REVIEW ARTICLE

Accepted 12 July 2002
Revised 26 June 2002
Received 28 November 2001
Indian J. Pharm. Sci., 2002, 64(5): 423-432

Multidrug Resistance: The Role of the ATP-Binding Cassette Superfamily Transport Protein, P-glycoprotein

A. A. JOSHI* AND C. L. VISWANATHAN
Bombay College of Pharmacy, Kalina, Mumbai-400 098.

Multidrug resistance is a condition in which cells exhibit resistance to drugs to which they have never been exposed to previously. The main factor contributing to this is the overexpression of an energy-dependant multidrug transporter, P-glycoprotein. P-glycoprotein is also involved in the normal physiological functions. P-glycoprotein is widely distributed in the body and found in organs like lower gastrointestinal tract, kidney, liver, pancreas, testes and most importantly, the blood-brain barrier. Although, many mechanisms have been promulgated which involve P-glycoprotein in multidrug resistance, there is no satisfactory mechanism that reconciles the entire phenomenon related to multidrug resistance. Efforts have also been taken to isolate, characterize and spectroscopically analyze P-glycoprotein. Based on the available data, there are many agents, which are proven to be the substrates of P-glycoprotein. Moreover, there also are inhibitors to P-glycoprotein that enhance the intracellular levels of many drugs that are effluxed by P-glycoprotein.

Clinical oncologists were the first to observe that cancers treated with multiple different anticancer drugs developed cross-resistance to many other cytotoxic agents to which they had never been exposed. This resulted in effectively eliminating the possibility of curing these tumors with chemotherapy[1]. In many cases, cells grown in tissue culture from such multidrug resistance tumors demonstrated patterns of resistance *in vitro* similar to those seen *in situ*. This observation suggested that multidrug resistance (MDR) was in many, but not all, cases the result of heritable changes in cancer cells causing altered levels of specific proteins, or mutant proteins, that allowed cancer cells to survive in the presence of many different cytotoxic agents. These genetic alterations that conferred resistance to cytotoxic drugs, affected cell dynamics, susceptibility of cells to apoptosis, uptake and efflux of drugs, or repair of drug-induced damage (usually to DNA). Although several mechanisms are possible for drug resistance[1], the best-studied mechanism of MDR is related to the overexpression of an energy-dependent multidrug transporter, or P-glycoprotein[1].

STRUCTURE OF P-GLYCOPROTEIN (P-gp)

P-gp is a member of the large ATP- binding cassette superfamily of transport proteins also called traffic ATPases[1-3]. This 170-kDa phosphorylated and glycosylated plasma membrane protein was first described by Juliano and Ling in 1976[4]. Humans have two MDR genes, MDR1 and MDR3 (also called MDR2), and rodents have three genes, namely mdr1a (also called mdr3), mdr1b (also called mdr1) and mdr2. Only the MDR1 gene (responsible for P-gp synthesis) in humans and the mdr1a and mdr1b genes in rodents appear to be involved in drug transport and development of drug resistance[5]. The human MDR3 and murine mdr2 genes encode a P-gp which does not seem to have a role in drug transport, but has a role in the secretion of phosphatidy choline into bile and has been shown to be a phosphatidylcholine flippase[6-7].

Structurally, MDR P-gp (fig. 1) is a transmembrane protein, which is comprised of two monomeric units each of which is 1280 amino acids long and consists of 2 homologous halves of 610 amino acids joined by a flexible linker of region of 60 amino acids. Fig. 1 is a schematic representa-

---

*For correspondence

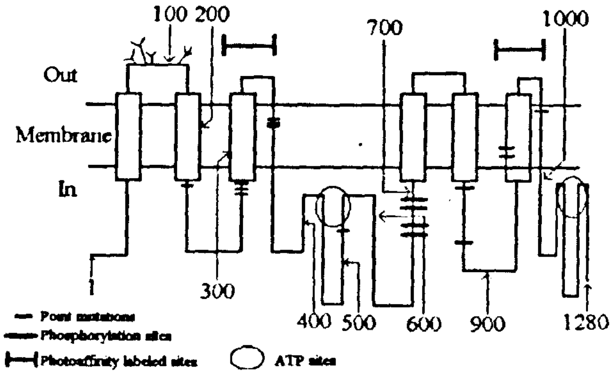


Fig. 1: Schematic representation of P-glycoprotein.

A hypothetical two-dimensional model of P-gp based on hydropathy analysis of the amino acid sequence and its functional domains.

tion of one monomer of P-gp. Thus, there are six transmembrane helices contributed by each monomer that span the plasma membrane in the hydrophobic domains and ATP binding domains. Since P-gp is a dimer, each half has an N-terminal hydrophobic domain containing six transmembrane domains followed by a hydrophilic domain containing a nucleotide binding site[8]. Each of the nucleotide binding sites can bind ATP and its analogs and both sites are essential since inactivation of either site inhibits substrate-stimulated ATPase activity. In other words, it suggests that coupling of ATPase activity to transport requires interaction of the two monomer units to yield a functional dimer[1]. Each nucleotide binding site consists of three conserved regions called Walker A, B and C motifs[9]. Biochemical characterization of both human and hamster purified P-gps have determined that both sites are capable of hydrolyzing ATP, but not simultaneously, the stoichiometry of ATP hydrolysis is 1 mol of ATP/mol of P-gp[10-12] and that drug binding and ATP hydrolysis are intimately coupled.

From a bird's eye view of the plasma membrane, P-gp appears as a donut-shaped structure with 6-fold symmetry, a diameter of 10 nm and a large central pore of about 5 nm in diameter. From a side view, it has a thickness in the plane of the plasma membrane of about 8 nm. Since the depth of the plasma membrane lipid layer is about 4 nm, about half of the molecule is within the plasma membrane. The central pore is closed at the inner (cytoplasmic) end, forming an aqueous chamber within the membrane that is open from this chamber to the lipid phase. Two lobes of about 3 nm are exposed at the cytoplasmic end and probably correspond to the nucleotide bindings[13].

Several studies and experiments have been carried out to unravel the structure of P-gp by employing complex and sophisticated techniques. One of these techniques include the use of cysteine-scanning mutagenesis and thiol-modification techniques[14]. This experiment takes into account that the protein contains seven endogenous cysteine residues at positions 137, 431, 717, 956, 1074, 1125 and 1227 (fig. 2). All of the cysteines were mutated to alanine[15], resulting in a Cys-less P-gp. The Cys-less P-gp was a useful tool for determining the topology of P-gp at the cell surface. Moreover, it has also been helpful to prove that both nucleotide-binding domains (NBDs) may need to bind ATP to couple drug binding to ATPase activity[16]. Moreover, identification of residues in drug-binding site of a human P-gp using a thiol reactive substrate, dibromobimane has been studied[17].

MECHANISM OF ACTION OF P-GLYCOPROTEIN

Although several models for P-gp function have been proposed, there is still no clear understanding at a molecular level of how the multidrug transporter lowers intracellular accumulation of many drugs such as anticancer drugs and protease inhibitors. The current models for P-gp function are based on whether the transporter directly or indirectly mediates drug transport. The altered partitioning model by Roepe[18,19] proposes that overexpression of the P-gp leads to alteration of electrical membrane potential ($\Delta\Psi_0$), intracellular pH (pH_i) and other biophysical characteristics of the cell. According to this model, P-gp indirectly promotes decreased intracellular level of intracellular drug accumulation.

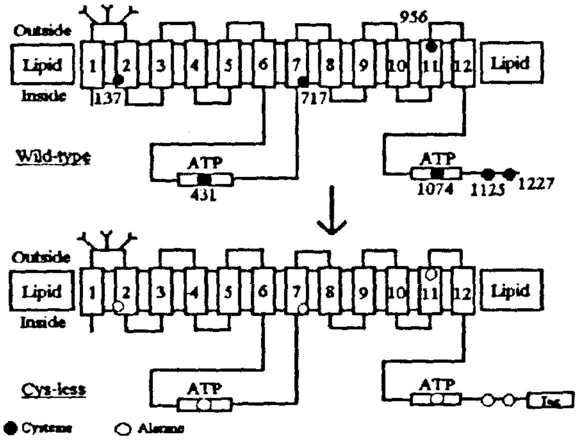


Fig. 2: Construction of P-gp.

Wild-type P-gp contains seven cysteine residues that were mutated to alanine resulting in the Cys-less P-gp. A tag was added to the COOH-end to facilitate detection by immunoblot analysis.

On the other hand, according to the pump model[20], the energy of ATP hydrolysis by P-gp is utilized for the removal of drugs from cell membranes and cytoplasm analogous to the ion-translocating pumps. P-gp is proposed to recognize substrates through a complex substrate recognition region or regions and directly pump drugs out of the cell by using molecular mechanisms that are not well understood.

The majority of experimental data strongly supports the drug pump model because evidence for a direct interaction of many of the substrates or reversing agents with the transporter has been obtained, including drug binding studies[21,22], photoaffinity labeling experiments[23-25], demonstration of drug-stimulated ATPase activity in direct proportion to the ability of the P-gp to transport these drugs[26], and a variety of amino acid substitutions in P-gp that alter its substrate specificity. Recent work on the stoichiometry of drug transport and ATP hydrolysis indicates that the hydrolysis of 1-3 molecules of ATP is required for the transport of one molecule of the drug[27].

However, this mechanism has been criticized by Zhu[28]. According to Zhu, this purported function for P-gp violates two fundamental principles that typically define an energy-dependant transporter, namely, the law of substrate specificity and the law of energy coupling and supply. Firstly, over a hundred structurally unrelated lipophilic compounds (usually with log P values between 10 and 1000) are known to be P-gp substrates, and this list is still increasing. Secondly, taking into consideration such a plethora of varied and structurally unrelated substrates, it is difficult to conceptualize that a membrane transporter could effectively transport many (but not all) of these lipophilic substrates after selectively recognizing them. Finally, if one assumes that P-gp is a direct pump for many lipophilic drugs, then the translocation by P-gp of these lipophilic compounds against facile, fast passive diffusion would consume such an incredible amount of cellular energy that it would impose an impossible burden on the energy-generating system of the cells resulting in seemingly futile energy cycling. For instance, the estimated energy consumption for P-gp-mediated canalicular transport of 1.7 nM of daunomycm in the human liver alone could be as high as 5000 calories per day[28].

On this basis, Zhu has put up a working hypothesis, which attempts to fill the loopholes of the pump model. This hypothesis, termed as "handle hypothesis", suggests that a common structure must be present in all the substrate molecules, which could be recognized by P-gp. With respect to this, Zhu has proposed that lipophilic compounds with diverse chemical structures could all become substrates for P-gp after they undergo enzymatic biotransformation. This metabolic process could add a common structure moiety (a common handle) to these structurally unrelated molecules and thus render them selectively recognizable by P-gp. This idea is represented in fig. 3. If some organic molecules (such as molecule B in this diagram) already contain suitable functional groups in their structure for metabolic conjugation, then they can directly form water-soluble conjugates without prior phase I oxidative metabolism. It is important to note that the drug-metabolizing enzymes for both oxidative and conjugative metabolism are almost universally present in mammalian cells and that the level of these enzymes could be sustainably induced during continuous exposure to chemical inducers such as lipophilic anti-cancer agents. In case of, say, molecule A, a handle will be attached to it when it undergoes phase I and II metabolism, such that it will be easily recognized by P-gp. Moreover, the proposed handle hypothesis agrees with the fundamental laws mentioned earlier that govern an energy-dependent membrane transporter, i.e., substrate specificity and energy coupling and supply. However, till date, there is no direct evidence that phase II metabolism is essential for drug efflux.

LOCATION AND FUNCTION OF P-GLYCOPROTEIN IN NORMAL PHYSIOLOGY[1]

Tissue localization of P-gp suggests a normal physiological role in protection against xenobiotics. After it was proved that P-gp was responsible for conferring resistance to some anticancer agents, doubts of it being involved in the normal physiological functions were raised. The first hint of the possible endogenous functions came from studies in which monoclonal antibodies to P-gp were used to localize the protein in frozen sections of human tissues. All positive

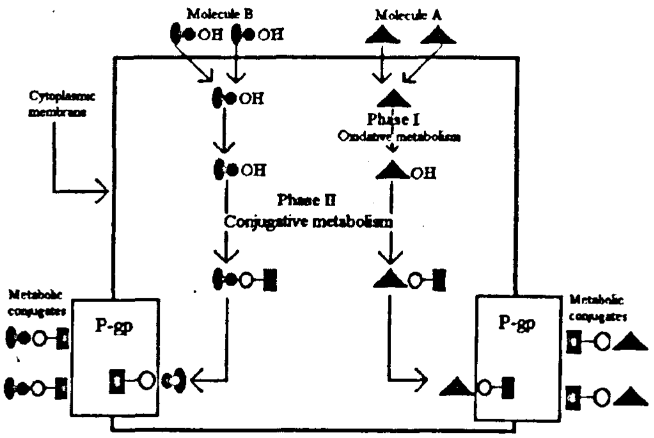


Fig. 3: Handle hypothesis proposed by Zhu.

tissues showed plasma membrane localization of positive cell types. In epithelial cells of the lower gastrointestinal tract (jejunum, ileum and colon), high levels of P-gp were located only on the mucosal surface of these tissues that suggested a function to prevent uptake of substrates and perhaps to facilitate excretion across the mucosa of the GI tract[29]. In kidney and liver, P-gp was present on the brush border and biliary face, respectively, of proximal tubule cells and hepatocytes, consistent with a role of P-gp in excretion of xenobiotics and endogenous metabolites into the urine and bile. P-gp was also found on the apical surface of pancreatic ductules. One of the most interesting localizations for P-gp was on the luminal surface of capillary endothelial cells in the brain and testes.

Several other cell types and tissues express P-gp. There is P-gp in the placenta, probably in more than one cell type[30], which is suggestive of a role for P-gp in protecting the fetus from toxic xenobiotics. Human adrenal cortex is rich in P-gp. These localizations in steroid-secreting glands suggest that P-gp might be involved in secretion of steroids, or in protecting the plasma membranes of steroid-secreting cells from the toxic effects of high steroid concentrations. Some hematopoietic cells express P-gp, albeit at lower levels than are seen in epithelial cells, brain capillary endothelial cells and adrenal cortical cells. CD34-positive bone marrow stem cells express enough P-gp to account for this phenotype. Some T cells and macrophages appear to express P-gp, but the physiological significance of this is not yet known.

LOCALIZATION AND ACTIVITY OF P-GLYCOPROTEIN IN THE BLOOD-BRAIN BARRIER[31]

Interest in the possible function of P-gp in the blood-bram barrier was triggered by the findings of Cordon-Cardo *et al.*[32] showing that several monoclonal antibodies recognizing P-gp specifically stained blood capillaries in brain and to a lesser extent in testis, but not capillaries in most other tissues, or in the choroid plexus of the brain. Based on the known drug transport properties of P-gp in the blood-brain barrier, this would limit the entry of potentially toxic compounds from the blood into the brain by pumping them actively back into the blood[32]. However, this would also lead to subtherapeutic levels of drugs intended for CNS penetration in the brain cells. The evidence to support this statement is seen by the fact that the levels of quinidine rise dramatically if P-gp is blocked by SDZ PSC 833[33]. This data suggests that the net uptake of quinidine is increased by the order of 15.7 times if P-gp is blocked. Additional information could be obtained by the studies performed by Schinkel *et al.*[34] They showed that mouse mdr1a P-gp is abundant in blood-brain barrier and its absence in mdr1a (-/-) [mdr1a gene knock out] leads to highly increased levels of drugs ivermectin, vinblastine, digoxin and cyclosporin A. It is interesting to note that mdr1a in mice is homologous to MDR1 of humans and such an observation indicates an advantage or disadvantage from a CNS drug delivery point of view.

P-GLYCOPROTEIN SUBSTRATES AND INHIBITORS

Crude and purified P-gp preparations exhibit both basal and drug-stimulated ATPase activities. The basal activity is believed to be due to endogenous lipid or other endogenous substrates, such as hydrophobic peptides. The profile of the drug-stimulated ATPase activity is thought to reflect the nature of interaction of P-gp with drug substrates. Based on their effect on ATPase activity of human P-gp, a number of compounds have been classified into three distinct classes:

I. Class I agents: This class of compounds stimulate ATPase activity at lower concentrations, but inhibit the activity at higher concentrations, e.g. vinblastine, verapamil and paclitaxel.

II. Class II agents: This class of compounds enhance ATPase activity in a dose-dependant manner without any inhibition e.g. bisantrene, valinomycin and tetraphenylphosphonium.

III. Class III agents: This class of compounds inhibits both basal and verapamil-stimulated ATPase activity, e.g. cyclosporin A, rapamycin and gramicidin D[1].

To gather further information on interaction between P-gp and its substrates, 167 compounds were analyzed in multidrug resistant cells of colon carcinoma. The compounds were prospectively defined as P-gp substrates if the cytotoxicity was increased $\leq$ 4-fold by the addition of cyclosporin A (CsA) and antagonists of P-gp if inhibition of efflux increased rhodamine accumulation by 4 times. Among the 84 compounds that met either criterion, 35 met the criterion of P-gp substrates, 42 met the criterion of P-gp antagonists and seven met the criterion for both the categories. Thus compounds interacting with P-gp fall into two distinct groups: one comprising cytotoxic compounds that are transported and have poor or no antagonistic activity and a second group of compounds which show good antagonistic activity, but no evidence of significant transport. In other words, the second category of drugs bind to P-gp and inhibit the transport of other drugs as well[35].

The chemicals transported by P-gp have very diverse structures, the only communality being that they are hydrophobic amphipathic molecules (molecules having two sides with characteristically different properties) that are not negatively charged and that they are between 200-1800 kDa in size. These include anticancer drugs such as doxorubicin, daunorubicin, vinblastine, vincristine, actinomycin D, paclitaxel, teniposide and etoposide; immunosuppressive drugs like cyclosporin A and FK 506; steroids like aldosterone, hydrocortisone, cortisol, corticosterone and dexamethasone; HIV protease inhibitors such as amprenavir, indinavir, nelfinavir, ritonavir and saquinavir; the antihistamine terfenadine; cardiac drugs like digoxin and quinidine; lipid lowering drug lovastatin; the dopamine antagonist domperidone; the antiemetic ondansetron; the antidiarrheal loperamide; the antigout agent colchine; the antibiotic erythromycin; the antitubercular agent rifampicin; the antihelminthic agent ivermectin and the fluorescent dye rhodamine 123. Under certain circumstances, P-gp may also be able to transport hydrophilic negatively charged compounds, such as methotrexate[36].

P-gp inhibitors[37-42] include the immunosuppressant cyclosporin A and its non-immunosuppressive analog PSC 833 (valspodar); the calcium channel blocker verapamil, the HIV protease inhibitors[43,44] like nelfinavir, ritonavir and saquinavir[45] and possibly indinavir; the progesterone antagonist mifepristone (RU 486); the antiarrhythmic agent quinidine[35]. the sedative midazolam; the anti-estrogen tamoxifen; the peptide chemosensitizers reversins 121 and 205; the acridonecarboxamide derivative GF 120918 and the cyclopropyldibenzosuberane LY 335979. Moreover, it has been shown that various MDR type substrates and chemosensitizers compete at a common drug binding site present in P-gp. This study was carried out by using gramicidin D and valinomycin[46]. As noted earlier, there are six transmembrane domains present in the plasma membrane. It is worthwhile to note that drug-stimulated ATPase activity requires the movement of transmembrane (TM) segments 6 and 12. TM 6 and TM 12 are particularly important because they directly connect the two transmembrane domains to their respective ATP binding domains. Both TM segments interact and undergo essential conformational changes during drug-binding or during ATP hydrolysis. These conformational changes are particularly important during drug-stimulated ATPase activity since cross-linking these two domains leads to the destruction of drug-stimulated ATPase activity. Drug-stimulating activity could be restored by breaking the disulfide bond by dithiothreitol[47]. In addition. Loo and Clarke showed that the interface between TM 6 and TM 12 forms part of the drug-binding pocket in P-gp[48].

RAPID PURIFICATION AND SPECTROSCOPIC ANALYSIS OF P-GLYCOPROTEIN

The methods listed above for characterizing the interaction between the transmembrane and ATP binding domains are time consuming and do not yield sufficient enzyme to directly measure function. In absence of direct assay, it is possible that the response of the cell to cytotoxic agent could also involve activation of endogenous pump(s) or involve other mechanisms of drug resistance. Therefore, it would be an achievement in itself if a method of purification ofP-gp in considerable quantity would be developed.

An approach that has been applied successfully to soluble proteins is metal-chelate chromatography of proteins containing a histidine tag. In case of P-gp, nickel chromatography was employed. To purify P-gp by this technique, its cDNA was transiently expressed in HEK 293 cells as they yield high quantities of P-gp. More than 80% of P-gp could be extracted from membranes with the detergent n-dodecyl-β-D-maltoside. Ionic detergents or inclusion of phospholipids during purification procedure prevented binding of P-gp to the column. Other non-ionic detergents [Triton X-100 or $C_{12}E_8$ (octaethylene glycol dodecyl ether)] inactivated P-gp, while the use of octyl glucoside was less effective in solubilizing P-gp. When purified P-gp was subjected to immunoblot analysis with antibody against human P-gp, the 170 kDa was the only band that was visible. After one round of purification, greater than 50% of the eluted proteins was P-gp-$(His)_{10}$. A second round of purification yielded more than 80 % ofP-gp, with yields of 6-12 μg. If regenerated, rather than new, nickel column is used, then a contaminating band of 56 kDa was visible[49]. A Fourier Transform Attenuated Total Reflection Infra Red Analysis was carried out on the reconstituted P-gp in proteoliposomes[50]. A quantitative evaluation of the secondary structure and a kinetic of $^2H/H$ exchange of P-glycoprotein were performed in the presence and in the absence of MgATP, MgATP-verapamil and MgADP. These provided evidence that a large fraction of P-gp was inaccessible to the aqueous medium. Addition of MgATP induced an increased accessibility to the solvent of a population of amino acids, while addition of MgATP-verapamil resulted in the substraction of a part of the protein from access to the aqueous solvent. No significant changes were observed by addition of MgADP or verapamil alone. The secondary structure was affected by the addition of ligands. The variations observed in the $^2H/H$ exchange rate in course of P-gp interactions with the above ligands represented tertiary structure changes.

MULTIDRUG RESISTANCE (MDR) AND P-GLYCOPROTEIN: AN INSIGHT INTO CHEMOSENSINZERS

The major difficulty with current cancer chemotherapeutic drugs is the clinically acquired or nonintrinsic resistance of cancer cells to these drugs. The most common mechanism for drug resistance in cancer chemotherapy, now termed MDR, is defined as the ability of cancer cells exposed to a single drug to develop resistance to a diverse range of structurally and functionally unrelated drugs. These drug include the anthracyclines (doxorubicin and daunomycin), the vinca alkaloids (vincristine and vinblastine), podophyllotoxins and actinomycin D. Previously, scientists were puzzled at the underlying mechanism of this phenomenon. It is now generally believed that the MDR phenotype is mainly related to an increased production of cell surface glycoprotein, now commonly known as P-glycoprotein[51]. A large number of non-cytotoxic compounds known as chemosensitizers or MDR modulators can sensitize resistant cells for the action of cytotoxic drugs. Chemosensitizers include calcium channel blockers, calmodulin antagonists, steroids and cyclic peptides. Most reversing agents block drug transport by acting as competitive or noncompetitive inhibitors[52] and by binding either to drug interaction sites[53] or to other modulator binding sites, lead to allosteric changes.

The effectiveness of P-gp reversing agents in chemosensitizing multidrug-resistant cancer cells has stimulated a serious effort to define a common pharmacophore necessary to circumvent P-gp mediated MDR. Since most of these chemosensitizing compounds and their structural analogs are highly hydrophobic and traverse the plasma membrane with relative ease, their effect on drug accumulation and drug resistance can be conveniently determined in intact multidrug-resistant cells. However, interpretation of data from such experiments must be done carefully. Chemosensitivity to an antineoplastic drug could be restored by mechanisms independent of P-gp, and because some of the P-gp modulators are substrates of the pump, higher concentrations of those compounds might be required to potentiate accumulation of cytotoxic drugs in cells overexpressing P-gp.

Mechanistically, P-gp modulators are either high-affinity substrates of the pump or are efficient inhibitors of ATP-dependant transport by P-gp. The strength of modulator interactions could also be determined by measuring their ability to compete for binding of photoactivatable substrate analogs to P-gp. In general, it is the concentration required for half maximal stimulation or inhibition of P-gp-ATPase activity than does the extent of stimulation or inhibition, but there are some tripeptides that stimulate P-gp-ATPase activity but poorly inhibit drug transport.

Using one of these assays, several groups have significantly contributed to the knowledge of structure-activity relationships of P-gp substrates and modulators. Although enough structural diversity exists among the P-gp modulators so that no consensus structure can be defined, within each class of drugs, SAR studies have made it possible to define certain chemical features of these compounds that seem to be essential for functional interaction with P-gp.

STRUCTURAL REQUIREMENTS OF P-GP INHIBITORS'

As mentioned above, there are no common features in the compounds, which block the P-gp. Analysis of each individual class of compound/s provides an insight to the structural requirements of some of the P-gp inhibitors. Some of them are described below:

Verapamil:

There have been studies in the SAR of verapamil. Based on the ability to reverse doxorubicin resistance, the key structural domains of verapamil and its analogs for MDR reversal activity can be assigned to the motifs at the R_1 and R_2 position, (fig. 4). In this, the N-phenethyl ring (in position R) also seems to be dispensable, because replacement of it with an aliphatic chain exerts no detectable effect on anti-MDR activity. However, the 8-methylnanone [-$CH(CH_3)_2$] at R_2 seems to play a key role in reversing MDR mediated by P-gp. Carbon extension of this group (as in anipamil) significantly reduces the reversal activity. Also, replacement of 7-CN in R_1 position with the 7-methoxylamino group results in loss of reversal potency. It is interesting that the reversal property of verapamil analogs clearly lacks enantio-selectivity.

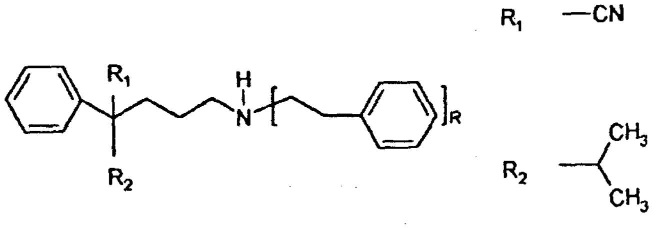
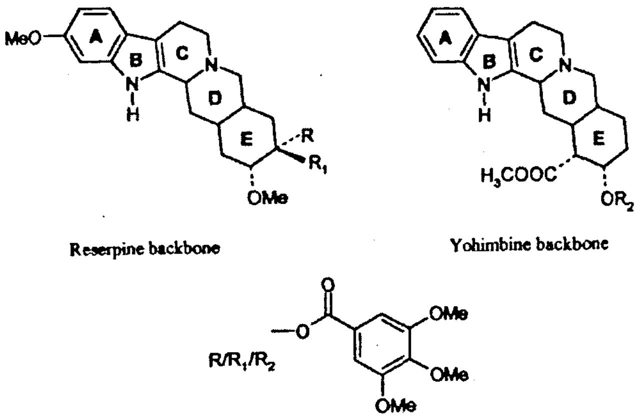


Fig. 4: P-gp ligands with verapamil nucleus.


Reserpine backbone

Yohimbine backbone

Fig. 5: Reserpine and Yohimbine backbone.

Reserpine:

The naturally occurring alkaloid reserpine possesses strong modulatory potency against MDR, whereas the structurally related compound, yohimbine lacks it[54] (fig. 5). Both compounds contain two aromatic domains and a basic nitrogen atom linked through a fused polycyclic array of three six-membered rings (rings C, D and E) and an ester bond. The trimethoxylbenzoyl group either at R (as in reserpine) or at R$_1$ (as in 18-epireserpine) positions of the reserpine backbone or at the R$_2$ position of yohimbine (trimethyl benzoylyohimbine) is important in increasing chemosensitivity to vinblastine as well as in the ability to compete with ^{125}I-labeled NASV (a photoactivable vinblastine analog) binding to P-gp. In general, for both reserpine and yohimbine analogs, compounds with a pendent benzoyl group beside the basic indolo-piperidine ring system with certain conformational constraints are the most effective in chemosensitization of P-gp-mediated vinblastine resistance. An overlay of the verapamil and trimethyl benzoylyohimbine reveals that verapamil can achieve a thermodynamically possible conformation similar to that of its yohimbine analog. This conformation of verapamil is also similar to that of vinblastine, which is the preferred one for this substrate. It is interesting that the difference in stereochemical configuration of the trimethoxybenzoyloxy substituents in reserpine, epireserpine and trimethoxybenzoyloxy yohimbine has no effect on their apparent ability to modulate MDR; this suggests lack of stereoselectivity.

Staurosporine:

The importance of the type and relative position of aromatic rings for anti-MDR activity has also been recognized in the indolecarbazole alkaloid staurosporine[55] (fig. 6). Replacement of the indolocarbazole structure of staurosporine with the related bisindolylmaleimide system dramatically reduces its ability to reverse P-gp-mediated doxorubicin resistance. Therefore, MDR reversing ability of staurosporine analogs appears to be linked to the integrity of the indolecarbazole structure. Staurosporine and its benzoyl derivative (at the R$_2$ position) are potent inhibitors of cellular rhodamine efflux. It is interesting that the analogs of staurosporine that are better inhibitors of P-gp-mediated drug transport are not necessarily the ones that are efficiently transported by P-gp.

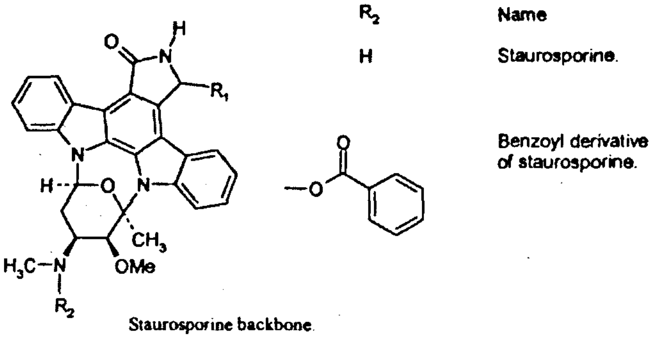
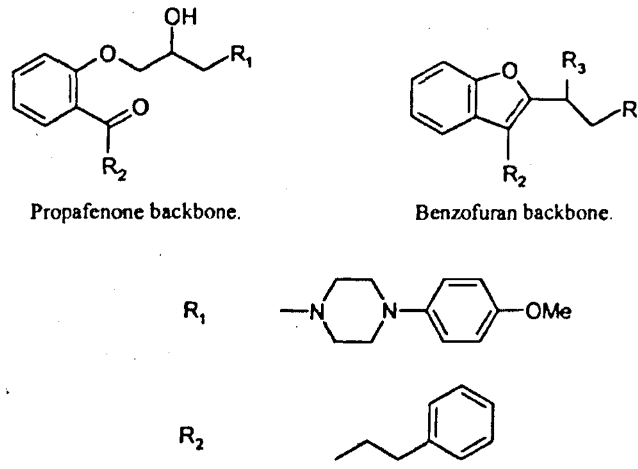

Staurosporine backbone.

Fig. 6: Staurosporine backbone.

Propafenone-related compounds:

No other compound has been subjected to structure-activity studies as thoroughly as the antiarrhythmic agent propafenone (fig. 7). Although a significant correlation exists between lipophilicity and biological activity within the structurally homologous series of propafenone analogs, modifications at critical positions of the molecule leads to decrease in activity, which cannot be related to a change in lipophilicity alone. Within the set of compounds tested, the phenylpropiophenone moiety is important for maintaining


Propafenone backbone.

Benzofuran backbone.

Fig. 7: Propafenone backbone.

high chemosensitizing activity to daunorubicin and colchicine resistance. Structural modification leading to a benzophenone derivative as well as incorporation of the carbonyl C atom into a benzofuran moiety results in a decrease of anti-MDR activity. Removal of the phenyl ring at the R_2 portion leads to a compound with only one benzene moiety that shows almost complete loss of active. Decrease in activity could be observed even when the ethylene moiety between the two aromatic rings is removed, which suggests that an optimal distance has to be maintained between the aromatic rings[56]. Within an extended set of analogs, modification in the ortho position of the ether oxygen decreases modulator activity, in addition, the type of oxygen used (carbonyl, alcohol and ether) also influences the interaction of these compounds with P-gp. This interaction may be mediated through hydrogen bond formation. Repositioning of the ortho acyl substituent aromatic ring to obtain a meta or para analog decreases P-gp inhibitory potential in the following order: ortho>meta>para[57].

Different substitutions at the nitrogen atom indicate that this part plays a major role in activity. Compounds with a tertiary amino group at R_1 have greater potency than propafenone itself does. Incorporation of the nitrogen into a cyclic nonaromatic ring structure further enhances the modulatory potency, and the highest activity can be achieved with the arylpiperazines containing three aromatic rings and a piperazine moiety[57]. However, insertion of oxygen into the cyclic nitrogen-containing sub-structure dramatically decreases the reversing potency.

Phenoxazines:

Several different classes of compounds with tricyclic ring nuclei have been studied to identify important structural features responsible for anti-MDR activity. Among these compounds, which include phenoxazine, phenothiazine, phenoxazone, resurfin acetate, xanthene, xanthene carboxylic acid, acridine carboxylic acid and 1,10-phenanthroline, phenoxazine proved to be the most active. Hydrophobicity does not correlate with the ability of this series of compounds to modulate the accumulation of vincristine and vinblastine (fig. 8). These data indicate that the presence of a -NH group at position 10 and a highly electronegative element, such as oxygen, at position 5 of the tricyclic ring nucleus are important determinants for modulating MDR activity.

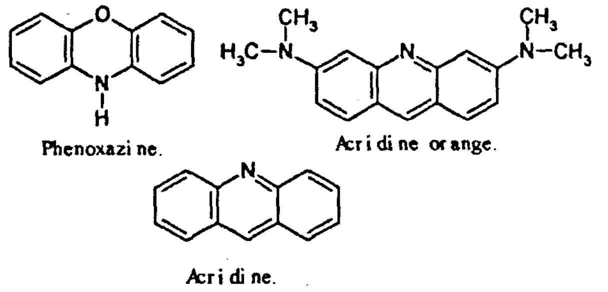


Fig. 8: Phenoxazine backbone.

Phenothiazines and thioxanthenes:

Knowledge of the anti-MDR features of tricyclic ring containing compounds has been further studied by Ford *et al.* [58,59] with derivatives of phenothiazine and thioxanthene (fig. 9). Overall, $-CF_3$ or -Cl at position 2 in the nucleus, a paramethyl substituted piperanzyl side chain and a distance of 4 carbons between these two domains are optimal for anti-MDR activity. In general, thioxanthenes are more hydrophobic than their corresponding phenothiazine derivatives because of the substitution of a carbon for nitrogen in the cyclic ring. Of all thioxanthene derivatives tested, flupentixol has been the most effective agent[58].

Overall, in modulator binding to P-gp, a clear lack of conserved elements of molecular recognition is apparent, which complicates the structural definition of MDR pharmacophore. Furthermore, much evidence suggests that the drug binding sites on P-gp are multiple and complex. Nevertheless, information compiled from various structure-activity studies can be used to outline a minimum requirement for anti-MDR activity. Because P-gp is able to recognize drug molecules directly from the membrane bilayer, the overall hydrophobicity of the modulators seems to be an

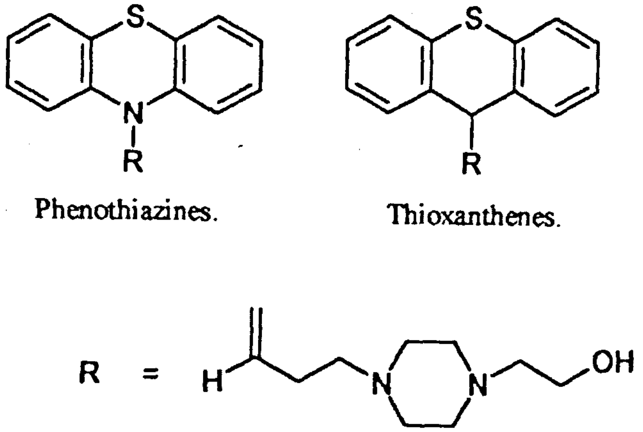


Fig. 9: Phenothiazine and thioxanthene backbone.

important, but not the sole, requirement for chemosensitizing activity. Because aromatic groups largely contribute to the hydrophobicity of a compound, planar ring structures seem to be a hallmark of anti-MDR compounds.

Apart from the planar aromatic domain, presence of a basic nitrogen atom located within an extended side chain of the aromatic ring structure also seems to play a determining role in the interaction of modulators with P-gp. Tertiary amino groups increase considerably the anti-MDR potency of a compound compared with primary and secondary amines. The chemosensitizing activity increases even more if the nitrogen atom is incorporated into a nonaromatic ring structure (as in propafenone analogs, phenothiazines and thioxanthenes).

SUMMARY

Although a wealth of information on the structure-function relationship of P-gp has been generated in recent years, it is still unclear how P-gp works as a drug efflux pump or about its role in normal physiology. Further insights into the mechanistic aspects will be provided by the resolution of the three-dimensional structure of P-gp. Similarly, the development of new technology such as techniques for molecular dissection and transgenic animals should make it possible to answer questions about the physiological and pharmacological role of P-gp. These studies should, in the next 5-10 years, provide better understanding not only of P-gp but also of many other ATP-binding cassette transporters and facilitate the treatment of human diseases in which these transporters play a major role.

REFERENCES:

1. Ambudkar, S.V., Dey, S., Hrycyna, C.A., Ramchandra, M., Pastan, I. and Gottesman, M.M., **Annu. Rev. Pharmacol. Toxicol.**, 1999, 60, 361.
2. Doige, C.A. and Ames, G.F.L., **Annu. Rev. Microbiol.**, 1993, 47, 291.
3. Higgins, C.F., **Annu. Rev. Cell. Biol.**, 1992, 8, 67.
4. Juliano, R. and Ling, V., **Biochim. Biophys. Acta.**, 1976, 455, 152.
5. Fardel, O. and Lecureur, V., **Gen. Pharmacol.**, 1996, 27, 83.
6. Smit, J. and Schinkel, A.H., **Cell**, 1993, 75, 451.
7. Schinkel, A.H., **Adv. Drug Del. Rev.**, 1999, 36, 179.
8. Gottesman, M.M. and Pastan, I., **Curr. Opin. Genet. Develop.**, 1996, 6, 610.
9. Hrycyna, C.A, Ramchandra, M., Ambudkar, S.V., Ko, Y.H. and Pedersen, P.L., **J. Biol. Chem.**, 1998, 273, 16631.
10. Urbatsch, I.L., Sankaran, B. Bhagat, S. and Senior, A.E., **J. Biol. Chem.**, 1995, 270, 26956.
11. Urbatsch, I.L., Sankaran, B., Weber, J. and Senior, A.E., **J. Biol. Chem.**, 1995, 270, 19383.
12. Rosenberg, M.F., Callaghan, R., Ford, R.C. and Higgins, C.F., **J. Biol. Chem.**, 1997, 272, 10685.
13. Loo, T.W. and Clarke, D.M., **Biochim. Biophys. Acta.**, 1999, 1461, 315.
14. Loo, T.W. and Clarke, D.M., **J. Biol. Chem.**, 1995, 270, 843.
15. Loo, T.W. and Clarke, D.M., **J. Biol. Chem.**, 1995, 270, 22957.
16. Zhou, Y.M., Pastan, I. and Gottesman, M.M., **Mol. Pharmacol.**, 1999, 56, 997.
17. Loo, T.W. and Clarke, D.M., **J. Biol. Chem.**, 1997, 272, 31945.
18. Roepe, P.D., **Biochim. Biophys. Acta.**, 1995, 1241, 385.
19. Wadkins, R.M. and Roepe, P.D., **Int. Rev. Cytol.**, 1997, 171, 121.
20. Gottesman, M.M. and Pastan, I., **J. Biol. Chem.**, 1988, 263, 12163.
21. Callaghan, R., Berridge, G., Ferry D.R. and Higgins, C.F., **Biochim. Biophys. Acta.**, 1997, 1328, 109.
22. Cornwell, M.M., Gottesman, M.M. and Pastan, I., **J. Biol. Chem.**, 1986, 262, 7921.
23. Ramchandra, M., Ambudkar, S.V., Dey, S., Hrycyna, C.A. and Chen, D., **Biochemistry**, 1998, 37, 5010.
24. Bruggemann, E.P., Germann, U.A., Gottesman, M.M. and Pastan, I., **J. Biol. Chem.**, 1989, 264, 15483.
25. Greenberger, L.M., Lisanti, C.J., Silva, J.T. and Horwitz, S.B., **J. Biol. Chem.**, 1991, 266, 20744.
26. Ambudkar, S.V., **Methods Enzymol.**, 1998, 292, 504.
27. Ambudkar, S.V., Cardarelli, C.O., Pashinsky, I. and Stein, W.D., **J. Biol. Chem.**, 1997, 172, 21260.
28. Zhu, B.T., **Mol. Carcinogenesis**, 1999, 25, 1.
29. Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M.M. and Pastan, I., **Proc. Natl. Acad. Sci. USA**, 1987, 84, 7735.
30. Sugawara, I., Kataoka, I., Morishita, Y., Hamada, H. and Tsunio, T., **Cancer Res.**, 1988, 48, 1926.
31. Tsuji, A. and Tarnai, I., **Adv. Drug Del. Res.**, 1997, 25, 287.
32. Cordon-Cardo, C., O'Brien, J.P., Casals, D., Rittman-Grauer, L., Biedler, J.L., Melamed, M.R. and Bertino, J.R., **Proc. Natl. Acad. Sci. USA**, 1989, 86, 695.
33. Kusuhara, H., Suzuki, H., Terasaki, T., Kakee, A., Lemaire, M. and Sugiyama, Y., **J. Pharmacol. Exp. Ther.**, 1997, 283, 574.
34. Schinkel, A.H., Wagenaar, E., Mol, C.A.A.M. and van Deemter, L., **J. Clin. Invest.**, 1996,97,2517.
35. Scala, S., Akhmed, N., Rao, U.S., Paull, K., Lan, L., Dickstein, B., Lee, J., Elgemeie, G.H., Stein, W.D. and Bates, S.E., **Mol. Pharmacol.**, 1997, 51, 1024.
36. Srinivas, R. and Middlemas, D., **Antimicrob. Agents Chemother.**, 1998, 42, 3157.
37. Fardel, O. and Courtois, A., **Anticancer drugs**, 1996, 7, 671.
38. Drach, J., Gsur, A., **Blood**, 1996, 88, 1747.
39. Huisman, M. and Smit, J., **AIDS**, 2000, 14, 237.
40. Mayor, U. and Wagenaar, E., **J. Clin. Invest**, 1997, 100, 2430.
41. Twentyman, P. and Bleehen, N., **Eur. J. Cancer**, 1991, 7, 1639.
42. Yumoto, R. and Murakami, T., **J. Pharmacol. Exp. Ther.**, 1999, 289, 149.
43. Lee, C.G.L., Ambudkar, S.V., Dey, S., Pastan, I., Cardarelli, C.O., Ramchandra, M. and Jeang, K.T., **J. Clin. Invest**, 1998, 101, 287.
44. Choo, E.F., Leake, B., Wandel, C., Imamura, H., Wood, A.J.J.,

Wilkinson, G.R. and Kim, R.B., **Drug Disp. Metabolism,** 2000, 28, 655.
45. Kim, A.E., Dintaman, J.M., Waddell, D.S. and Silverman, J.A., **J. Pharmacol. Exp. Ther.,** 1998, 286, 1439.
46. Borgnia, M.J., Eytan, G.D. and Assaraf, Y.G., **J. Biol. Chem.,** 1996, 271, 3163.
47. Loo, T.W. and Clarke, D.M., **J. Biol. Chem.,** 1997, 272, 20986.
48. Loo, T.W. and Clarke, D.M., **J. Biol. Chem.,** 1997, 272, 31945.
49. Loo, T.W. and Clarke, D.M., **J. Biol. Chem.,** 1995, 270, 21449.
50. Sonveaux, N., Shapiro, A.B., Goormaghtigh, E., Ling, V. and Ruysschaert, J., **J. Biol. Chem.,** 1996, 271, 24617.
51. Klopman, G., Shi, L.M. and Ramu, A., **Mol. Pharmacol.,** 1997, 52, 323.
52. Garrigos, M., Mir, L.M. and Orlowski, S., **Eur. J. Biochem.,** 1997, 244, 664.
53. Ambudkar, S.V., Dey, S., Ramchandra, M., Pastan, I. and Gottesman, M.M., **Proc. Natl. Acad. Sci. USA,** 1997, 94, 10594.
54. Pearce, H.L., Safa, A.R., Bach, N.J., Winter, M.A. and Cirtain, M.C., **Proc. Natl. Acad. Sci. USA,** 1989, 86, 5128.
55. Budworth, J., Davies, R., Malkhandl, J., Gant, T.W. and Ferry, D.R., **Brit. J. Cancer,** 1996, 73, 1063.
56. Chiba, P., Burghofer, S., Richter, E., Tell, B. and Moser, A., **J. Med. Chem.,** 1995, 38, 2789.
57. Chiba, P., Ecker, G., Schmid, D., Drach J. and Tell, B., **Mol. Pharmacol.,** 1996, 49, 1122.
58. Ford, J.M., Bruggemann, F.P., Pastan, I., Gottesman, M.M. and Hait, W.N., **Cancer Res.,** 1990, 6, 1748.
59. Ford, J.M., Prozialeck, W.C. and Hait, W.N., **Mol. Pharmacol.,** 1989, 35, 105.