vinorelbine
Lignans	Different chemical structure	Etoposide, Teniposide

2. Resistance to Cytotoxic Drugs

A major problem in cancer chemotherapy is drug resistance. Drug resistant cells can be classified into two major classes including (1) cells resistant to a single class of drugs with the same mechanism of action and (2) cells resistant to chemically diverse drugs with multiple mechanisms of action. The latter phenomenon is known as multidrug resistance (MDR) (Sawicka *et al.*, 2004). Although numerous factors have been implicated in the development of MDR, a large body of evidence strongly supports an important role for energy-dependent pump systems that either exclude or extrude chemotherapeutic agents from cells, rendering less cellular accumulation of anticancer drugs (Higgins *et al.*, 1997; Stavrovskaya, 2000; Sauna *et al.*, 2001). Figure 1 illustrates the transport proteins implicated in MDR. P-glycoprotein (P-gp), which is the human MDR1 gene product, was the first ATP-dependent system that was identified in MDR (Juliano and Ling, 1976).

However, the overexpression of P-gp is not the only cause of MDR. Another member of the transmembrane transporters include the proteins which belong to the ATP-binding cassette (ABC) superfamily such as the MDR-associated protein 1-9 (MRP1-MRP9) (Gottesman *et al.*, 2002), and the mitoxantrone resistance (MXR) associated proteins (e.g. breast cancer resistance protein, BCRP) (Sauna *et al.*, 2001). These transporters function as a drug-export pump, resulting in reduction of intracellular drug level.

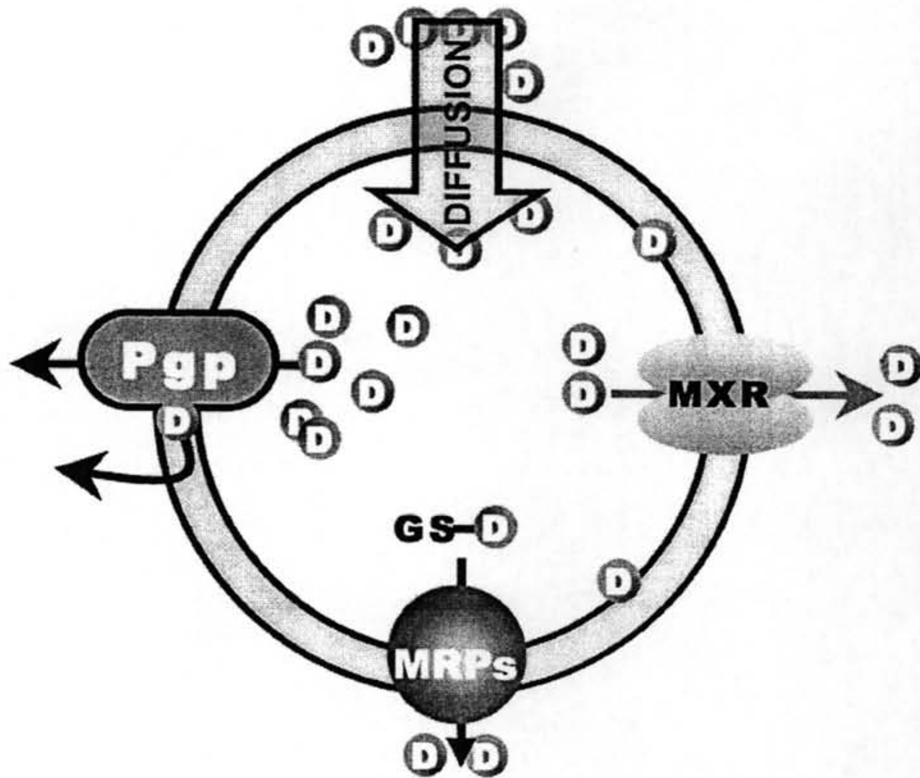


Figure 1 Role of ABC transporters in the development of MDR phenotype in cancer cells. Most natural product drugs (D) enter the cell by diffusion. They may be pumped out by P-gp and MRP using ATP. Drugs complex with glutathione (GSH) may also be transported out of cells by MRP1-4. MXR (or ABCP or BCRP) also efflux drugs in an energy-dependent manner (Sauna *et al.*, 2001).

3. P-glycoprotein (P-gp)

3.1 Structure of the multidrug resistance P-glycoprotein

P-glycoprotein (P-gp) is the most typical transmembrane efflux pump with a molecular weight of 150-170 kDa. P-gp belongs to the ATP-binding cassette (ABC) family of transporter. As shown in Figure 2, P-gp is glycosylated at the first extracellular loop and composed of 12 hydrophobic transmembrane domains (TMDs) and 2 nucleotide-binding domains (NBD). One NBD connects two TMDs with a hydrophilic NBD loop. TDMs form channels for substrate efflux, whereas, NBDs in the interior of cytoplasm participate in the binding and hydrolysis of ATP. Upon ATP binding, the TMDs reorganize into three compact domains in order to transform the lipid bilayer into the central pore of the transporter (Higgins *et al.*, 1997; Germann and Chamber, 1998; Ambudkar *et al.*, 1999; Ozben, 2006).

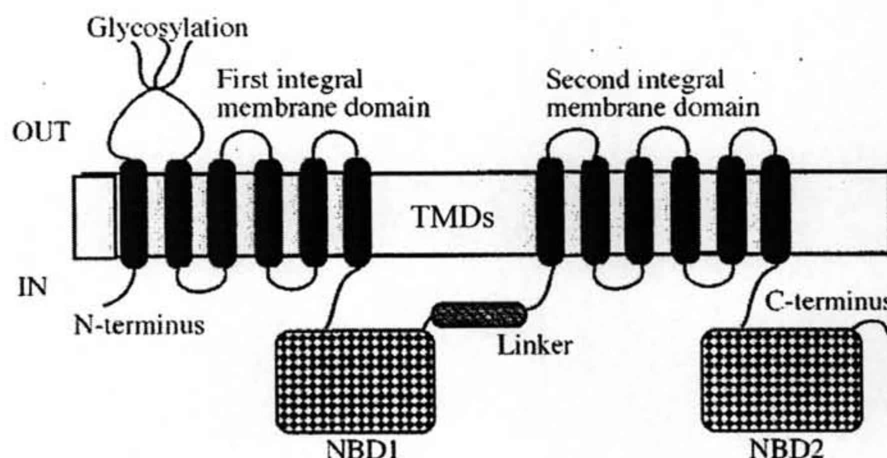


Figure 2 Topological map and domain organization of P-glycoprotein (Higgins *et al.*, 1997)

Recent studies indicated that there are at least four distinct drug-binding sites (DBSs) on P-gp which can be classified as transport and modulating sites for substrate or inhibitors (Wang *et al.*, 2003).

3.2 Physiological function and localization of P-gp

P-gp is expressed constitutively in many normal human tissues. Normal physiological roles of P-gp have been speculated in absorption, distribution, and excretion of xenobiotic. Endogenous substrates of P-gp include lipids and steroid hormones such as sphingomyelin, phosphatidylcholine, corticosterone, and testosterone. In epithelial cells of the lower GI tract (jejunum, ileum, and colon), high levels of P-gp are located only on the mucosal surface suggesting its function to prevent uptake of substrate and perhaps to facilitate excretion across the mucosa. In kidney and liver, P-gp is presented on the brush border of proximal tubule and hepatocytes, which is consistent with a role for P-gp in excretion of xenobiotics and endogenous metabolites into the urine and bile. P-gp is also found on the apical surface of pancreatic ductules and on the luminal surface of capillary endothelial cells in the brain and testes (Matheny *et al.*, 2001; Stavroskaya, 2000; Larsen *et al.*, 2000).

The expression of P-gp increases markedly in tumors that are derived from tissues that normally express P-gp. In this regards, the tumors become resistance to certain cytotoxic agents before chemotherapy is initiated. In some tumors, the expression of P-gp may be at the low level before chemotherapy, and the expression increases substantially during the chemotherapy. Consequently, those tumors become MDR.

3.3 Mechanism of P-gp-induced drug efflux

Various models have been proposed to explain the mechanism of P-gp induced extrusion of xenobiotics. The most extensive-studied models include pore model, flippase model and hydrophobic vacuum cleaner (HVC) model (Verma *et al.*, 2003) (Figure 3). In pore model, drug associates with P-gp in the cytosolic compartment then it is transported out of the cell through a protein channel. In the flippase model, drug is embedded in the inner leaflet of the plasma membrane, bind to P-gp within the plane of membrane then it is translocated to the outer leaflet of the bilayer. Subsequently, that drug passively diffuses into extracellular fluid. The hydrophobic vacuum model combines the features of pore and flippase models.

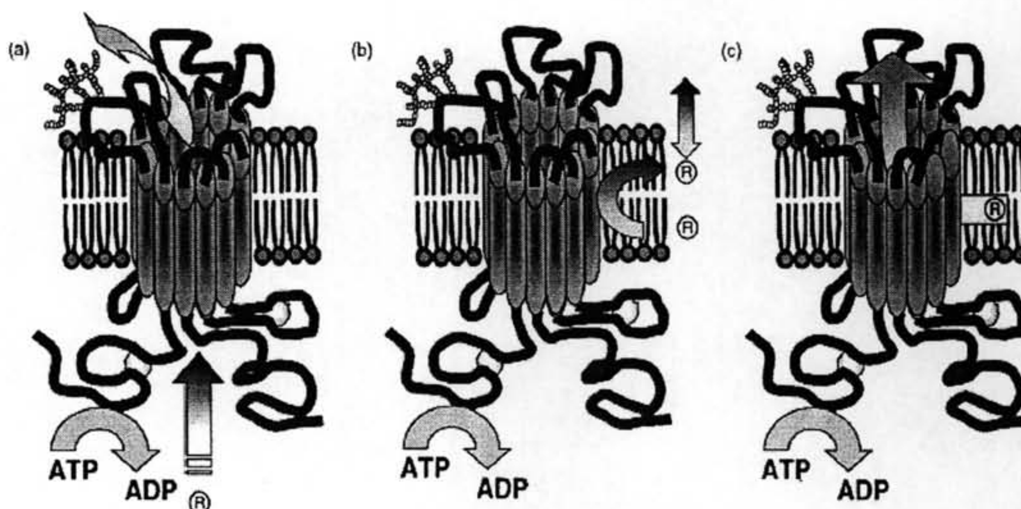


Figure 3 Models proposed to explain the mechanism of drug efflux by P-gp. (a) Pore model, (b) flippase model and (c) hydrophobic vacuum cleaner model.

3.4 Agents that interact with P-gp

A variety of chemicals with different molecular structure can bind to P-gp and become P-gp substrates or P-gp inhibitors. Furthermore, a number of compounds can affect the expression of P-gp (Matheny *et al.*, 2001).

P-gp modulators can be divided into three categories (Wang *et al.*, 2003) as the followings:

1. High-affinity substrates of the pump
2. Efficient inhibitors of ATP hydrolysis-coupled P-gp transport
3. Partial substrates or inhibitors, depending on the type of its interaction with P-gp

3.4.1 P-gp substrates

Several chemicals with structural differences have been identified as P-gp substrates. Generally, the known P-gp substrates share certain common features including hydrophobicity with a molecular mass of 300-2000 Da, and a positive charge at pH 7 (Sarkadi and Miller, 1997; Ambudkar *et al.*, 1999). Examples of P-gp

substrates are anticancer drugs (*Vinca* alkaloids, anthracyclines, and epipodophyllotoxins), antibiotics, and other cytotoxic agents (Table 2) (German and Chamber, 1998; Sauna *et al.*, 2001).

Table 2 Cytotoxic agents known as P-gp substrates (Germann and Chambers, 1998).

Groups of compound	**Drugs**
1. Anti-cancer drug	
Anthracyclines	Daunorubicin, Doxorubicin, Idarubicin, Mitroxantrone
Vinca alkaloids	Vincristine, Vinblastine
Epipodophyllotoxins	Etoposide (VP-16), Teniposide (VM-24)
Taxanes	Paclitaxel, Docetaxel, Taxotere
Miscellaneous	Dactinomycin, Mithramycin, Trimetrexate, Mitomycin, Topotecan
2. Other cytotoxic compounds	
Antibiotics	Actinomycin D
Antimicrotubule agents	Cochicine, Podophyllotoxin
Protein synthesis inhibitors	Puromycin, Emetine
DNA intercalators	Ethidium bromide
Toxic peptides	Vanilomycin, Gramicidin, N-acetyl-leucyl-leucyl-norleulinal (ALLN)

3.4.2 P-gp inhibitors

A large number of non-cytotoxic compound known as chemosensitizers or MDR modulators can enhance the response of drug-resistant cells to cytotoxic drugs. These sensitizers may exert their actions through inhibition of P-gp-mediated cytotoxic drug transport. It is possible that the sensitizers can interfere with substrate binding, ATP hydrolysis, ATPase activity, and inhibition of protein kinase C (PKC) (Ambudkar *et al.*, 1999; Wang *et al.*, 2003).

The structural definition of the MDR pharmacophore is complicated due to conserved elements of molecular recognition. P-gp is able to recognize drug molecules directly from membrane bilayer. Hence, hydrophobicity appears to be an important property of P-gp modulators. Aromatic groups largely contribute to the hydrophobicity of a molecule. In addition, anti-MDR compounds contain planar ring structures and basic nitrogen atom within an extended side chain of the aromatic ring. The presence of tertiary amino groups, in comparison with primary and secondary amine, increases the anti-MDR potency considerably. Moreover, the chemosensitizing activity increases even more if the nitrogen atom is incorporated into a non aromatic ring (Wiese and Pajeva, 2001).

Although most of the P-gp inhibitors share a basic structure pattern comprising a cationic protonable site linked to an aromatic lipophilic part by spacer of variable length, the establishment in structure activity relationship (SAR) has been difficult due to the diversity of chemical structure of inhibitors. Generally, the inhibitors of P-gp may be grouped into seven classes based on their molecular structure or pharmacological actions. They are (1) calcium or sodium channel blockers, (2) calmodulin antagonists, (3) protein kinase C inhibitor (PKCIs), (4) flavonoid and steroidal compounds, (5) indole alkaloids and polycyclic compounds, (6) cyclic peptides and macrolide compounds, (7) miscellaneous compounds (Table 3).

Development of P-gp inhibitors

One mechanism to overcome MDR toward anticancer drugs is inhibition of MDR transporters such as P-gp. This may achieve by administration of non-toxic compounds that inhibit function of ABC transporters. The compounds that would reverse resistance against anticancer drugs are called MDR inhibitors, MDR

modulators, MDR reversal agents, or chemosensitizers. They can be divided into three generations as follows: (Krishna and Mayer, 2000; Liscovitch and Lavie, 2002; Mahadevan and List, 2004; Ozban, 2006).

First-generation MDR inhibitors

First-generation MDR inhibitors have other pharmacological activities and have not been specifically developed for inhibiting MDR. Most of these compounds are also substrates for ABC transporter. However, their affinities toward ABC transporters are low thus the high dose is required. Consequently, its application as MDR modulators is quite limited due to an unacceptable high toxicity and side effects.

Verapamil is first-generation MDR drugs. It can both stimulate (if the pump is inactive) and inhibit (if actively pumping) the activity of P-gp, and that verapamil binding of many chemotherapeutic agent as well as other modulators to P-gp, indicating that verapamil acts through the mechanism of competitive inhibition on P-gp binding. Subsequent studies also demonstrated that many other MDR chemosensitizers behave in a similar manner to that of verapamil, with different potencies or acting on different sites of P-gp molecule (Wang *et al*., 2003).

Second-generation MDR inhibitors

Second-generation chemosensitizers have been designed to reduce the side effects of the first generation drugs. However, most of these compounds and anti-cancer drugs are substrates for cytochrome P450 3A4 and several anticancer drugs are substrates of both ABC transporter and cytochrome P450 3A4. The pharmacokinetic interaction between anticancer drugs and the MDR modulators may occurs, leading to an unanticipated increase in concentration and unacceptable side effects of anti-cancer drugs.

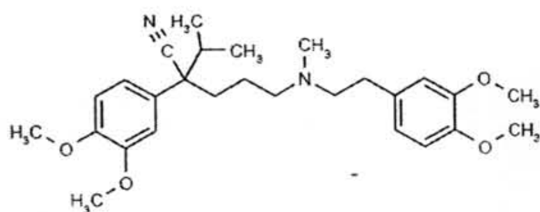
Third-generation MDR inhibitors

Third-generation molecules have been developed to overcome the limitations of the second generation MDR modulators. They are not metabolized by cytochrome P450 3A4. Thus, they have no influence on the plasma pharmacokinetics of

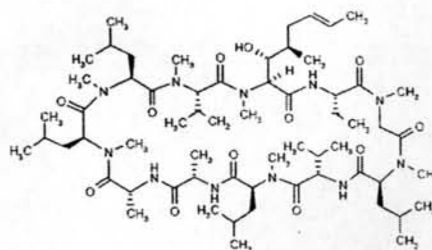
anticancer drugs. Third-generation agents specifically and potently inhibit P-gp without effects on other ABC transporters. These compounds offer significant improvements in chemotherapy without a need for chemotherapy dose reductions.

Table 3 Examples of MDR reversing agents (Krishna and Mayer, 2001)

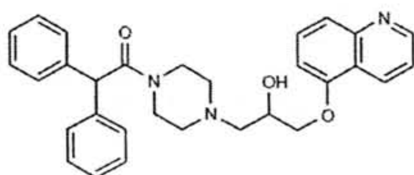
Category	Examples
1. calcium channel blockers	verapamil, nifedipine, diltiazem, beperidil
2. calmodulin inhibitors	trifluperazine, prochlorperazine, *trans*-flupenthixol
3. coronary vasodilators	dipyridamole, amiodarone
4. indole alkaloids	vindoline, reserpine
5. quinolines	chloroquine, quinine
6. lysomotropic agent	nigericin, monexin
7. hormones and antihormones	progesterone, tamoxifen
8. cephalosporins	cefoperazone, ceftriaxone
9. anticancer drug analogs	N-acetyldaunorubicin, C-20'-vinblastine
10. cyclosporins	cyclosporine A
11. protein kinase inhibitors	staurosporine, H-87
12. surfactants and lipids	cremophor-EL, Tween 80, PS
13. antibodies	MRK16, UIC-2
14. structure analogs of first generation	dexverapamil, emopamil, gallopamil, Roll-2933, dexniguldipine
15. cyclosporine A analogs	PSC-833
16. others	Ro44-5912
17. cyclopropyldibenzosuberane	LY335979
18. acridonecarboxamide	GF120918
19. diketopiperazine	XR9051
20. diarylimidazole	OC144-093



Verapamil



Cyclosporine A



MS-209

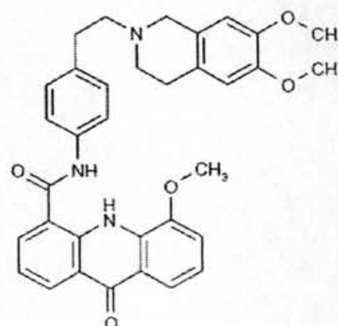


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Figure 4 Some structures of MDR reversing agents

3.4.3 Mechanism of P-gp inhibitors

Most of the P-gp inhibitors or reversing agents block trans-membrane transport by acting as competitive or noncompetitive inhibitors. These inhibitors can bind to substrate binding sites or allosteric sites. However, none of the known current modulators inhibit the binding of ATP to its binding site. For example, verapamil is a competitive inhibitor without the influence on catalytic cycle of P-gp. Cyclosporine A can inhibit transport function through interfering with substrate recognition and ATP hydrolysis. In addition, some P-gp inhibitors such as safingol may target at the posttranslation of P-gp protein. However, mutational analyses have shown that phosphorylation of P-gp is not essential for its transport function (Ambudkar *et al.*, 1999; Wiese and Pajeva, 2001).

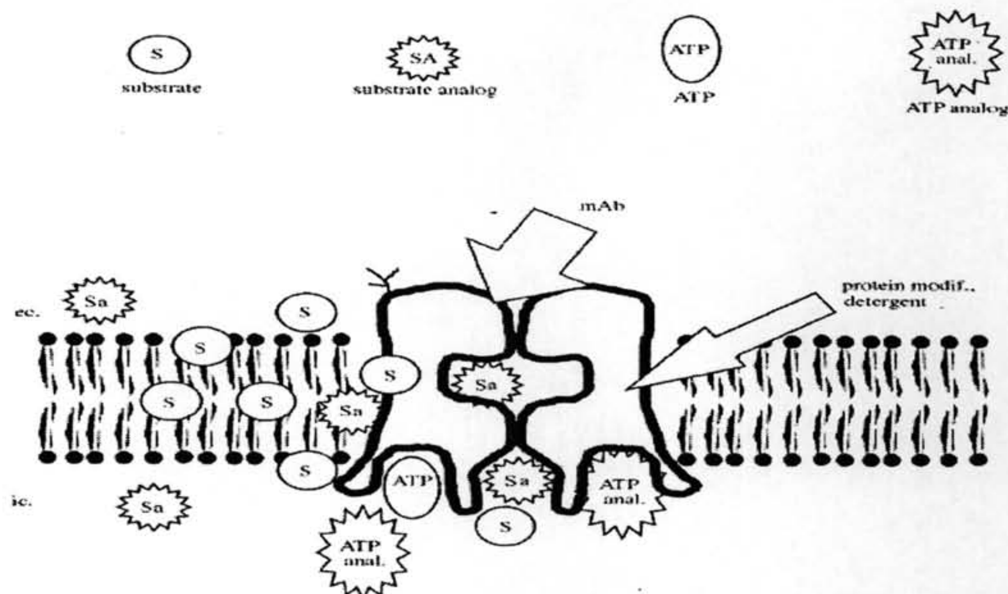


Figure 5 Inhibitors of the multidrug transporters and their possible models of action (Sarkadi and Muller, 1997).

Many possible modes of action of MDR inhibitors have been proposed as in Figure 5. Compounds like substrates analogs of P-gp may inhibit P-gp mediated drug transport reversibly or irreversibly by occupying the substrate-interacting domain of P-gp. In addition, those ATP analogs bind irreversibly to the specific ATP-binding domains of the transporters, and inhibit its function. Monoclonal antibodies reacting extracellular functional domains may selectively inhibit changes in protein conformation. Agents which modify lipid-protein interactions or protein structure, if effectively targeted, could also be developed as clinically applicable MDR inhibitors.

4. Natural products as potential P-gp inhibitors

4.1 Quinine and quinoline derivatives

Several quinine and quinoline derivatives (Figure 6) have been identified as P-gp inhibitors. For example, quinine increased the remission rate and survival in P-gp positive hematological malignancies treated with intensive chemotherapy (Avendano and Menendez, 2002). MS-209, a quinolone derivative (Figure 4) with high affinity to P-gp and MRP, inhibit P-gp function at low nanomolar concentrations in the *in vitro* model. Currently, MS-209 is in Phase III trial in Japan for the treatment

of breast cancer and Phase I trials in breast and lung cancer in Europe (Liscovitch and Lavie, 2002).

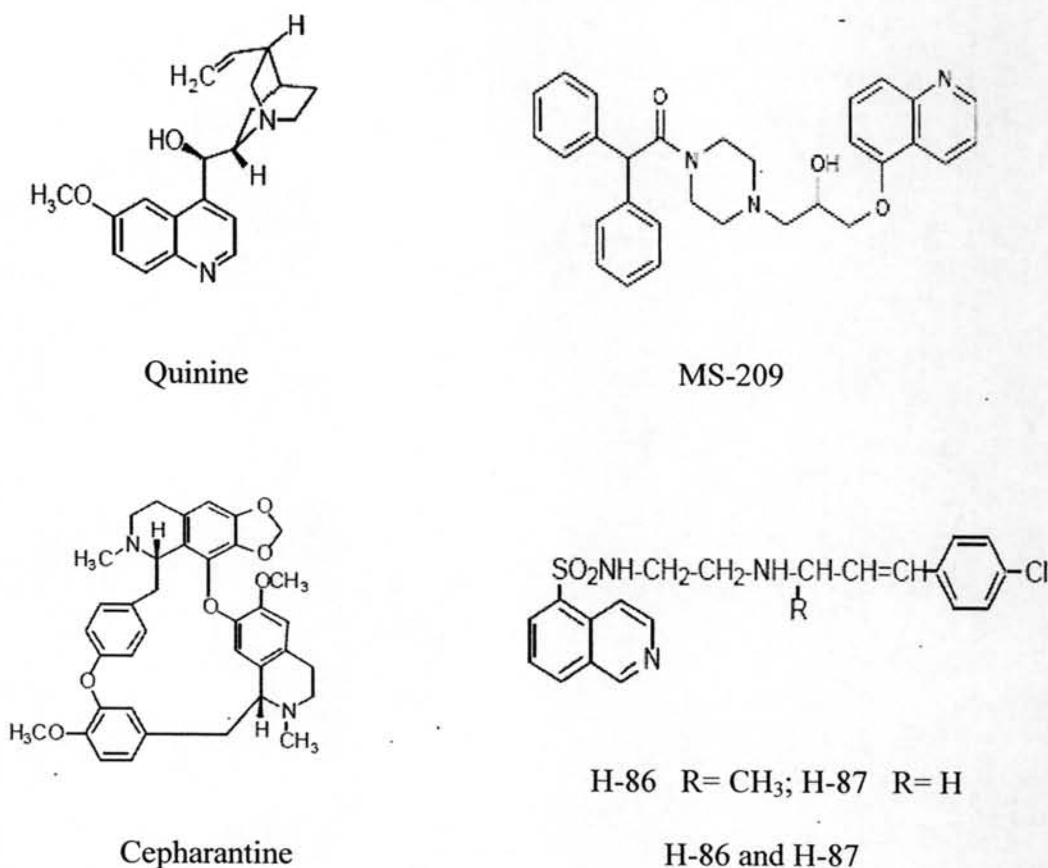


Figure 6 Structure of quinine and quinoline derivatives (Avendano and Menendez, 2002; Liscovitch and Lavie, 2002).

Most of the derivatives of isoquinoline alkaloids have anti-MDR properties. For example, cepharantine and related bisbenzylisoquinoline, tetrandine, elicit anti-MDR properties in various degrees, which are due to interaction with P-gp in P-gp expressing cell lines (Hirai *el al.*, 1995; Wang *et al.*, 2003; Fu *et al.*, 2004) and another isoquinoline-5-sulfonamide derivatives such as H-86 and H-87 are also capable of helping the cells to overcome overcoming resistance to vinblastine treatment. This effect is P-gp-dependent and correlated to the inhibition of the *mdr1* gene expression by inhibition of the cAMP-dependent protein kinase (Miyamoto *et al.*, 1990; Kim *et al.*, 1993).

4.2 Tetrahydroisoquinoline alkaloids

Some tetrahydroisoquinoline alkaloids have been shown their anti-MDR activities (Avendano and Menendez, 2002). The molecular structures of tetrahydroisoquinoline alkaloids are similar to those of isoquinoline derivatives, containing the quinone ring (1) and aromatic core (2) (Figure 7). The members of tetrahydroisoquinolines include potent cytotoxic agents that display a range of antitumor activities, antimicrobial activity, and other biological properties.

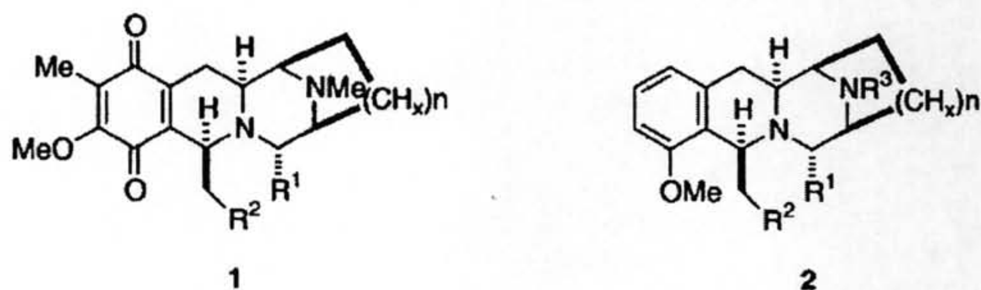


Figure 7 General structure of tetrahydroisoquinoline (Scott and Williams, 2002).

The tetrahydroisoquinolines from natural products can be classified into the saframycin, naphthyridinomycin/bioxalomycin, and quinocarcin/tetrazomine families. Furthermore, the saframycin family can be classified into four groups as follows: (1) saframycins, (2) renieramycins, (3) safracins, and (4) ecteinascidins (Figure 8) (Scott and Williams, 2002).

Saframycins and renieramycins are bistetrahydroisoquinolines containing two tetrahydroisoquinolines groups with different the side chains. Saframycins consist of a pyruvamide side chain, whereas, renieramycins contain an angelate ester side chain. Ecteinascidins are bistetrahydroisoquinoline alkaloids possess two units, unit A and B of tetrahydroisoquinoline, and the side chain is a ten-membered sulfide-containing lactone ring attached with unit C of a tetrahydroisoquinoline or β-carboline or chain of carbon (Figure 8).

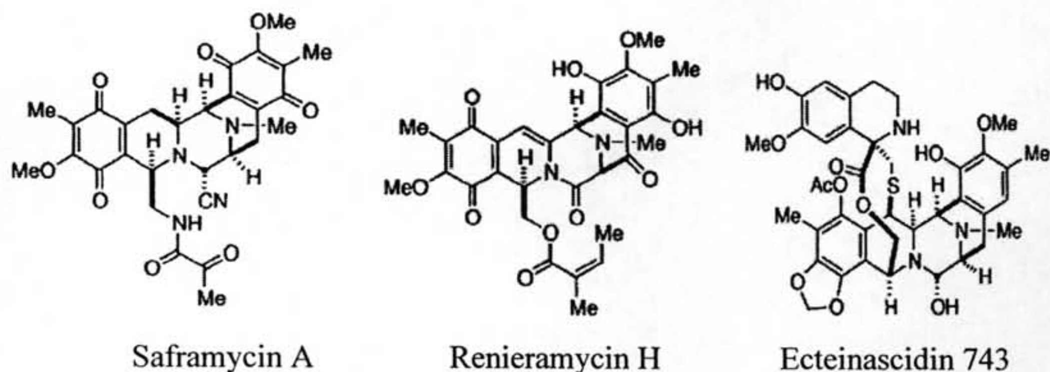


Figure 8 Examples of saframycins, renieramycins and ecteinascidins (Scott and Williams, 2002).

The isolation of the ecteinascidins (ET) was first reported by Reinhart *et al.* in 1990. Examples of the ET compounds include ET729, ET743, ET745, ET759A, ET759B, and ET770. ET743 is an example of tetrahydroisoquinoline alkaloid that consists of three tetrahydroisoquinoline units (Figure 8). ET743 elicits the potent anti-cancer activity (Table 4) and, its effectiveness has been extended to a variety of human tumor xenografts implanted in nude mice (Fricker, 2001). This compound is a potent DNA-alkylating agent, selectively alkylating the N-2 amino group of guanine residue in the minor groove of DNA. ET743 has a unique mechanism of action because it kills cancer cells by poisoning transcription-coupled nucleotide excision repair (Pommier *et al.*, 1996).

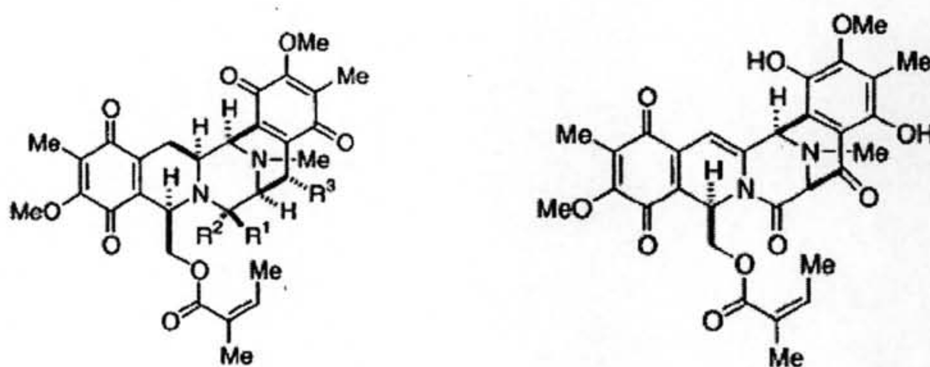
Table 4 The anti-tumor activity of ET743 against several tumor cell lines (Scott and Williams, 2002).

Tumor type	**IC_{50} (nM)**
P388 leukemia	0.34
L1210 leukemia	0.66
A549 lung cancer	0.26
HT29 colon cancer	0.46
MEL-28 melanoma	0.50

Furthermore, ET743 belongs to the fourth-generation of MDR1 inhibitors (Liscovitch and Lavie, 2002; Mahadevan and List, 2004). It has been shown that ET743 can potentiate the activity of vincristine and doxorubicin by down-regulating P-gp/*MDR1* gene (Kanzaki *et al.*, 2002).

In 1982, Frincke and Faulkner isolated four new natural products from the sponge *Reniera* sp. that possess structures similar to that of the saframycins. These compounds were named renieramycin A-D (Scott and William, 2002) (Figure 9).

Renieramycins are isoquinoline marine natural products that are structurally related to other isoquinoline natural products such as saframycins, naphthyridinomycin, and ecteinascidins. Renieramycins can be isolated from marine sponges belonging to genera *Reniera, Xestospongia, Haliclona, Cribrochalina*, and *Neopetrosia*. These natural products are of interest as new antineoplastic active compounds. It has been shown that reneiramycin A-D, H, and I have moderate antimicrobial activities, while renieramycin G contains moderate MIC activity against KB and LoVo cell lines (Oku *et al.*, 2003; Saito *et al.*, 2004).



Renieramycin A $R^1=R^2=H$, $R^3=OH$

B $R^1=R^2=H$, $R^3=OMe$

C $R^1=R^2=O$, $R^3=OH$

D $R^1=R^2=O$, $R^3=OEt$

Renieramycin H

Figure 9 Structures of Renieramycins

Renieramycin M ($C_{31}H_{33}N_3O_8$) is a new tetrahydroisoquinoline compound (Figure 10) which can be isolated from a blue sponge, *Xestospongia* sp., growing around Sichang Island in the Gulf of Thailand (Suwanborirux *et al.*, 2003). This compound is dark yellow prisms, showing melting point 194.5-197 °C (Amnuoypol, 2004).

The structure of Renieramycin M have carbon skeleton consisting of two quinone rings. As a member of the saframycin families, Renieramycin M has a high potential as cytotoxic and antitumor activity against several cancerous cell lines (Table 5) (Suwanborirux *et al.*, 2003; Saito *et al.*, 2004).

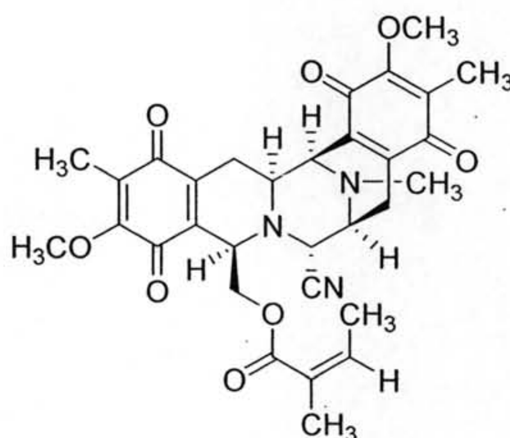


Figure 10 Structure of Renieramycin M

Table 5 Activity of Renieramycin M against several tumor cell lines (Suwanborirux *et al.*, 2003)

Tumor type	**IC_{50} (nM)**
HCT116	7.9
QG56	19
NCI-H460	5.9
DLD1	9.6

HCT116 = human colon carcinoma; QG56 = human lung carcinoma; NCI-H460 = human lung carcinoma; DLD1 = human colon carcinoma

5. Assessment of P-gp interaction

There are three approaches to investigate the influence of compounds on P-gp function (Wang *et al.*, 2003). These approaches can be performed in the *in vitro* assays and enable to classify compounds as being substrate or inhibitor of P-gp. The potential P-gp modulators can be evaluated in the systems as follows:

1. Compound being transported across polarized cell monolayer expressing P-gp on the apical membrane
2. Compound being uptake into cells over-expressing P-gp
3. Compound being directly bind to P-gp using inside-out membrane vesicles or reconstituted P-gp

5.1 *In vitro* screening models for P-gp interaction

There are a number of *in vitro* experiments to determine whether compounds of interest can interact with P-gp and modulate P-gp functions.

5.1.1 Growth inhibition assay

The classical method for the *in vitro* determination of the effect of an MDR modulator is to perform tumor cell killing experiments in well-defined *MDR1*-expressing tumor cell lines. This method determines the IC_{50} values of the cytotoxic agent in the absence and presence of a modulator, respectively. As known, the IC_{50} values indicate the concentrations yielding 50% cell destruction and can be used to compare cytotoxicity and MDR reversing activity of the studied drug (Weise and Pajeva, 2001).

Another parameter is an MDR ratio (Fold reversal, Fold sensitization, Dose-modifying factor, or Degree of potentiation) which is a ratio between the IC_{50} value of the cytotoxic agent in absence and presence of relatively nontoxic concentration of modifier. This parameter is most widely used to show the activity of MDR reversal agents (Avendano and Menendez, 2002).

$$\text{Fold reversal of MDR} = \frac{IC_{50\ \text{of cytotoxic drug}}}{IC_{50\ \text{of cytotoxic drug + modulator}}}$$

These parameters have been well established in the MDR reversal studies to evaluate the general cytotoxicity of the antitumor agent of which effect being modulated. However, direct drug modulating effects on MDR1 can be further studied in intact cells or various pump preparations to determine the function of these MDR1 proteins. For intact cell studies, the most widely used methods are the radioactively labeled or fluorescent dye transport measurements. These include estimation of labeled vincristine or doxorubicin uptake as well as the increase of cellular fluorescence during accumulation of fluorescent MDR1 substrates, e.g. anthracyclines, verapamil-derivatives, rhodamine 123, Hoechst dyes, or Fluo-3 (Quesada *et al.*, 1995; Sarkadi and Muller, 1997; Wiese and Pajeva, 2001).

5.1.2 Cell-based drug accumulation assay

In cellular drug accumulation assays, the accumulation of P-gp substrates in over-expressed P-gp cells is restricted by P-gp mediated-efflux of this compound back into the extracellular fluid. Thus, the P-gp modulating activities of these compounds can be evaluated based on the accumulation of a drug or P-gp marker in the cells expressing high levels of P-gp in comparison with the accumulation in the cells relatively deficient of P-gp activity. In general, these assays are performed in cell lines with high resistance to cytotoxic P-gp substrates such as vinblastine. In the experiment, the accumulation of P-gp substrate in the presence or absence of a known P-gp inhibitor is determined. Drug accumulation in the P-gp expressing cells is then compared with accumulation in cells from the low P-gp-expression parental cell line. Since P-gp transports its substrates out of the cell, it is likely that accumulation of P-gp substrates is less in P-gp expressing cells than in the P-gp deficient cells. In addition, the similar procedure may be applied under conditions where P-gp is inhibited (Quesada *et al.*, 1995; Sarkadi and Muller, 1997; Hochman *et al.*, 2002).

5.1.3 Inhibition of P-gp efflux

The principle of this method is a measurement of the pure binding affinity of the modulator to the putative receptor on the protein. Along with other data such as cytotoxic endpoint, this approach can estimate of the relative contribution of the protein-mediated transport to the whole MDR modulating effect.

5.2 Cell culture in the studies of P-gp interaction

As mentioned above, the inhibitory potency of P-gp modulator can be studied in P-gp substrate accumulation model, in which the amount of substrate taken up by cells expressing P-gp being compared in the presence and absence of inhibitors (Varma *et al.*, 2003). Several cell culture models with highly expressed P-gp protein have been used for these studies such as Caco2 cells, LLC-PK_1 and LLC-MDR_1 cell lines.

Potential drug interactions can occur at the level of P-gp. These may be due to either compound being a potent inhibitor or a substrate of P-gp. The interactions can lead to the variability of drug accumulation and pharmacokinetics including absorption, distribution, and elimination. Thus, it is important to assess the intrinsic property of new compounds as being a P-gp inhibitor and/or as being a P-gp substrate. Generally, compounds being substrate may act as an inhibitor under certain conditions (Hochman *et al.*, 2002); for example, the competition between two substrates for one substrate binding domain. However, this may not be the case for P-gp substrates because most of P-gp substrates have relatively low affinity for the protein and cannot significantly inhibit P-gp function at reasonable working concentration.

6. LLC-PK_1 and LLC-MDR_1 cell lines

The LLC-PK_1 cells are a proximal tubular renal cell line derived from pig kidney epithelial cells (Hull *et al.*, 1976). This cell line has a structure and function similar to those of renal proximal tubular cells (Decorti *et al.*, 2001). As a model for human P-gp, LLC-MDR_1 cells are generated by transfection of the porcine kidney epithelial cell LLC-PK_1 with the human *MDR1* gene. The LLC-MDR_1 have P-gp

activity more than parental cells as determined by transepithelial transport and cellular accumulation of P-gp substrate (Tanigawara *et al.*, 1992; Tanaka *et al.*, 1997; Van der Sandt *et al.*, 2000; Decoti *et al.*, 2001; Yamazaki *et al.*, 2001). Table 6 shows example of experimental model for P-gp modulators using LLC-PK_1 and LLC-MDR_1 system.

Table 6 Examples of P-gp studies using LLC-PK_1 and LLC-MDR_1 cells.

Method	P-gp substrate	Test compound	Reference
1. Transepithelial transport and cellular accumulation	[H^3]vinblastine	Steroid hormone and polychlorobiphenyl	Fujise *et al.*, 2002
2. Transepithelial transport and cellular accumulation	doxorubicin, vinblastine	Cadmium	Kimura *et al.*, 2005
3. Transepithelial transport	[H^3]daunorubicin, [H^3]vinblastine	Cepharanthin	Hirai *et al.*, 1995
4. Transepithelial transport and cellular accumulation	Rhodamine 123	Cannabinoids	Zhu *et al.*, 2006
5. Cellular accumulation	Rhodamine 123	Pluronic block copolymers	Batrakova *et al.*, 2001
6. Cellular accumulation	Calcein	Antiepileptic drugs	Weiss *et al.*, 2003
7. Cellular accumulation	Rhodamine 123, Daunorubicin	*Kaempferia parviflora* extracts	Patanasethanont *et al.*, 2006