[Frontiers in Bioscience 2, d427-437, September 15, 1997]

MULTIPLE TRANSPORT PROTEINS INVOLVED IN THE DETOXIFICATION OF ENDO- AND XENOBIOTICS

Yogesh C. Awasthi[1,2], Sanjay Awasthi[3], and Piotr Zimniak[4]

Departments of [2]Human Biological Chemistry & Genetics and [3]Internal Medicine, University of Texas Medical Branch, Galveston, Texas; and [4]Department of Internal Medicine and Biochemistry & Molecular Biology, University of Arkansas for Medical Sciences, and McClellan VA Hospital, Little Rock, Arkansas

Received 9/3/97 Accepted 9/8/97

TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Transport and detoxication mechanisms
- *3.1. Transport and Phase III detoxication mechanisms*
- *3.2. Efflux of xenobiotic and drugs from mammalian cells*
- *3.3. Transport of xenobiotics/metabolites by erythrocytes*
- *3.4.. Heterogeneity of GSH-conjugates transporters*
- *3.5. ATP-dependence of GSH-conjugate transporter(s)*
- *3.6. Structural properties of erythrocyte transporters*
- *3.7. Functional relatedness between drug efflux pumps and erythrocyte GSH-conjugate transporter, DNP-SG ATPase*
- *3.8. Transport mechanisms in liver*
- *3.9. Drug efflux pumps; Pgp and MRP*
- *3.10. P-glycoprotein*
- *3.11. Multiple drug resistance associated protein (MRP)*
- *3.12. Does MRP transport only anionic conjugates?*
- *3.13. MRP and DNP-SG ATPase*
- *3.14. Multi-specific organic anion transporter (MOAT)*

4. Future directions
5. Acknowledgment
6. References

1. ABSTRACT

Transport mechanisms involved in the exclusion of xeno- and endobiotic toxins from the cellular environment play a crucial role in protecting cells from toxicity of these compounds. A transporter designated as dinitrophenyl S-glutathione ATPase (DNP-SG ATPase) present in human erythrocyte membrane has been characterized in our laboratory. The unique functional features of this transporter include its ability to mediate ATP-dependent transmembrane movement of organic anions such as glutathione conjugates, as well as weakly cationic amphiphilic compounds such as doxorubicin and other substrates of P-glycoprotein. The substrate specificity profile of DNP-SG ATPase overlaps with those of the drug efflux pumps, P-glycoprotein, multidrug resistance associated protein (MRP), and the multi-specific organic anion transporters (MOAT) despite its distinct structural properties from these transporters. Possible functional interrelationships among these transporters is discussed in this review and it is proposed that analogous to the phase I and phase II drug metabolizing enzymes the xeno- and endobiotic transporters may belong to several distinct gene families members of which share overlapping catalytic properties. Their functional diversity covering a wide range of substrate affinities provides protection from structurally diverse xeno- and endobiotic toxicants.

2. INTRODUCTION

Living organisms defend themselves from the toxicants present in the environment through biotransformation of these compounds to relatively non-toxic metabolites and their subsequent elimination through transport. Most cells are equipped with a multitude of phase I and phase II biotransforming enzymes (1). In phase I, reactive groups such as -OH, -NH_2, or >O are introduced/exposed on relatively hydrophobic xenobiotics so that they can be conjugated to hydrophilic-compounds such as GSH, glucuronate, sulfate, etc. by the phase II enzymes, and the resultant products (usually less toxic, more hydrophilic) can be excreted through active/facilitated transport processes across the cellular membrane. Likewise, electrophiles (both hydrophobic and water soluble) can be conjugated to the abundant nucleophiles such as GSH by phase II enzymes, and the conjugation products can be transported out of cells. Whereas extensive studies have been conducted on biotransformation enzymes resulting in the identification of numerous isozymes belonging to the superfamilies of phase I (2,3) and phase II enzymes (4-8) in mammalian tissues including humans, much less information is available on the enzymes which are involved in the active transport of xenobiotics and/or their metabolites. A concept that the transport of xenobiotic metabolites may be considered as phase III of the

detoxification mechanisms has been propagated recently (9-11).

Cells resist chemical aggression of environmental toxins by, 1) warding off the aggression through repulsion by force, 2) capturing or converting the toxins into relatively harmless entities and, 3) expulsion of the converted entities in order to avoid any possible long term harmful effects. In general, all cells are equipped with defense mechanisms involving these three discrete steps. Bacteria and parasites are known to defend against toxins by exclusion mechanisms as exemplified by the acquisition of drug resistance by the malaria parasite, *Plasmodium falciparum* (12). In humans, the significance of these exclusion mechanisms was not fully recognized until the relatively recent discoveries of drug efflux pumps, including the P-glycoproteins (Pgp) and multidrug resistance associated proteins (MRP), which are overexpressed in multidrug-resistant cancer cells (13,14). Capturing of toxicants is carried out in cells through their binding to certain abundant proteins such as albumin and ligandin (15, review) which trap and often inactivate these compounds, while their conversion to relatively harmless molecules is catalyzed by a multitude of phase I (2,3) and phase II (4-8,15-18) enzymes. The efflux of the metabolites of xenobiotics is carried out by plasma membrane transporter proteins, thus ensuring a safe environment for the cellular components. As expected, all these steps require energy. The drug exclusion pumps require ATP while phase I reactions require the reducing equivalents of NADPH. Some of the phase II enzymes (e.g. glucuronosyl and sulfotransferases) require energy for the activation of substrates, while others, such as glutathione (GSH) S-transferases (GST), require energy for the synthesis of GSH. Phase I and phase II enzymes are often induced by the invading chemicals. Overexpression of the drug efflux pumps, Pgp (13,19) and MRP (14,20), in cancer cells exposed to gradually increasing drug concentrations suggests that the transporters are also induced by xenobiotics. When the toxicants evade or overwhelm the cellular defense mechanisms, they cause toxicity which may eventually result in cell death. of CYP450s (2,3) and GSTs (4,5,8,15-18) have been well characterized. The transport mechanisms which flank the biotransformation processes in the simplified description of cellular defense mechanisms given above are rather poorly understood at present. The importance of the transport mechanisms in cellular defense against xenobiotics has been underscored by the discoveries of drug transporters overexpressed in multidrug resistant cancer cells. These drug efflux pumps have been covered in detail in several excellent review articles (13,19,20). In this mini-review, our current understanding of the structure and function of transport mechanisms for xenobiotics and their metabolites in mammalian tissues including humans is summarized. As pointed out above, studies on cellular defense mechanisms have largely focused on phase I and phase II biotransforming enzymes. Multiple forms of these enzymes (e.g. CYP450s, glutathione S-transferases, UDP-glucuronosyltransferases, and sulfotransferases) are expressed in mammals in a tissue-specific manner. The structural and functional properties of these enzymes have been extensively studied. In particular, a large number of isozymes belonging to the gene superfamilies

3. TRANSPORT AND PHASE III DETOXICATION MECHANISMS

3.1. Transport and Phase III detoxication mechanisms

implies that it is operative after phase II and that only the products of phase II biotransformation (e.g. the conjugates GSH, glucuronate, sulfate) are transported through these mechanisms. Recent studies, however, strongly suggest that some of these transporters, such as dinitrophenyl S-glutathionyl ATPase (DNP-SG ATPase) and MRP, can mediate the transport of the unmodified xenobiotics/drugs prior to their metabolism by phase I and/or phase II enzymes, as well as their phase I and phase II metabolites (20,21). Thus, some of these transport mechanisms may in fact be flanking the biotransformation mechanisms on both sides. DNP-SG ATPase, the GSH-conjugate transporter of human erythrocytes, can transport not only GSH-conjugates but also doxorubicin, its phase I metabolites, and other substrates of P-glycoprotein without their biotransformation (21). Likewise, MRP (the multidrug resistance associated protein overexpressed in some multidrug resistant cancer cells) might mediate the transport of parent drugs and xenobiotics without their biotransformation (20) as well as the transport of GSH and glucuronic acid conjugates of the endo- and xenobiotics generated during the phase II reaction of biotransformation (22-24). Thus, detoxification mechanisms involving transporters appear to be operative both pre- and post-biotransformation reactions, and the term phase III detoxification mechanisms for these processes would not be appropriate. Recently, it has been suggested that the transport mechanisms (e.g. GS-X pump) for xenobiotic metabolites should be viewed as phase III of the detoxification processes (9). This concept seems logical except that the term phase III

3.2. Efflux of xenobiotic and drugs from mammalian cells

Much of our current understanding on the mechanisms of transport of drugs/xenobiotics and/or their conjugates in mammalian tissues stems from studies with liver and erythrocytes. Liver is the major organ involved in the biotransformation of drugs/xenobiotics, and is the site of formation of potentially toxic endogenous metabolites which must be exported. Erythrocytes, on the other hand, are the major targets for the exposure of environmental toxins finding their way into circulation. Both tissues must heavily rely on the transport mechanisms for their defense, and it is not surprising that the transport of xenobiotics/metabolites in these tissues has attracted a great deal of attention of investigators in this area. In addition to these two models, cultured cancer cells have also been extensively used to study the mechanisms of the transport of drugs. Therefore, in this mini-review we will focus on the mechanisms of xenobiotic transport in erythrocytes and liver, and discuss their possible interrelationships with the transport mechanisms characterized so far in multidrug resistant cancer cells.

3.3. Transport of xenobiotics/metabolites by erythrocytes

Erythrocytes lack ER and phase I biotransforming enzymes such as CYP450, but are relatively rich in GSH (25) which constitutes the major defense against electrophilic xeno-/endobiotics. Nucleophilic GSH readily conjugates these electrophiles non-enzymatically, or through reactions catalyzed by GSTs which are abundant in erythrocytes (26-28). In most tissues, GSH-conjugates are further metabolized to mercapturic acids. Erythrocytes lack the complete battery of mercapturic acid pathway enzymes (29) and are therefore unable to further metabolize the GSH-conjugates. Whereas GSH-conjugates are usually less toxic than the parent compounds, in some instances the toxic effects of xenobiotics are potentiated through GSH-mediated biotransformation (30,31). Furthermore, the accumulation of GSH-conjugates could lead to impairment of the glycolytic and pentose pathway enzymes of erythrocytes, and therefore these conjugates must be expelled to avoid long term toxic effects. During xenobiotic exposure GSH can be also oxidized to GSSG, and its accumulation may also cause toxicity primarily through its interaction with cytosolic proteins such as hemoglobin. The mechanisms for the transport for GSSG and GSH-conjugates are, therefore, important for the defense of erythrocytes against xenobiotics.

ATP-dependent transport of GSSG was first demonstrated by Srivastava and Beutler (32). Characteristics of this transport system, studied in inside out vesicles (IOVs) prepared from erythrocytes, suggested that it had two components, one with high affinity (K_m 0.1 mM), and another with low affinity (K_m 7.3 mM) for GSSG (33). Electrophilic xenobiotics such as 1-chloro-2,4-dinitrobenzene (CDNB) are rapidly conjugated to GSH within erythrocytes in a reaction catalyzed by GST (34). Energy-dependent efflux of the GSH-conjugates of CDNB (dinitrophenyl S-glutathione, DNP-SG) from erythrocytes has been demonstrated (35,36). The transport of GSH-conjugates has been extensively studied (37-43) using IOVs prepared from erythrocyte membranes, and it has been established that the transport of DNP-SG by erythrocytes is mediated by an ATP-dependent primary active process (38). Similar to the system for GSSG transport, the system specific for xenobiotic-GSH conjugates in erythrocytes also seem to have more than one component. While the exact interrelationship between the mechanisms mediating the transport of GSH conjugates and GSSG is still incompletely understood, the available evidence suggests that these two compounds may be transported preferentially by distinct transporters with overlapping substrate specificities.

3.4. Heterogeneity of GSH-conjugates transporters

It has been shown that the DNP-SG transporter from erythrocytes has at least two kinetically distinct components which exhibit different pH profiles and inhibition patterns by organic anions (40-43). One of the components has high, and the other low affinity for DNP-SG. The high- and low-affinity systems have K_m M and 1.6 mM, respectively (43). Both of these components are competitively inhibited by various polyvalent anions, including GSH-conjugates, conjugates of steroid hormones, bile salts, and bilirubin suggesting a wide range of substrate specificities for these systems. Glucuronidated and sulfated drugs and taurocholate inhibit only the low affinity system, while anionic dyes fluorescein, indocyanine green and bromosulphophthalein inhibit the high affinity system competitively and the low affinity system non-competitively. The kinetic properties of these components suggest that the low K□ values for DNP-SG of 3.9 $_m$system may be more important for the transport of GSH-conjugates and polyvalent organic anions, whereas the high K_m component is important for the transport of monovalent organic anions. It has been suggested that the transport of GSSG and DNP-SG from erythrocytes is mediated by separate systems (38,44). However, Akerboom *et al*. (42) have shown that the high affinity uptake of DNP-SG by erythrocyte IOVs was competitively inhibited by GSSG (K_i M), and that DNP-SG competitively inhibited the low K□ 88 $_m$ component of GSSG uptake. The high K_m DNP-SG uptake system was not inhibited by GSSG, which would suggest the existence of a common high affinity transporter for DNP-SG and GSSG in erythrocytes. The transport of the GSH-conjugate of bimane also revealed similar kinetic characteristics to that of DNP-SG (40). The low and high K_m transport systems for DNP-SG have also been observed in IOVs prepared from rat, pig, and bovine erythrocytes, and such heterogeneity has also been observed in GSH-conjugate transporter purified from human erythrocytes (45) and bovine lens (46). The results of these studies are consistent with the presence of a multicomponent transport system in erythrocytes for the efflux of GSH-conjugates and other organic anions. The transport system in erythrocyte membranes is capable of actively transporting various organic anions including GSH-conjugates, GSSG, conjugates of bilirubin, and anionic organic dyes (32,33,35-46). In addition, versatile transporters such as DNP-SG ATPase (21) and MRP (47) are also present in erythrocyte membranes, which in addition to the transport of organic anions can also mediate the transport of weakly cationic hydrophobic compounds.

3.5. ATP-dependence of GSH-conjugate transporter(s)

Studies demonstrating that GSSG (48) and DNP-SG (49) could stimulate ATP hydrolysis by erythrocyte membrane vesicles provided a direct link between the transport of these compounds and ATP hydrolysis. IOVs prepared from erythrocyte membranes manifested GSSG stimulated Mg^{++} dependent ATPase activity (48). In another study, IOVs from erythrocyte membranes catalyzed ATP hydrolysis which was stimulated by DNP-SG (49). This ATPase, designated as DNP-SG ATPase, required Mg^{++}, was saturable with respect to DNP-SG as well as ATP, and was distinct from other membrane ATPases including GSSG ATPase (48) and the inorganic cation pumps (49). The K_m values of human erythrocyte DNP-SG ATPase for ATP and DNP-SG were found to be about 1.7 mM and 0.05 mM, respectively. ATP hydrolysis by this enzyme was found to be stimulated not only by DNP-SG but also by other GSH-conjugates including S-(methyl)-, S-(n-propyl)-, S-(n-pentyl)-, and S-(n-decyl)glutathione, and the glutathione-conjugates of 9,10-epoxy stearic acid and p-

nitrobenzyl alcohol. ATP hydrolysis by DNP-SG ATPase was also stimulated by endogenously generated conjugates or their analogs such as bilirubin ditaurate, lithocholic acid-3-O-sulfate, and lithocholic acid 3-O-glucuronide (45). These studies suggested a functional relationship of the hepatic transport system responsible for secretion of bile acid and bilirubin conjugates into bile with the erythrocyte transporters involved in excretion of organic anions into plasma. This contention was supported by studies showing that proteins immunologically similar to DNP-SG ATPase were present in other tissues including liver, kidney (50), and skeletal muscle and lung (51,21).

3.6. Structural properties of erythrocyte transporters

At least two distinct organic anion transporters have been partially characterized in erythrocytes (21,52) but their complete structures remain unknown. Presence of MRP, whose primary structure is derived from its cDNA sequence (14), has also been demonstrated in erythrocyte membrane (47). The purification of GSSG stimulated Mg^{++} ATPase activity from human erythrocytes yielded an enzyme composed of two non-identical subunits having Mr values of 82 kDa and 62 kDa (52). This enzyme had GSSG stimulated Mg^{++} ATPase activity, and could be functionally reconstituted in liposomes capable of ATP-dependent active transport of GSSG. It showed immunological similarity with the corresponding enzyme purified from cytoplasmic membranes of human hepatocytes suggesting inter-relatedness between the erythrocyte transporter(s) and those of liver.

The protein responsible for the DNP-SG stimulated ATPase activity of erythrocyte membranes (49) has also been purified (21,45,50,51). Apparently homogenous DNP-SG ATPase showed the presence of a single band with an Mr value of 38 kDa upon SDS-PAGE (20,49). DNP-SG, other GSH-conjugates, and the conjugates of bile acids, bilirubin, and steroid hormones stimulated ATP hydrolysis by DNP-SG ATPase (45). However, GSSG did not stimulate ATP-hydrolysis by this enzyme suggesting that GSSG-Mg++ ATPase and DNP-SG ATPase are distinct transporters. Our unpublished studies with highly purified preparations of DNP-SG ATPase suggest it to be a versatile transport protein. The purified enzyme could be reconstituted in proteoliposomes which were capable of ATP dependent transport of DNP-SG and doxorubicin (DOX), saturable with respect to both these compounds as well as ATP. The K_m values for ATP and for DNP-SG were similar to those observed for the ATP hydrolysis. DNP-SG ATPase did not show immunological cross-reactivity with the two best characterized xenobiotic efflux pumps, Pgp (13) and MRP (14). On the other hand, DNP-SG ATPase showed immunological similarity to the multispecific organic anion transporter (MOAT), a 90 kDa transporter capable of transporting DNP-SG across rat liver canalicular membranes (53,54). Taken together, these studies suggest that a number of structurally and functionally related transporters protect erythrocytes and other cells from xenobiotics and their metabolites. These transporters have varying degrees of structural and functional relatedness and overlapping substrate specificities for a wide range of compounds. Furthermore, these transporters may be expressed in a tissue specific manner, a situation that is analogous to that found for phase I and phase II enzymes.

The occurrence of multiple transporters in the plasma membrane with some shared catalytic properties is consistent with the general conclusion that the enzymes involved in detoxification processes occur as families in which members have overlapping but distinct kinetic properties. Perhaps this is best exemplified by the GST gene superfamily in which isozymes belonging to six families (alpha, mu, pi, Theta, Sigma and microsomal) have overlapping yet distinct kinetic properties (4,5,8,18). Members of these families are expressed in a species- and tissue-specific manner, probably to meet the particular needs of the organisms for protection against exogenous and endogenous toxicants. By analogy, the plasma membrane transporters of organic compounds which play an important role in cellular detoxification mechanisms may be expected to belong to several interrelated families with members having overlapping transport capabilities. This idea is corroborated by the fact that more than one transporter exists within the MRP (20) and MDR (19) subfamilies of the ABC-transporter super family; moreover, impairment of expression of one transport protein may lead to a compensatory increase in the amount, and presumably function, of another (55). At least three transporters belonging to the MDR (19), two belonging to the MRP family (20) have been characterized. It is expected that other sub-families of these transporters will be identified in future when the erythrocyte transporters discussed above are completely characterized in terms of their structure and functions.

3.7. Functional relatedness between drug efflux pumps and erythrocyte GSH-conjugate transporter, DNP-SG ATPase

Kinetic studies with human erythrocyte DNP-SG ATPase (21) provided evidence for the functional relatedness of erythrocyte GSH-conjugate transporter (DNP-SG ATPase) with P-glycoprotein (Pgp). West (56) had first propagated the idea that Pgp, the drug efflux pump overexpressed in some MDR cancer cells (57-61), and the GSH-conjugate transporter of erythrocyte membranes (38) may be related. Initially, this proposal did not gain wide support because of the apparently distinct properties of these transporters. The studies by Awasthi *et al*. (21) for the first time showed that DNP-SG ATPase, the GSH-conjugate transporter of human erythrocytes, catalyzed ATP hydrolysis stimulated by DOX as well as by DNP-SG. Moreover, ATP hydrolysis by DNP-SG ATPase was stimulated not only by DOX but also by its metabolites and other drugs (21) conventionally considered to be the substrates of Pgp (19,59). These studies for the first time suggested that the erythrocyte GSH-conjugate transporter(s) was/were capable of catalyzing ATP dependent efflux of not only organic anions but also of weakly cationic compounds, and that the amphiphiles such as DOX and vincristine could also be transported by this enzyme. Transport studies with the IOVs enriched with purified DNP-SG ATPase showed that ATP-dependent transmembrane transport of DOX and DNP-SG was

saturable with respect to time, sensitive to the osmolarity of the assay medium, and temperature dependent (21). Subsequently, studies with the other drug efflux pump MRP have also shown that it can mediate the transport of GSH-conjugates (22-24) as well as the weakly cationic or neutral amphiphilic compounds such as vincristine (62) and aflatoxin B_1 (63).

The substrate specificity of DNP-SG ATPase overlaps not only with that of Pgp but also of MRP. Pgp catalyzes the transport of hydrophobic drugs (60,64-66); however, glutathione- and glucuronic acid conjugates which are substrates of MRP (22-24,62,63,67) are not transported by Pgp. Direct evidence for MRP-mediated transport of DOX and other Pgp substrates is lacking, but the overexpression of MRP in multidrug-resistant cells selected for DOX resistance (14), and transport studies with inside out vesicles prepared from MRP overexpressing cells suggest that MRP can transport at least some of the Pgp substrates (62). Kinetic properties of DNP-SG ATPase studied in reconstituted proteoliposomes show that the enzyme is capable of transporting both DOX and glutathione conjugates (21). This suggests that versatile drug efflux pumps other than Pgp and MRP are expressed in normal human tissues, and perhaps play an important role in the protection against xeno- and endobiotic toxins.

3.8. Transport mechanisms in liver

At least four ATP dependent processes involving the transport of organic molecules are known to be mediated by the canalicular domain of the hepatocyte plasma membrane (68). These are: (i) the transport of bile acids by a hitherto unidentified transporter, (ii) transport of phosphatidylcholine by the product of the murine mdr-2 gene and its human counterpart MDR2/3 (69,70), and transport of (iii) hydrophobic drugs and (iv) non-bile organic anions. Of these, the transport of the latter two classes of compounds is probably more relevant to the protection against toxicity than the transport of bile acids and phospholipids which are mainly involved in the process of bile formation. The liver canalicular efflux pumps specific for hydrophobic drugs and for non-bile organic anions are often thought (71-74) to be related to the anti-cancer drug pumps Pgp (13) and MRP (20), respectively. This is likely to be an oversimplification; as discussed below, the specificities of Pgp and MRP overlap, and additional transporters with related activities exist.

3.9. Drug efflux pumps; Pgp and MRP

Studies with drug resistant cancer cells have led to the characterization of two distinct xenobiotic export pumps belonging to the Pgp and the MRP groups of ABC transporters, respectively. Pgp and MRP share only about 15% homology but both have been shown to confer multidrug resistance phenotype to cancer cells. In humans two genes (MDR1 and MDR2) related to Pgp have been characterized. Only the MDR1 gene confers multidrug resistance and is gene product, Pgp is involved in the transport of chemotherapeutic drugs (see Ref. 19). The product of the MDR2 gene has been shown to transport phosphatidylcholine and is perhaps not involved in the transport of xenobiotics (69,70). Both these genes have been localized on the long arm of chromosome 7. In mice three MDR genes have been found (reviewed in Ref. 19). Out of these, mdr1 and mdr3 (also referred to as mdr1a and mdr1b) gene products are involved in the transport of drugs, while the mdr2 gene product is orthologous to the human MDR2 and is a phospholipid translocator (68). Similar to MDR genes, at least two MRP related genes have been characterized (reviewed in Ref. 20). The canalicular form of MRP (MRP2) is predominantly expressed in liver. While a genetic defect associated with defective MDR-1 expression has not been reported, the absence of functional MRP2 has been associated with TR^- /GY rat mutants with impaired transport of bilirubin conjugates (74). Likewise, the Dubin-Johnson syndrome in humans has been associated to defective MRP2 protein (75). Thus, MRP and its related transporters seem to play an important physiologic role in defending the cellular environment from the endo- and/or xenobiotic toxins.

3.10. P-glycoprotein

Pgp, a 170 kDa membrane protein which catalyzes the energy dependent transport of anticancer chemotherapeutic agents and other hydrophobic compounds was first characterized in multidrug resistant cancer cells (13,19,57-61,64-66). Overexpression of Pgp has been associated with the MDR phenotype in a number of cancer cell lines (19,76). The protein has been purified from overexpressing cancer cells, and reconstitution studies have shown that it can catalyze ATP-dependent transport of vinblastine, and that this transport was inhibited by other substrates of Pgp (66). Pgp has a unique structure formed by two highly homologous halves, each of which contain six transmembrane segments and one nucleotide binding fold. The proteins have been detected in a number of MDR cancer cells as well as in normal tissues including brain, testis, intestine, and liver (19), suggesting a role in extrusion of toxins ingested with food as well as their metabolites. This suggested role of Pgp in the excretion of xenobiotics from normal tissues is consistent with the results of studies in which mice lacking mdr1a (functionally homologous to human MDR-1) gene show impaired transport of Pgp substrates (e.g. vinblastine) when challenged with these drugs (77). The physiological significance of Pgp in normal tissues is, however, not completely understood, and double knock out mice with both mdr-1a and mdr1b genes disrupted appear to have a normal phenotype (77). As pointed out earlier in this article, the human erythrocyte glutathione conjugate transporter (DNP-SG ATPase) can also mediate ATP-dependent transport of DOX, a substrate of Pgp, and it is also present in human liver (49). Despite overlapping substrate specificities, Pgp and DNP-SG ATPase are distinct as indicated by their structural and immunological properties.

3.11. Multiple drug resistance associated protein (MRP)

MRP, a 190 kDa protein cloned from a multidrug-resistant small cell lung cancer cell line selected for DOX resistance, also belongs to the ABC family of transporters (14,20). It has only about 15% structural identity with Pgp. The MRP polypeptide has an estimated molecular weight of about 171 kDa which is modified to a

190 kDa mature glycoprotein (78). The drug resistance of MRP overexpressing cells to anthracylines and vinca alkaloids suggests overlapping substrate specificities between Pgp and MRP. However, there are remarkable differences in the kinetic properties of these two transporters. MRP can mediate the transport of GSH conjugates, including the physiological substrates leukotriene C_4 (19,22-24), GSSG (79), and sulfate and glucuronate conjugates (67) such as 17 -estradiol 17-(beta-D glucuronide). Furthermore, several organic anions can competitively inhibit the transport of leukotriene C_4 or 17 -estradiol (beta-D glucuronide). This suggests the ability of MRP to transport other organic anions generated in phase II biotransoformation reactions. Pgp and its homologs apparently lack this ability. Whereas Pgp-mediated ATP-dependent transport of vinblastine, DOX and related drugs has been demonstrated in reconstituted vesicles (64-66), whether MRP can mediate transport of these drugs without their biotransformation has been debated. Photoaffinity labeling by the photoreactive analogs of DOX and vinblastine has been shown for Pgp (64); MRP doses appear to bind these compounds (80,81). The inhibition profiles of Pgp and MRP are also different. Verapamil and cyclosporin which are effective inhibitors of Pgp-mediated transport of chemotherapeutic drugs have a minimal effect on drug transport by MRP overexpressing cells (82,83).

3.12. Does MRP transport only anionic conjugates?

The rather unique transport characteristics of MRP seem to defy the paradigm that a single transporter should not be able to catalyze the transport of anionic as well as cationic organic compounds. It is well established that MRP can catalyze ATP-dependent transport of organic anions such as GSH-conjugates, glucuronides, sulfates, GSSG, and leukotriene C_4 (20,22-24,62,67). While direct evidence for MRP-mediated transport of DOX is lacking, the protein was cloned from multidrug resistant cells selected for DOX resistance (14). MRP-overexpressing cells show decreased accumulation of chemotherapeutic drugs such as DOX or vincristine which suggest that MRP perhaps can mediate transport of weakly cationic or neutral amphiphiles. In fact, MRP-mediated transport of vincristine (62) and aflatoxin B_1 (63) has been demonstrated when GSH is present in the medium. Based on the observations that the depletion of GSH in MRP over expressing multidrug resistant cells could reverse DOX resistance of these cells, it has been suggested that MRP transports the anionic GSH-conjugates rather than the unmodified drugs (85), and it has been hypothesized that MRP is a GS-X pump (10,47,84). Reversal of resistance to DOX of MRP overexpressing cells upon GSH depletion (84), overexpression of MRP in cisplatin resistant cells (10), the ability of MRP to transport organic anions (22-24,67), and the GSH requirement for ATP-dependent transport of vincristine by MRP overexpressing inside out membrane vesicles (62) may argue in favor of the hypothesis that MRP is indeed a GS-X pump capable of transporting only organic anions. This hypothesis, however, appears to be flawed for the following reasons. There is no evidence for the formation of GSH-conjugates of DOX, vincristine and related drugs, and the appearance of GSH-conjugates in the medium of MRP overexpressing cell has not been observed when cells are exposed to these drugs. While it has been shown that the ATP-dependent transport of vincristine occurs only in the presence of physiological concentrations of GSH in the medium (62), GSH-mediated biotransformation of vincristine and formation of its GSH-conjugate under these conditions is highly unlikely. Moreover, it has been shown that inside-out vesicles enriched in MRP can mediate ATP-dependent uptake of the GSH-conjugate of aflatoxin B_1 as well as its unmodified form (63). In these studies (63), the transport of unmodified aflatoxin B_1 was stimulated by GSH without conjugate formation. Inhibition studies suggested that aflatoxin B_1 and its GSH-conjugates were recognized by similar or overlapping binding sites. The question as to how GSH modulates the ATP-dependent transport of vincristine or aflatoxin B_1 by MRP remains to be resolved, but it seems clear that conjugation of hydrophobic drugs to GSH is not an obligatory prerequisite for transport by MRP. It has been suggested that cellular GSH is required to keep MRP in a conformational state that allows the transport of neutral or positively charged molecules (85). This idea is consistent with the results of studies showing the MRP mediated transport of calcein or its acetomethoxy ester from tumor cells is not inhibited by cellular GSH depletion but is inhibited by probenecid and vincristine (86,87).

3.13. MRP and DNP-SG ATPase

The substrate specificity profile of MRP appears to be similar to that of the erythrocyte GSH-conjugate transporter DNP-SG ATPase. Studies with reconstituted liposomes containing purified DNP-SG ATPase show its ability to mediate the transport GSH-conjugates as well as unmodified doxorubicin (21). However, the ATP-dependent transport of DOX in vesicles reconstituted with DNP-SG ATPase is not affected by GSH or GSSG, suggesting differences in the kinetic properties of MRP and DNP-SG ATPase. The presence of MRP has been recently demonstrated in human erythrocyte membranes (47), and it has been suggested that MRP is the GS-X pump of these membranes. For the reasons discussed above, the latter contention is not supported by available evidence. Similar but non-identical functional characteristics, and widely distinct structural and immunological properties of MRP and DNP-SG ATPase demonstrate that these two distinct transporters can mediate transport of GSH-conjugates and other xenobiotics. Likewise, kinetic properties of MRP are distinct from the GS-X pump characterized in cisplatin resistant HL-60 cells (10). Further studies are needed to determine the kinetic properties of MRP in isolated systems because the studies with transfected cells or transgenic animals may not adequately define the substrate profile of MRP, especially when it is becoming increasingly evident that a number of related transporters are co-expressed in tissues. For a direct comparison of substrate specificity profiles of MRP with those of other transporters including Pgp, MOAT (53,54), DNP-SG ATPase (21), and GS-X pump (10), the kinetics of transport of GSH-conjugates, other organic anions, and substrates of Pgp, e.g. DOX and vinblastine, should be studied in reconstituted liposomes containing MRP of unquestionable purity. Such studies will also address the controversy about the ability of MRP to transport unmodified drugs as discussed above.

3.14. Multi-specific organic anion transporter (MOAT)

There appears to be a functional overlap between MRP, GSH-conjugate transporter(s), and MOAT. Unlike MRP2, the other hepatic transporters listed above have not been cloned and sequenced, and their identity and degree of relationship(s) among them remain unclear. However, the kinetic characteristics of a transporter that could participate in the ATP-dependent efflux of non-bile acid organic anions from the hepatocyte into bile, termed MOAT, have been studied in detail (53,54). A 90 kDa protein which catalyzes ATP-hydrolysis in the presence of DNP-SG has been purified from rat canalicular membranes (53,54). This transporter binds ATP and, upon reconstitution into proteoliposomes, catalyzes ATP-dependent transport of DNP-SG. The molecular weight of this transporter suggests that it is distinct from Pgp, MRP2, and DNP-SG ATPase. The purified transporter could be phosphorylated by protein kinase C, and the phosphorylation enhanced both its DNP-SG stimulated ATPase activity and ATP-dependent DNP-SG transport following reconstitution. Another peptide with an Mr value of 37 kDa has been observed in rat liver canalicular membranes (88). Similar to the 90 kDa MOAT, this peptide is also immunologically similar to human erythrocyte DNP-SG ATPase, and it has been suggested that it may be a fragment of MOAT. MOAT appears to be present in TR^-/GY mutants indicating that it is distinct from the canalicular form of MRP (MRP2), the absence of which has been linked to TR^-/GY phenotype.

The ability of MRP to actively transport chemotherapeutic drugs, GSH-conjugates as well as the cholestatic conjugated estrogens points towards a functional interrelationship between DNP-SG ATPase of human erythrocytes and MRP. Likewise, the functional similarities between the hepatic transporters MRP2, MOAT, and erythrocyte DNP-SG ATPase suggest that these transporter proteins are members of closely related families. Thus, it appears that transporters of xenobiotics which constitute an important component of overall cellular detoxification mechanisms exist as family of enzymes, members of which have overlapping yet distinct catalytic properties. In this respect, detoxifying transporters resemble other detoxifying or biotransforming enzymes, e.g. CYP-P450 and GST. Several members of Pgp and MRP families with distinct structural and catalytic properties are already known, and other transporters with varying degrees of interrelatedness are perhaps waiting to be discovered.

4. FUTURE DIRECTIONS

Recent advances in molecular techniques have led to the identification of a number of genes for transporter proteins belonging to the ABC family both in prokaryotes and eukaryotes (89-92). These transporters are distinct from other ATP binding proteins such as inorganic cation pumps, and may be involved in active transport of structurally divergent xeno- and endobiotics. According to a recent study utilizing the expressed sequence tags database, the number of genes identified in the ABC gene family in humans now stands at 33 (92). Whether or not all these genes correspond to functional transporters of xenobiotics and are relevant to detoxification mechanisms remains to be determined. Whereas the primary structure of these transporters can be determined by approaches such as those described in (92), the usefulness of functional predictions based on sequence comparisons is limited. Transfection of cultured cells with cDNAs yields important functional information, but is not free from uncertainties, as discussed previously. It is therefore likely that a full functional characterization of the newly identified ABC transporters can be obtained only through a combination of molecular and transfection techniques with classical approaches of studying their catalytic and kinetic properties in isolated reconstituted systems. Recombinant proteins, especially expressed in bacterial systems, could be unsuitable for reconstitution studies because of posttranslational modifications that may be required for function. The usefulness of recombinant proteins needs therefore to be validated in each case, and in some situations fully functional, mature proteins can perhaps be obtained only by their purification from tissues. At present, concerted efforts are being made to characterize transporter proteins through molecular cloning, determination of mechanisms regulating their expression, and development of transgenic animals. These approaches were undoubtedly instrumental in an unparalleled advance in our knowledge of transport proteins. However, the molecular approaches have overshadowed the need for an understanding of the precise kinetic properties of these transporters. The latter information can be obtained by studies of reconstituted model systems obtained with purified transporters in the absence of interfering activities (93). Such knowledge, when coupled with information gained from cloning approaches, will synergistically enhance our understanding on the physiological roles of this important group of proteins.

5. ACKNOWLEDGMENT

Supported in part by grants GM32304 (YCA), CA63660 (SA), and VA Merit Review (PZ).

6. REFERENCES

1. R T Williams: Detoxication Mechanisms. In: The metabolism and detoxication of drugs toxic substances and other organic compounds. John Wiley & Sons, NY (1959).
2. M Ingleman-Sundberg, & I Johansson: The molecular genetics of the human drug metabolizing cytochrome P450. In: Advances in Drug Metabolism. Eds. Pacifici G.M. Fracchia G.N. European Commission Luxembourg, pp. 543-85 (1995).
3. D. R. Nelson, T. Kamataki, D. J. Waxman, F. P. Gungerich, R. W. Estabrook, R. Feyereisen, F. J Gonzalez, M. J. Coon, I. C. Gunsalus, O. Gotoh, K. Okuda & D. W. Nebert: The P450 superfamily, update on new sequences, gene mapping, accession numbers, early trivial names of enzymes and nomenclature. *DNA Cells Biol.* 12, 1-51 (1993).

4. J. D. Hayes & D. J. Pulford: The glutathione S-transferase supergene family: Regulation of GST and contribution of the isozymes to cancer chemoprevention and drug resistance. *Crit. Rev. Biochem. Mol. Biol.* 30, 445-600 (1995).
5. B. Mannervik & U. H. Danielson: Glutathione S-transferases structure and catalytic activity. *CRC Crit. Rev. Biochem.* 23, 283-337 (1988).
6. G M Pacifici & C De'Santi: Human Sulfotransferases. Classification and metabolic profile of the major isoforms. The point of view of the clinical pharmacologist: In: Advances in Drug Metabolism. Eds. Pacifici G M & Fraccihia G N) European Commission, Luxembourg, pp. 311-49 (1995).
7. G. J. Mulder: Glucuronidation and its role in regulation of biological activity of drugs. *Ann. Rev. Pharmacol. Toxicol.* 32, 25-49 (1992).
8. Y. C. Awasthi, R. Sharma & S. S. Singhal: Human glutathione S-transferases. *Int. J. Biochem.* 26, 295-308 (1994).
9. T. Ishikawa: ATP dependent glutathione S-conjugate transport pump. *Trends in Biochem. Sci.* 17, 463-8 (1992
10. T Ishikawa & K Akimaru: Transport of glutathione S-conjugates from cancer cells: Function and structure of GS-X pump. In: Glutathione S-transferases structure function and clinical implications. Eds: Vermeulen, N P E, Mulder, G J, Nieuwenhuyse, H, Peters, W H M, Vanbladeren, P J) Taylor & Francis, London, pp. 199-211 (1996).
11. P. Zimniak, S. Awasthi & Y. C. Awasthi: Phase III detoxification system. *Trends in Biochem. Sci.* 18, 164-6 (1993).
12. S. J. Foote, J. K. Thompson, A. F. Cowman & D. J. Kemp: Amplification of the multidrug resistance gene in some chloroquine-resistant isolates of P. falciparum. *Cell* 57, 921-30 (1989).
13. J. A. Endicott & V Ling: The biochemistry of P-glycoprotein-mediated multidrug resistance. *Ann. Rev. Biochem.* 58, 137-71 (1989).
14. S. P. C. Cole, G. Bharadwaj, J. H. Gerlach, J. E. Mackie, C. E. Grant, K. C. Almquist, A. J. Stewart, E. U. Kurz, A. M. V. Duncan & R. G. Deeley: Over expression of a transporter gene in a multidrug resistant cancer cell line. *Science* 258, 1650-4 (1992).
15. W. B. Jakoby: The glutathione S-transferases: A group of multifunctional detoxification proteins. *Adv. Enzymol.* 46, 383-414 (1978).
16. T. H. Rushmore & C. B. Pickett: Glutathione S-transferases, structure, regulation, and therapeutic implications. *J. Biol. Chem.* 268, 11475-8 (1993).
17. S. Tsuchida & K. Sato: Glutathione transferases and cancer. *CRC Crit. Rev. Biochem. Mol. Biol.* 27, 337-84 (1992.
18. T. M. Buetler & D. L. Eaton: (1992) Glutathione S-transferases: Amino acid sequence comparison, classification, and phylogenetic relationship. *Environ. Carcinogen. Ecotoxicol. Rev.*C10, 181-203 (1992).
19. K. V. Chin, I. Pastan & M. M. Gottesman: Function and regulation of human multidrug resistance gene. *Adv. Cancer Res.* 60, 157-80 (1993).
20. D. Lauitier, Y. Canitrot, R. G. Deeley & S. P. C. Cole: Multidrug resistance mediated by multidrug resistance protein (MRP) gene. *Biochem. Pharmacol.* 52, 967-77 (1996).
21. S. Awasthi, S. S. Singhal, S. K. Srivastava, P. Zimniak, K. K. Bajpai, M. Saxena, R. Sharma, S. A. III Ziller, E. Frenkel, S. V. Singh, N. G. He & Y. C. Awasthi: ATP-dependent transport of doxorubicin, daunomycin and vinblastine in human tissues by a mechanism distinct from the P-glycoprotein. *J. Clin. Invest.* 93, 958-65 (1994).
22. I. Leier, G. Jedlitschky, U. Bucholz, S. P. C. Cole, R. G. Deeley & D. Keepler: The MRP gene encodes an ATP-dependent export pump for leukotriene C_4 and structurally related conjugates. *J. Biol. Chem.* 269, 27807-10 (1994).
23. M. Muller, C. Meijer, G. J. R. Zaman, P. Borst, R. J. Scheper, N. H. Mulder & P. L. M. Jansen: Over expression of the gene encoding the multidrug resistance associated protein results in increased ATP-dependent glutathione S-conjugate transporter. *Proc. Nat. Acad. Sci. USA* 91, 13033-7 (1994).
24. G. Jedlitschy, I. Leier, U. Buchholz, K. Basnouin, G. Kurz & D. Keepler: Transport of glutathione, glucuronate, and sulfate conjugates by the MRP-gene encoded conjugate export pump. *Cancer Res.* 56, 988-94 (1996).
25. E. Beutler, O. Duron & B. M. Kelly: Improved method for determining blood glutathione. *J. Lab. Clin. Med.* 61, 882-90 (1963).
26. C. J. Marcus, W. H. Habig & W. B. Jakoby: Glutathione S-transferase from human erythrocytes, non-identity with the enzymes from liver. *Arch. Biochem. Biophys.* 188, 287-93 (1978).
27. Y. C. Awasthi & S. V. Singh: Purification and Characterization of a new form of glutathione S-transferase from human erythrocytes. *Biochem. Biophys. Res. Commun.* 125, 1053-60 (1984).
28. S. V. Singh, G. A. S. Ansari & Y. C. Awasthi: Anion exchange high-performance liquid chromatography of Glutathione S-transferases: Separation of the minor isoenzymes of human erythrocyte, heart, and lung. *J. Chromatogr.* 361, 337-45 (1986).
29. S. K. Srivastava, Y. C. Awasthi, S. P. Miller, A.Yoshida & E. Beutler: Studies on gamma-glutamyl transpeptidase in human and rabbit erythrocytes. *Blood.* 47, 645-50 (1976).
30. P. J. Van Bladern, A. Vander Gen, A., D. D. Briemer & G. R. Mohn: Stereoselective activation of vicinal dihalogen compounds by glutathione conjugation. *Biochem. Pharmacol.* 28, 2521-4 (1979).
31. R. J. Graves, C. Coutts & T. Green: Methylene chloride induced liver damage; an interspecies comparison. *Carcinogenesis* 16, 1919-26 (1995).
32. S. K. Srivastava & E. Beutler: The transport of oxidized form of glutathione from human erythrocytes. *J. Biol. Chem.* 244, 9-16 (1969).
33. T. Kondo, G. Dale & E. Beutler: Studies on glutathione transport utilizing inside out vesicles prepared from human erythrocytes. *Biochim. Biophys. Acta* 645, 132-6 (1981).
34. Y. C. Awasthi, H. S. Garg, D. D. Dao, C. A. Partridge & S. K. Srivastava: Enzymatic conjugation of

erythrocyte glutathione with 1-Chloro-2,4-dinitrobenzene: The fate of glutathione conjugate in erythrocytes and the effect of glutathione depletion on hemoglobin. *Blood.* 58, 733-8 (1981).
35. P. G. Board: Transport of glutathione S-conjugate from human erythrocytes. *FEBS Letts.* 124, 163-5 (1981).
36. Y. C. Awasthi, G. Misra, D. K. Rassin & S. K. Srivastava: Detoxification of xenobiotics by glutathione S-transferases erythrocytes: Transport of the conjugate of glutathione and 1-chloro-2,4-dinitrobenzene. *British J. Hematology.* 55, 419-25 (1983).
37. T. Kondo, M. Murao & M. Taniguchi: Glutathione S-conjugate transport using inside out vesicles from human erythrocytes. *Eur. J. Biochem.* 238, 551-4 (1982).
38. E. F. LaBelle, S. V. Singh, S. K. Srivastava & Y. C. Awasthi: Dinitrophenyl glutathione efflux from human erythrocytes is primary active ATP-dependent transport. *Biochem. J.* 238, 443-9 (1986).
39. E. G. Eckert & P. Eyer: Formation and transport of xenobiotic glutathione S-conjugate in red cells. *Biochem. Pharmacol.* 35, 325-9 (1986).
40. L. Pulaski & G. Bartosz:. Transport of bimane-S-glutathione conjugate in human erythrocytes. *Biochim. Biophys. Acta* 1268, 279-84 (1995).
41. L. Pulaski & G. Bartosz: Effect of inhibitors on the transport of dinitrophenyl-S-glutathione in human erythrocytes. *Biochem. Biol. Internat.* 36, 935-42 (1995).
42. T. P. Akerboom, G. Bartosz & H. Sies: Low and high K_m transport of dinitrophenyl glutathione in inside out vesicles from human erythrocytes. *Biochim. Biophys. Acta* 1103, 115-9 (1992).
43. G. Bartosz, H. Sies & T. P. Akerboom: Organic anions exhibit distinct inhibition patterns on the low-K_m and high-K_m transport of S-(2,4-dinitrphenyl) glutathione through the human erythrocyte membrane. *Biochem. J.* 291, 171-4 (1993).
44. E. F. LaBelle, S. V. Singh, S. K. Srivastava & Y. C. Awasthi: Evidence for different transport systems for oxidized glutathione and S-dinitrophenyl glutathione in human erythrocytes.*Biochem. Biophys. Res. Commun.* 139, 538-44 (1986).
45. S. S. Singhal, R. Sharma, S. Gupta, H. Ahmad, P. Zimniak, A. Radominska, R. Lester & Y. C. Awasthi: The anionic conjugates of bilirubin and bile acids stimulate ATP hydrolysis by S-(dinitrophenyl) glutathione ATPase of human erythrocyte. *FEBS Lett.* 281, 255-7 (1991).
46. K. Kumari, N. H. Ansari, S. Saxena, Y. C. Awasthi, & S. K. Srivastava: Low and high K_m forms of dinitrophenylglutathione-stimulated ATPase in bovine lens. *Exp. Eye Res.* 57, 243-7 (1993).
47. L. Pulaski, G. Jedlitschky, I. Leier & U. Bucholz: Identification of the multidrug resistance protein (MRP) as the glutathione S-conjugate export pump of erythrocytes. *Eur. J. Biochem.*241, 644-8 (1996).
48. T. Kondo, Y. Kawakami, N. Taniguchi & E. Beutler: Glutathione disulfide stimulated Mg2+ ATPase of human erythrocyte membrane. *Proc. Nat. Acad. Sci. USA* 84, 7373-7 (1987).
49. E.F. LaBelle, S. V. Singh, H. Ahmad, L. Wronski, S. K. Srivastava & Y.C. Awasthi: A novel dinitrophenyl glutathione stimulated ATPase is present in human erythrocyte membranes.*FEBS Lett.* 228, 53-6 (1988).
50. R. Sharma, S. Gupta, S. V. Singh, R. D. Medh, H. Ahmad, E. LaBelle & Y. C. Awasthi: Purification and characterization of dinitrophenylglutathione ATPase of human erythrocytes and its expression in other tissues. *Biochem. Biophys. Res. Commun.* 171, 155-61 (1990).
51. M. Saxena, S. S. Singhal, S. Awasthi, S. V. Singh, E. F. Labelle, P. Zimniak & Y. C. Awasthi: Dinitrophenyl S-glutathione ATPase purified from human muscle catalyzes ATP hydrolysis in the presence of leukotrienes. *Archives Biochem. Biophys.* 298, 231-7 (1992).
52. T. Kondo, K. Miyamoto, S. Gasa, N. Taniguchi & Y. Kawakami: Purification and characterization of glutathione disulfide-stimulated Mg2+-ATPase from human erythrocytes. *Biochem. Biophys. Res. Commun.* 162, 1-8 (1989).
53. S. Pikula, J. B. Hayden, S. Awasthi, Y. C. Awasthi & P. Zimniak: Organic anion-transporting ATPase of rat liver: I. Purification, photoaffinity labeling, and regulation by phosphorylation.*J. Biol. Chem.* 269, 27566-73 (1994).
54. S. Pikula, J. B. Hayden, S. Awasthi, Y. C. Awasthi & P. Zimniak: Organic anion-transporting ATPase of rat liver: II. Functional reconstitution of active transport and regulation by phosphorylation. *J. Biol. Chem.* 269, 27574-9 (1994).
55. I. C. West: What determines the substrate specificity of the multi-drug-resistance pump? *Trends Bio. Sci.* 15, 42-6 (1990).
56. T. Hirohashi, K. Niinuma, K. Ito, H. Suzuki, K. Kume, T. Shimizu & Y. Sugiyama: Characterization of ATP-dependent organic anion transport system on rat canalicular membrane maintained in EHBR. *Hepatology*, 24, 203A (1996).
57. K. Dano: Active outward transport of daunomycin in resistant Ehrlich ascites tumor cells. *Biochim. Biophys. Acta* 323, 466-83 (1973).
58. N. Kartner, D. Everden-Porelle, G. Bradley & V. Ling: Detection of P-glycoprotein in multidrug-resistant cell lines by monoclonal antibodies. *Nature (London)* 316, 820-3 (1985).
59. M. C. Willingham, M. M. M. Cornwell, C. O, Cardarelli, M. M. Gottesman & I. Pastan: Single cell analysis of daunomycin uptake and efflux in multidrug-resistant and -sensitive KB cells: effects of verapamil and other drugs. *Cancer Res.* 46, 5941-6 (1986).
60. M. Horio, M. M. Gottesman & I. Pastan: ATP-dependent transport of vinblastine in vesicles from human multidrug-resistant cells. *Proc. Nat. Acad. Sci. USA* 85, 3580-4 (1988).
61. C-J. Chen, E. Chin, K. Udea, D. Clark, I. Pastan, M. M. Gottesman & I. B. Robinson: Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug-resistant human cells. *Cell* 47, 381-9 (1986).
62. D. W. Loe, K. C. Almquist, R. G. Deeley & S. P. C. Cole: Multidrug resistance protein (MRP)-mediated transport of leukotriene C_4 and chemotherapeutic agents in membrane vesicles: Demonstration of glutathione

dependent vincristine transport. *J. Biol. Chem.* 271, 9675-82 (1996).
63. D. W. Loe, R. K. Stewart, T. E. Massey, R. G. Deeley & S. P. C. Cole: ATP-dependent transport of aflatoxin B_1 and its glutathione conjugates by the product of the multidrug resistance protein (MRP) gene. *Mol. Pharmacol.* 51, 1034-41 (1997).
64. S. R. Schlemner & F. M. Sirotnak: Functional studies of P-glycoprotein in inside out membrane vesicles derived from murine erythroleukemia cells over expressing MDR3. Properties and kinetics of the interaction of vinblastine with P-glycoprotein and evidence for its active mediated transport. *J. Biol. Chem.* 269, 31059-66 (1994).
65. F. J. Sharom, X. Yu & C. A. Doige: Functional reconstitution of drug transport and ATPase activity in proteoliposomes containing partially purified P-glycoprotein. *J. Biol. Chem.* 268, 24197-202 (1993).
66. S. V. Ambudkar: Purification and reconstitution of functional human P-glycoprotein. *Bioenerg. Biomembrane* 27, 23-9 (1995).
67. D. W. Loe, K. C. Almquist, S . P. C. Cole & R. G. Deeler: ATP-dependent 17 -estradiol 17(-D-glucuronide) transport by multidrug resistance protein: inhibition by cholestatic steroids. *J. Biol. Chem.* 271, 9683-9 (1996).
68. D. Keppler & I. M. Arias: Hepatic canalicular membrane. Introduction: Transport across the hepatocyte canalicular membrane. *FASEB J.* 11, 15-8 (1997).
69. S. Ruitz & P. Gross: Phosphatidylcholine translocase: a physiological role for mdr2 gene. *Cell* 77, 1071-83 (1994).
70. J. J. M. Smit, A. H. Schinkel, R. P. J. Oude Elfrink, A. K. Goren, E. Wagenaar, L. van Deemter, C. A. A. M. Mol, R. Ottenhoff, N. M. T. Vander Lugt, M. A. VanRoom, M. A. VanderValk, G. J. A. Ofterhaus, A. J. M. Borns & P. Borst, P. Homozygous disruption of mdr2 P-glycoprotein gene leads to a completed absence of phospholipid from bile and to liver disease. *Cell*75, 451-61 (1993).
71. I. M. Arias, M. Che, Z. Gatmaitan, C. Leveille, T. Nishida & M. St Pierre: The biology of the bile canaliculus. Hepatology, 17, 318-29 (1993).
72. M. Buchler, J. Konig, M. Brom, J. Kartenbeck, H. Spring, T. Horie & D. Keppler: cDNA cloning of the hepatocyte canalicular isoform of the multidrug resistance protein, cMrp, reveals a novel conjugate export pump deficient in hyperbilirubinemic mutant rats. *J. Biol. Chem.*, 271, 15091-8 (1996).
73. C. R. Leveille-Webster & I. M. Arias: The biology of the P-glycoproteins. *J. Membr. Biol.*, 109, 89-102 (1995).
74. C. C. Paulusma, P. J. Bosma, G. J. R. Zaman, C. T. M. Bakkar, M. Otter, G. L. Scheffer, R. J. Scheper, P. Borst & R. P. J. Ouede Elferink: Congenital jaundice in rats with a mutation in multidrug resistance associated protein. *Science* 271, 1126-8 (1996).
75. J. Kartenback, U. Lenschner, R. Mayer & D. Keppler: Absence of canalicular isoform of the MRP gene encoded conjugate export pump from the hepatocytes in Dubin-Johnson syndrom. *Hepatology* 23, 1061-6 (1966).
76. M. M. Gottesman: How cancer cells evade chemotherapy: Sixteenth Richard and Hinda Rosenthal Foundation Award Lecture. *Cancer Res.* 53, 747-754 (1993).
77. P. Borst & A. H. C. Schinkel: What have we learnt thus far from mice disrupted with P-glycoprotein genes. *Eur. J. Cancer* 32A, 985-990 (1996).
78. K. C. Almquist, D. W. Loe, D. R. Hipfner, J. E. Mackie, S. P. C. Cole & R. G. Deeley: Characterization of the 190 kDa multidrug resistance protein (MRP) in drug selected and transfected human tumor cells. *Cancer Res.* 55, 102-10 (1995).
79. I. Leier, G. Jedlitschky, U. Buchholz, M. Center, S. P. C. Cole, R. G. Deeley & D. Keppler: ATP-dependent glutathione disulfide transport mediated by the MRP gene-encoded conjugate export pump. *Biochem. J.* 314, 433-7 (1996).
80. S. P. C. Cole, K. E. Sparks, K. Fraser, D. W. Loe, C. E. Grant, G. M. Wilson & R. G. Deeley: Pharmacological characterization of multidrug resistant MRP-transfected cells. *Cancer Res.* 54, 5902-5910 (1994).
81. T. McGrath, C. Latoud, S. T. Arnold, A. R. Safa, R. L. Felsted & M. S. Center: Mechanisms of multidrug resistance in HL60 cells: Analysis of resistance associated membrane proteins and levels of MDR gene expression. *Biochem. Pharmacol.* 38, 3611-9 (1989).
82. S. P. C. Cole, H. F. Downes, & M. L. Slovak: Effect of calcium antagonists on the chemosensitivity of two multidrug resistant human tumor cell lines which do not over express P-glycoprotein. *Br. J. Cancer* 59, 42-6 (1989).
83. M. A. Barr, T. Rhodes, M. S. Center & P. R. Twentyman: Chemosensitization and drug accumulation effects of cyclosporin A., PCS-833 and verapamil in human MDR human large cell cancer cells over expressing a 190 k membrane protein distinct from P-glycoprotein. *Eur. J. Cancer* 20A, 408-15 (1993).
84. G. J. R. Zaman, J. Lankelma, O. van Tellingen, J. Beijnen, H. Dekker, C. Paulusma, R. P. J. Dude Elfernik, F. Bass & P. Borst: Role of glutathione in the export of compounds from cells by the multidrug-resistance associated protein. *Prot. Nat. Acad. Sci. USA* 92, 7690-7694 (1995).
85. J. J. Boxterman, M. Heijn & J. Lankelma: How does MRP/GS-X pump export doxorubicin? *J. Nat. Cancer Inst.* 88, 466-7 (1996).
86. Z. Hollo, L. Homolya, T., Hegedus & B. Sarkadi: Transport properties of the multidrug resistance-associated protein (MRP) in human tumour cells. *FEBS Lett.* 383, 99-104 (1996).
87. N. Feller, H. J. Boxterman, D. C. Wahrer & H. M. Pinedo: ATP-dependent efflux of calcein by the multidrug resistance protein (MRP): no inhibition by intracellular glutathione depletion.*FEBS Lett.* 368, 385-8 (1995).
88. P. Zimniak, S. A. Ziller III, I. Panfil, A. Radominska, H. Wolters, F. Kuipers, R. Sharma, M. Saxena, M. T. Moslen, M. Vore, R. Vonk, Y. C. Awasthi & R. Lester: Identification of an anion-transport ATPase that catalyzes glutathione conjugate-dependent ATP hydrolysis in canalicular plasma membranes from normal rats and rats with conjugated hyperbilirubinemia (GY mutant). *Archives Biochem. Biophys.* 292, 534-8 (1992).

89. C. F. Higgins: ABC transporters from microorganisms to man. *Ann. Rev. Cell Biol.* 8, 67-113 (1992).
90. N. Lomri, J. G. Fitz & B. J. Scharschmidt: Hepatocellular transport: Role of ATP-binding cassette proteins. *Seminars in Liver Disease* 16, 201-10 (1996).
91. M. J. Fath & R. Kolter: ABC transporters: Bacterial exporters. *Microbiol. Rev.* 57, 995-1017 (1993).
92. R. Allikmets, B. Gerrard, A. Hutchinson & M. Dean: Characterization of the human ABC superfamily: Isolation and mapping of 21 new genes using the expressed sequence tags database. *Human Molec. Genetics,* 5, 1649-55 (1996).
93. E. Racker, B. Violand, S. O'Neal, M. Alfonzo & J. Telford: Reconstitution, a way of biochemical research: some new approaches to membrane-bound enzymes. *Arch. Biochem. Biophys.,* 198, 470-477 (1979).