Minireview

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The Multidrug Transporter, a Double-edged Sword

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The elucidation of the means by which tumor cells develop resistance to the toxic effects of potent chemotherapeutic agents has revealed a great deal about mechanisms of drug uptake, metabolism, and extrusion, as well as providing basic insights into cellular processes such as regulation of gene expression and gene amplification. One perplexing problem facing the cell biologist and oncologist has been the tendency of cultured cells and tumors in patients to exhibit simultaneous resistance to multiple chemically unrelated chemotherapeutic agents that are of natural product origin. Recent work from several laboratories has shown that this multidrug resistance is due to a multidrug transport protein, known as P-glycoprotein or P170, which is encoded by the *MDR*1 gene in the human (also reviewed in Refs. 1–4). This multidrug transporter directly uses the energy of ATP to extrude a large variety of drugs from cells. The identification of this transport protein in cells selected for multidrug resistance in tissue culture has made it possible to begin to examine the function of the multidrug transporter in normal tissues. As detailed in the following review, this transporter, whose expression was identified as a mechanism of drug resistance in cancer cells, apparently may also function as a means of protecting animals against toxic substances present in their diets.

One Kind of Multidrug Resistance Results from Energy-dependent Drug Efflux

Tissue culture cells can be selected for resistance to a variety of hydrophobic natural product drugs such as colchicine, doxorubicin (Adriamycin), actinomycin D, or vinblastine. Increasing the concentration of the selecting agent in multiple small single steps results in high level cross-resistance to these agents as well as many other drugs including other anthracyclines, *Vinca* alkaloids, and epipodophyllotoxins (1, 4–9). These drugs have no obvious common chemical features other than their hydrophobicity due to the presence of aromatic rings and a tendency to be positively charged at neutral pH. The primary physiological characteristic of the cell lines resistant to these agents is that they accumulate far less drug than parental drug-sensitive cell lines because they have an energy-dependent drug efflux system (5, 10–12). Highly multidrug-resistant cell lines express large amounts of a 170,000-dalton glycoprotein, called P-glycoprotein or P170, in their membranes (5).

In the human KB (HeLa) carcinoma cells studied in our laboratory, and in many other cultured cells, the activity of the drug efflux system increases with each step which selects for greater multidrug resistance. This suggests that the acquisition of this transport system is the major means by which KB cells become cross-resistant to multiple drugs (1, 4). However, not all cell lines resistant to multiple drugs express this drug efflux system. Using different cell systems and selecting agents, other laboratories have described a less common alternate form of multidrug resistance involving anthracyclines and epipodophyllotoxins, but not vinblastine. This cross-resistance is not attributable to drug efflux but may be due to an alteration in the killing pathway for these drugs involving topoisomerase II (13–15). Cells may also acquire cross-resistance to different alkylating agents in a single step via increased activity of glutathione *S*-transferase, but it is unclear whether this resistance extends to other drugs (3, 16). In addition, cross-resistance to some of the natural product drugs may occur because they have common targets in the cell, such as tubulin for the anti-microtubule agents (17). The discussion in this review will be limited to the broadest form of multidrug resistance which results from the energy-dependent drug efflux system for natural product cytotoxic agents.

A striking characteristic of multidrug-resistant cell lines is that they become fully drug-sensitive when cultured in the presence of certain other less cytotoxic drugs, such as verapamil and quinidine (11, 18). Analysis of the chemistry of these and many other reversing agents suggests that they are also hydrophobic, positively charged, and similar in size to the cytotoxic drugs to which multidrug-resistant cells are resistant (19). At least in the case of verapamil, these reversing agents are substrates for the multidrug transport system (20) and thus appear to act by inhibiting the multidrug transporter (see below).

Identification and Cloning of the MDR1 Gene

To clone the gene or genes encoding the human multidrug transport system, human multidrug-resistant KB cells were selected for resistance to high levels of colchicine, vinblastine, or doxorubicin (Adriamycin) (8, 9). These cells contain many minute and double minute chromosomes, and by in-gel renaturation analysis, amplified DNA segments common to all these cell lines were identified in their genomic DNA (21). By homology with similarly amplified sequences in multidrug-resistant Chinese hamster cells (22), two related human genomic sequences were isolated from multidrug-resistant KB cells. These were designated *MDR*1 and *MDR*2 (23). *MDR*1 and *MDR*2 are genetically linked and map to chromosome 7q (24, 25).

Expression of a 4.5-kilobase mRNA, corresponding to the *MDR*1 gene only, was found to correlate with drug resistance in many different multidrug-resistant cells (26). Using the genomic *MDR*1 probe, overlapping cDNA segments for the entire *MDR*1 RNA were obtained and sequenced (27, 28). This full-length cDNA encodes the cell surface 170,000-dalton P-glycoprotein (29). In parallel work, similar *mdr* cDNAs have been cloned and completely sequenced from the mouse (30). Based on overexpression of the *mdr* RNA in multidrug-resistant cells, cDNA clones from the mouse and hamster have also been obtained (31, 32). Antibodies to the *mdr* gene product, P-glycoprotein, have been used to isolate hamster *pgp* (*mdr*) clones (33).

The human *MDR*1 cDNA encodes a 1280-amino acid protein with 12 predicted transmembrane domains (28). The first half of the molecule shows 43% identity with the second half, suggesting that two halves were generated by a gene duplication event. The regions of greatest identity in the two halves of the P170 molecule are also very homologous to similar sequences in the ATP-binding subunits of bacterial transport proteins (28, 30, 33, 34). A model of the multidrug transporter, based on the deduced amino acid sequence of the human protein, is shown in Fig. 1. The orientation of the protein in the membrane is based on evidence that monoclonal antibody C219 recognizes a cytoplasmic COOH-terminal domain of P-glycoprotein (35), and on the assumption that the ATP binding domains are cytoplasmic.

The deduced sequences of mouse and human P-glycoproteins are 80% identical. As shown in Fig. 1, the regions of greatest sequence identity occur in the intracytoplasmic parts of the protein, around the putative ATP binding regions, as well as in other cytoplasmic regions. These other regions of sequence identity may be functionally conserved, perhaps for drug binding. Glycosylation sites in the first extracytoplasmic loop are not conserved between species, nor are many of the amino acids in the amino or carboxyl terminus, nor is the linker region connecting the two homologous halves of the molecule. A recent comparison of *MDR*1 sequences from colchicine- and vinblastine-selected multidrug-resistant KB cells demonstrated the presence of a valine at position 185 in the colchicine-selected cells, but a glycine at this site in vinblastine-selected cells and in the wild-type gene (36). An *MDR*1 cDNA expression vector (see below) with a valine introduced at position 185 instead of a glycine changed the resistance of transfected cells to favor colchicine over vinblastine (36). These results show that there was a mutation in the *MDR*1 gene during multistep drug selection in colchicine which changed the specificity of the multidrug transporter to favor colchicine over vin-

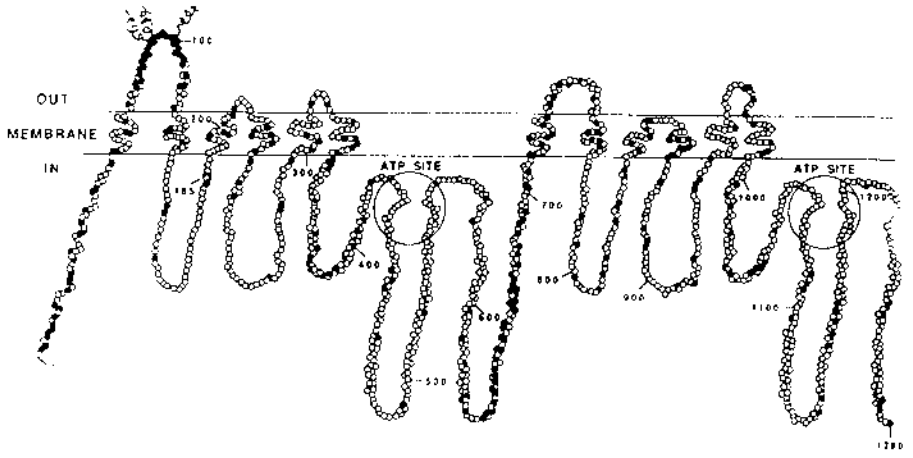


FIG. 1. **Model of the human multidrug transporter.** The ATP binding sites, based on sequence homology to bacterial transporters (34) are *circled*, putative *N*-linked sugars are shown as *curly lines*, the glycine to valine mutation at position 185 associated with preferred colchicine resistance (36) is shown as a *cross-hatched square*, and amino acids which differ between the mouse *mdr*1 sequence (30) and the human *MDR*1 sequence (28) are shown as *filled-in circles*.

blastine. These results also suggest that the cytoplasmic domain around position 185 may be involved in drug binding. Similar mutations might account for the observed variation in the relative resistance to different drugs at different steps in selection, with cells usually having the greatest resistance to the selecting drug (1–4, 26).

As noted above, two *MDR* genes have been identified in the human genome. Expression of only *MDR*1 is associated with drug resistance. Transcripts for the second *MDR* gene, termed *mdr*3 by Van der Bliek *et al.* (37), have been found in human liver, but the function of the gene is not known. The sequence of *mdr*3 appears to be the same as *MDR*2 of Choi *et al.* (23, 36). There appear to be three linked *mdr*-related genes in mouse and hamster (4, 38–40). In the hamster, two of these genes, *pgp*1 and *pgp*2, are similar to *MDR*1, with *pgp*1 having the closest sequence to this human gene (38). The two sequences which have been described in the mouse, represented by λ clones DR11 and DR29, and termed *mdr*1 and *mdr*2, respectively, by Gros *et al.* (40), are similar to *pgp*2 and *MDR*2 (*mdr*3 of Ref. 37), respectively (4, 40, 41). Selection of mouse J774.2 cells for multidrug resistance results in the expression of several different molecular weight forms of P-glycoprotein. Most of the variants are due to post-translational glycosylation, but at least two of the precursors appear to be different gene products, suggesting that the expression of either one of these two genes may result in drug resistance (42). An additional complication is evidence for alternate splicing of some hamster *pgp* mRNAs (38) and the human *MDR*2 (also known as *mdr*3) mRNA (37). The clarification of the relationship among the three rodent *mdr* genes and the two human *MDR* genes must await the isolation and complete sequencing of all of the genes or their cDNAs. Transfection of the cDNAs into tissue culture cells will determine which genes confer drug resistance and which do not. Preliminary results indicate that the mouse *mdr*2 gene does not confer multidrug resistance (40) as does the mouse or human *MDR*1 genes ((41, 43) see below).

Most of the human *MDR*1 gene has been isolated on a set of overlapping cosmids, encompassing approximately 150 kilobases of genomic DNA.[1] The major promoter for expression of the *MDR*1 gene has been isolated and sequenced (44). The sequence shows no TATA box, but there is a CAAT box and a GC-rich region upstream from the major start site of transcription. This upstream region acts as a promoter in constructions using CAT as a reporter gene (44). In certain drug-resistant cell lines, this major promoter for the *MDR*1 gene is part of a transcript which initiates from a second promoter found further upstream from the protein coding sequences (44). The factors regulating transcription initiation from these two promoters have not been determined.

Evidence That the MDR1 Gene Encodes a Multidrug Transporter

Full-length cDNAs for the human *MDR*1 gene or the mouse *mdr*1 gene have been cloned into expression vectors and transfected into drug-sensitive cell lines. These cDNAs are able to confer drug resistance on the transfected cells (41, 43), suggesting that expression of the *MDR* gene is limiting for the development of multidrug resistance. However, this does not prove that the product of the *MDR*1 gene is the transporter itself. Protein sequence analysis (see above) shows that the P-glycoprotein has the expected properties of a transporter, including multiple transmembrane domains and putative ATP binding sites. Within resistant cells, as expected for a transport protein, P-glycoprotein is found abundantly in plasma membranes, but not in coated pits (45). This location is consistent with recent data which indicate that *N*-linked glycosylation of P-glycoprotein is a slow process and that the half-life of P-glycoprotein within the plasma membrane is greater than 24 h (46).

TABLE I

Inhibitors of vinblastine transport into isolated vesicles

The ID_{50} is the concentration of the agent which inhibits vinblastine transport by 50%.

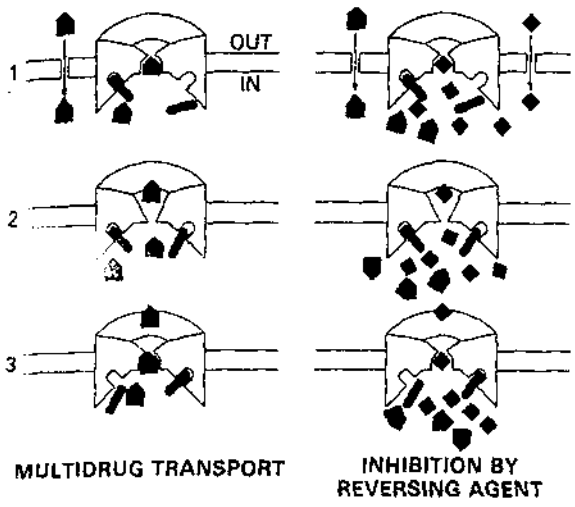
Agent	ID_{50}
	μM
AMP-PNP	500
Vanadate	10
Vinblastine	2
Vincristine	2
Actinomyin D	4
Daunomycin	30
Colchicine	160
Puromycin	170
Verapamil	3
Quinidine	3

The photoaffinity label *N*-(*p*-azido-3-^{125}I-salicyl)-*N'*-(β-aminoethyl)vindesine is an analog of vinblastine which has been used to demonstrate directly that P-glycoprotein binds drugs (47), a property also consistent with its role as a transporter. Labeling of P-glycoprotein by N-(*p*-azido-3-^{125}I-salicyl)-*N'*-(β-aminoethyl)vindesine is inhibited by daunomycin and vinblastine, and by verapamil, quinidine, and many but not all other drugs which reverse multidrug resistance (47–49). One reversing agent, [^{3}H]azidopine, has been shown to bind directly to P-glycoprotein (50). 8-Azido-[^{32}P]ATP also labels P-glycoprotein (51), as is expected from the presence of ATP binding sites predicted by sequence analysis.

Membrane vesicles prepared from multidrug-resistant cells, but not from drug-sensitive cells, will transport [^{3}H]vinblastine (52). The accumulation of [^{3}H]vinblastine within mixed sucrose gradient-purified plasma membrane vesicles is ATP-dependent, osmotically sensitive, and occurs against a concentration gradient. Inhibitors of this system are shown in Table I. The nonhydrolyzable ATP analog AMP-PNP[2] is a competitive inhibitor of ATP in this transport process. Vanadate, a general ATPase inhibitor, is a noncompetitive inhibitor of transport. These results suggest that ATP must be hydrolyzed during this transport process. All the drugs to which multidrug-resistant cell lines are resistant inhibit transport to varying extents, as shown in Table I. Daunomycin is a moderately potent competitive inhibitor (52), while the more water-soluble drugs, colchicine and puromycin, are weaker inhibitors. These results suggest that all of these drugs share a single site for transport but do not rule out the possibility that there is more than one site on the transporter for

[1] D. Clark, K. Ueda, I. Pastan, and M. M. Gottesman, unpublished data.

[2] The abbreviation used is: AMP-PNP, adenyl-5′-yl imidodiphosphate.



Cartoon of multidrug transport. The *panel* on the *left* s[hypothetical steps in the transport of a hydrophobic cy[natural product such as vinblastine (*orange*). In step 1, the drug enters the cell by diffusion through the membrane and binds to the transporter. In step 2, as ATP (*red*) is hydrolyzed to ADP (*green*), the transporter undergoes an allosteric change and the drug is moved outside of the cell. In step 3, the system recycles and is ready for transport of another molecule. Two equivalent ATP binding sites, only one of which needs to be occupied for transport, and one drug binding site are assumed. The *panel* on the *right* shows how a reversing agent, such as verapamil (*blue*) can interfere with transport by acting as a competitive inhibitor (and probably a substrate) of the transporter.

binding. Several agents which reverse drug resistance have also been tested, and all inhibit transport in this system. Verapamil and quinidine, which are particularly potent, also appear to be competitive inhibitors of transport. In intact cells, verapamil is transported by the multidrug transporter (20) in a process which hydrolyzes ATP (53). The concepts derived from these transport and binding studies have been integrated into the schematic model for transport shown in Fig. 2. Although P-glycoprotein is post-translationally modified by phosphorylation, the effect of this modification on transport is unclear (54).

The definitive proof that P-glycoprotein is a multidrug transporter will require the purification to homogeneity of a functional protein and reconstitution into a purified vesicle system where transport can be demonstrated. Hamada and Tsuruo (55) have recently used antibody affinity columns to purify the protein, which has some residual ATPase activity, but reconstitution has not yet been demonstrated.

Specialized Retroviral Vectors for the Multidrug Transporter

The isolation of expression vectors for the multidrug transporter (41, 43) has made it possible to confer drug resistance on most cultured cell lines. Since the multidrug transporter can be expressed in virtually any drug-sensitive cell, the gene can be used as a dominant selectable marker to introduce other genes into cultured cells (56). Under continued selective pressure, cell lines containing the *MDR*1 cDNA and a second unselected gene will amplify both the *MDR*1 cDNA and the unselected gene, allowing expression of very high levels of the second gene product (56). Although this is a useful system for cell culture studies, there are several advantages to the creation of high-titer infective retroviral vectors. For example, viral infections are much easier to perform than are transfections, and it is possible to infect virtually all of the cells in a population with a retroviral vector. Retroviral vectors have been constructed for both mouse and human *mdr* genes (57, 58). In both cases, a very high frequency of *MDR*1 expression was obtained. After infection of Madin-Darby dog kidney cells with the *MDR*1 retroviral vector, the multidrug transporter was predominantly expressed on the apical surface of these polarized cells (where it is expressed in normal kidney tubule cells, see below), indicating that the cDNA contains all of the information necessary for polarized expression (58). These results indicate that an *MDR*1 retroviral vector can be used to infect normal cells and that transferred transporter should be localized properly within these cells.

Retroviral vectors carrying the *MDR*1 gene show some promise for use in gene therapy. Recent experiments indicate that mouse bone marrow can be efficiently infected in culture with the *MDR*1 retroviral vector. A high percentage of the resulting granulocyte-macrophage colony forming units are multidrug-resistant in an *in vitro* assay.[3] If selection of resistant marrow *in vivo* proves to be feasible, the *MDR*1 gene may be a useful selectable marker for introducing other cloned genes into bone marrow. *MDR*1 retroviral vectors might also be useful as a means of conferring multidrug resistance on the bone marrow of patients undergoing intensive chemotherapy.

Expression of the MDR1 Gene in Normal Tissues and in Tumors

Studies on expression of *MDR*1 mRNA in normal tissues indicate substantial expression in colon, small intestine, kidney, liver, and adrenal with very low level expression in most other tissues (59). P-glycoprotein is located on the biliary surface of hepatocytes and small biliary ductules, on the brush border of renal proximal tubule cells, on the lumenal surface of pancreatic ductules, on the lumenal surface of the mucosa of the small and large intestine, and diffusely in both medulla and cortex of the adrenal (60). *mdr* RNA is found in a similar location in the gastrointestinal tract in the hamster by *in situ* hybridization (61). In the mouse, an *mdr*1-related gene is also expressed at high levels in secretory epithelial cells in the gravid uterus (62). This localization is consistent with a role for P-glycoprotein as a transporter in most of these organs. Liver, kidney, and bowel are the sites from which cytotoxic natural products present in the diet, or introduced by the chemotherapist, are removed from the body. Hence, the *MDR*1 gene product could be functioning as a multidrug transporter in these locations to protect animals from a variety of cytotoxic compounds present in their diets. In rat liver, levels of *MDR*1 RNA increase after hepatectomy (63, 64) or after treatment with chemical carcinogens (64), supporting the idea that expression of the *MDR*1 gene is a response to toxic insult. In the adrenal gland or the pregnant uterus, the *MDR*1 gene product may also be involved in transport of endogenous metabolites.

Adenocarcinomas derived from adrenal, kidney, liver, and bowel are known to be intrinsically resistant to a broad range of chemotherapeutic agents, including the drugs to which multidrug-resistant cells are resistant. These tumors express easily detectable levels of *MDR*1 mRNA (59),[4] suggesting that their multidrug-resistant phenotype is related to the presence of the multidrug transporter. For kidney tumors, this concept is supported by evidence that unselected cell lines derived from kidney cancers express *MDR*1 RNA and are multidrug-resistant, and this resistance can be reversed by verapamil and quinidine (65).

Additional published and unpublished evidence from many laboratories for the expression of the *MDR*1 gene in other tumor types, especially tumors which have undergone selective pressure by chemotherapy *in vivo*, suggests that expression of the multidrug transporter may play a significant role in determining the resistance of many tumors to chemotherapy. The accumulation of more data on tumors, the development of clinical protocols for prospective study of expression of the *MDR*1 gene product during the development of drug resistance in patients, and the successful application in animal models and patients of agents which reverse drug resistance or specifically target multidrug-resistant cells, such as immunotoxins (66), will be needed to prove the clinical relevance of basic research on the multidrug transporter. Meanwhile, the continued genetic and biochemical study of the multidrug transporter will provide an opportunity to learn more about basic transport processes in normal cells and tissues.

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[3] J. R. McLachlin, M. A. Eglitis, K. Ueda, P. W. Kantoff, I. Pastan, W. F. Anderson, and M. M. Gottesman, unpublished data.

[4] L. Goldstein, M. M. Gottesman, and I. Pastan, unpublished data.

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