BIOCHEMICAL, CELLULAR, AND PHARMACOLOGICAL ASPECTS OF THE MULTIDRUG TRANSPORTER

Suresh V. Ambudkar,* Saibal Dey,* Christine A. Hrycyna,* Muralidhara Ramachandra,†-2 Ira Pastan,† and Michael M. Gottesman

*Laboratory of Cell Biology and †Laboratory of Molecular Biology, Division of Basic Sciences, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892; e-mail: ambudkar@helix.nih.gov; deys@helix.nih.gov; chrycyna@helix.nih.gov; murali@canji.com; pasta@helix.nih.gov; and mgottesman@nih.gov

KEY WORDS: multidrug resistance, P-glycoprotein, drug transport, ATP hydrolysis, cancer chemotherapy, chemosensitizer

ABSTRACT

Considerable evidence has accumulated indicating that the multidrug transporter or P-glycoprotein plays a role in the development of simultaneous resistance to multiple cytotoxic drugs in cancer cells. In recent years, various approaches such as mutational analyses and biochemical and pharmacological characterization have yielded significant information about the relationship of structure and function of P-glycoprotein. However, there is still considerable controversy about the mechanism of action of this efflux pump and its function in normal cells. This review summarizes current research on the structure-function analysis of P-glycoprotein, its mechanism of action, and facts and speculations about its normal physiological role.

1 The US Government has the right to retain a nonexclusive, royalty-free license in and to any copyright covering this paper.
2 Present Address: Canji, Inc, San Diego, CA 92121.
INTRODUCTION

The Phenomenon of Multidrug Resistance

Clinical oncologists were the first to observe that cancers treated with multiple different anticancer drugs tended to develop cross-resistance to many other cytotoxic agents to which they had never been exposed, effectively eliminating the possibility of curing these tumors with chemotherapy. In many cases, cells grown in tissue culture from such multidrug-resistant tumors demonstrate patterns of resistance in vitro similar to those seen in situ. This observation suggests that multidrug resistance (MDR) is in many, but not all, cases the result of heritable changes in cancer cells causing altered levels of specific proteins, or mutant proteins, which allow cancer cells to survive in the presence of many different cytotoxic agents. These genetic alterations that confer resistance to cytotoxic drugs can affect cell cycle dynamics, susceptibility of cells to apoptosis, uptake and efflux of drugs, cellular drug metabolism, intracellular compartmentalization of drugs, or repair of drug-induced damage (usually to DNA). Although examples of many of these kinds of MDR have been documented in cultured cells (1, 2), clinical data proving that these mechanisms are responsible for MDR in vivo are generally lacking.

The best-studied mechanism of MDR is that due to overexpression of an energy-dependent multidrug efflux pump, known as the multidrug transporter, or P-glycoprotein (P-gp). P-gp was first detected as a surface phosphoglycoprotein overexpressed in cultured cells selected for MDR (3) and was subsequently cloned from mouse and human cells based on amplification of the MDR locus (4, 5). Overexpression of mouse mdr1a and mdr1b cDNAs (5), and of human MDR1 cDNA (6) in cultured cells and in mouse bone marrow (7), confers resistance to many cytotoxic anticancer drugs, as well as to many other hydrophobic pharmacological agents.

One of the mysteries of this transport system, which is discussed in detail below, is how a single transport system can recognize so many different substrates. The availability of monoclonal antibodies and specific molecular probes for P-gps in rodents and humans has made possible studies of the expression and localization of the multidrug transporter in normal tissues (8–12). Recent studies of mice with insertional inactivation of the mdr1a and mdr1b gene [reviewed by Schinkel (13)] confirmed (a) that mdr1 genes play an important role in normal absorption and excretion of many commonly used pharmacological agents and xenobiotics and (b) that they play a key role in regulating cellular and tissue levels of these agents. Thus, studies of the multidrug transporter have important implications for the understanding of energy-dependent transport processes, as well as for the pharmacology and toxicology of many commonly used drugs and compounds ingested in the diet.
STRUCTURE-FUNCTION ANALYSIS

Mutational Analyses of P-gp

P-gp is a member of the large ATP-binding cassette superfamily of transport proteins also called traffic ATPases (14, 15). P-gp is composed of two homologous halves, each containing six transmembrane domains and an ATP binding/utilization domain, separated by a flexible linker polypeptide (Figure 1). ATP binding and hydrolysis appear to be essential for the proper functioning of P-gp, including drug transport (16).

Although a number of experimental approaches have been used to help elucidate the mechanism of action of human P-gp, including study of the purified protein (17–21), the study of P-gp mutants has been one of the most widely employed methods. Generally, these mutants have been generated either by in vivo drug selection or by site-directed mutagenesis techniques followed by in vivo and in vitro biochemical characterization. These P-gp mutants fall roughly into three categories: (a) misprocessed biosynthetic mutants, (b) mutants that affect substrate specificity, and (c) mutants that abrogate the function of the transporter (1; for reviews, see 22–25).

Figure 1  A hypothetical two-dimensional model of human P-glycoprotein (P-gp) based on hydrophathy analysis of the amino acid sequence and its functional domains. (Small circles) Amino acid residues; (solid circles) the positions of mutations that alter the substrate specificity of P-gp (for clarity not all mutations are shown; for a complete list see Table 1). (Large circles) ATP sites; Walker A, B, and C regions are indicated. (Squiggly lines) N-linked glycosylation sites; (circled P) phosphorylation sites. (Bars above the model) Regions labeled with photoaffinity analogues. (Adapted from Reference 96.)
The mutations in mammalian P-gps that affect substrate specificity are described in Table 1. These mutations are clustered predominantly in the transmembrane domains, mainly 5, 6 and 11, 12, but they are also found throughout the rest of the molecule, including in the soluble intra- and extracellular loops and the ATP binding/utilization domains. Transmembrane domains 5, 6 and 11, 12 and the extracellular loops connecting them were determined by photoaffinity labeling with P-gp substrate analogs to be the major sites of drug interaction (see Figure 1) (26–30). 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 autonomous or interdependent. As suggested by the mutational data presented in Table 1, other regions may also play supporting roles, either directly or indirectly, in defining the drug binding domains.

Since P-gp is composed of two homologous halves, an important mechanistic question raised by the mutational data has been whether the two halves operate independently or in concert. Drug resistance is not conferred on drug-sensitive NIH3T3 cells that coexpress the two halves of P-gp, even though stable expression of each half-molecule has been detected (31). However, on expression of these molecules in insect (Sf9) cells using baculovirus vectors encoding each half separately, low-level reconstitution of drug-stimulated ATPase activity was achieved; this suggests that coupling of ATPase activity to transport requires interaction of the two halves (31). Deletion of the central core of the linker region of human P-gp results in a protein that is expressed at the cell surface at levels similar to the wild-type protein, but it is not functional for either transport or drug-stimulated ATPase activity (32). Furthermore, replacement of the deletion with a peptide with a predicted flexible secondary structure was sufficient for restoring the functional properties of the molecule. These data suggest that interaction 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 interaction of the two halves, likely specifically for the proper interaction of the two ATP binding sites.

Many of the mutations that result in nonfunctional but properly processed P-gp molecules lie within the ATP binding/utilization domains. Site-directed mutagenesis of the consensus sequences of the nucleotide binding domains suggest that both nucleotide binding domains are essential for the proper functioning of P-gp (33–36). Biochemical characterization of both human and hamster purified P-gps have determined (a) that both sites are capable of hydrolyzing ATP, but not simultaneously, (b) that the stoichiometry of ATP hydrolysis is 1 mol of ATP/mol of P-gp (17, 36, 37), and (c) that drug binding and ATP hydrolysis are intimately coupled (20).
Table 1 List of mutations in human, mouse, and hamster P-glycoproteins that affect substrate specificity

<table>
<thead>
<tr>
<th>aa mutation</th>
<th>Region</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>H61R, F, K, M, W, Y</td>
<td>TM 1</td>
<td>Human MDR1</td>
<td>149, 150</td>
</tr>
<tr>
<td>G64R</td>
<td>TM 1</td>
<td>Human MDR1</td>
<td>150</td>
</tr>
<tr>
<td>L65R</td>
<td>TM 1</td>
<td>Human MDR1</td>
<td>150</td>
</tr>
<tr>
<td>Δaa78–97</td>
<td>EC 1</td>
<td>Human MDR1</td>
<td>151</td>
</tr>
<tr>
<td>Q128H&lt;sup&gt;a&lt;/sup&gt;</td>
<td>TM 2</td>
<td>Mouse mdr1</td>
<td>152</td>
</tr>
<tr>
<td>R138H</td>
<td>IC 1</td>
<td>Mouse mdr1</td>
<td>152</td>
</tr>
<tr>
<td>Q139H, R</td>
<td>IC 1</td>
<td>Mouse mdr1</td>
<td>152</td>
</tr>
<tr>
<td>Q141V</td>
<td>IC 1</td>
<td>Human MDR1</td>
<td>19, 153</td>
</tr>
<tr>
<td>Q145H</td>
<td>IC 1</td>
<td>Mouse mdr1</td>
<td>152</td>
</tr>
<tr>
<td>E155G, K</td>
<td>IC 1</td>
<td>Mouse mdr1</td>
<td>152</td>
</tr>
<tr>
<td>F159I</td>
<td>IC 1</td>
<td>Mouse mdr1</td>
<td>152</td>
</tr>
<tr>
<td>D174G</td>
<td>IC 1</td>
<td>Mouse mdr1</td>
<td>152</td>
</tr>
<tr>
<td>S176G, P</td>
<td>IC 1</td>
<td>Mouse mdr1</td>
<td>152</td>
</tr>
<tr>
<td>K177I</td>
<td>IC 1</td>
<td>Mouse mdr1</td>
<td>152</td>
</tr>
<tr>
<td>N179S</td>
<td>IC 1</td>
<td>Mouse mdr1</td>
<td>152</td>
</tr>
<tr>
<td>N1835/G185V</td>
<td>IC 1</td>
<td>Human MDR1</td>
<td>154</td>
</tr>
<tr>
<td>G183D</td>
<td>IC 1</td>
<td>Mouse mdr1</td>
<td>152</td>
</tr>
<tr>
<td>G185V</td>
<td>IC 1</td>
<td>Human MDR1</td>
<td>155–157</td>
</tr>
<tr>
<td>G187V</td>
<td>IC 1</td>
<td>Human MDR1</td>
<td>153</td>
</tr>
<tr>
<td>A192T</td>
<td>TM 3</td>
<td>Mouse mdr1</td>
<td>152</td>
</tr>
<tr>
<td>F204S</td>
<td>EC 2</td>
<td>Mouse mdr1</td>
<td>152</td>
</tr>
<tr>
<td>W208G</td>
<td>EC 2</td>
<td>Mouse mdr1</td>
<td>152</td>
</tr>
<tr>
<td>K209E</td>
<td>EC 2</td>
<td>Mouse mdr1</td>
<td>152</td>
</tr>
<tr>
<td>L2101</td>
<td>TM 4</td>
<td>Mouse mdr1</td>
<td>152</td>
</tr>
<tr>
<td>T211P</td>
<td>TM 4</td>
<td>Mouse mdr1</td>
<td>152</td>
</tr>
<tr>
<td>I214T</td>
<td>TM 4</td>
<td>Mouse mdr1</td>
<td>152</td>
</tr>
<tr>
<td>P223A</td>
<td>TM 4</td>
<td>Human MDR1</td>
<td>158</td>
</tr>
<tr>
<td>G288V</td>
<td>IC 2</td>
<td>Human MDR1</td>
<td>153</td>
</tr>
<tr>
<td>I299M, T319S, L3221, G324K, S351N</td>
<td>TM 5, EC3, IC 3</td>
<td>Human MDR1</td>
<td>159</td>
</tr>
<tr>
<td>F335A</td>
<td>TM 6</td>
<td>Human MDR1</td>
<td>19</td>
</tr>
<tr>
<td>ΔF335</td>
<td>TM 6</td>
<td>Human MDR1</td>
<td>160</td>
</tr>
<tr>
<td>V338A</td>
<td>TM 6</td>
<td>Human MDR1</td>
<td>161</td>
</tr>
<tr>
<td>G338A, A339P</td>
<td>TM 6</td>
<td>Hamster PGY1</td>
<td>162, 163</td>
</tr>
<tr>
<td>A339P</td>
<td>TM 6</td>
<td>Hamster PGY1</td>
<td>163</td>
</tr>
<tr>
<td>G341V</td>
<td>TM 6</td>
<td>Human MDR1</td>
<td>161</td>
</tr>
<tr>
<td>K356R, Q</td>
<td>N-NBD</td>
<td>Human MDR1</td>
<td>164</td>
</tr>
<tr>
<td>ERGA → DKGT</td>
<td>N-NBD</td>
<td>Mouse mdr3</td>
<td>165</td>
</tr>
<tr>
<td>aa 522–525</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T578C</td>
<td>N-NBD</td>
<td>Mouse mdr3</td>
<td>165</td>
</tr>
</tbody>
</table>

(Continued)
Another part of the table appears as follows:

<table>
<thead>
<tr>
<th>aa mutation</th>
<th>Region</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>G802V</td>
<td>IC 4</td>
<td>Human MDR1</td>
<td>153</td>
</tr>
<tr>
<td>G830V</td>
<td>IC 4</td>
<td>Human MDR1</td>
<td>19, 153</td>
</tr>
<tr>
<td>P866A</td>
<td>TM 10</td>
<td>Human MDR1</td>
<td>158</td>
</tr>
<tr>
<td>F934A</td>
<td>TM 11</td>
<td>Mouse mdr3</td>
<td>166</td>
</tr>
<tr>
<td>G935A</td>
<td>TM 11</td>
<td>Mouse mdr3</td>
<td>166</td>
</tr>
<tr>
<td>I936A</td>
<td>TM 11</td>
<td>Mouse mdr3</td>
<td>166</td>
</tr>
<tr>
<td>F938A</td>
<td>TM 11</td>
<td>Mouse mdr3</td>
<td>166</td>
</tr>
<tr>
<td>S939A</td>
<td>TM 11</td>
<td>Mouse mdr3</td>
<td>166</td>
</tr>
<tr>
<td>S939F</td>
<td>TM 11</td>
<td>Mouse mdr3</td>
<td>167, 168</td>
</tr>
<tr>
<td>S941F</td>
<td>TM 11</td>
<td>Mouse mdr3</td>
<td>167, 168</td>
</tr>
<tr>
<td>T941A</td>
<td>TM 11</td>
<td>Mouse mdr3</td>
<td>166</td>
</tr>
<tr>
<td>Q942A</td>
<td>TM 11</td>
<td>Mouse mdr3</td>
<td>166</td>
</tr>
<tr>
<td>A943G</td>
<td>TM 11</td>
<td>Mouse mdr3</td>
<td>166</td>
</tr>
<tr>
<td>Y946A</td>
<td>TM 11</td>
<td>Mouse mdr3</td>
<td>166</td>
</tr>
<tr>
<td>S948A</td>
<td>TM 11</td>
<td>Mouse mdr3</td>
<td>166</td>
</tr>
<tr>
<td>Y949A</td>
<td>TM 11</td>
<td>Mouse mdr3</td>
<td>166</td>
</tr>
<tr>
<td>C952A</td>
<td>TM 11</td>
<td>Mouse mdr3</td>
<td>166</td>
</tr>
<tr>
<td>F953A</td>
<td>TM 11</td>
<td>Mouse mdr3</td>
<td>166</td>
</tr>
<tr>
<td>F983A</td>
<td>TM 12</td>
<td>Human MDR1</td>
<td>169</td>
</tr>
<tr>
<td>L975A, V981A, F983A</td>
<td>TM 12</td>
<td>Human MDR1</td>
<td>169</td>
</tr>
<tr>
<td>M986A, V988A, Q990A</td>
<td>TM 12</td>
<td>Human MDR1</td>
<td>169</td>
</tr>
<tr>
<td>V991A</td>
<td>TM 12</td>
<td>Human MDR1</td>
<td>169</td>
</tr>
<tr>
<td>V981A, F983A</td>
<td>TM 12</td>
<td>Human MDR1</td>
<td>169</td>
</tr>
<tr>
<td>L975A, F983A</td>
<td>TM 12</td>
<td>Human MDR1</td>
<td>169</td>
</tr>
<tr>
<td>L975A, V981A</td>
<td>TM 12</td>
<td>Human MDR1</td>
<td>169</td>
</tr>
<tr>
<td>F978A</td>
<td>TM 12</td>
<td>Human MDR1</td>
<td>19</td>
</tr>
</tbody>
</table>

aa, amino acid; EC, extracellular loop; IC, intracellular loop; TM, transmembrane domain; NBD, nucleotide binding/utilization domain.

As per the nomenclature system proposed by the human gene nomenclature committee of HUGO.

Single letter designations represent amino acid residues. Numbers represent amino acid in the primary sequence and the letter following the number represents the residue.

Taken together, these mutational data suggest that the two halves of human P-gp interact to form a single transporter and that the major drug binding domains reside in or near transmembrane domains 5, 6 and 11, 12. It is also clear that both ATP sites are necessary for a functional molecule and that, in fact, interaction between the ATP sites and the drug binding domains is essential for drug transport. It is clear, however, that the next major breakthrough in understanding the mechanism of action of human P-gp will occur with the generation of high-resolution two-dimensional and three-dimensional structures. Electron microscope and single-particle image analyses have met with some preliminary success (38), but it has proven difficult to purify large
Biochemical Aspects of P-gp

VACCINIA VIRUS-BASED TRANSIENT EXPRESSION SYSTEM FOR FUNCTIONAL CHARACTERIZATION OF P-GP

Most of the earlier studies of P-gp utilized mammalian cells subjected to drug selection with or without introduction of MDR1 cDNA. Interpretations of the results from such studies have been a subject of contention because of the pleiotropic effects of drug selection on cellular functions, including possible activation of endogenous drug resistance mechanisms. Therefore, a vaccinia virus-T7 RNA polymerase hybrid transient expression system that does not involve drug selection has been adapted for functional expression of P-gp (39). In this system, the wild-type or mutant MDR1 cDNAs constructed in an expression plasmid under the control of bacteriophage T7 promoter can be expressed and analyzed rapidly by transfection of cells infected with a recombinant virus encoding T7 RNA polymerase. Because high levels of P-gp can be achieved within 48 h posttransfection, there is no need to generate a recombinant vaccinia virus for each mutant. P-gp expressed in this infection-transfection protocol localizes at the cell-surface, binds and mediates energy-dependent transport of drugs in intact cells, and exhibits in membrane preparations ATPase activity stimulated by drugs.

SUBSTRATES OF P-GP

P-gp confers resistance against a wide spectrum of compounds that are hydrophobic, amphipathic natural product drugs (Table 2). These compounds include not only anticancer drugs, but also therapeutic agents such as HIV-protease inhibitors (40, 41). These compounds are chemically diverse, some of them may carry a positive charge at physiological pH and, because all are hydrophobic, they enter cells by passive diffusion (24).

REVERSAL OF MDR BY CHEMOSENSITIZERS

A large number of noncytotoxic compounds known as chemosensitizers or MDR modulators sensitize resistant cells for the action of cytotoxic drugs. Chemosensitizers include calcium channel blockers, calmodulin antagonists, steroids, cyclic peptides, and drug analogs. Efforts are ongoing in several laboratories to understand the structural and functional basis for the inhibition of P-gp–mediated transport by chemosensitizers or modulators (see below). It is believed that a clear understanding of drug binding sites and the mechanism by which modulators inhibit P-gp function will aid in the development of better chemosensitizers for clinical use.

Inhibition of drug transport could potentially result from the blockage of specific recognition of the substrate, binding of ATP, ATP hydrolysis, or coupling of ATP hydrolysis to translocation of the substrate. Most reversing agents block
drug transport by acting as competitive or noncompetitive inhibitors (42) and by binding either to drug interaction sites (43) or to other modulator binding sites (43; S Dey, M Ramachandra, I Pastan, MM Gottesman & SV Ambudkar, submitted for publication), leading to allosteric changes. On the other hand, none of the known modulators inhibit ATP binding. Modulators such as verapamil are substrates of the transporter and hence inhibit the transport function in a competitive manner without interrupting the catalytic cycle of P-gp (44). Reversing agents such as cyclosporin A inhibit transport function by interfering with both substrate recognition (45) and ATP hydrolysis (20). Because ATP hydrolysis is required for transport (33, 34), modulators that inhibit ATPase activity are unlikely to be transported by P-gp. In addition to a direct interaction with P-gp resulting in inhibition of transport, it has also been postulated that some of the modulators, such as safingol (46), may regulate P-gp function by

Table 2  Selected substrates of P-glycoprotein

<table>
<thead>
<tr>
<th>Category</th>
<th>Substances</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anticancer drugs</td>
<td>Vinca alkaloids (vincristine, vinblastine)</td>
</tr>
<tr>
<td></td>
<td>Anthracyclines (doxorubicin, daunorubicin, epirubicin)</td>
</tr>
<tr>
<td></td>
<td>Epipodophyllotoxins (Etoposide, Teniposide)</td>
</tr>
<tr>
<td></td>
<td>Paclitaxel (taxol)</td>
</tr>
<tr>
<td></td>
<td>Actinomycin D</td>
</tr>
<tr>
<td></td>
<td>Topotecan</td>
</tr>
<tr>
<td></td>
<td>Mitramycin</td>
</tr>
<tr>
<td></td>
<td>Mitomycin C</td>
</tr>
<tr>
<td>Other cytotoxic agents</td>
<td>Colchicine</td>
</tr>
<tr>
<td></td>
<td>Emetine</td>
</tr>
<tr>
<td></td>
<td>Ethidium bromide</td>
</tr>
<tr>
<td></td>
<td>Puromycin</td>
</tr>
<tr>
<td>Cyclic and linear peptides</td>
<td>Gramicidin D</td>
</tr>
<tr>
<td></td>
<td>Valinomycin</td>
</tr>
<tr>
<td></td>
<td>N-Acetyl-leucyl-leucyl-norleucine</td>
</tr>
<tr>
<td></td>
<td>Yeast a-factor pheromone</td>
</tr>
<tr>
<td>HIV protease inhibitors</td>
<td>Ritonavir</td>
</tr>
<tr>
<td></td>
<td>Indinavir</td>
</tr>
<tr>
<td></td>
<td>Saquinavir</td>
</tr>
<tr>
<td>Other compounds</td>
<td>Hoechst 33342</td>
</tr>
<tr>
<td></td>
<td>Rhodamine 123</td>
</tr>
<tr>
<td></td>
<td>Calcein-AM</td>
</tr>
</tbody>
</table>
affecting such posttranslational modifications as phosphorylation. However, mutational analyses have established that the phosphorylation of P-gp is not essential for its transport function (47).

ATPase Activity of P-gp  Crude and purified P-gp preparations exhibit both basal and drug-stimulated ATPase activities. The basal activity is believed to be due to endogenous lipid or other endogenous substrates, such as hydrophobic peptides. It is likely that uncoupled ATPase activity also contributes to the basal activity. The profile of the drug-stimulated ATPase activity is thought to reflect the nature of interaction of P-gp with drug substrates (39, 48). Based on their effect on ATPase activity of human P-gp, we categorized a number of compounds—including anticancer drugs, reversing agents, and hydrophobic peptides—into three distinct classes (49). Class I agents (e.g. vinblastine, verapamil, and paclitaxel) stimulate ATPase activity at low concentrations but inhibit the activity at high concentrations. Class II compounds (e.g. bisantrene, valinomycin, and tetrophenylphosphonium) enhance ATPase activity in a dose-dependent manner without any inhibition. In contrast, Class III compounds (e.g. cyclosporin A, rapamycin, and gramicidin D) inhibit both basal and verapamil-stimulated ATPase activity. In general, most compounds known to be transported by P-gp stimulate ATPase activity. Among the reversing agents, some (e.g. verapamil) stimulate the activity whereas others (e.g. cyclosporin A) inhibit ATP hydrolysis. Such a differential effect of reversing agents on ATP hydrolysis suggests more than one mechanism for inhibition of P-gp–mediated transport. P-gp–ATPase activity has also been known to be affected by the lipid environment (50, 51), and the effect of lipid may explain differential degrees of basal and drug-stimulated ATPase activities of P-gp in different preparations.

Purified and reconstituted P-gp shows a drug-stimulated ATPase activity of approximately 5–22 μmol/min per mg of protein (20, 21, 52–55), which is equivalent to that observed for other ion-translocating ATPases. ATPase activity is Mg$^{2+}$ dependent, although other divalent cations support the activity to a lesser extent. Among the nucleotides, ATP is preferred, with a $K_m$ for MgATP hydrolysis ranging from 0.3 to 1.4 mM, whereas ADP inhibits the activity in a competitive manner with a $K_i$ of approximately 0.3 mM. Because of the high $K_m$ for MgATP and the high $K_i$ for MgADP, it has been suggested that drug transport could be sensitive to ATP depletion under certain conditions (56). In the presence of verapamil, the apparent $K_m$ for ATP remains the same, indicating that drug binding does not alter the affinity for ATP (20, 54). Inhibitors of other ion-translocating ATPases, e.g. sodium azide, oligomycin, ouabain, and ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, do not inhibit P-gp–ATPase activity. Vanadate inhibits P-gp–ATPase activity, but at
a concentration of approximately 10 µM, much higher than that required for inhibition of P-type ATPases (50–500 nM). ATPase activity of P-gp is also inhibited by sulfhydryl reagents such as N-ethylmaleimide, indicating the presence of important cysteine residue(s) in the catalytic domains. Inhibition by N-ethylmaleimide can be blocked by ATP and is not reversible with dithiothreitol. The cysteines that are modified by N-ethylmaleimide within human P-gp have been identified as Cys-431 and Cys-1074 in the Walker A regions of ATP binding sites in N- and C-terminal halves, respectively (34).

VANADATE-INDUCED TRAPPING OF NUCLEOTIDES TO ANALYZE ATP HYDROLYSIS AND COUPLING OF ATP HYDROLYSIS TO DRUG BINDING  Sodium ortho-vanadate, an analog of inorganic phosphate, inhibits the catalytic cycle of P-gp by forming a stable, noncovalent complex with MgADP at the catalytic site (36, 37). As expected, similar to vanadate, aluminum fluoride and beryllium fluoride also form stable complexes with P-gp-MgADP and inhibit P-gp–ATPase activity (57). On trapping of vanadate, the binding affinity for nucleotide increases dramatically, and the resulting P-gp–MgADP–vanadate complex has a significantly longer half-life than the P-gp–MgADP–phosphate intermediate. As a result of vanadate-induced trapping, only a single turnover of the enzyme occurs, and the resulting inhibited P-gp–MgADP–vanadate complex resembles the normal catalytic transition state, P-gp–MgADP–phosphate. The finding that vanadate trapping of nucleotide at one site per molecule, either at the N- or C-terminal ATP binding/utilization site, is sufficient to completely inhibit ATPase activity has confirmed earlier observations that both nucleotide binding sites are catalytically active (34). On the basis of these results, Senior and colleagues (56, 58) have proposed an alternate catalytic site model for the action of P-gp. According to this alternate catalytic site model, although both sites are capable of binding ATP, only one site participates in the catalysis at a given time, and the conformation of this catalytic site precludes the other site from hydrolyzing ATP. We utilized the vanadate-induced photochemical cleavage of the peptide bond to determine whether both ATP-binding/utilization sites can hydrolyze ATP simultaneously (17). Similar to other ATPases, a photochemical peptide bond cleavage occurs within the Walker A nucleotide binding domain consensus sequence [GX_4 GK (T/S)] when the molecule is trapped with vanadate in an inhibited catalytic transition state (P-gp–MgADP–vanadate) and exposed to ultraviolet light. Immunoblot analysis of the resultant products revealed that little-to-no degradation of P-gp occurred in the absence of vanadate. In the presence of vanadate, products resulting from cleavage at either of the ATP sites, but not both sites, were obtained. These results indicate that both the
N- and C-terminal ATP sites can hydrolyze ATP, and ATP is not hydrolyzed simultaneously by both sites. Consistent with these results, mutations in either ATP site prevented vanadate-induced trapping of ADP at both sites (59). 

Experiments utilizing the vanadate-trapping technique and photoaffinity labeling have also provided insights into the mechanism by which ATP hydrolysis is coupled to drug binding. Catalytic ligands including ATP and vanadate lead to a considerable reduction in azidopine labeling of P-gp in crude membranes from colchicine-resistant Chinese hamster ovary cells (51). Using a purified human P-gp preparation that was devoid of contaminating ATPases to assess the effects of binding and hydrolysis of ATP on interaction with drugs (20), we found that vanadate inhibited photoaffinity labeling of P-gp with substrate analogs [125I]iodoarylazidoprazosin and [3H]azidopine under strict ATP hydrolysis conditions in a concentration-dependent manner. Vanadate-induced inhibition of photoaffinity labeling did not occur in P-gp mutants incapable of binding and/or hydrolyzing ATP because of single amino acid change(s) in N-, C-, or both nucleotide binding/utilization sites (CA Hrycyna, M Ramachandra, I Pastan & MM Gottesman, unpublished data). Because vanadate-trapped P-gp is known to resemble the ADP- and phosphate-bound catalytic transition state, these findings reveal that ATP hydrolysis results in a conformation with reduced affinity for substrates.

DRUG TRANSPORT BY PURIFIED P-GP IN A RECONSTITUTED SYSTEM Many of the earlier drug transport studies were performed using plasma membrane vesicles from multidrug-resistant mammalian cells expressing P-gp and yeast secretory vesicles enriched in P-gp. Results from these studies indicated that drug transport is inhibited by reversing agents and requires ATP hydrolysis. However, because these studies were performed with crude membrane vesicles, it could not be conclusively determined that P-gp alone is sufficient for drug transport. P-gp–mediated drug transport has now been demonstrated in reconstituted proteoliposomes with partially (60) and homogeneously purified P-gp preparations (53, 61). These studies have clearly established that P-gp by itself acts as an ATP-driven drug efflux pump. Sharom and colleagues (60) demonstrated ATP-dependent uptake of colchicine that was inhibited in the presence of substrates such as verapamil and daunorubicin in lipid vesicles reconstituted with a partially purified P-gp preparation from Chinese hamster ovary cells. Shapiro & Ling (61) used reconstituted liposomes consisting of purified hamster P-gp and demonstrated MgATP-dependent, chemosensitizer-inhibitable transport of Hoechst 33342, a fluorescent substrate of P-gp. During Hoechst 33342 transport, no large pH changes occurred in P-gp–containing liposomes, indicating that alteration in pH does not contribute to P-gp–mediated
drug transport (62, 63). In their later studies, these authors measured the kinetics of Hoechst 33342 by P-gp-enriched plasma membrane vesicles from Chinese hamster ovary cells. Because Hoechst 33342 is fluorescent when bound to the membrane, but not when in the aqueous medium, it was possible to determine the movement of the dye out of the membrane by monitoring the fluorescence intensity. The initial specific rate of transport was directly proportional to the amount of dye in the lipid phase and inversely proportional to the concentration in the aqueous phase, demonstrating that P-gp extracts Hoechst 33342 from the lipid membrane (64).

When P-gp from the human carcinoma multidrug-resistant cell line, KB-V1, was purified and reconstituted in proteoliposomes, it exhibited high levels of drug-stimulated ATPase activity as well as ATP-dependent [3H]vinblastine accumulation (52, 53). Both the ATPase and vinblastine transport activities were inhibited by vanadate. Additionally, [3H]vinblastine transport in proteoliposomes was inhibited by verapamil and daunorubicin but not by camptothecin, which is not recognized by P-gp. ATP-dependent transport of [3H]daunorubicin and [3H]vinblastine has also been shown in proteoliposomes with purified P-gp from colchicine-resistant Chinese hamster ovary cells (65, 66).

STOICHIOMETRY OF ATP HYDROLYSIS TO DRUG TRANSPORT It is clear from the biochemical studies described above that drug transport by P-gp is coupled to ATP hydrolysis. The accurate measurement of stoichiometry of ATP hydrolysis and drug transport is essential to assess the physiological relevance of P-gp drug pump function. Earlier attempts to measure stoichiometry by using partially or homogeneously purified P-gp in a reconstituted system indicate at minimum 50 ATP molecules hydrolyzed per molecule of substrate transported (61, 60). This value seems to be too high to account for the level of observed drug resistance. Eytan et al (67) observed 0.5–0.886 Rb⁺ complexed valinomycin molecules transported per ATP molecule hydrolyzed. Recently, Shapiro & Ling (68) measured stoichiometry of rhodamine 123 transport and ATP hydrolysis by Chinese hamster P-gp in membrane vesicles. At saturating rhodamine 123 concentrations and subsaturating (0.3 mM) levels of ATP, the stoichiometry was 0.83. However, at high ATP concentrations (1.5 mM), the coupling ratio decreased to 0.57. The reason for this discrepancy is not known. We estimated the stoichiometry of coupling of vinblastine transport to vinblastine-stimulated ATP hydrolysis by human P-gp in NIH 3T3 transfectants. We observed that the turnover number for vinblastine efflux and vinblastine-stimulated ATPase activity at 37°C was 1.4 and 3.5 s⁻¹, respectively (69). These data indicate that 2–3 molecules of ATP are hydrolyzed for every molecule of vinblastine transported out of the cell. Thus, the coupling ratio appears to be in the range of 1 to 3, which is similar to other ion-translocating pumps.
THE Catalytic CYCLE OF P-GP COUPLED TO DRUG TRANSPORT  As stated above, the drug binding sites and ATP binding/utilization domains interact with each other during substrate-stimulated ATP hydrolysis. It is also clear from photoaffinity labeling of the P-gp–MgADP complex in the presence of vanadate that during the transition state, P-gp exhibits decreased affinity for drugs. Recent work on drug binding to P-gp indicates the presence of at least two nonidentical substrate interaction sites (43). By incorporating these observations with the alternate catalytic cycle for ATP hydrolysis by P-gp proposed by Senior and colleagues (70, 58), we present a model for the catalytic cycle of P-gp coupled to drug transport (Figure 2). In this model, the N- and C-halves of transmembrane domains form two distinct drug-binding sites, which are part of the drug translocation pathway. The ON-site may be closer to the inner leaflet of the lipid bilayer. Drug binding to the ON-site is also affected during ATP hydrolysis, as is evident from photoaffinity labeling of the P-gp–MgADP complex in the

![Figure 2](image_url)
presence of vanadate. On the other hand, drug binding to the OFF-site is unaffected by modulators such as cis-flupentixol, and it is less sensitive to vanadate trapping (43). In this model, ATP and substrate can bind to P-gp independently (Figure 2, steps I and II). On ATP hydrolysis, a conformational change decreases the affinity of the ON-site to the drug, and as a result, the drug is moved from the ON-site to the OFF-site (Figure 2, step III). Subsequently, the drug is translocated from the OFF-site to the external medium. The release of the drug from OFF-site may take place either prior to or simultaneously with the release of P_i. Trapping vanadate at the P_i site (P-gp–MgADP–Vi) (step IVA, Figure 2) stabilizes this conformational form in the cycle (P-gp–MgADP–P_i). The formation of this transition state intermediate, which exhibits low affinity for substrate, also suggests that the final step in this cycle is the release of ADP (subsequent to step IV, Figure 2, but not shown). Currently, it is not clear whether there is additional energy required for the movement of drug from the OFF-site to the medium. Whether the second ATP site has exactly the same effect as the first site, or is involved in another step in the complete catalytic cycle, is unclear.

**Pharmacological Aspects of P-gp:**

**The Pharmacophore Structure**

The effectiveness of P-gp reversing agents in chemosensitizing multidrug-resistant cancer cells has stimulated a serious effort to define a common pharmacophore necessary to circumvent P-gp–mediated MDR. Since most of these chemosensitizing compounds and their structural analogs are highly hydrophobic and traverse the plasma membrane with relative ease, their effect on drug accumulation and drug resistance can be conveniently determined in intact multidrug-resistant cells. However, interpretation of data from such experiments must be done carefully. Chemosensitivity to an antineoplastic drug could be restored by mechanisms independent of P-gp (71, 72), and because some of the P-gp modulators are substrates of the pump, higher concentrations of those compounds might be required to potentiate accumulation of cytotoxic drugs in cells overexpressing P-gp (72). Also, the potency of a reversing agent often depends on the cytotoxic drug for which resistance is being sensitized. For example, verapamil is a potent reversor of daunorubicin, paclitaxel, and vinblastine resistance, whereas cyclosporin A is a better inhibitor of colchicine resistance (73).

Mechanistically, P-gp modulators are either high-affinity substrates of the pump or are efficient inhibitors of ATP-dependent transport by P-gp. The ability of a modulator to stimulate or inhibit the ATPase activity in isolated membrane preparations indicates direct interaction of the compound with P-gp. The strength of modulator interactions can also be determined by measuring their ability to compete for binding of photoactivatable substrate analogs to P-gp.
However, these modulatory effects do not necessarily indicate effectiveness as a chemosensitizer. In general, it is the concentration required for half maximal stimulation or inhibition of the P-gp–ATPase activity or drug transport that correlates better with the reversing ability than does the extent of stimulation or inhibition, but there are some tripeptides, as is discussed below, that stimulate P-gp–ATPase activity but poorly inhibit drug transport (74).

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

COLCHICINE An extensive study on the P-gp substrate colchicine and its analogs has been carried out to define the essential features of this alkaloid required for its efficient transport by P-gp (75). Structurally, colchicine contains two aromatic rings, which together with a third ring structure form the phenyltropolone backbone of the molecule, with four methoxy groups (at R₁, R₂, R₃, and R₅ positions) and an acetamido group (at R₄ position) attached to the periphery (Figure 3). Although the methoxy groups of the two aromatic rings (ring A and ring C) seem to be dispensable for interaction with P-gp, the nitrogen atom of the acetamido group at C7 position is critical for P-gp recognition. It has been suggested that the nitrogen atom at the C7 position either participates in hyperconjugation of the Π-aromatic electrons of the C-ring or the C7−NH group functions as a hydrogen bond donor in the interaction with P-gp. Replacement of the seven-carbon C-ring with a six-carbon aromatic ring of the tropolone (as in allocolchicine) significantly affects interaction of the molecule with P-gp, which suggests that the integrity of the seven-carbon C-ring is essential. In addition, the calculated molar refractivity of the colchicine analogs indicates a minimal size requirement (overall size greater than the 9.7 calculated molar refractivity threshold) of the colchicine analogs for efficient interaction with P-gp. Taken together, the overall size of the colchicine analogs and specific molecular groups appears to be of more significance in determining whether they are P-gp substrates rather than their calculated effective lipophilicity.

VERAPAMIL Similar structure-activity relationships have been analyzed among structural analogs of the P-gp modulator, verapamil (76). Based on their ability to reverse doxorubicin resistance, the key structural domains of verapamil for MDR reversal activity can be assigned to the motifs at the R₅ and R₆ position (76). As in the case of colchicine, the methoxy groups in the phenyl
Figure 3 (Continued)
Figure 3. The backbone structures of selected P-glycoprotein (P-gp) substrates and modulators. (Bold letters) The R groups critical for recognition by P-gp of each class of compounds (wherever applicable); (insets) their structures.
rings (in positions $R_2$ and $R_3$, $R_9$ and $R_{10}$) are not important because replacement or incorporation of additional methoxy groups in the phenyl rings (in $R_1$ and $R_4$; see Figure 3) have no effect on the ability of the molecules to reverse P-gp function. Similarly, the 1-phenyl ring (in position $R_4$) also seems to be dispensable, because replacement of it with an aliphatic chain (−$C_8H_{17}$, −$C_{10}H_{21}$ or −$C_{12}H_{25}$; compounds LU49940, LU48895, or LU51903, respectively) exerts no detectable effect on anti-MDR activity. However, the −8-methyl-nanone [−$CH(CH_3)_2$] at $R_6$ position seems to play a key role in reversing MDR mediated by P-gp. Carbon extension of this group (as in anipamil) significantly reduces the reversal activity. Also, replacement of 7-cyan (−CN) in $R_5$ position with the 7-methoxylamino (−$CH_2NH_2$) group (as in LU46605) results in loss of reversal potency. It is interesting that the reversal property of verapamil analogs clearly lacks enantio-selectivity.

RESERPINE The naturally occurring alkaloid reserpine possesses strong modulatory potency against MDR (77, 78), whereas the structurally related compound yohimbine lacks it (79). Both compounds contain two aromatic domains and a basic nitrogen atom linked through a fused polycyclic array of three six-membered rings (rings C, D, and E) and an ester bond (Figure 3). The trimethoxybenzoyl group either at $R_2$ (as in reserpine) or at $R_3$ (as in 18-epireserpine) positions of the reserpine backbone or at the $R$ position of yohimbine (trimethyl benzoylyohimbine) is important in increasing chemosensitivity to vinblastine as well as in the ability to compete with $^{125}$I-labeled NASV (a photoactivatable vinblastine analog) binding to P-gp (80). In general, for both reserpine and yohimbine analogs, compounds with a pendent benzoyl group beside the basic indolo-piperidine ring system with certain conformational constraints are the most effective in chemosensitization of P-gp–mediated vinblastine resistance. An overlay of the verapamil and trimethyl benzoylyohimbine reveals that verapamil can achieve a thermodynamically possible conformation similar to that of this yohimbine analog (80). This conformation of verapamil is also similar to that of vinblastine, which is the preferred one for this substrate. It is interesting that the difference in stereochemical configuration of the trimethoxybenzoyloxy substituents in reserpine, epireserpine, and trimethoxybenzoyl-yohimbine has no effect on their apparent ability to modulate MDR; this suggests lack of stereoselectivity.

STAUROSPORINE The importance of the type and relative position of aromatic rings used to determine anti-MDR activity has also been recognized in the indolecarbazole alkaloid staurosporine (81) (Figure 3). Replacement of the indolocarbazole structure of staurosporine with the related bisindolylmaleimide system dramatically reduces its ability to reverse P-gp–mediated doxorubicin resistance. Therefore, MDR reversing ability of staurosporine analogs appears
to be linked to the integrity of the indolecarbazole structure. Staurosporine and its benzoyl derivative (at the R₂ position), CGP41251, are potent modulators of cellular rhodamine 123 efflux. It is interesting that the analogs of staurosporine that are better inhibitors of P-gp mediated drug transport are not necessarily the ones that are efficiently transported by P-gp.

PROPafenone-Related Compounds No other compound has been subjected to structure-activity studies as thoroughly as the antiarrhythmic agent propafenone (Figure 3). Although a significant correlation exists between lipophilicity and biological activity within the structurally homologous series of propafenone analogs, modification at critical positions of the molecule leads to decrease in activity, which cannot be correlated to a change in lipophilicity alone (82). Within the set of compounds tested, the phenylpropiofenone moiety is important for maintaining high chemosensitizing activity to daunorubicin and colchicine resistance. Structural modification leading to a benzophenone derivative as well as the incorporation of the carbonyl C atom into a benzoafuran moiety results in a decrease of anti-MDR activity (83). Removal of the phenyl ring at the R₂ position leads to a compound with only one benzene moiety that shows almost complete loss of activity (83). Decrease in activity can be observed even when the ethylene moiety between the two aromatic rings is removed, which suggests that an optimal distance has to be maintained between the aromatic rings (84). Within an extended set of analogs, modification in the ortho position of the ether oxygen decreases modulatory activity. In addition, the type of oxygen used (carbonyl, alcohol, and ether) also influences the interaction of these compounds with P-gp. This interaction may be mediated through hydrogen bond formation in which a hydrogen bond acceptor close to C1 seems to be essential. Repositioning of the ortho acyl substituent aromatic ring to obtain a meta or a para analog decreases P-gp inhibitory potential in the following order: ortho > meta > para (82).

Different substitutions at the nitrogen atom indicate that this part of the molecule plays a major role in activity (82, 83). Compounds with a tertiary amino group at R₁ have greater potency than propafenone itself does. Incorporation of the nitrogen into a cyclic nonaromatic ring structure further enhances the modulatory potency, and the highest activity can be achieved with the arylpiperazines containing three aromatic rings and a piperazine moiety (82). However, insertion of oxygen into the cyclic nitrogen-containing substructure dramatically decreases the reversing potency (83).

PhenoXazine Several different classes of compounds with tricyclic ring nuclei have been studied to identify important structural features responsible for anti-MDR activity. Among these compounds, which include phenoxazine, phenothiazine, phenoxazone, resurfin acetate, xanthene, xanthene carboxylic acid,
acridine carboxylic acid, and 1,10-phenanthroline, phenoxazine proved to be the most active agent for sensitizing MDR cells to vincristine and vinblastine (72). Hydrophobicity does not correlate with the ability of this series of compounds to modulate the accumulation of vincristine and vinblastine. Xanthene, the most hydrophobic compound, shows marginal effect on the accumulation of $[^3H]$vincristine in MDR cells. These data indicate that the presence of a $-\text{NH}$ group at position 10 (see Figure 3) and a highly electronegative element, such as oxygen, at position 5 of the tricyclic ring nucleus are important determinants for modulating MDR activity.

**QUINACRINE AND CHLOROQUINE** A tricyclic ring containing compounds such as acridine, acridine orange, and quinacrine potentiates drug cytotoxicity with similar efficiency, emphasizing the relative importance of the aromatic part of the acridine structure over the side groups (85). Chloroquine, which is a synthetic indole alkaloid related to acridine compounds, has a dicyclic instead of tricyclic ring system with an amino derivative at position 4. The fact that primaquine, an 8-amino compound, enhances vinblastine cytotoxicity with an efficiency similar to chloroquine, further indicates that the position of the side group in this particular class of compounds seems to be flexible in determining anti-MDR activity.

**PHENOTHIAZINES** Knowledge of the anti-MDR features of tricyclic ring-containing compounds has been further extended in studies by Ford et al (86, 87) with derivatives of phenothiazine and thioxanthene (Figure 3). Results from their studies indicate that increasing hydrophobicity of the phenothiazine and thioxanthene nucleus increases their potency against MDR. Thus, the $-\text{CF}_3$-substituted compounds at position C2 are the most potent drugs, whereas $-\text{OH}$ substituted compounds are the least (87). The distance between the amino group in the side chain and the tricyclic ring nucleus is also important for antagonism of MDR. In addition, the type of amino group significantly influences the chemosensitizing potency. Tertiary amines are more potent than primary or secondary amines, and piperazinyl amines are more potent than noncyclic groups (87). Overall, $-\text{CF}_3$ or $-\text{Cl}$ at position 2 in the nucleus, a paramethyl substituted piperazinyl side chain, and a distance of 4 carbons between these two domains are optimum for anti-MDR activity.

In general, thioxanthene derivatives are more hydrophobic than their corresponding phenothiazine derivatives because of the substitution of a carbon for nitrogen in the cyclic ring. Of all thioxanthene derivatives tested, flupentixol has been the most effective MDR reversing agent (86). Because of an exocyclic double bond, the side chain of flupentixol can assume either a cis or a trans configuration with respect to the tricyclic ring nucleus. The stereoisomers
locked in the trans configuration show three- to five-fold higher anti-MDR activity than the cis isomers. The reason behind this stereoselective potency has been recently addressed in our laboratory. In isolated membrane preparations, P-gp-mediated ATP hydrolysis and photoaffinity labeling of P-gp with the substrate analog [125I]iodoarylazidoprazosin ([125I]IAAP) show clear inhibition in the presence of trans (E)-flupentixol, whereas both activities are stimulated by the cis isomer of the compound. Because substrate recognition and ATP hydrolysis are essential steps for drug transport, the stereospecific effect of flupentixol on the functional aspects of P-gp indicates distinct mechanisms of inhibition by the two isomers (S Dey, M Ramachandra, I Pastan, MM Gottesman & SV Ambudkar, submitted for publication).

**PRENYLCYSTEINES** Linear and cyclic peptides comprise a completely different class of P-gp substrates or modulators. The fact that expression of P-gp in yeast cells, devoid of a-factor transporter (STE6) function, can complement (although not completely) a-factor secretion, suggested the ability of P-gp to transport prenylated peptides (88). Mature a-factor is a dodecapeptide posttranslationally modified by a 15-carbon farnesyl group attached to a cysteine residue at the C-terminal end. Prenylcysteines and their methyl esters corresponding to the C terminus of prenylated proteins have been tested for their ability to stimulate ATPase activity of P-gp (89, 90). S-farnesylcysteine (FC) and S-geranylgeranyl-cysteine (GGC) have no effect on P-gp–ATPase, whereas their methyl derivatives FCME and GGCME stimulate this activity in a concentration-dependent manner (89). Therefore, these results clearly indicate that prenylcysteines functionally interact with P-gp and that methylation of the α-carbon is an important determinant in this interaction. It is also clear that in this interaction P-gp does not discriminate between 15-carbon farnesyl and 20-carbon geranylgeranyl isoprenoids on the sulfhydryl group of cysteine. Acetylation of the amino group of FC (giving rise to AFC) increases the hydrophobicity of the molecule to a comparable extent, as does methylation of the C-terminal end (FCME), but it fails to stimulate the ATPase activity of P-gp, which suggests a specific effect of methylation on interaction with P-gp that is not due to a simple increase in hydrophobicity (89). Prenylcysteines containing carboxyl derivatives such as methyl ester, methylamide, amide, and even a bulky methylated glycine residue can activate the ATPase activity of P-gp efficiently (90). Simply amidating the carboxyl group (to produce FCA) results in a prenylcysteine equipotent with FCME, indicating that the methyl group itself is not recognized by P-gp (90). The most likely explanation is that methylation plays an indirect role in the interaction by blocking the negative charge on free carboxylate, which otherwise proves detrimental for binding of prenylcysteines to P-gp. This is further supported by the fact that when
the carboxyl derivative is a glycine, which possesses a free carboxylate itself, the molecule is inactive in interaction with P-gp unless the carboxylate of the attached glycine is methylated. Consistent with this result, the dipeptide γ-glutamyl-farnesylcysteine (γ-Glu-F-Cys) activates the P-gp–ATPase activity only in its carboxyl-methylated form γ-Glu-F-Cys-OMe. This also suggests that the position of the free amino group required for the optimal substrate activity is flexible, because the dipeptide γ-Glu-F-Cys-OMe is as potent as the parent compound FCME (90).

Acetylation of the amino group of FCME (producing AFCME) essentially abolishes its stimulatory effect. AFCME inhibits the basal ATPase activity (at concentrations >20 µM) of the multidrug transporter and interferes with the functional interaction of other substrates with P-gp. Therefore, modification of the amino group of prenylcysteines to eliminate their charge characteristics promotes interaction with P-gp in a way that would inhibit ATP hydrolysis instead of stimulating it. This also suggests an important role of the nitrogen atom of prenylcysteines in the interaction with P-gp (90).

Cysteine methylester itself does not stimulate P-gp ATPase activity, which suggests a critical role for the isoprenoid moiety. Similarly, the isoprenoid farnesyl that has the cysteine residue replaced with an −OH group is equally ineffective (89). Therefore, the major determinants of prenylcysteine interaction with P-gp will include the isoprenoid moiety, the carboxyl methyl group, and the positive charge of the protonated amino group. Thus, although the prenylcysteine methyl esters are structurally distinct from other P-gp substrates, they contain the characteristic cationic and hydrophobic moieties believed to be required for functional interaction of substrates and modulators with the multidrug transporter.

PEPTIDES Understanding of peptide-P-gp interaction has been further extended in studies involving small tripeptides like pepstatin and leupeptin to cyclic dodecapeptides like cyclosporin A. The importance of methylation, overall hydrophobicity, and the presence of charged residues in determining substrate properties of the linear peptides are apparent from these studies. Protease inhibitors, like acetyl-leucyl-leucyl-norleucinal (ALLN) and acetyl-leucyl-leucyl-methioninal (ALLM), are both transport substrates for P-gp (91) and strong activators of its ATPase activity (92). Although blocking the N and C terminus of these tripeptides seems to have a positive effect on stimulation of ATPase activity (as in ALLN and ALLM), presence of a charged arginine residue at the C-terminal end of leupeptin appears to eliminate this stimulatory effect. Because leupeptin inhibits transport of other substrates (colchicine) by P-gp, it suggests that the presence of charged residue does not eliminate interaction with P-gp; rather, it alters the nature of the interaction. In fact, the nature
of the charged residues at the C-terminal end largely determines their effect on P-gp–mediated colchicine transport (93).

Calpeptin (benzoyloxycarbonyl-leucyl-norleucinal), which has a more hydrophobic aromatic blocking group at the N terminus, stimulates P-gp–ATPase activity with higher affinity than ALLN or ALLM. Also methylation, which contributes to the overall hydrophobicity of the peptide molecule, seems to induce a stimulatory effect on ATPase activity of the chemotactant peptide, formyl-methionyl-leucyl-phenylalanine (FMLP), which by itself is a poor substrate. The apparent stimulatory effect of methylesterification on ATPase activity suggests that the C-terminal aldehyde group is not an absolute requirement for being a P-gp substrate. Pepstatin (isovaleryl-valyl-valyl-statiny-alanyl-statine), a pentapeptide, also activated P-gp–ATPase activity, indicating no steric limitation on interaction of peptides more than three amino acid residues in length (92).

GENERAL REMARKS In modulator binding to P-gp, a clear lack of conserved elements of molecular recognition is apparent, which complicates the structural definition of the MDR pharmacophore. Furthermore, much evidence suggests that the drug binding sites on P-gp are multiple and complex. Nevertheless, information compiled from various structure-activity studies can be used to outline a minimum requirement for anti-MDR activity. Because P-gp is able to recognize drug molecules directly from the membrane bilayer, the overall hydrophobicity of the modulators seems to be an important, but not the sole, requirement for chemosensitizing activity. It is reasonable to state that hydrophobicity of a molecule aids interaction with P-gp by improving its chance of interaction. Because aromatic groups largely contribute to the hydrophobicity of a compound, planar ring structures seem to be a hallmark of anti-MDR compounds. However, one should not underestimate the potential of these ring structures to be involved directly in interaction with P-gp.

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

Mechanism of Action of P-gp

Although several models for P-gp function have been proposed, there is still no clear understanding at a molecular-level of how the multidrug transporter lowers intracellular accumulation of anticancer drugs. The current models for
P-gp function are based on whether the transporter directly or indirectly mediates drug transport. The altered partitioning model by PD Roepe and coworkers (reviewed in 94, 95) proposes that overexpression of the P-gp leads to alteration of electrical membrane potential ($\Delta \psi_o$) and intracellular pH ($pH_i$) and to other biophysical characteristics of the cell. These alterations in the biophysical parameters of the cell then perturb the intracellular level of anticancer drugs. Thus, according to this model, P-gp indirectly promotes decreased intracellular drug accumulation. On the other hand, according to the pump model (96), the energy of ATP hydrolysis by P-gp is utilized for the removal of drugs from cell membranes and cytoplasm analogous to the ion-translocating pumps. The pump recognizes substrates through a complex substrate recognition region or regions and directly pumps drugs out of the cell by using molecular mechanisms that are not yet well understood.

The majority of experimental data strongly supports the drug pump model because evidence for a direct interaction of many of the substrates or reversing agents with the transporter has been obtained, including drug binding studies (65, 97), photoaffinity labeling experiments (20, 28, 98), the demonstration of drug-stimulated ATPase activity in direct proportion to the ability of P-gp to transport these drugs (99), and a variety of amino acid substitutions in P-gp that alter its substrate specificity (see Figure 1 and Table 1). Recent work on the stoichiometry of drug transport and ATP hydrolysis indicates that the hydrolysis of 1–3 molecules of ATP is required for the transport of one molecule of the drug (69). This is similar to that observed with other ion-transporting pumps.

Arguments supporting the altered partitioning model include the finding of increased intracellular pH ($pH_i$) and altered membrane potential ($\Delta \psi_o$) in some drug-resistant cells overexpressing P-gp (100, 101). These observations alone are not sufficient to explain the mechanism of action of P-gp because these changes are not found in all MDR1-expressing cells (102, 103), and these changes are not of sufficient magnitude to account for the up to several hundred–fold increases in drug resistance of some multidrug-resistant cells (reviewed in 25). The experiments with yeast membrane vesicles or with purified P-gp in phospholipid vesicles demonstrate that the drug transport can occur even in the absence of electrochemical gradients (61, 104). Thus, it is likely that these changes in biophysical properties of the cell might well be epiphenomena associated with the prolonged selection of cells in cytotoxic drugs or they might be secondary to the action of P-gp itself, which transports many positively charged as well as neutral hydrophobic substrates. This latter point has been best resolved by developing high-level transient expression systems, such as vaccinia-based expression, in which functional P-gp can be studied in the absence of prior drug selection (39) (see also above). The altered partition model has not accounted for the role of substrate-stimulated ATP hydrolysis by P-gp, for the alteration in substrate specificity by a change in just a single amino
Acid, or for how change in electrochemical properties of the membrane results in decreased retention of neutral molecules such as colchicine. It is, however, likely that in some multidrug-resistant cells, pH gradient and membrane potential may contribute to MDR, but that these electrochemical gradients are not in themselves sufficient to account for the high level of drug resistance seen in many multidrug-resistant cells expressing P-gp. Thus, P-gp seems to function as a primary pump wherein the energy from the hydrolysis of ATP is used for drug transport, which is not linked to co- or countertransport of anions or cations.

Both the above-described models deal with the energetics of P-gp drug transport function; however, they do not explain how the drugs are pumped out of the cell. A variety of chemically diverse lipophilic substrates for transport by P-gp interact directly with the transporter, probably in the regions of transmembrane segments 5, 6 and 11, 12, which are photoaffinity labeled by substrate analogs (29, 30, 43), and these regions most likely represent ON- and OFF-sites (see Figure 2), although the nature of their interactions is still unknown. Because of the unusual kinetics of drug uptake and efflux, which suggest that drugs are detected and ejected before they reach the cytoplasm, and because of some experiments indicating that substrate drugs can be removed directly from the plasma membrane (105–107), we have proposed that P-gp is a "hydrophobic vacuum cleaner" (24, 106), whose primary mode of action is to detect and remove its hydrophobic substrates directly from the lipid bilayer. Another version of this model suggests that P-gp acts as a flippase (108), carrying its substrate from the inner leaflet of the lipid bilayer to the outer leaflet. This model is supported by the finding that the MDR2 gene product (a close homolog of P-gp) is a phosphatidylcholine translocase (flippase) essential for extrusion of phosphatidylcholine from the hepatic plasma membrane into the bile (109, 110). P-gp, the MDR1 gene product, also transports phospholipids such as phosphatidylethanolamine or phosphatidylcholine (111, 112). In addition to accounting for the known experimental observations regarding the action of P-gp, this model may explain the rather broad substrate recognition of P-gp owing to the different rules governing hydrophobic interactions in a lipid bilayer. Further elucidation of the hydrophobic vacuum cleaner or flippase models will require use of biophysical techniques and substrates conjugated with spin probes.

ROLE OF P-GP IN NORMAL PHYSIOLOGY

Tissue Localization of P-gp Suggests a Normal Physiological Role in Protection Against Xenobiotics

The identification of P-gp as an energy-dependent pump, which could confer resistance to hydrophobic compounds cytotoxic to cancer cells, raised the
question of the normal functions of P-gp. The first hint as to what these functions might be came from studies in which monoclonal antibodies to P-gp were used to localize the protein in frozen sections of human tissues. All positive tissues show plasma membrane localization of positive cell types. In epithelial cells of the lower gastrointestinal tract (jejunum, ileum, and colon), high levels of P-gp are located only on the mucosal surface of these tissues, which suggests a function to prevent uptake of substrates and perhaps to facilitate excretion across the mucosa of the GI tract (11). In kidney and liver, P-gp is present on the brush border and biliary face, respectively, of proximal tubule cells and hepatocytes, consistent with a role for P-gp in excretion of xenobiotics and endogenous metabolites into the urine and bile. Some P-gp is also found on the apical surface of pancreatic ductules. One of the most interesting localizations for P-gp is on the luminal surface of capillary endothelial cells in the brain and testes, consistent with a role for P-gp in forming the blood-brain barrier (12, 113). Studies in which P-gp was introduced into renal epithelial cells in vitro and in which these cells were subsequently allowed to form polarized monolayers demonstrated apical expression of the human P-gp introduced into the cells (114), and the monolayers acquired the ability to transfer P-gp substrates across the polarized intact monolayers (115).

Several other cell types and tissues express P-gp. There is P-gp in the placent, probably in more than one cell type (10, 116), which suggests a role for P-gp in protecting the fetus from toxic xenobiotics. In rodents, the pregnant endometrium has glands that are very positive for P-gp (117, 118), and human adrenal cortex is rich in P-gp. These localizations in steroid-secreting glands suggest that P-gp might be involved in secretion of steroids, or in protecting the plasma membranes of steroid-secreting cells from the toxic effects of high steroid concentrations. One observation consistent with this result is that progesterone is a P-gp inhibitor (119), and other steroids, specifically corticosterone, are transported by epithelial monolayers expressing P-gp (120). Mouse adrenal Y-1 cells, in which one copy of the mdr1b gene has been inactivated by insertional mutagenesis, show reduced steroid secretion (121). However, as noted below, mdr knockout mice are viable, so steroid secretion cannot be absolutely dependent on P-gp function.

Some hematopoietic cells express P-gp, albeit at lower levels than are seen in epithelial tissues, brain capillary endothelial cells, and adrenal cortical cells. CD34-positive bone marrow stem cells, long known to be rhodamine dull, express enough P-gp to account for this phenotype (increased efflux of the mitochondrial laser dye rhodamine 123) (122) and possibly to explain some of their resistance to chemotherapy, which eliminates more differentiated hematopoietic cell types. Some T cells and macrophages appear to express P-gp (123–125), but the physiological significance of this is not yet known (see below).
Mice in Which mdr Genes Have Been Insertionally Inactivated Have Major Alterations in Pharmacokinetics and Tissue Distribution of Substrate for P-gp

Firm evidence for the function of P-gp in mice came from studies in which mdr genes were inactivated by insertional mutagenesis (126–128). Rodents have two mdr1 genes, termed mdr1a and mdr1b, both of which have been inactivated individually and simultaneously (129). Loss of either or both genes has no effect on viability, fecundity, or life span of mice. In contrast, the related gene, mdr2, is essential for transport of phosphatidylcholine from hepatocyte membranes into bile, and mdr2 knockout mice develop progressive cirrhosis because of inadequate formation of bile micelles (110). The viability of the complete mdr1 knockout makes a strong argument for the feasibility of pharmacological strategies to improve cancer therapy in which P-gp is mostly, or completely, inhibited in the human.

The phenotype of the mdr1b knockout mouse appears to be the same as the mdr1a mdr1b double knockout, which suggests that under laboratory conditions, mdr1a contributes little to pharmacokinetics of drugs and xenobiotics and does not compensate in a significant way for the loss of mdr1b, the major drug transporter in this rodent. However, this result does not mean that mdr1a does not have some other, as yet undetermined, function; its tissue-specific expression in the mouse is somewhat different from that of mdr1b (13), which suggests that it may have a function as yet undetected under controlled conditions in the laboratory.

Mice lacking functional mdr1 gene(s) show a striking sensitivity to toxicity of some drugs to the central nervous system, which suggests in some cases almost complete abrogation of the blood-brain barrier for these drugs and in other cases partial elimination of this barrier. These results are entirely consistent with the previously observed high level of expression of MDR1 on the luminal surface of capillary endothelial cells in the human brain. Drugs tested so far include ivermectin, an anthelminthic that normally does not enter the rodent brain in significant amounts but is present in the brain at levels greater than 100-fold higher in mdr1 defective mice and that at these concentrations is lethal in mdr1 knockout mice. Digoxin also appears to be a substrate for the P-gp–dependent blood-brain barrier, as is loperamide, a semisynthetic opioid sold as an over-the-counter remedy for diarrhea (130, 131); brain levels of both drugs are much higher in mdr knockout mice than in normal mice. Levels of the anticancer drug vinblastine are elevated more than twofold in the brain of the mdr1 knockout mice, which suggests a role for P-gp in preventing this and perhaps other anticancer drugs from entering the central nervous system.

Analysis of the blood-brain barrier function of P-gp is complicated by the fact that P-gp is also involved in absorption and excretion of its substrates. Mice
without P-gp absorb much more of the anticancer drug taxol, when this drug is given orally, than do normal mice, in which oral absorption is limited (132). When doses of anticancer drugs, such as vinblastine, are given intravenously, clearance in bile and urine is reduced (131). Similar results are seen in man when potent inhibitors of P-gp, such as the cyclosporin A analog PSC833, are given intravenously (133) or orally (134). The net effect of reduced clearance is to increase serum levels for longer periods of time so that the area under the curve after intravenous administration is significantly increased. However, this pharmacokinetic effect can be taken into account when studying brain levels of drugs, and an independent effect on blood-brain barrier for several drugs can still be demonstrated (135).

**The Physiological Role of P-gp in Health and Disease; Therapeutic Implications of Pharmacological Inhibition of P-gp**

As indicated in the preceding sections, strong morphological and genetic evidence exists demonstrating a role for P-gp in absorption, distribution, and excretion of certain hydrophobic, amphipathic drugs and xenobiotics in mice and probably in humans. These effects are illustrated schematically in Figure 4,

![Figure 4](image-url)

*Schematic representation of the effect of P-glycoprotein (P-gp) action on the bioavailability of drugs. (Solid arrows) Drug path; (dotted arrows and solid arrow to urinary excretion) P-gp activity. Although not shown here, P-gp is also present in the adrenal gland. This figure is reproduced from Reference 148 by copyright permission of the American Society for Clinical Investigation.*
in which P-gp can be seen to serve as a barrier (a) to entry of these toxic compounds into the body, (b) to remove drugs from the circulation once they have entered, (c) to keep drugs from leaving the circulation into tissues that are especially sensitive to their toxicities, and (d) at a cellular level to protect cells if drugs have left the circulation to enter the interstitial space.

This view of the protective function of P-gp has profound implications for the study of drug pharmacokinetics and drug delivery to target tissues, especially given the very broad specificity of the multidrug transporter. Modern pharmacology aims to design drugs that can easily transit the plasma membrane, usually because of hydrophobic properties; many of these newly created drugs, if positively charged or neutral, will be substrates for detection and extrusion by P-gp or related transporters. The MRP family of transporters (reviewed in 136) can transport anionic drugs with similar properties. Thus, knowledge of whether a newly designed drug is a substrate for P-gp will be important in determining the likelihood of oral absorption, the pharmacokinetics of uptake and excretion, and the penetrance of the drug into brain, germ cells, and the fetus.

Inhibition of P-gp could be a useful intervention to improve oral uptake of drugs; reduce drug excretion, thereby reducing dosing requirements; and allow penetration of drugs into privileged sites in the body, such as the brain and some hematopoietic cells. The importance of P-gp in MDR in cancer suggests that cytotoxic drug delivery to cancer cells can be improved by inhibition of P-gp, perhaps to afford a significant therapeutic advantage and improved treatment of cancer patients. Clinical trials to thoroughly test this hypothesis are currently under way in many medical centers.

Similarly, gene therapy allows delivery of cDNAs encoding the multidrug transporter into particular target tissues and cells to protect them against the toxic effects of chemotherapy. Such a strategy could prove useful during chemotherapy of cancer, by protection of bone marrow and other drug-sensitive tissues. The feasibility of this approach has already been demonstrated in transgenic mice expressing the human P-gp gene (7) and in gene transfer experiments of human P-gp into mouse bone marrow (137–139). Vectors for delivery of P-gp have been developed and clinical trials to test this hypothesis are also underway (140). If delivery of P-gp to specific target tissues is feasible, and expression of P-gp protects these cells from cytotoxic drugs in humans, then the MDR1 gene could be used as a selectable marker for introduction of other, therapeutic genes into specific cell types, such as bone marrow (141, 142).

It is of interest to note that the cytochrome P450 metabolic system, especially the enzyme cytochrome P450 3A, has substrate specificity similar to that of P-gp (143, 144) and has almost certainly evolved to improve handling of hydrophobic, amphipathic drugs similar to those transported by P-gp. Although
cytochrome P450 has been a focus of interest with respect to drug interactions, P-gp, with similar substrate specificity, probably also serves as an important bottleneck for handling of many toxic drugs and xenobiotics. Thus, two drugs that are transported by P-gp will compete for this transport, resulting in increased oral absorption of both, in decreased excretion, and in redistribution, possibly into the central nervous system and other cells that express P-gp (41). This kind of drug interaction by competition for transport can be used as a tool to inhibit the multidrug transporter (when the inhibitor drug has little or no other pharmacologic effect), or it may be a cause of unfavorable, or unexpected, drug interactions.

As noted earlier, P-gp is expressed in CD34-positive bone marrow stem cells and in some B cells, T cells, and macrophages. The physiologic role of P-gp in this context is not known, but some authors have speculated that P-gp is involved in excretion of endogenous cytokines, especially those that lack obvious signal sequences for secretion (145). If this proves to be the case, then manipulation of P-gp levels in this setting could serve as a tool to alter immune function. One speculation, that P-gp can substitute for the peptide transport function ascribed to the TAP transport system (146), appears not to be valid (147). In this setting, however, delivery of hydrophobic drugs to circulating immune system cells can be significantly affected by levels of P-gp in these cells. For example, most of the new HIV protease inhibitors are P-gp substrates (40, 41, 148), and variable responsiveness of patients to these drugs, despite adequate blood levels and sensitive viruses, could be attributable to P-gp–based cellular resistance.

Finally, the full range of endogenous and exogenous substrates for P-gp has not yet been explored. Hardly a week goes by without another publication identifying a new substrate for P-gp, and it is entirely possible that there are whole classes of endogenous substrates that have not yet been identified. As noted above, a role for P-gp in handling specific steroids seems likely but has not yet been demonstrated in an intact animal model. Other low-molecular-weight biologically significant peptides, lipids, and other small molecules may also prove to be substrates for P-gp. This is certainly an area worthy of much thorough investigation.

PERSPECTIVE

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

Literature Cited

transition state using orthovanadate reveals cross-talk between the two ATP sites. J. Biol. Chem. 273:16631–34


73. Cardarelli CO, Aksentijevich I, Pastan I, Gottesman MM. 1995. Differential effects of P-glycoprotein inhibitors on NHI3T3 cells transfected with wild-type (G185) or mutant (V185) multidrug transporters. *Cancer Res.* 55:1086–91


87. Raymond M, Gros P, Whetley M,
MULTIDRUG RESISTANCE IN CANCER

113. Cordon-Cardo C, O’Brien JP, Casals


CONTENTS

Cytochrome P-450 3A4: Regulation and Role in Drug Metabolism, F. Peter Guengerich


The Pineal Gland and Melatonin: Molecular and Pharmacologic Regulation, Jimbo Borjigin, Xiaodong Li, Solomon H. Snyder

Regulation of Gene Expression by Reactive Oxygen, Timothy P. Dalton, Howard G. Shertzer, Alvaro Puga

Induction of Cytochrome P4501A1, James P. Whitlock Jr.

Cytotoxicity of Short-Chain Alcohols, R. C. Baker, R. E. Kramer


Regulation and Inhibition of Phospholipase A2, Jesús Balsinde, María A. Balboa, Paul A. Insel, Edward A. Dennis

Inhibition of Nitric Oxide Synthase as a Potential Therapeutic Target, Adrian J. Hobbs, Annie Higgs, Salvador Moncada

Genetic Regulation of Glutamate Receptor Ion Channels, Scott J. Myers, Raymond Dingledine, Karin Borges

Redox Regulation of c-Ha-ras and Osteopontin Signaling in Vascular Smooth Muscle Cells: Implications in Chemical Atherogenesis, Kenneth S. Ramos

Metallothionein: An Intracellular Protein to Protect Against Cadmium Toxicity, Curtis D. Klaassen, Jie Liu, Supratim Choudhuri

Cyclins and Cell Cycle Checkpoints, D. G. Johnson, C. L. Walker

New Insights into Dopaminergic Receptor Function Using Antisense and Genetically Altered Animals, David R. Sibley

Beta-Adrenergic Receptors and Receptor Signaling in Heart Failure, Steven R. Post, H. Kirk Hammond, Paul A. Insel

Biochemical, Cellular, and Pharmacological Aspects of the Multidrug Transporter, Suresh V. Ambudkar, Saibal Dey, Christine A. Hrycyna, Muralidhara Ramachandra, Ira Pastan, Michael M. Gottesman

Teratology of Retinoids, Michael D. Collins, Gloria E. Mao

Excitatory Amino Acid Transporters: A Family in Flux, R. P. Seal, S. G. Amara