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P-GP Modulators: Strategies Overcoming MDR in Chemotherapy

Dipika Wanve, Aboli Pawar, Divit Shetty
Address - Government College Of Pharmacy,Amravati -444604
Address - Government College Of Pharmacy,Amravati -444604
Address - Government College Of Pharmacy,Amravati -444604

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ABSTRACT:
Multi drug resistance (MDR) is major issue responsible for chemotherapy. Membrane transporter proteins play vital role in cause of MDR among which ABC transporters are the prototype to be targeted in drug resistant cancer treatment. Efflux mediated by ABC transporters have a great impact over accumulation of drug in tissue. The first identified and the prototype of ABC transporters-P-glycoprotein (P-gp) is present all over body which play a role in regulatory mechanism like protecting tissue from unnecessary cytotoxicity. Their prominent expression on Blood Brain Barrier (BBB) limits the penetration of drug in CNS disorders which indicates need of P-gp inhibition to obtain therapeutic effect. Similarly poor chemotherapeutic outcomes due to overexpression of these membrane transporters have become major issue of concern. For overcoming this issue, the discovery of P-gp inhibitors have become effective strategy. P-gp inhibitors, RNA interference, nanomedicines and combination delivery of drugs are new strategies to cope with this issues.

Keywords: ABC transporter, efflux transporter, P-gp, BBB, Multidrug Resistant (MDR) chemotherapy

I. INTRODUCTION:

Transporters that mediate the transport of drugs from tissue back to the bloodstream are called as Efflux transporters. These transporters belong to ATP binding cassette (ABC) superfamily, while those which are involved in the uptake of drug or other molecules in tissue from blood included in SLC (Solute Linked Carriers) superfamily. These transporters are present all over the body over various organs like the liver, digestive tract, kidney, testis, blood cells, placenta, and CNS. They play a vital role in drug absorption and distribution in organs that are protected by Blood-organ barriers like Blood-Brain Barrier, Blood Placenta Barrier, etc.[1][2] They provide global protection to CNS but not involved in any specific function. BBB represents the main crossing point between blood and the CNS by regulating efflux and influx of molecules.[1] Moreover, many genetic disorders may occur as a result of mutations in these membrane transporters such as cystic fibrosis (ABCC7), immune deficiency (ABCB2 and ABCB3), intrahepatic cholestasis of pregnancy (ABCB11), persistent hypoglycemia of infancy (ABCC8), retinal degeneration, etc. [2]

This article emphasizes on one important superfamily of membrane transporters and their functions such as a carrier for a drug (specifically of P-GP), their role in BBB, how they affect the transport of anticancer agents and penetration across the BBB and studies related to the anticancer drugs efficacy and to improve chemotherapy by inhibiting P-GP.

ABC TRANSPORTERS:

ATP-binding cassette (ABC) transporters are membrane-bound proteins that act as a channel to transport molecules and many drugs across the cell membrane. This large superfamily includes many members which act as carriers for many molecules. These are found in all living species including bacteria to humans. As these ABC transporters mainly involve efflux transporters which serve as a barrier for many drug molecules, it uses hydrolyzed ATP to transport molecules either with or against the concentration gradient. ABC transporters are all-encompassing membrane-bound proteins that use ATP hydrolysis to drive the transport of a wide range of lipophilic, amphipathic substrates across the membrane. ABC carriers are of critical importance. In many of these carriers, several physiological functions and defects are associated with severe inherited disorders. 49 genes are encoding ABC transporter in humans and these are subdivided into seven subfamilies, labeled as A-G.[2][33]

Two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs) make up the structural makeup of these transporters. TMDs

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make pathways across the membrane for translocation while NBDs hydrolyze ATP and utilize its energy for transport. In humans, ABC transporters are present on almost every cell, in the liver, the digestive tract, kidney but most predominantly expressed in barriers such as blood-brain barrier (BBB), blood-spinal cord barrier, blood-placenta barrier, blood-testis barrier, and blood-cerebrospinal fluid barrier. These transporters transport many waste products, organic cations, anions, xenobiotics, and many therapeutic agents from the brain back to blood. Therefore, ABC transporters act as a protective barrier in brain capillaries and in endothelial cells of choroid plexus. As they are responsible for many therapeutic agent's effluxes, multidrug resistance occurs causing failure of the treatment of severe CNS disorders. Multidrug resistance-associated protein 1(MRP1) [encoded ABCC1] at basolateral membrane domain, MRP2 [encoded ABCC2], the breast cancer resistant protein (BCRP) [encoded ABCG2], and MDR1 (aka P-GP) [encoded ABCB1] located at the luminal membrane, are some important members of ABC superfamily and best studied in BBB.[33][2] A major cause of treatment failure can be due to drug-drug interactions causing variation of ABC transporter activity, genetic polymorphism, and overexpression (as in cancer cells). We will further focus on the ABCB1 subfamily, its expression, and activity on cancer cells and BBB and development of CNS pharmacoresistant.

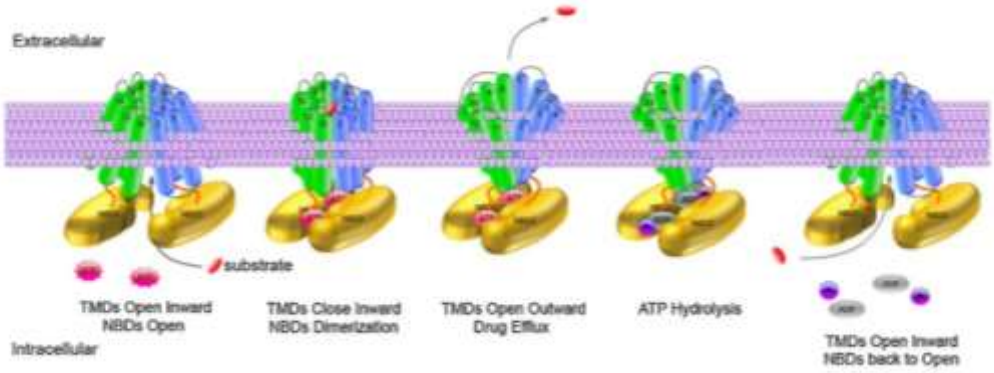

Figure no.1: Catalytic cycle of P-glycoprotein drug efflux (1) "TMDS Open Inward/NBDs Open": P-glycoprotein stays in the ground state, substrate binds at the cytoplasmic side of the TMDS: (2) "TMDs Closed Inward/NBDs Dimerization": two Mg-ATP molecules bind to the ATP binding pocket of NBDs, which triggers the dimerization of two NBDs as a 'sandwich dimer:(3) 'TMDs Open Outward/Drug Efflux": binding of Mg-ATP stimulates the conformational change of TMDs by switching from inward to outward conformation, substrate is released to the extracellular environment:(4) "ATP Hydrolysis": after ATP hydrolysis. 2 ADP and 2 phosphates are released from the NBDs and the energy were provided for the NBD dissociation and reset to the ground state (Wilkens, 2015).

ABC in the brain:

ABC transporters were discovered in the early 1970s and their further demonstration for its expression on BBB contributed as an important part of barrier function. Both in physical and psychological conditions, efflux transporters are challenging field for research as it comprises of complex regulation at BBB. In 1970, it was discovered as a prototype transporter involved in multidrug-resistance (MDR) of cancer cells and was detected to be present on endothelial cells of BBB. The first ABC transporter detected on BBB was P-gp transporter.[3][2]

BBB is a selective interface between vascular and CNS and plays a vital role in maintaining hemostasis. The recently invented concept of the neurovascular unit introduces the functional interaction between the neuronal and non-neuronal cells. The BBB is created by the interaction between capillary endothelial cells which are not fenestrated and tightly sealed tight junctions that have minimal pinocytosis. The endothelial capillary cells form a polarized barrier similar to that located in the proximal tubule of the retina or renal, which regulates molecular diffusion via the BBB, and restricts the entry of xenobiotic through intercellular, tight junctions through paracellular pathways. Permeability for many solutes is inversely related to size (most extremely low permeability of macromolecules) and directly related to lipophilicity. As a matter of fact, for many small, uncharged molecules, passive permeability for octanol/water partition coefficient increases in vivo blood-brain barriers, possibly suggesting diffusion through the lipid-like foundation of cell surface membranes.[3][4][5] The P-

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gp transporters expressed on BBB are shown below in the figure.

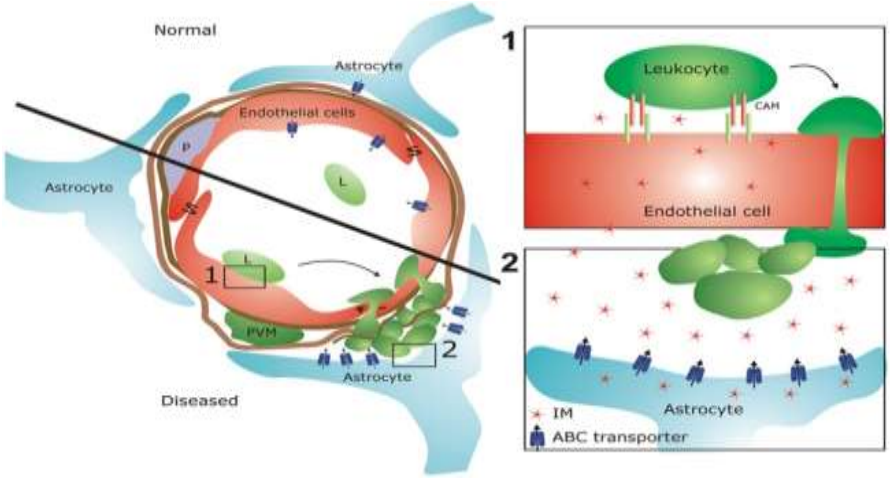

Fig.2- ABC transporters on BBB.[8]

P-gp is identified as an important determinant of the drug distribution to, and elimination from CNS. The outcome of the functions of this P-gp includes reducing or avoiding neurotoxic adverse effects of the drugs that otherwise would penetrate the brain. But this transporter may also limit the penetration of beneficial drugs that are used for CNS disorders.[5] In one reported study, epileptogenic brain tissue surgically resected from patients with medically intractable epilepsy was studied and they demonstrated that transporters such as P-glycoprotein (PGP) and members of the multidrug resistance-associated protein (MRP) family are overexpressed in capillary endothelial cells and astrocytes. They were found to be acting as an active defense mechanism, preventing the penetration of many lipophilic substances in the brain. Along with the overexpression of multidrug transporters, their functional polymorphism may play an important role in pharmacoresistant. The reports study also demonstrated that several AEDs like phenytoin, phenobarbital, carbamazepine, lamotrigine, and felbamate are substrates for either P-gp or MRP or both.[6] As these transporters are majorly responsible for the resistance of many drugs acting on CNS, modulation of ABC efflux transporters on BBB have a great impact on improving drug penetration in drug-resistant CNS disorders. Studies have demonstrated novel approaches to modulate P-gp function, nanoparticulate system drug delivery to CNS, and use of immunoliposomes to improve the penetration of drugs across the BBB thus improving their therapeutic outcomes.[5][6][7]

P-GP TRANSPORTERS ON DIFFERENT ORGANS:

P-gp [ABCB1] and other members of this family that are considered as multidrug transporters are breast cancer resistance protein 1BCRP [ABCG2], multidrug resistance protein MRP1 [ABCC1]. They are involved in the reduction of intracellular concentration of anticancer agents done through ATP dependent effluxing, limiting their therapeutic efficacy. P-gp has a crucial role in natural cellular detoxification. Deletion of the MDR gene in transgenic mice, concludes the absence of P-gp from BBB endothelium, which is related to the increase in brain intoxication by Ivermectin. Induction or inhibition of P-gp activity causes altered drug pharmacokinetics and response, further altering therapeutic outcomes. In cancer cells, multidrug resistance (MDR) can occur due to overexpression of P-gp and has shown disease progression in first-line chemotherapy.[1][2][3]

There was an experimental study done on the expression of P-gp in an endothelial cell at BBB and other blood tissue barrier sites. This study was performed by the immunocytochemical technique and immunohistochemical technique. In the immunocytochemical technique, human melanoma cell line BRO was transfected with a human MDR1 cDNA. Mouse monoclonal antibodies HYB-241 and HYB-612 were used. While in histochemical studies tissue of normal adult man was obtained from pathology specimen within 1-2 hours of resection and also autopsy specimens collected within 10 hours of death. Normal specimens from each organ were taken. The monoclonal antibodies HYB-241 and HYB-612 bind to an extracellular epitope of P-gp while C219 helps to identify the internal portion of the

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molecule. These antibodies were tested for immunoreactivity on cells that were multidrug-resistant due to transfection with the MDR1 gene. The transfected cells were found to be more reactive than parental cell lines. The P-gp expression was detected on most of the secretory glands like sweat glands of the skin, linings of trachea and lungs, thyroid follicles, pancreas cells (acinar cells), endometrium, cells of mammary gland and prostate. The most prominent site of expressions of P-gp was detected on the endothelial cells of blood capillaries of the blood-brain barrier (BBB), while on other hand endothelial cells of capillaries of the choroid plexus were found to be nonreactive and also there was no staining on other organs like kidney and placenta. [9]The prominent expression on BBB was concluded as a possibility of the proteins that may be the operative component of the system. This study concluded that patterns of expression of the endothelial cell indicate a physiological role for P-gp in the regulation of certain molecules in specified anatomic compartments. It also may take part in the explanation of the failure of systemic chemotherapy. A well-documented phenomenon is the relapse of acute lymphocytic leukemia in the testes or the meninges following remission achieved by systemic chemotherapy. It is thought to be the product of the malignant cells that survive induction chemotherapy due to the failure of such drugs to enter the test or stroke fluid.[9][49] The expression of P-gp in the test capillary network Distribution of P-gp on various organs and systems obtained by the study is given in the following figure:

Tissue type	HYB-241	C219
Nervous system		
Frontal cortex	+	+
Hippocampus	+	+
Cerebellum	+	+
Spinal cord	+	+
Choroid plexus	−	−
Meninges	−	−
Peripheral nerves	−	−
Sympathetic ganglia	−	−
Reproductive system		
Testes	+	+
Rete testis	−	−
Ovaries	−	−
Uterus	±	±
Skin		
Papillary dermis	+	+
Reticular dermis	−	−
Hematopoietic system		
Lymph node	−	−
Spleen	−	−
Thymus	−	−
Gastrointestinal system		
Stomach	−	−
Colon	−	−
Repiratory system		
Bronchi	−	−
Lung	−	−
Urinary system		
Kidney	−	−
Ureter	−	−
Urinary bladder	−	−
Placenta	−	−

Analysis of fresh frozen tissue sections by immunoperoxidase techniques. Endothelial cells of arterioles and larger blood vessels were unreactive in these microanatomical locations. +, Homogeneously stained; ±, heterogeneously stained; −, undetectable immunoreactivity.

Table 1- Distribution of P-gp in human capillary endothelial cells detected by HYB241 and C219 mouse mAbs.[9]

P-GP: REGULATION AN EXPRESSION ON CANCER CELLS:

The first member of ABC superfamily to be identified in human is P-gp. It is a 170 kDa membrane-bound protein encoded by the MDR1 gene (labeled as ABCB1). Many studies have demonstrated that P-gp is one of the most important efflux transporters present on many different parts of the body which extrudes many lipophilic molecules, certain cytokines and also any therapeutic agents. By pumping the drugs out of the cells, P-gp confers the ability to withstand lethal doses of some cytotoxic drugs and reduces their cytotoxicity. This ability produces a negative correlation between the expression of P-gp and chemosensitivity or survival in leukemias, lymphomas, osteogenic sarcoma, small cell lung

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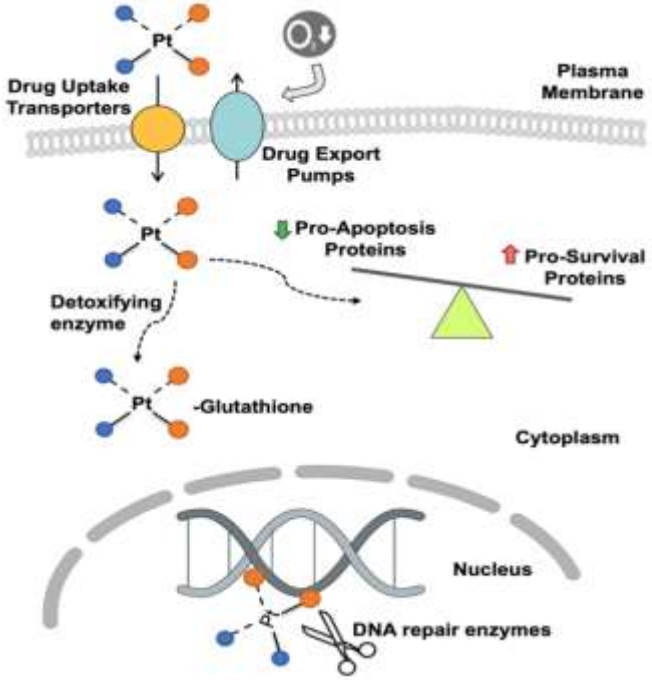
cancer, breast cancer, and pediatric solid tumors.[10][33]

High levels of gene and MDR1 protein expression were detected in the medulla, adrenal glands, kidney, colon, jejunum as well as endothelium of BBB capillaries while MDR2 isoform has been located in the liver. In the case of mice MDR1- adrenal gland, placenta, and travis uterus; MDR2 restricted to liver and muscles whereas MDR3 to lungs, digestive tube, and BBB.[11][13]

P-gp expression is high in tumors that are arises from carrier expressing tissues such as colon, adrenal glands, liver, pancreas, kidney. Its intermediate expression is seen at the diagnosis of some neuroblastomas, some hematological malignancies, and soft tissue carcinoma. While low-level expressions are found on the lungs, stomach, ovary, breast, lymphoma, melanoma, leukemia, and multiple melanomas. Some may show elevation of expressed levels after the chemotherapy. The markers for further P-gp positivity in cancer are Multi-drug Resistance associated Protein (MRP) and glutathione 'S' transferase Pi expression. P-gp expression has a relation with the failure of cancer treatment and poor prognosis. A strong evidence-based correlation has been notified between the overexpression or increased levels of MDR1 and the relapsed in case of soft tissue sarcoma in pediatrics, neuroblastomas, and lymphoblastic leukemias. Earlier P-gp was believed to be the only protein able to confer MDR in mammalian tumor cell lines but later reports on tumor cell line displaying MDR even in absence of P-gp overexpression pointed at existence of other MDR conferring proteins.[27][18]



MOC	Mechanism	Gene change
1A	Drug uptake	⇩ SLC22A3
		⇩ SLCO1B1
		⇩ SLCO1B3
		⇩ SLC31A1
1B	Drug efflux	⇧ ABCB1
		⇧ ABCG2
		⇧ ABCC1
		⇧ ABCC2
2	Drug metabolism	⇧ GSTM
4	DNA repair	⇧ ERCC1
		⇧ XPA
5A	Apoptosis	⇩ BAX
		⇩ BAD
5B	Survival	⇧ BIRC5
		⇧ BCL2
		⇧ BCL-X
6	Microenvironment	⇧ ADAM17
7	EMT	⇧ TWIST

Figure no.2: Mechanisms for resistance of chemotherapy

MOLECULAR BASIS OF P-GP MEDIATED MDR:

P-gp can accommodate smaller and larger molecular or several molecules simultaneously due to their large and versatile drug-binding region encompassing multiple and overlapping binding sites. Recently described mouse P-gp structure also correlates the continuous changes in surface topology occurring due to the opening and closing of the two halves of P-gp with rotation and translation of individual helix in TM domains.[21][42] The drug-binding region of P-gp is mostly made of hydrophobic & aromatic residues to which substrate bind via hydrophobic and Van der Waals interaction. Many Pgp substrates or inhibitors are typically lipid-soluble and amphipathic molecules. There are a few polar side chains identified in addition to aromatic (tyrosine and tryptophan) residue and these may mediate additional H-bond interaction with ligands.[21][41]

As P-gp extrudes drugs from the inner leaflet of the plasma membrane, it has been proposed to function as a "hydrophobic vacuum cleaner". P-gp surface facing the inner leaflet of membrane bilayer was observed to have an additional binding site for QZ-Val in the co-crystal

structure. This site was predicted to represent a substrate entry point. The additional QZ binding peptide appears to correlate with the structure and activity of ligands, and a greater number of drugs needed to be used for scrutinizing this structure-activity correlation. Overall, the recent structural determinations started a molecular understanding of how P-gp mediates MDR.[21][41]

P-GP SUBSTRATES AND INHIBITORS:

Substrates of P-gp:

P-gp transporter has several substrates that differ not only in size and form but also in specific chemical properties. The subjection of a substrate to P-gp efflux is its interaction with the bilayer lipid membrane, and a wide variety of cationic, lipophilic, and planar drugs are protein substrates despite their structural dissimilarities. This describes the wide distribution of structural complexity, or the non-specific existence of P-gp in the functional context (Higgins, Gottesman, 1992). There is nevertheless one systemic unifying function that is widely shared among all P-gp substrates, all of which have spatially different hydrophilic and hydrophobic molecules. Many clinically useful agents are reconstituted by P-gp substrates. Anticancer products, various Pharmacotherapeutic agents acting on the central nervous system, the cardiovascular system, and antimicrobials are substrates for the P-gp transporter.Multiple chemicals can bind to P-gp at the same time with a highly flexible drug binding pocket. Examples are verapamil and TMEA may be binding to the different regions P-gp pockets typical drug-binding (Loo et al., 2003a). Fluorescence studies have shown that LDS-751 and R123 can simultaneously bind and interact with one another in a non-competitive way to the R-site of P-gp (Lugo and Sharom 2005). In yet another study the reversal of inhibitors was caused by the mutation of residues that forms hydrogen bonds with P-gp inhibitors and their stimulatory activity on ATP binds and hydrolysis (Chufan et al., 2016).[12][13]

Paclitaxel is one of the most important anticancer agent effective against multiple types of solid tumors which works by enhancing tubulin polymerization, binding to microtubules, stabilizing microtubule dynamics, inducing metaphasic, or anaphasic mitotic block. It has found to have low oral bioavailability and poor aqueous solubility upon oral administration. Paclitaxel plasma concentration decreases rapidly after administration but then decreases after more than 24 h and stays above 0.05 μM.[50] The intestinal uptake of Paclitaxel is found to be hampered by the efflux of a drug due to the active P-gp transporter (MDR1/ABCB1), also via its metabolism by CYP450.[14]]P-gp are expressed not only on intestine but also at several organs like kidney, liver, secretory glands, and more importantly on BBB. P-gp mediated efflux reduces the accumulation of substrates in the cells and also its overexpression causes the complete resistance and loss of sensitivity. It also shows the effect on brain by increasing its efflux and reducing its therapeutic efficacy. Paclitaxel is a substrate of MDR1 P-gp (Gottesman and Patson, 1993). A reported in-vitro study has shown that paclitaxel when given with P-gp inhibitors like cyclosporine, verapamil, PSC833 results in reversal of resistance in P-gp expressing cells. This information is considered to be more useful in the clinical view for combining P-gp inhibitors with paclitaxel.[14][15] According to the study performed to determine the kinetics of P-gp-mediated efflux and how it contributed to the overall efflux of paclitaxel at a concentration range of 1-1500 nM. Human breast BC19 (P-gp rich) carcinoma cell, that is an MDR1-transfected variant of MCF7 (P-gp -negative) cells were used. Uptake and efflux of Paclitaxel is found to be both intracellular and extracellular in one of the monolayer cell cultures study. Results showed that BC19 when treated with PSC833 , enhanced the intracellular concentration of paclitaxel identical to that of MCF7 cells and reduced intracellular and extracellular drug concentrations. This decreasing the difference at high concentrations concludes, the P-gp mediated efflux in cancer cells and also lowered intracellular free drug than in extracellular concluded the efflux in BC19 with active carrier-mediated transport. The efflux highly depends on the concentration of Paclitaxel. All taxanes have shown similar efflux by P-gp which can be recovered or reversed by using P-gp inhibitors along with chemotherapy.[15]

Similarly, a study on docitaxel was done to determine the importance of its distribution in the brain. Docitaxel 33 mg/kg was administered in wild type and P-gp knockedout mice. Controll group received docitaxel and single dose cyclosporine A or valspodar(25mg/kg)/ elasidar(25 mg/kg). Results showed low brain concentration of docetaxel in wild type mice while in knockedout mice it was 4-9 folds higher at several points. On administration of these drugs to wild type mice results in significant rise in brain docitaxel value. The study concluded that the docitaxel penetration in brain is limited by P-gp and can be enhanced significantly with P-gp inhibitor. Valspodar and elacidar are found to be equally effective in co administration with docitaxel. They have shown

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increased docitaxel concentration in brain and are more effective then cyclosporine A. The increased dose of P-gp inhibitor enhances the drug penetration and rentation of docitaxel in brain. Valspodar upto 25 mg show max effect above which it shows prominent side effects ataxia. P-gp is less efficient in protecting brain against docitaxel then paclitaxel as they show 11 fold differences with paclitaxel in brain penetration between knockout and wild type mice. While 6.2 fold in case of docitaxel and thus paclitaxel found to be weaker substrate for P-gp than docetaxel. Thus in case of brain tumors, docitaxel can be more efficient with elacidar that allow full dose administration of dicitaxel.[16][51]

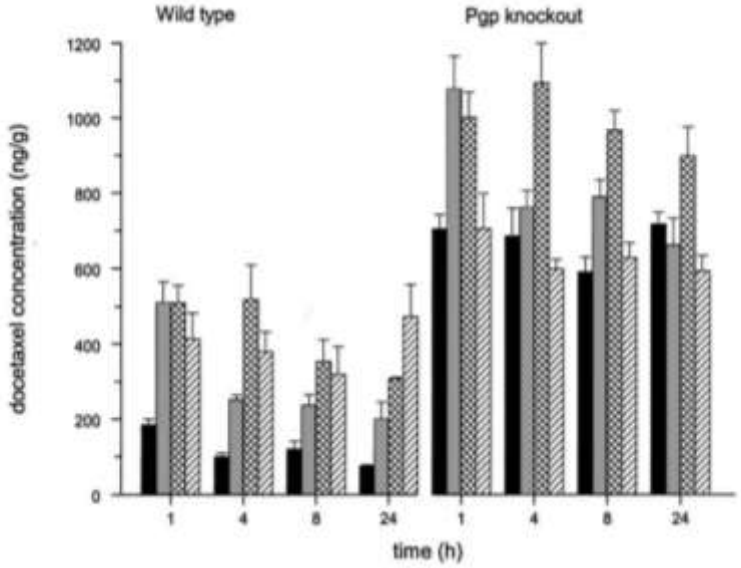

Fig no 3: Brain concentrations of docetaxel in wild-type and Pgp knockout mice at 1, 4, 8 and 24 h after administration of 33 mg/kg docetaxel alone or combined with 50 mg/kg cyclosporine A , 25 mg/kg valspodar and 25 mg/kg clacridar . Each bar represents the mean+SEM for 4-6 mice.

INHIBITORS OF P-GP :

In chemotherapy one of the main barriers is P-gp. P-GP inhibition is one of the important strategies in overcoming the difficulties associated with P-GP. The inhibition of this efflux pump is mainly done to avoid MDR occurring due to the efflux of therapeutic agents. There are three mechanisms by which P-GP can be inhibited: 1) blockage of drug binding site either competitively, non-competitively or allosterically; 2) disturbing ATP hydrolysis; 3) altering the integrity of membrane lipids.[17]

Inhibitors of P-GP are classified into 3 generations as follows:

Generation	Example	Specificity	Limitations
First-generation	Verapamil, cyclosporine A, reserpine, quinidine, yohimbine, tamoxifen, and toremifene	Non-selective and low binding affinities.	They serve as substrates for various enzyme and transporter systems. Pharmacological activity exists in them.. They are transported by P-gp.
Second generation	Dexverapamil, dexniguldipine, valspodar (PSC 833), and Dofequidar fumarate (MS-209)	Higher specificity then first-generation inhibitors but interact with other systems.	They serve as substrates for the ABC transporters and the CYP 3A4 enzyme.
Third generation	Cyclopropyldibenzosuberane zosuquidar (LY335979), laniquidar (R101933), mitotane (NSC-38721), biricodar (VX-710), elacridar (GF120918/GG918), ONT-093,	Highest specificity that specifically and potently inhibits P-gp function.	No limitations like the first and the second-generation inhibitors.

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	tariquidar (XR9576), and HM30181		

Table 2: Generations of P-gp inhibitors with examples, specification, and limitations

Monoclonal antibodies can also be effective in tumor cell MDR. UIC2 monoclonal antibody can recognize and inhibit P-GP mediated transport only in the presence of certain inhibitors like vinblastine, cyclosporine A, and PSC 833 (valspodar).[17][19] As the absorption of many drugs (which require small dose or have slow dissolution and diffusion rate) get decreases by the interference of P-GP mediated efflux, P-GP inhibitor is co administered with that drug to improve its absorption by preventing its efflux.The inhibition of P-gp by the modulators is cycled repeatedly, preventing drug efflux, when the inhibitor molecules are effluxed, they quickly bind to the P-gp binding sites again.. P-gp inhibitors not only improve absorption, but also improve drug distribution, metabolism, and excretion as P-GP is present in many organs like kidney tubules, bile ductle. As the P-GP inhibitor reduces biliary excretion and the clearance big substrate, they can also increase the half-lives of the substrate. Tariquidar is one of the potent inhibitors of P-gp. It exhibits high affinity for P-GP efflux transporter, although it is a substrate of BCRP (breast cancer resistance protein), that is another ABC transporter. A study was reported to address that TQR is a potent and non-competitive inhibitor of P-gp. They investigated TQR and it's interaction with the human P-gp and mouse P-GP.[19][20]

The human adenocarcinoma cell line KB-3-1 and it's P-GP expressing subline KB-8-5-11 and it's mouse P-GP expressing subline 3T3 C3M. Various assays were performed in this study. In the cytotoxicity assay, the ability of TQR to reverse the resistance of human and mouse P-GP to cytotoxic substrate paclitaxel was determined. For these cells were seeded in well (4000 per well) in media, dilutions were made with paclitaxel in DMEM and P-gp inhibitor was added to each well. Half maximal inhibitory concentration (IC50) was measured. Resistance rate (RR) calculated from dividing mean IC50(concentration of cytotoxic drug required to decrease cell viability by 50%) of resistance cell line by that of parental cell line's IC50. In flowcytometry, cells were incubated in Rh123 containing medium user untreated, and TQR treated condition and efflux of fluorescent P-GP substrate Rh123 was measured using FACS Caliber flow cytometer. Another radioactivity assays were performed in which cells were seeded in medium containing parent and P-gp expressing cell with or without TQR and incubated in radioactive medium, radioactivity was expressed as percent accumulation compared with untreated control cells. ATPase assay was performed to determine whether TQR affect human and mouse P-GP differently. In this, membrane vesicles with ATPase buffer were incubated in different concentrations of QTR and ATP hydrolysis was measured.[19][20]

Results of this study showed that 1) in human cells IC50 of paclitaxel significantly decreased in presence of 10nM, 100nM and 1uM TQR than with alone paclitaxel and in mouse cells it decreases after 100nM and 1uM TQR. The difference in responses may be attributed to the inherent difference between human and mouse P-GP. 2)The concentration of TQR from 10nM-100nM were examined and found that 40nM TQR significantly increased cellular accumulation of Rh123 in human P-gp expressing cell as compared to untreated cells and similar pattern seen in P-GP expressing cells in the mouse.

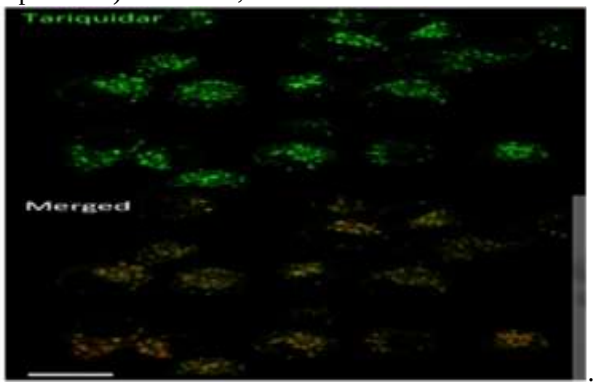

Fig.4- Cellular accumulation of Rh123 in treated and untreated human P-gp expressing cells.[19]

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3) The ATPase activity of P-gp decreased in the presence of increasing TQR concentration below the basal rate for both man and mouse P-gp.1 µM TQR elicited a 50% decrease in ATP hydrolysis. Thus study concludes TQR is a potent inhibitor of P-GP[19][20]

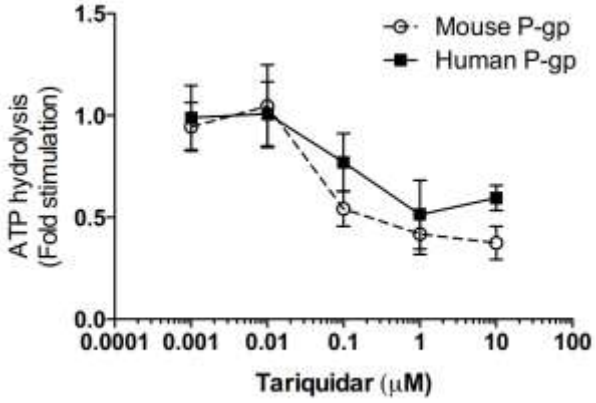

Fig.5- Graph of ATP hydrolysis with increasing concentrations of TQR.[19]

Table.3- Substrates and inhibitors of P-gp.

Substrates	Inhibitors
Antacids: Cimetidine	Immunosuppressant: Cyclosporine, Sirolimus, Tacrolimus
Antibiotics: Tetracycline, Rifampin	Opioids: Loperamide, Domperidone, Morphine, Pentazocine Methadone, Asimadoline, Fentanyl
Betareceptorr antagonist: Bunitrol, Carvedilol, Celiprolol, Taninolol, Reserpine	Steroids: Dexamethasone, Methylprednisone, Methylprednisolone, Hydrocortisone, Costisole, Corticosterone
Anticancer Agents: Paclitaxel, Daunorubicine, Vinblastine, Actinomycine, Docetaxel, Etoposide, Imatinib, Temiposide	Divers inhibitors: Verapamil, Valspodar, Cyclosporin, Ketoconazole
HIV Protease inhibitor: Amprenavir, Indinavir, Nelfinavir, Saquinavir Ritonavir	Others: Colchicine, Itraconazole, Phenothiazine, Ivermectin
Calcium Channel Blocker: Diltiazem, Mibefradile	
Histamine antagonist: Fexofenadine, Terfenadine	

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Transporter	Name	Gene Symbol	Substrates
P-glycoprotein	P-gp	ABCB1	amphipatic cations and organic compounds
Multidrug Resistance Protein	MRP-1	ABCC1	hydrophilic anion compounds, large molecules
	MRP-4 MRP-5	ABCC4 ABCC5	small polar compounds, nucleoside analogues
Breast-Cancer-Resistance Protein	BRCP	ABCG2	partially overlap with those of P-gp

P-GP INHIBITORS IN CANCER CHEMOTHERAPY:

P-gp as an efflux pump inhibits drug accumulation inside the tumor due to their overexpression on cancer cells further developing resistance of these drugs to cancer cells. Anticancer drugs get extruded before they reach their intended target and thus administered drugs cannot produce the desired outcome. Chemotherapeutic agents must reach a cytotoxic concentration in the cancer cells for effective chemotherapy. Several approaches have been taken up to overcome this P-gp mediated drug resistance and improve therapeutic strategies. To overcome this difficulty, chemotherapeutic agent administered concurrently with an inhibitor of P-gp. P-gp inhibitors like verapamil, cyclosporine restrain extrusion by P-gp, and improve the target activity of the drug. Other strategies are also involved in improving chemotherapy like anti-P-gp monoclonal antibodies. In resistant human myelogenous leukemia cell lines, greater cytotoxicity was shown by Vincristine-loaded lipid nanoparticles conjugated to an anti-P-gp monoclonal antibody (MRK-16).[17]

Several studies show that overexpression of P-gp in cancer cells can be either intrinsic or acquired upon drug treatment, depending upon the tissue of origin. In one reported study, they extended their investigation of the reversal of MDR by P-gp inhibitors to cancer of different origins using both two-dimensional cell culture and spheroid-microtumor assays. They showed that the addition of P-gp inhibitors (resazurin, Curcumin, or verapamil) previously found into a prostate cancer cell lines that over express P-gp cause reversal of the MDR phenotype. It was discovered that the presence of P-gp inhibitors significantly increased the MDR cell line's sensitivity to the chemotherapeutic agent.. They showed the comparison of IC50 values for the paclitaxel or vinblastine in the presence and absence of P-gp inhibitors for the two ovarian cancer cell lines and IC50 values of both paclitaxel and vinblastine found to be decreased in presence of P-gp inhibitors than with alone paclitaxel or vinblastine. Cell mortality was found to be increased in both two-dimensional and spheroid cultures when inhibitors were used in combination with chemotherapeutic agents.[19][22] The study also demonstrated that co-administration of P-gp inhibitors with chemotherapeutic agents cause an increase in the accumulation of calcein-AM (fluorescent P-gp transport substrate) due to significant increase in micro tumor penetrations in two-dimensional cell culture studies. This study demonstrated that, inhibitors directly blocked the pumping action of P-gp but not pump substrate itself.[22]

In this study, the effect of P-gp inhibitors on cell proliferation upon exposure to chemotherapy was also determined by treating cells with vinblastine/ paclitaxel and inhibitor alone and vinblastine/ paclitaxel in combination with P-gp inhibitor and cell colonies were assessed by staining. And it was concluded that in the presence of inhibitor and chemotherapeutic agent combination, no cell colonies observed, that is a combination of both inhibits cell proliferation. P-gp inhibitors were also assessed for their effect on

cancer cell migration in the presence of chemotherapeutic agents. For this, the MDR prostate cell line was used and wound healing assay was performed. P-gp inhibitor alone was taken as a control. In the presence of vinblastine, reduction of the area of the scratch wound was observed which indicates the ability of MDR cancer cells to migrate into a wound site. When P-gp inhibitors were used with chemotherapeutic agents, wound healing was not observed suggesting that cancer cell migration was inhibited. These properties of P-gp inhibitors studied in this study make them an important promising part of future drug development studies.[22] A major focus of clinical and laboratory studies of drug transporters have aimed at improving cancer therapy. The experimental study done on optimization of chemotherapy by increasing its systemic exposure was performed using cyclosporine, (a P-gp inhibitor or P-gp modulating agent) and results have shown to improve the efficacy of systemic chemotherapy in pediatric retinoblastoma patient with a high dose of cyclosporine. Cyclosporin A is an effective drug that anticipates blocking efflux of vincristine, etoposide that is P-gp mediated.[23] Another study done on thiosemicarbazones derivative including 3 synthesized compounds 8a, 10a and 17a focused on its collateral sensitivity towards P-gp overexpressing tumor cells causing the cytotoxicity of tumor cells. They show similar effects as the potent P-gp inhibitors PSC833 or Tariquidar. They are reported to show potential P-gp inhibition on BBB and GB levels and have promised to increase doxorubicin and rhodamine123 uptake along with increased doxorubicin permeability across BBB monolayer. With 10a derivative, doxorubicin plasma levels reduced and were progressively detectable in the brain.[24]

For optimal chemotherapy, the drug needs to achieve cytotoxic levels in cancer cells and its efficacy to reach all the viable tumor cells depends on the optimal delivery of drugs through the vascular system. Also, it depends on drug penetration in tumor cells that are deprived of blood vessel supply, with tortuous and leaky vessels, large inter capillary distances, and intermittent blood flow. Thus for an anticancer drug like doxorubicin, the fluorescence study of drug penetration has shown decreased intracellular concentration of doxorubicin in the tumor (mice) and breast cancer (human). Many anticancer agents such as vinca-alkaloids (vincristines, taxenes) show similar mechanisms for their resistance like doxorubicin. The sensitivity of the P-gp efflux pump for these drugs and also the high affinity for them is responsible for its resistance. Valspodar along with cisplatin in ovarian cancer resistant have passed all the clinical trials and have been effectively used in combination.[47][31][25] It has shown maximum cancer cell cytotoxicity in ovarian cance.[30][31] The study of vaspodar and cisplatin combination over the cell death, cell viability and oxidative stress condition demonstrated that the potential of cytotoxic drug is depended on proteins that are involved in defensive mechanisms of cell.[31][47]A data also demonstrate strongly that Valspodar can increase the cellular absorption of daunorubicin by interacting with Pgp in vivo in leukemia (U. Tidefelt, J. Liliemark, A. Gruber, E. Liliemark, B. Sundman-Engberg, G. Juliusson, L. Stenke, A. Elmhorn-Rosenborg, L. Möllgård, S. Lehman, D. Xu, A. Covelli, B. Gustavsson, C. Paul).[26]

A reported study explains that the distribution of doxorubicin over the P-gp overexpressed cancer cells is more when given in combination with P-gp inhibitors. It also studied that first-generation inhibitor verapamil as well as second-generation inhibitor valspodar both alter drug penetration in both human xenograft and murine tumor, which leads to improving doxorubicin uptake in tumor cells but restricted to cells that are rich in blood vessel supply.[27] This study suggested that P-gp inhibitors may apply to other membrane-based drug efflux proteins such as multidrug resistance protein1 (MRP1), and also highlight its importance in drug distribution studies in Novel drug delivery strategies.[27] Tamoxifen, a competitive estradiole inhibitor have been used in US, since a decade for treating postmenopausal breast cancer. It is considered as a potent P-gp inhibitor by experimental study (R Callghan and C O Higgins).[28][29] The study was conducted in relapsed and drug resistant cancer patient along with 300 mg etoposide to focus on its potential modulator activity. Tamoxifen 20-40 mg was administered in patients. Among 26 patients, one patient with relapsed lymphoma and one with adenocarcinoma showed partial response. Effects and concentration of tamoxifen and its metabolite N-desmethyltamoxifen and didesmethyltamoxifen were studied with sufficient level of cytotoxicity in cancer cell. But in vitro study has failed to show effect that neither tamoxifen nor verapamil were able to produce cytotoxicity in cancer cell line by increasing etoposide level in drug. But the cytotoxic levels were found to be increased for doxorubicin and vinblastine.[] A study also reveals the tamoxifen metabolites desmethyltamoxifen along with specific and high affinity P-gp inhibitor tariquidar and zosuquidar show synergistic effect

by enhancing ceramide chemotherapeutic efficacy.[30] This is done by increasing its cytotoxic levels in multidrug resistant AML. Expression of P-gp in older patients with AML tends to increase and probably associated with poor induction chemotherapy and thus it was discovered that (Ruoping Tang, Anne-Marie Faussat, Jean-Yves Perrot, Zora Marjanovic, Simy Cohen, Thomas Storme, Hamid Morjani, Ollivier Legrand & Jean-Pierre Marie).[31][52] Along with ceramaide in cancer cell, the effect of tamoxifen and its metabolite alone was found to be limited. But, when administered with tariquidar, cyclosporine A and zosuquidar demonstrated more efficacious in producing cytotoxicity.[30]. About 70% ovarian cancer is diagnosed at advanced stage and cells at stage I and II show more prominent resistant and is responsible for failure of chemotherapy. So there is necessity of MDR modulator and valspodar is more effective MDR modulator in combination with anticancer agent cisplatin in ovarian cancer.[31][30][][32]

A data about reported C6-ceramide – tamoxifen combination with activation of apoptotic factor causing ultimate cytotoxic response, suggested the induction of caspase-independant apoptosis in relevance with cancer therapy. The entire study concluded that ceramide is not a substrate of P-gp and also not found to be retended with p-gp inhibitor. But it has found to enhance ceramide driven apoptosis and reveals mechanism underlying cytotoxic responses.[32]

OTHERS APPROACHES TO IMPROVE CHEMOTHERAPY WITH ABC TRANSPORTER MODULATORS:

There are several resistance mechanisms among which Efflux transporter induced resistance is one of the important reason of resistance to chemotherapy. For improvising the chemotherapeutic effect in case of MDR, combinations of multiple drugs play important role. Combination drugs that have occurred via different mechanisms and thus multi-drug resistance can be avoided. The combination treatment not only lessens the drug resistance but also augment cidal effect of anticancer drug. This therapy optimizes therapeutic outcomes to a greater extent. The earlier discovered potential anticancer drugs and their mechanisms have revealed new possible targets of drug development.[33]

Designing of new drug or optimizing existing drug to evade P-gp mediated efflux can be achieved by either by identification of new lead compounds or the chemical modification of existing anticancer drugs. [21][34]A substantial research was made on vinca alkaloids and a derivative of vinblastine called Vinflunine having a fluorinated modification at the C20 position was shown to have decrease susceptibility to P-gp mediated efflux with lower in vitro neurotoxicity and enhanced bioavailability. [34] In 2009, EMA approved Vinflunine as the second-line treatment for urothelial cancer, and remains under investigation for different cancer treatment in several clinical trials.[36]

Involvement of anti-P-gp monoclonal antibody with the chemotherapeutic agents could be another strategy in preventing P-gp efflux. In resistant human myelogenous leukemia cell, Vincristine loaded lipid nanoparticles conjugated with an anti-P-gp monoclonal antibody (MRK-16) had shown greater cytotoxicity.[17][44] Also a study made by Naito's group emphasized on the increased chemosensitivity of MDR K562/ADM cells to vincristine and Doxorubicin when a combination of MRK-16 (monoclonal antibody) and cyclosporine A were used (Naito et al)., 1993.[38] Again MRK-16 blocked actinomycin D and Vincristine efflux, on the other hand MRK-17 inhibited MDR cell proliferation.[39] The study by God et al reported the combine use of UIC2 monoclonal antibody and cyclosporine A (first generation P-gp inhibitor) and concluded that UIC2 monoclonal antibody and a class of modulator used at low concentration together can be an effective strategy for blocking P-gp efflux in vivo.[17][45]

Some other novel promising approaches includes cancer vaccines, antisense oligonucleotide, and small-interfering RNA (siRNA) which specifically decrease the expression of P-gp, but still these are not enough to improve the outcome for many cancer patients.[35][33][43] The leukotriene LTD4 receptor antagonist MK571 and a fungal toxin fumitremorgin C (FTC) were found to be a specific modulators for inhibiting MRP1 and ABCG2. [33]Co-administration of MDR modulator with anticancer have resulted in significant cytotoxicity in tumor cells. They also have found to limit chemosencetization to MDR tumors. This problem is also associated with MDR blockade of P-gp on healthy tissues like kidney, liver. Their blockade, inhibit the normal clearance of anticancer drug in healthy tissue. While the combination of MDR modulators with anticancer have found to cause toxicity and more side effects.[39][33]

So, alternative approach for this can be liposomes. Liposomes are the carriers that are utilized to provide tumor selective drug delivery that cannot be provided by encapsulated drug. This substitution focuses on the liposomal anticancer

agent that avoid P-gp blockade on healthy cells and rather effectively accumulate anticancer agent in target tumor cells that is with optimal selective localization. A non-capsulated drug doxorubicin in its liposomal forms can increase selective localization in tumor. A comparative study between capsulated and non-capsulated doxorubicin show that, non-capsulated one alters doxorubicin clearance in liver and also reduced its exposure to kidney. And thus it increase cytotoxic level in selective tumor cells.[40]

II. CONCLUSION AND PERSPECTIVE:

Clinical studies have shown the failure of chemotherapy due to drug resistant phenomenon. Hence overcoming multidrug resistance(MDR) issue in cancer have become prime necessity. The advanced studies have been made to clearly understand the mechanism behind this resistance which revealed the action of efflux transporters (ABC transporters). In ABC transporter P-gp found to be most prominent and active efflux transporter due to their presence all over the body with effective efflux mechanism. Most of the cancer cells shows overexpression of P-gp which lead to natural drug resistance. Several effective chemotherapeutic agents show sensitivity towards P-gp which provide access for their efflux and failure of treatment. Thus P-gp modulators are among the promising approaches for overcoming these obstacles.

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