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Molecular genetic analysis and biochemical characterization of mammalian P-glycoproteins involved in multidrug resistance

Christine A. Hrycyna

A variety of human cancers become resistant or are intrinsically resistant to treatment with conventional drug therapies. This phenomenon is due in large part to the overexpression of a 170 kDa plasma membrane ATP-dependent pump known as the multidrug resistance transporter or P-glycoprotein. P-glycoprotein is a member of the large ATP binding cassette (ABC) superfamily of membrane transporters. This review focuses on the use of structure–function analyses to elucidate further the mechanism of action of mammalian P-glycoproteins. Ultimately, a complete understanding of the mechanism is important for the development of novel strategies for the treatment of many human cancers.

Key words: ABC superfamily / chemotherapy / P-glycoprotein / multidrug resistance / mutational analysis

The successful treatment of cancer depends greatly upon the effectiveness of cytotoxic anticancer drugs such as the *Vinca* alkaloids, the anthracyclines, the epipodophyllotoxins, taxol, and actinomycin D. These natural products have several different cytotoxic targets including microtubules, DNA, and RNA polymerase. Although these compounds share little to none of the same chemistry, they all are amphipathic molecules with planar aromatic rings that preferentially partition to organic solvents.[1] Unfortunately, most cancers are either intrinsically resistant to any initial treatment with these therapeutic compounds or acquire resistance to a broad spectrum of these agents over time, a phenomenon called multidrug resistance.[2–4]

It has become well established that this broad-based resistance results largely, but not solely, from the overexpression of a number of members of the ATP binding cassette (ABC) superfamily of membrane transporters,[5,6] including the multidrug transporter P-glycoprotein (P-gp) and members of the MRP family (multidrug resistance-associated proteins).[7] Recently, a newly identified gene product alternatively known as ABCG2/MXR1/ABCP1/BCRP has also been implicated in the resistance phenotype of drug-selected cells in culture.[8–10] Members of the P-gp family of transporters implicated in multidrug resistance have been cloned from mouse (*mdr*1 and *mdr*3), human (*MDR*1), rat, hamster and other mammalian cells. Overexpression of the MDR genes from human and mouse has been found to be sufficient for conferring the multidrug resistant phenotype to both cells in culture and in mouse bone marrow.[11–14] Clinically, many different human cancers express the *MDR*1 gene at levels sufficient to confer multidrug resistance. In fact, based on an analysis of several hundred different human cancers, it can be estimated that approximately 50% of human cancers will express the *MDR*1 gene at some time during therapy.[15] (Details about the cloning and genetic analysis of MDR genes can be found in reviews by Schoenlein[16,17] and Gottesman *et al.*[4])

The sequence information derived from cloning of the genes led to a working model for the structure of P-gp, now one of most thoroughly characterized proteins in the ABC protein superfamily. P-gp is a 1280 amino acid (170 kDa) ATP-dependent plasma membrane-associated protein

From the Department of Chemistry, Purdue University, 1393 Brown Building, West Lafayette, IN 47907-1393, USA.
E-mail: hrycyna@purdue.edu

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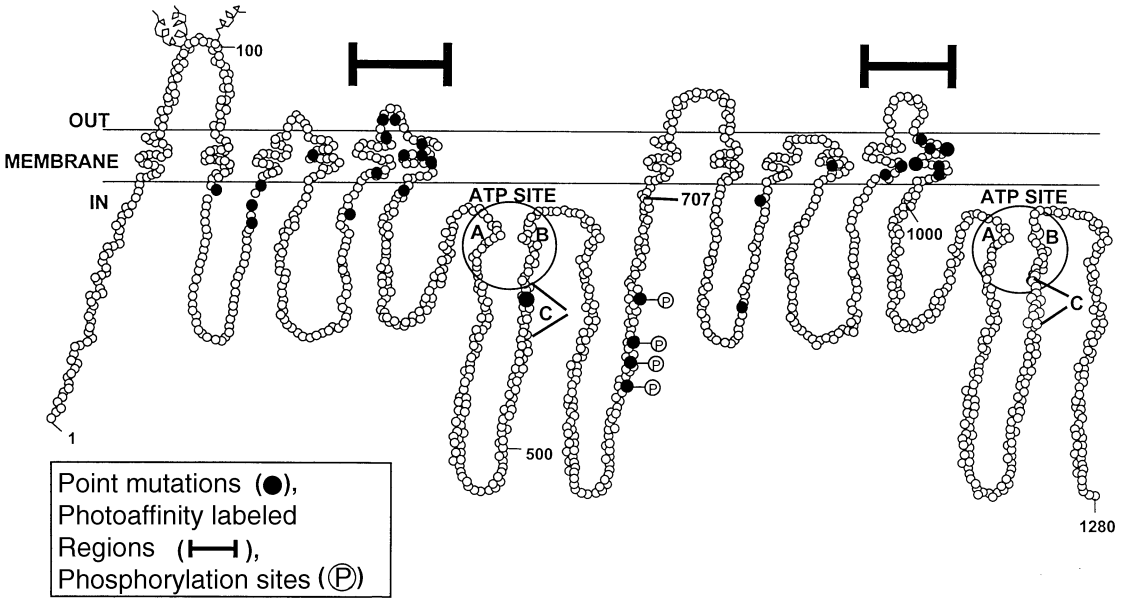

Figure 1. Two-dimensional hypothetical model of human P-gp structure based on a hydropathy plot analysis of primary amino acid sequence. The ATP binding/utilization domains are circled with the Walker A, B, and 'linker dodecapeptide' or 'signature sequence' (LSGGQ) motifs are designated by the letters 'A', 'B' and 'C'. Putative glycosylation sites are represented by squiggly lines. The regions known to bind photoaffinity drug analogues are designated by the heavy dark bars and the serine residues that are known to be phosphorylated are shown as darkened circles with an attached and encircled 'P'. Each circle represents an amino acid residue. The full circles show many of the positions of mutations that change substrate specificity in human P-gp (see Table 1). Two amino acids corresponding to residues Y949 and Y953 from mouse Mdr3 are included as they are conserved in all mammalian P-gp's and change substrate specificity of the mouse transporter.[78] (Adapted from References 3 and 97.)

comprised of 12 transmembrane domains and two ATP binding/utilization sites. The protein is arranged in two homologous halves connected by a flexible linker region. Each half contains six transmembrane domains and two cytosolically localized ATP binding/utilization domains[3,4] (Figure 1). Despite speculation concerning possible topological variations in this model in which some transmembrane domains are thought to be a part of large extracellular loops, other studies suggest that the original 12-transmembrane domain model remains the most likely. Loo and Clarke[18] have provided additional evidence for this model by assessing the reactivity of reintroduced cysteine residues to membrane permeant and impermeant sulfhydryl group reagents in a cysteine-less mutant of human P-gp. Additional characteristics of P-gp include at least two confirmed substrate binding regions localized to the transmembrane segments, specifically transmembrane domains 5, 6 and 11, 12, several phosphorylation sites in the linker region of the protein that joins the two halves of the protein and three *N*-linked glycosylation sites located in the first extracytoplasmic loop (Figure 1).

P-glycoprotein is an energy-dependent pump that reduces accumulation of drugs within cells. Although ATP binding and hydrolysis are essential for the proper functioning of P-gp,[19] how the energy of ATP hydrolysis is transduced in the system to result in the efficient transport of a large variety of structurally unrelated hydrophobic and amphipathic molecules, including many chemotherapeutic agents, remains the subject of intensive ongoing research. One proposed model suggests that the P-gp transporter pump may reduce intracellular drug concentrations by acting as a 'hydrophobic vacuum cleaner' effectively increasing drug efflux and decreasing drug influx by the recognition and removal of compounds from the plasma membrane before they reach the cytosol or intracellular target to elicit their cytotoxic effects.[3,20] Another model being explored is the 'flippase' model in which P-gp would detect a drug within the inner leaflet of the plasma membrane and 'flip' it into into the extracellular space or into

Table 1. List of mutations in human, mouse and hamster P-gp's that affect substrate specificity[f]

aa[a] Mutation	Region[b]	Source[c]	Reference
Δ aa 78–97	EC 1	human *MDR*1 (ABC20)[d]	78
Q128H[e]	TM 2	mouse *mdr*3	79
R138H	IC 1	mouse *mdr*3	79
Q139H, R	IC 1	mouse *mdr*3	79
G141V	IC 1	human *MDR*1	25, 80
Q145H	IC 1	mouse *mdr*3	79
E155G, K	IC 1	mouse *mdr*3	79
F159I	IC 1	mouse *mdr*3	79
D174G	IC 1	mouse *mdr*3	79
S176F, P	IC 1	mouse *mdr*3	79
K177I	IC 1	mouse *mdr*3	79
N179S	IC1	mouse *mdr*3	79
N183S/G185V	IC 1	human *MDR*1	81
G183D	IC1	mouse *mdr*3	79
G185V	IC 1	human *MDR*1	82–84
G187V	IC 1	human *MDR*1	80
A192T	TM 3	mouse *mdr*3	79
F204S	EC 2	mouse *mdr*3	79
W208G	EC 2	mouse *mdr*3	79
K209E	EC 2	mouse *mdr*3	79
L210I	TM 4	mouse *mdr*3	79
T211P	TM 4	mouse *mdr*3	79
I214T	TM 4	mouse *mdr*3	79
P223A	TM 4	human *MDR*1	85
K285T	IC 2	human *MDR*1	1
G288V	IC 2	human *MDR*1	80
I299M, T319S, L322I, G324K, S351N	TM 5, EC3, IC 3	human *MDR*1	86
Δ V334	TM 6	human *MDR*1	1
F335A	TM 6	human *MDR*1	25
Δ F335	TM 6	human *MDR*1	87
V338A	TM 6	human *MDR*1	88
G338A, A339P	TM 6	hamster *PGY*1	89, 90
A339P	TM 6	hamster *PGY*1	90
G341V	TM 6	human *MDR*1	88
K536R,Q	N-NBD	human *MDR*1	91
ERGA→DKGT (aa 522–525)	N-NBD	mouse *mdr*3	92
T578C	N-NBD	mouse *mdr*3	92
G812V	IC 4	human *MDR*1	80
G830V	IC 4	human *MDR*1	25, 80
P866A	TM 10	human *MDR*1	85
F934A	TM 11	mouse *mdr*3	93
G935A	TM 11	mouse *mdr*3	93
I936A	TM 11	mouse *mdr*3	93
F938A	TM 11	mouse *mdr*3	93
S939A	TM 11	mouse *mdr*3	93
S939F	TM 11	mouse *mdr*3	94, 95
S941F	TM 11	mouse *mdr1*	94, 95
T941A	TM 11	mouse *mdr*3	93
Q942A	TM 11	mouse *mdr*3	93

Table 1—*continued*

aa[a] Mutation	Region[b]	Source[c]	Reference
A943G	TM 11	mouse *mdr*3	93
Y946A	TM 11	mouse *mdr*3	93
S948A	TM 11	mouse *mdr*3	93
Y949A	TM 11	mouse *mdr*3	93
C952A	TM 11	mouse *mdr*3	93
F953A	TM 11	mouse *mdr*3	93
F983A	TM 12	human *MDR*1	96
L975A, V981A, F983A	TM 12	human *MDR*1	96
M986A, V988A, Q990A, V991A	TM 12	human *MDR*1	96
V981A, F983A	TM 12	human *MDR*1	96
L975A, F983A	TM 12	human *MDR*1	96
L975A, V981A	TM 12	human *MDR*1	96
Δ F978	TM 12	human *MDR*1	1
F978A	TM 12	human *MDR*1	25

[a] aa, amino acid.
[b] EC, extracellular loop; IC, intracellular loop; TM, transmembrane domain; NBD, nucleotide binding/utilization domain.
[c] cDNA source.
[d] As per the nomenclature system proposed by HUGO, the human gene nomenclature committee.
[e] Single letter designations represent amino acid residues. Numbers represent the amino acid in the primary sequence and the letter following the number represents the residue.
[f] Adapted from Reference 4.

the outer leaflet where it can diffuse away from the cell.[21] The overall predicted topological similarities between members of the ABC superfamily and their strict dependence upon ATP binding and hydrolysis for function suggest that the mechanism of action of P-gp, when fully defined, may serve as the paradigm for many ABC transporters.

Although the overall predicted topology of members of the ABC superfamily is relatively well conserved, the similarities between these proteins are most striking and apparent in the nucleotide binding/utilization domains. In fact, the greatest similarity between the two homologous halves of P-gp itself lies in the ATP binding domains.[3] Each of these domains is characterized by several consensus motifs including the Walker A and Walker B motifs. The A nucleotide binding fold consensus sequence is G-$(X)_4$-G-K-(T)-$(X)_6$-I/V and the B binding fold consensus sequence is R/K-$(X)_3$-G-$(X)_3$-L-(hydrophobic)$_4$-D where X is any amino acid.[22] Significant homology is also observed in the 'linker dodecapeptide' or 'signature' region just preceding the Walker B motif defined by the consensus sequence LSGGQ.[23,24] These observations suggest that these entire sections of the polypeptide chain may be important in both nucleotide binding and in the energy transduction process that drives the transport of substrates out of the cell.

Stable and transient expression systems for P-gp

In order to study wild-type P-gp or mutant variants, it is important to achieve high expression levels of the protein. One way to achieve this goal in mammalian cells transfected or transduced with P-gp constructs is through long-term drug selection with MDR substrates. The imposition of such selection schemes has always been a point of controversy because of the unknown effects drug selection could have on host cell functions. The data must necessarily be interpreted with the understanding that other endogenous drug resistance mechanisms may possibly be activated or selected for in the presence of the cytotoxic agents used for selection. Another method of expressing larger quantities of protein is through transient expression systems. Recently, a number of versatile expression systems have been developed that offer the opportunity to study wild-type and mutant P-gp in their native forms without concern about pleiotropic cellular effects due to drug selection.

A system has been developed in which human P-gp mutant proteins tagged with 10 histidine residues at the C-terminus are transiently expressed in HEK 293 cells[25] and subsequently purified by nickel-chelate chromatography. This methodology resulted in significant enrichment of P-gp allowing for quantitative assessment of the ATPase activities of these proteins and correlation of these activities to their drug-resistant phenotypes. However, the expression levels do not appear high enough to allow for direct assessment of function in whole cells. An alternative approach to the study of human P-gp, reviewed by Evans *et al.*,[26] has been to express the wild-type and mutant forms of the protein in heterologous expression systems such as in the yeasts *S. cerevisiae* and *P. pastoris* and in insect cells infected with recombinant baculovirus vectors. These systems offer the advantages of ease of genetic selection and manipulation and, perhaps more importantly, they allow for a high level of expression of recombinant P-gp that if tagged with a 6–10 histidine residue epitope, can be purified in large quantities with relative ease. The baculovirus and *P. pastoris* systems have proven to be excellent systems for the overexpression of P-gp and for *in vitro* biochemical characterization, since recombinant P-gp represents a relatively large percentage of the membrane fraction.[27,28] However, the systems are not ideally suited for studies in intact cells. Infection with baculovirus causes disruption of the membrane making the cells permeable and leaky. Additionally, baculovirus is not well-suited for mutational studies because of the need to develop a new virus for each construct. However, new technological advances are making it easier to produce mutants much more rapidly.[29,30] On the other hand, the *P. pastoris* system has been used extensively to study a variety of purified mutant P-gp's *in vitro*.[27,31]

A vaccinia virus-bacteriophage T7 RNA polymerase hybrid expression system[32] has been adapted for exploring P-gp function in mammalian cells both in intact cells and for *in vitro* biochemical assays.[33–35] Cell lines (e.g. HeLa and human osteosarcoma) with little to no endogenous P-gp expression and low endogenous membrane-associated ATPase activity are used and the expression of human *MDR*1 is monitored by immunoblot analysis. Presence of P-gp on the cell surface in intact cells can be detected using a monoclonal antibody that recognizes an external epitope (e.g. MRK–16[36] or UIC2[37]) by fluorescence-activated cell sorting (FACS) analysis. P-gp function is also evaluated by fluorescent substrate efflux experiments as well as uptake of radioactive substrates in intact cells. *In vitro* assays have also been established to evaluate drug binding and drug-stimulatable ATPase activity using a variety of drug substrates.

This system has proven to be ideally suited to study virtually all aspects of P-gp function. Since all of the assays can be performed in a single cell line and relatively large amounts of protein can be expressed within 16–24 hours of transfection without having to impose any drug selection, this system is simple and extremely powerful. It is also well-suited for studying large numbers of chimeric/mutant constructs since the recombinant protein can be expressed by an infection and transfection procedure that eliminates the need to generate recombinant viruses for each mutant construct to be analyzed. One inherent drawback, however, of the vaccinia system is that the infected/transfected cells cannot be examined for changes in substrate specificity by cell proliferation assays.

The study of mutant and chimeric P-gp molecules that are either naturally occurring or artificially engineered has proven to be one of the most useful and informative approaches employed to aid in elucidating the mechanism of action of P-gp and in answering structure/function questions concerning substrate specificity and ATP utilization. Generally, these mutants have been generated either by *in vivo* drug selection or by site-directed mutagenesis techniques followed by expression in any number of the systems described above followed by *in vivo* and *in vitro* biochemical characterization. A large number of P-gp mutants located throughout the molecule have been studied and were found to have a variety of effects on transporter function ranging from inactivation to change of substrate specificity. Most commonly, these mutations are localized within the nucleotide binding/utilization domains and the substrate binding/interacting domains[4] (for other reviews, see References 1, 3 and 38).

Mutational analysis of the nucleotide binding/utilization domains of P-gp

Mutational analyses of the nucleotide binding domains of P-gp have shed important light on the functional nature of these regions. Many of the mutations that result in non-functional, but properly processed P-gp molecules lie within the nucleotide binding/utilization domains. Site-directed mutagenesis of the consensus sequences of the nucleotide bind-

ing domains suggest that both nucleotide binding domains are essential for the proper functioning of P-gp as inactivation of one ATP site abrogates activity of the protein.[3, 4, 25, 39–41] Biochemical characterization of the catalytic ATP hydrolysis cycles of both human and hamster purified P-gps have determined that both ATP sites are capable of hydrolyzing ATP but not simultaneously[42–44] and that drug binding and ATP hydrolysis are intimately coupled.[28] These data have led to a model of P-gp action originally proposed by Senior and colleagues[44–47] that suggests cross-talk between the two ATP sites and involves alternating catalysis of each ATP site. Although both sites are capable of hydrolyzing ATP, analysis of site-directed mutations made in the Walker B regions of human and mouse P-gp have suggested that under certain experimental conditions the two ATP sites can be distinguished, suggesting possible different structural or functional roles, conformational states or accessibilities of the two sites at any given time in the transport process.[48, 49] Recently, it has been shown that there may be two distinct roles for ATP hydrolysis in a single turnover of the catalytic cycle of P-gp, one in the transport of substrate and the other in effecting conformational changes to reset the pump for the next catalytic cycle.[50, 51] Biochemical characterization of LmrA, an ABC transporter that is closely related functionally to P-gp, has provided evidence for another model that also extends the original alternating catalysis model.[52] This two-site transport model suggests that the alternating hydrolysis of ATP by each of the ABC domains of one half of the transporter is coupled to drug efflux, operating similarly to a two-cylinder engine.[52]

Further analysis of the nucleotide binding domains has mapped out other important residues involved in the P-gp catalytic cycle. Mutational analysis of highly conserved carboxylate residues in the nucleotide binding domains of mouse Mdr3 demonstrated that these residues may play a role in steps after the transition state of the catalytic cycle, possibly in the release of Mg•ADP.[31] It has also been determined that conserved serine residues in the Walker A domains are essential for catalysis of ATP hydrolysis and are critical for attainment of the P-gp catalytic transition state, as measured by ATP hydrolysis and vanadate trapping of Mg-nucleoside diphosphate.[53] Further analysis of the conserved glutamine residues in the nucleotide binding domains suggest that the primary role of this glutamine residue is in interdomain signal communication between catalytic site reaction chemistry and drug binding sites.[54]

Mutational analysis of the drug binding domains of P-gp

Another compelling yet confounding issue concerning the mechanism of action of P-gp deals with the seeming lack of substrate specificity of the transporter. In other words, how can a single molecule function to transport such a wide variety of agents? Mutations in mammalian P-gp's that affect substrate specificity are described in Table 1 (adapted from Reference 1). These mutations are clustered predominantly in the transmembrane domains, mainly 5, 6 and 11, 12, but are also found throughout the rest of the molecule including the soluble intra- and extracellular loops and the ATP binding/utilization domains, suggesting that other regions may also play supporting roles, either directly or indirectly, in defining the drug binding domains.

Previously, transmembrane domains 5, 6 and 11, 12 and the extracellular loops (EC) connecting them have been determined biochemically to be the major sites of drug interaction by photoaffinity labeling with [^{3}H]-azidopine and [^{125}I]-6-AIPP-forskolin and [^{125}I]-iodoarylazidoprazosin, digestion with proteases or cyanogen bromide, and specific immunoprecipitation with anti-P-gp antibodies directed against epitopes containing these regions.[55–59] Recently, using systematic cysteine scanning mutagenesis followed by cross-linking and inhibition assays with dibromobimane, the picture of the binding domain of P-gp has been expanded to include other residues from transmembrane domains 4 and 10, as well as those from domains 5, 6, 11, and 12.[60–62] It is generally thought that these regions form the drug binding/interaction sites in the three-dimensional structure of P-gp. In fact, mounting biochemical evidence suggests that these regions in P-gp may form multiple non-identical sites of drug interaction.[63–69]

These data, supported by the mutational data, suggest that these regions are important determinants in the drug binding site(s) but do not offer any insight as to whether these sites are acting autonomously or are interdependent. However, using systematic cysteine scanning mutagenesis and oxidative cross-linking, evidence has been provided suggesting that the transmembrane segments important for drug binding must be in close proximity to each other and exhibit different conformational changes in response to drug binding or ATP hydrolysis.[70] Recently, using fluorescence spectroscopy techniques, it was determined that the nucleotide and drug binding

regions of P-gp appear to be packed together compactly, thus facilitating the coupling of ATP hydrolysis to drug transport.[63] In total, these results suggest a complex three-dimensional drug binding site or sites for the interaction of the multitude of substrates and inhibitors of P-gp. Clearly, this will be a field of immense interest and research over the next several years.

Interaction of the two halves of P-gp

An important mechanistic question raised by the mutational data has been whether the two halves of P-gp operate independently or in concert. Previously, it has been shown that drug resistance was not conferred on drug-sensitive NIH3T3 cells that co-expressed the two halves of P-gp, even though stable expression of each half-molecule was detected.[71] However, upon expression of these molecules in Sf9 insect cells using baculovirus vectors encoding each half separately, drug-stimulated ATPase activity was reconstituted suggesting that coupling of ATPase activity to drug transport requires interaction of the two halves.[71] Deletion of the central core of the linker region connecting the two halves of human P-gp results in a protein that is expressed at the cell surface comparably to the wild-type protein but is not functional for either transport or drug-stimulated ATPase activity.[72] Replacement of the deletion with a peptide with a predicted flexible secondary structure was found to be sufficient for restoring the functional properties of the molecule. These data suggest that communication of the two halves of P-gp is necessary for the coordinate functioning of the molecule and that a flexible linker region is sufficient for the proper orientation of the two halves, most likely because it allows for the proper interaction of the two ATP binding sites. In addition, expression of either of the two halves of P-gp[73] does not provide a functional multidrug transporter, suggesting that the two halves may cooperate in the intact full length protein and further corroborates the idea that both ATP sites are essential.

Taken together, these mutational data suggest that the two halves of human P-gp interact to form a single transporter and that the drug binding sites may reside in transmembrane domains 4, 5, 6 and 10, 11, 12. It is also clear that both ATP sites are necessary for a functional molecule and that, in fact, the ATP sites and the drug binding domains may interact with each other to form the functional transporter. Results from fluorescence energy transfer experiments have suggested that the two nucleotide binding domains of P-gp are relatively close together[74,75] and lie close to the membrane surface.[76] Presumably, the other transmembrane, cytosolic, and extracellular domains are involved either in correct folding of the transporter or may represent alternative overlapping substrate recognition sites.[4]

Clearly, the next major breakthrough in our understanding of the mechanism of action of P-gp will occur with the generation of high-resolution two-dimensional and three-dimensional structures. Electron microscopic and single particle image analysis results are encouraging and have met with some preliminary success.[77] New advances in membrane protein expression, purification and handling should help eliminate problems associated with the generation of adequate amounts of pure protein for crystallization. New breakthroughs in crystallization protocols should also aid in the generation of higher quality crystals that may lead to high-resolution structural information. However, in the meantime, studying mutant variants of the wild-type protein should continue to help advance our knowledge of the structure and function of the P-gp multidrug transporters.

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