EFFLUX TRANSPORTERS

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INTRODUCTION

- Efflux transporters are positioned so as to encounter potentially harmful substances face-to-face — in important places like the intestine, the placenta, and the blood-brain and blood-testes barriers, making them an excellent first line of defense.

- Embedded within a cell’s membrane, this protein protects a cell by ejecting a variety of molecules — in many cases, toxins — on contact. The cell might be a bacterium, in which case the “toxins” are antibiotics.

- With cancer cells, the “poisons” are chemotherapy drugs.
P-glycoprotein

- MDR proteins (P-gp/ABCB) belongs to super family of ATP-binding cassette (ABC) transporters.

- It is also known as Multiple drug resistance-1 or ABCB1

- It is the first human ABC transporter cloned and characterized through its ability to confer a MDR phenotype to cancer cells.

- The ABC genes represents the largest family of transmembrane proteins. This proteins binds to ATP and use the energy to drive the transport of various molecules across all the cell membranes thus they act as energy dependent efflux transporters.
• In humans two members of the MDR/Pgp gene family MDR1 and MDR3 exist. Whereas 3 members of the P-gp gene family MDR1A, MDR1B, and MDR2 are found in animals.

• The human MDR1/ Pgp extrudes a variety of drugs across the plasma membrane and widely distributed. Whereas the homologous of MDR3 /Pgp as a more restricted expression with highest expression in hepatocytes and is required for phosphatidylcholine secretion into bile. However the involvement of MDR3 in drug transport is very low.
DISTRIBUTION OF P-gp

- liver: biliary hepatocytes
- small biliary ductules
- pancreas: pancreatic ductules
- kidney: proximal tubules
- Adrenal gland: cortex & medulla
- BBB
- GI epithelium
- Intestinal mucosal cells
- Pregnant uterus
- Epithelium of bronchi
- Glands: mammary, prostate, salivary, sweat glands of skin
• Blood capillaries of the brain & testes

• The concentration of p-gp is usually high in the plasma membrane of cancer cells, where it causes MDR pumping lipophillic drugs out of the cell. Its expression on human tumors is most commonly detected in colon, renal, and adrenal carcinomas; rarely in lung and gastric carcinomas.

• Function: protection by extruding toxins out of organs
• Protect the critical organs such as brain & testes
STRUCTURE OF MDR/P-gp

- MDR/P-gp is a 170 KDa transmembrane protein, an energy dependent efflux transporter driven by ATP hydrolysis.

- It is also involved in actively extruding various structurally unrelated amphipathic compounds with a molecular weight greater than 500 Da.

- It is composed of 1280 amino acids.

- It is composed of two homologous and symmetrical halves (cassettes) each of which contain six hydrophobic TM domines, each having 610 amino acids & one is having c-terminal & other with amino terminal.
Structure of the drug transporting P.gp

Two homologous & symmetrical halves (Cassettes)

Alpha helical segments
Carboxyl terminal

Intercellular flexible linker polypeptide loop

ATP binding motif
Amino terminal

Extracellular

Transmembrane domains (T1-T12) with major substrate binding sites on T0 & T12

Walker A,B & Signature C motif on both the ATP binding sites

ATP binding motif
• These TM domains are connected by an intra cellular flexible linker polypeptide loop, approximately 60 amino acids in length.

• The flexible secondary structure of the connector region is sufficient for the coordinate functioning of the two halves of p-gp, which is likely required for the proper interaction of the two ATP binding sites.

• There are two ATP binding domains of p-gp located in the cytosal side. Each ATP binding domains are also known as nucleotide binding folds(NBFs) contains three regions; walker A,B and signature C motifs
• A motif directly involved with the binding of ATP, B motif serves to bind the Mg+2 ion.

• Mg+2 may play a role in stabilizing the ATP binding site.

• Signature C involved in transduction of the energy of ATP hydrolysis to the conformational changes in the TMD required for translocation/efflux of the substrate.

• Glysine residues in TM2&TM3-determine the substrate specificity.

• Amino acids in TM1-determine the suitable substrate size

• In addition to TM domains, even ATP-binding domains have drug binding sites.
MECHANISM OF DRUG TRANSPORTATION BY P-gp

• The ATP binding sites that are restricted to walker A motif of ATP binding domains, many substrate binding sites have been identified throughout the TM domain of p-gp. The major drug binding sites reside in (or) near TM6 and TM12.

• The drug-binding pocket is located in the interface between the two halves of the molecule and is closely associated with TMDs 6 and 12.

• After a drug enters the lipid bilayer it interacts with specific residues in the drug-binding pocket. ATP hydrolysis follows and a conformational change in the TMDs is coupled to drug
MECHANISM OF DRUG TRANSPORTATION BY p-gp
Flippase model of P-gp mediated drug transport
• The drug intercalates between the phospholipid bilayer of the plasma membrane before interacting with P-gp. Upon interaction with P-gp, the drug is flipped from the inner leaflet to the outer leaflet of the lipid bilayer from where it diffuses to the extracellular space. The flipping process is the fast step, and the entry of the drug from the inner leaflet to the cytosol is the slow step.

• A drug with a higher lipid partition coefficient will be more easily removed from the lipid bilayer by P-gp than one with a lower lipid solubility independent of their relative concentrations in the system.
Substrates of p-gp

• P-gp exhibits extremely broad substrate specificities.
• Anticancer drugs
• Immunosuppressives
• HIV protease inhibitors
• Analgesics
• Corticosteroids
• Calcium channel blockers
• Fluorescent dyes
• Antibiotics
• Antidepressants
• Cardiac glycosides
• Antiepileptic drugs
• others:
  loperamide
  colchicine
  domperidone
INHIBITION OF P-gp

Reversal Agents
Reversal agents are those that inhibit that p-gp mediated drug transport and increase the influx of the therapeutic agent they are co administered with it.

Mechanism of inhibitors
- Competitive inhibitors without interrupting the ATP hydrolysis Eg.itraconazole,verapamil
- Blockage of ATP hydrolysis Eg.vandate
- Interferring with both substrate recognition & ATP hydrolysis Eg.cyclosporine-A
- Allosteric mechanism Eg.cis-(z)-flupentixol
P-gp reversing agents have been classified as 1st, 2nd and 3rd generation according to their toxicity and specificity of action.

**First Generation Inhibitors**

- These are pharmacological actives having ability to inhibit p-gp and are currently in clinical use for other indication. eg; calcium channel blockers such as verapamil,
- Immunosuppressive – cyclosporine A
- Antibiotics such as cefoperazone
- Antihypertensives such as reserpine, quinidine, yohimbine
Second Generation Inhibitors

These agents are devoid of pharmacological activity (or) lesser pharmacological activity and usually have p-gp affinity. 
Eg; R – verapamil with lower calcium channel blocker activity. 
PSC833(VALSPODAR), a Cyclosporine A analog with little immunosuppressive activity.

Third Generation Inhibitors

• These agents inhibits p-gp with high specificity these agents are more potent as compared first and second generation inhibitors.
  Eg. LY 335979(zosuquidar)
  OC 144093 and
  XR 9576(Tariquidar)
PHARMACOKINETIC IMPLICATIONS OF p-gp

Absorption

- The role of efflux transporters in determining the permeability and overall bioavailability of drugs has gained considerable attention.
- HIV protease inhibitors and anti cancer drugs have been reported to be substrates for p-gp and it can significantly limit the oral bioavailability of these drugs, showed improved bioavailability in the presence of p-gp inhibitors.
• Thus it should be appreciated that both passive permeability and the p-gp efflux process operating in mutually opposite directions contribute to overall drug permeability and thus influence the bioavailability.

❖ Many important drug interactions by modulation of intestinal P- gp have been reported.

• eg; Quinidine increases the absorption and Plasma concentration of oral morphine, suggesting that intestinal p-gp affects the absorption and bioavailability of the oral morphine.

• SQV efflux is more during the ethanol consumption, ethanol consumption increases the p-gp expression.
Drug distribution - elimination

• P-gp found to be present in broad spectrum of tissues. BBB and Placental barriers are very important determinants of drug distribution and p-gp is an important component of these biological barriers. Thus it can influence the drug distribution of many therapeutic agents significantly.

• p-gp limits the central distribution of drugs that are beneficial to treat CNS diseases. Modulation of p-gp efflux transporters at the BBB forms a novel strategy to enhance the penetration of drugs into the brain.
• Drugs that are administered during pregnancy will enter to some degree in the circulation of the fetus via passive diffusion and active transporters located on the fetal and maternal side of the tropoblast layer, p-gp on the maternal side of the tropoblast layer mediates the active efflux of lipophillic drugs from the fetal compartment.

• Excretion of drugs and endogenous compounds into urine and bile is mainly mediated by ATP dependent transporters out of which p-gp mediates considerable hepatobiliary excretion of doxorubicin and other drugs. Involvement of p-gp in this process is strongly drug dependent.

• CYP3A4 and p-gp present in intestinal tract act as synergistically in elimination of drug molecules.
Multidrug resistance associated proteins

- A subfamily referred as ABCC, includes cystic fibrosis transmembrane regulator (CFTR), the sulfonylurea receptors (SUR1&2) & Multidrug resistance associated proteins (MRPs) & includes other 13 members of human “c”branch ATP- Binding Cassette (ABC) super family.

- All “C”branch proteins share conserved structural features in their Nucleotide binding domains (NBDs) that distinguish them from other ABC proteins.
• The multidrug resistance associated proteins refers to their potential role in clinical multidrug resistance a phenomenon that hinders the effective chemotherapy for tumours.

• MRPs can collectively confer resistance to natural product drugs & their conjugated metabolites, platinum compounds, folate anti-metabolites, nucleoside & nucleotide analogs, peptide based agents, & alkylating agents.

• MRPs are also primary active transporters of other structurally diverse compounds including glutathione, glucuronide, & sulfate conjugates of large no. of xenobiotics
MEMBRANE TOPOLOGY OF ABCC PROTEINS:

- **MRPs**
  - a) long MRPs (1,2,3,6,&7).
  - b) short MRPs (4,5,8,9,10).

- **MSD** - membrane spanning domains.
- **NBD** - nucleotide binding domains.
- **CL3** - cytoplasmic linker
MEMBERS OF MRP FAMILY

membrane localization & physiological roles of MRPs.

1) MRP1 (ABCC1)


- Tissue distribution of MRPs is quite variable. MRP1 widely expressed, in high levels in Lungs, testis, kidney, skeletal and cardiac muscles, & the placenta, it is also expressed in a cell specific manner in the brain, as blood-brain barrier & choroid plexus of the blood cerebrospinal fluid barrier.

- MRP1 localizes predominantly to the plasma membrane & traffics selectively to the basolateral component in other cells, with an exception in placental & brain micro vessel endothelial cells localizes in apical membranes.
• MRP1 confers resistance to a variety of natural products of anticancer drugs including vinca alkaloids, anthracyclines epipodophyllotoxins.

• MRP1 shows preferential transport of anionic compounds like glucuronide, glutathione (GSH)& sulfate conjugates typical conjugated substrates includes 2,4dinitro phenyl-s-glutathione (DNP-SG), estradiol-17β glucuronide(E217βG), estrone3- sulfates.

• MRP1 is one of the glutathione-s-conjugate (GS-X) pumps, a transporter able to transport drugs conjugated to GSH out of the cell, the ability of MRP1 to transport substrates like methotrexate (MTX) or Arsenite(H₃AsO₃).

\[ H_3\text{AsO}_3 + 3\text{GSH} \rightarrow \text{As(SG)}_3 + 3\text{H}_2\text{O} \]

Arsenite  Glutathione complex

• This complex is transported by MRP1, as indicated by the ability of H₃AsO₃ to induce increased GSH export from cells with elevated levels of MRP1.

• MRP1 transports certain cationic compounds like vincristine & etoposide only in the presence of reduced GSH via co-transport.
Drug resistance mediated by MRP1&2 requires continuing supply of GSH to allow export of unconjugated drug as indicated in fig. There is often a simultaneous increase in MRP1 expression & gamma- glutamyl cysteine synthetase in tumour cells.

Transport of conjugated compounds & oxidized form of GSH in addition to possible upregulation of GSH-synthesizing enzymes, strongly suggests a role of MRP1 in detoxification & phase III elimination of toxic endogenous metabolites.
Glu + Cys

\[ \gamma \text{-Glu-Cys-synthetase} \]

\[ \gamma \text{-Glu-Cys} + \text{Gly} \]

GSH synthetase

GSH

\[ \text{GST} \]

GS-X

MRP

Y

X

Y
• MRP1 shows high affinity for the inflammatory mediator leukotrieneC\textsubscript{4} (LTC\textsubscript{4}) and play a significant role in the immune responses.

• MRP1 in the placenta may protect the developing fetus from xenobiotics & help prevent the fetal accumulation of E\textsubscript{2} \textsubscript{17}\textbeta G.

**MRP2 (ABCC2):**

• MRP2 as more restricted tissue distribution than MRP1, which is localized in the liver, kidney, small intestine, colon, gallbladder, placenta, & lung,

• MRP2 is only MRP that is consistently found in apical membranes as in liver canalicular membranes, in renal proximal tubules & in intestinal epithelium. MRP2 highest expression in the gut villi of proximal jejunum. In this respect, MRP2 co localizes with the P-gp & BCRP.
• MRP2 confers resistance to cisplatin.
• MRP2 location & substrate specificity plays important role in excreting metabolites into the bile, this is supported by the observation that absence of MRP2 in canalicular membrane results in impaired efflux of bilirubin glucuronide into the bile & manifests clinically as Dubin-Johnson syndrome.

**MRP3 (ABCC3):**

• MRP3 highly expressed in intestine & kidney, gallbladder localized to basolateral side of hepatocytes & cholangiocytes & low levels in placenta, prostate & liver MRP3 expression in liver is higher in Dubin-Johnson syndrome patients for compensation of absence of MRP2.
• MRP3 over expressing cells do not transport by GSH conjugates like (LTC₄).
• Glucuronide conjugates (E₂17 βG) are preferentially transported & plays important role in protecting liver from accumulation of monovalent bile salts, Eg: cholate, taurocholate, &glycocholate which contribute to the enterohepatic circulation of bile salts & other toxic conjugated compounds.

• MRP3 is responsible for basolateral efflux of acetaminophen glucuronide from liver.

**MRP4 & MRP5: (ABCC4&ABCC5)**

• MRP4 expressed at moderate levels in the ovary, testis, lung & higher levels in prostate (tubuloacinar cells) & kidney (proximal tubule).

• MRP4 is localized to apical (in kidney & endothelial cells of brain) rather than basolateral membranes (prostate, choriod plexus).
• MRP5 expressed widely than MRP4, with highest levels in skeletal muscle, cardiac muscle

• MRP4&5 differ from MRP1,2,3 like proteins in their ability to transport nucleoside analogs& nucleotide analogs, cyclic nucleotides.

• Ability to transport cyclic nucleotides contribute to modulation in intracellular c AMP& c GMP levels.

• Co-localization of MRP5& phosphodiesterases in the smooth muscle cells of urinary tract is important when coupled with phosphodiesterases inhibitors (sildenafil) also inhibits MRP5 mediated c GMP efflux & then contribute to increase drug efficacy i.e. in raising C GMP levels in smooth muscle cells.

• MRP4 also transports prostaglandins PGE1,PGE2& inhibited by NSAIDS& thrombaxane.
MRP6(ABCC6):

- Tissue distribution of MRP6 is particularly important because of association between mutations in this proteins & degenerative connective tissue disease (pseudoxanthoma elasticum).

- MRP6 localized in basolateral membrane of human parenchymal cells of liver, proximal tubules in higher levels & in keratinocytes of skin, trachea in low levels.

- Over expression & amplification of MRP6 gene in tumour cells was found only with over expression of MRP1 gene.

- MRP6 transports a no. of GS-conjugated organic anions including LTC4, 2,4-dinitrophenyl glutathione.

- PXE –causing defects to NBD2 of protein, the disease associated with loss of its transport function than substrate specificity.
MRP7(ABCC10)

- A membrane topology similar to MRP1, 2, 3, 6.
- MRP7 mRNA detected in higher levels in tissues like colon, skin, & testes.
- Drug resistance to docetaxel, vincristine, & vinblastine.
- MRP7 mediates low affinity transport for $E_217\beta$ G.

MRP8(ABCC11):

- Highly expressed in breast cancer.
- MRP8 confers resistance to pyrimidine analogs 5’-fluro-5’-deoxyuridine, 5-flurouracil & 5’-fluro-2’-deoxyuridine.
- MRP8 mediates transport of $E_217\beta$ G, dehydroepiandrosterone 3-sulfate, & LTC$_4$, bile acids.

MRP9(ABCC12):

- New molecules of MRP family, expressed in variety of adult tissues like brain, testes & in fetal tissue of liver, spleen & lung.
INHIBITORS OF MRPs

• Most highly specific MRP1 inhibitor tricyclic isoxazoles

• High affinity substrates of MRP1&MRP2 (LTC4, S-decyl glutathione) are organic anions with substantial hydrophobic moiety of at least 1 or 2 negative charges act as potent competitive inhibitors.

• Dietary flavonoids (quercertin), synthetic flavonoids (flavopiridol) inhibits both MRP1&2.

• Organic anion transport inhibitors such as sulfinpyrazone, probenecid inhibits both MRP1,2&3

• Probenecid and phosphodiesterase inhibitors such as sildenafil inhibi MRP5
Transport cycle of MRP

Transport cycle of MRP comprises six steps.

1) Binding of substrate (LTC4).
2) Initial binding of ATP by NBD1.
3) Binding of second ATP.
4) ATP hydrolysis.
5) Recycling of NBD2.
6) Recycling of NBD1.
TRANSPORT CYCLE OF MRPs
Efflux transporters are active in a broad spectrum of human cancers including leukemia, breast, lung and ovarian cancers.

Acute myeloblastic leukemia cells found combined presence of MRP1 & p-gp/MDR.

Antiandrogen flutamide & its active metabolites effectively effluxed by MRP1 over expressing cells making no effect of systemic therapy.

Increased expression of MRP2 associated with cisplatin resistance in human colon carcinoma.
Over expression of P-gp and other efflux transporters in the cerebrovascular endothelium in the region of the epileptic focus lead to drug resistance in epilepsy failure of antiepileptic therapy.

Brain entry of risperidone and its active metabolite 9-OH risperidone is limited by P-gp failure of therapy in psychotics.
Chemotherapy drugs are introduced and transported into the interior of cancer cells that lack MDR. As a result, these cells die. Those cancer cells with MDR prevent the drugs from entering the cell and survive.

Cancer cells with MDR continue to replicate and maintain their immunity to the drugs.

Cancerous cell growth continues and can spread unchecked.
Because of its lack of MDR, bone marrow tends to be highly susceptible to cancer-killing drugs. Scientists have proposed a possible method of gene therapy.

1. Stem cells are removed from the patient.
2. These cells do not contain MDR protein pumps.
3. A piece of RNA with the genetic code for MDR is placed within a retrovirus which is then added to the stem cell sample from the patient.
4. The retrovirus attaches to the stem cell and feeds the RNA into the cell.
5. Using reverse transcriptase, the MDR genetic code is incorporated into the DNA of the stem cell.
6. As this cell reproduces the code for MDR is passed on to each new generation of stem cell.
7. The genetically enhanced stem cells can then be re-introduced to the patient. As the stem cells reproduce the number of cells containing MDR would increase, thus increasing the patient’s bone marrow resistance to chemotherapy treatment.
CONCLUSION

- Efflux transporters are distributed in a wide range of tissues, as to prevent the access of xenobiotics into them,
- It has become an impediment to the successful therapy of a number of diseases and syndromes by effecting the pharmacokinetics and pharmacodynamics of various therapeutic agents used to treat them. Numerous agents are being screened for their efficacy to reverse the action of efflux transporters. There is considerable interest in the search for new efflux transporters inhibitors that do not elicit significant toxicity at doses required for efflux transporters inhibition.
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