THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 16, Issue of April 19, pp. 9683–9689, 1996
Printed in U.S.A.
© 1996 by The American Society for Biochemistry and Molecular Biology, Inc.

ATP-dependent 17β-Estradiol 17-(β-D-Glucuronide) Transport by Multidrug Resistance Protein (MRP)

INHIBITION BY CHOLESTATIC STEROIDS*

(Received for publication, November 30, 1995, and in revised form, January 29, 1996)

Douglas W. Loe‡, Kurt C. Almquist‡§¶, Susan P. C. Cole‡§‖, and Roger G. Deeley‡§**

From the ‡Cancer Research Laboratories and §Department of Pathology, Queen's University, Kingston, Ontario, K7L 3N6 Canada

In addition to its ability to confer resistance to a range of natural product type chemotherapeutic agents, multidrug resistance protein (MRP) has been shown to transport the cysteinyl leukotriene, LTC_4, and several other glutathione (GSH) *S*-conjugates. We now demonstrate that its range of potential physiological substrates also includes cholestatic glucuronidated steroids. ATP dependent, osmotically sensitive transport of the naturally occurring conjugated estrogen, 17β-estradiol 17-(β-D-glucuronide) ($E_2$17βG), was readily demonstrable in plasma membrane vesicles from populations of MRP-transfected HeLa cells (V_{max} 1.4 nmol mg^{-1} min^{-1}, K_m 2.5 μM). The involvement of MRP was confirmed by demonstrating that transport was completely inhibited by a monoclonal antibody specific for an intracellular conformational epitope of the protein. MRP-mediated transport of LTC_4 was competitively inhibited by $E_2$17βG ($K_{i(app)}$ 22 μM), despite the lack of structural similarity between these two substrates. Competitive inhibition of [^{3}H]$E_2$17βG transport was also observed with a number of other cholestatic conjugated steroids. All of these compounds prevented photolabeling of MRP with [^{3}H]LTC_4, demonstrating that the cholestatic steroid and leukotriene conjugates compete either for the same or possibly overlapping sites on the protein. Consistent with the presence of overlapping but non-identical sites, studies using chemotherapeutic drugs to inhibit MRP-mediated $E_2$17βG transport indicated that daunorubicin had the highest relative potency of the drugs tested, whereas it was the least potent inhibitor of LTC_4 transport. Non-cholestatic steroids glucuronidated at the 3 position of the steroid nucleus, such as 17β-estradiol 3-(β-D-glucuronide), did not compete for transport of $E_2$17βG by MRP, nor did they inhibit photolabeling of the protein with [^{3}H]LTC_4. These data identify MRP as a potential transporter of cholestatic conjugated estrogens and demonstrate site-specific requirements for glucuronidation of the steroid nucleus.

Increased expression of multidrug resistance protein (MRP)[1] or its cognate mRNA has been found in drug selected cell lines from a wide range of tumor types (1–8). These cell lines have a phenotype similar in many respects to that conferred by P-glycoproteins. Transfection of MRP expression vectors into drug-sensitive cells has confirmed that MRP confers resistance to a spectrum of natural product type chemotherapeutic agents (9, 10). MRP, like the P-glycoproteins, is a member of the ATP-binding cassette superfamily of transmembrane transporters. However, unlike the P-glycoproteins, it has not been possible to demonstrate that MRP binds and actively transports unmodified forms of the drugs to which it confers resistance (11, 12, 55).

The ability of MRP to function as an ATP-dependent transporter has been investigated using plasma membrane vesicles prepared from drug-selected and MRP transfected cells (13–15, 55). These studies have shown that vesicles enriched in MRP display elevated levels of ATP-dependent, high affinity transport of cysteinyl leukotrienes. Evidence has also been presented that the protein can transport other organic glutathione conjugates (13, 14), as well as oxidized glutathione itself (16). In the accompanying article (55), we provide immunological confirmation that MRP is a primary active transporter of LTC_4 and demonstrate for the first time that in the presence of GSH, MRP can actively transport the *Vinca* alkaloid, vincristine.

ATP-dependent transport systems for organic GSH conjugates (17), GSSG (18), and other organic anions (19, 20) have been characterized functionally in a number of tissues. Studies with intact cells or membrane vesicle preparations indicate that at least one of the transporters involved, frequently referred to as the multispecific organic anion transporter (MOAT), has a broad substrate specificity that includes other organic anions in addition to glutathione conjugates (21). MOAT has been studied most extensively in bile canalicular membranes where it is believed to contribute to the hepatic clearance of a wide range of xenobiotic and endogenous organic anions, notably the glucuronidated conjugates of bile acids and bilirubin (22). The transporter is functionally defective in hepatocanalicular membranes of the TR^- mutant Wistar rat, the phenotype of which is similar to that observed in the human Dubin-Johnson syndrome (23, 24) and the mutant Corriedale

* This work was supported in part by a grant from the Medical Research Council of Canada (MRCC) (to S. P. C. C. and R. G. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Recipient of an MRCC graduate studentship.
‖ Career Scientist of the Ontario Cancer Foundation.
** Stauffer Research Professor of Queen's University. To whom correspondence and reprint requests should be addressed: Cancer Research Laboratories, Rm. 314, Botterell Hall, Queen's University, Kingston, Ontario, Canada K7L 3N6. Tel.: 613-545-2981; Fax: 613-545-6830.

[1] The abbreviations used are: MRP, multidrug resistance protein; AMP-PCP, β,γ-methyleneadenosine 5′-triphosphate; AMP-PNP, adenosine 5′-[β,γ-imido]triphosphate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); $E_2$17αE, 17α-ethinyl-17β-estradiol; $E_2$3$SO_4$17βG, 17β-estradiol 3-sulfato-17-(β-D-glucuronide); $E_2$3βG, 17β-estradiol 3-(β-D-glucuronide); $E_3$3βG, 16α,17β-estriol 3-(β-D-glucuronide); $E_3$16αG, 16α,17β-estriol 16-(β-D-glucuronide); $E_2$17βG, 17β-estradiol 17-(β-D-glucuronide); $E_3$17βG, 16α,17β-estriol 17-(β-D-glucuronide); glycolithocholate-3-sulfate, 3α-hydroxy-5β-cholan-24-oic acid *N*-[carboxymethyl-]amide 3-sulfate; LTC_4, leukotriene C_4; mAb, monoclonal antibody; MOAT, multispecific organic anion transporter; PAGE, polyacrylamide gel electrophoresis.

This is an Open Access article under the CC BY license.

sheep (21, 25). All of these congenital conditions are associated with chronic conjugated hyperbilirubinemia. In the TR^- rat model, although hepatocanalicular MOAT activity is markedly reduced, organic anion transport in other tissues appears to be normal (26). Whether this indicates the existence of structurally distinct hepatic and non-hepatic forms of the transporter, or is attributable to a liver-specific defect in its expression has not been established.

Recent immunohistological data indicate that subcellular localization of MRP, or an MRP-related protein, may be abnormal in the livers of TR^- rats (27). This suggests that MRP could be the transporter functionally described as MOAT. However, although MRP mRNA can be detected in both rodent and human liver, its levels are low when compared with other tissues such as muscle, testes, heart, and lung (1, 28–30). To further define the substrate specificity of MRP, we have used plasma membrane vesicles from drug-selected and MRP-transfected cells, to examine ATP dependent transport of [^{3}H]17β-estradiol 17-(β-D-glucuronide) ($E_2$17βG). This estradiol metabolite is formed in the liver and subsequently excreted into bile (31, 32). Increases in the levels of $E_2$17βG and some other conjugated estrogens have been implicated in the development of cholestasis during the later stages of pregnancy, a condition which occurs with exceptional frequency in the Dubin-Johnson syndrome (33). Consequently, the potential for other known cholestatic and non-cholestatic compounds to inhibit MRP-mediated $E_2$17βG transport has also been determined. Finally, we have examined the relative abilities of several natural product drugs to which MRP confers resistance to inhibit transport of the conjugated steroid and determined the influence of GSH and glucuronate on their potencies.

EXPERIMENTAL PROCEDURES

Materials—$E_2$17βG, $E_2$3βG, $E_3$3βG, $E_3$16αG, $E_3$17βG, $E_2$3$SO_4$17βG, glycolithocholate-3-sulfate, $E_2$17αE, taurocholic acid, glycocholic acid, LTC_4, AMP, AMP-PNP, AMP-PCP, ATPγS, GTP, CTP, and UTP were purchased from Sigma. ATP was purchased from ICN Biochemicals (St. Laurent, PQ, Canada). Chemotherapeutic agents were obtained as described (55). Creatine phosphate and creatine kinase were purchased from Boehringer Mannheim (Dorval, PQ, Canada). [14,15,19,20-^{3}H]LTC_4 (128 Ci mmol^{-1}) and [6,7-^{3}H]$E_2$17βG (49 Ci mmol^{-1}) were purchased from DuPont NEN (Mississauga, Ontario, Canada) and Amplify™ was from Amersham (Oakville, Ontario, Canada). All other reagents were from Sigma. Isolation and purification of the murine MRP-specific mAbs (QCRL-1 and -3) used in this study have been described (34).

Cell Culture—The human small cell lung cancer cell line, H69, the doxorubicin-selected multidrug-resistant cell line, H69AR, and the drug-sensitive revertant cell line, H69PR, have been described (35, 36). The production and maintenance of MRP transfected (T14) or vector transfected (C6) HeLa cell populations were as described (12). The 8226/Dox40 myeloma cell line was provided by Dr. W. Dalton (Arizona Cancer Center, Tucson, AZ) (37). All cell lines were cultured in RPMI 1640 medium supplemented with 4 mM L-glutamine and 5% defined bovine calf serum (HyClone Laboratories, Logan, UT), in the absence of antibiotics.

Membrane Vesicle Preparation and Transport of $E_2$17βG—Plasma membrane vesicles were prepared as described (38, 39, 55). ATP-dependent transport of $E_2$17βG into membrane vesicles was measured at 37 °C in a 120-μl reaction volume containing 20–40 μg of vesicle protein in the presence of [^{3}H]$E_2$17βG (50 nM; 80 nCi/reaction). At indicated times, 20-μl samples were removed, diluted into 1 ml of ice-cold transport buffer (50 mM Tris-HCl, 250 mM sucrose, pH 7.5), and filtered under vacuum through glass fiber filters (type A/E; Gelman Sciences, Montreal, PQ) presoaked in transport buffer. Filters were washed twice with 5 ml of transport buffer, dried, and subjected to liquid scintillation counting. All data were corrected for the amount of [^{3}H]$E_2$17βG which remained bound to the filter in the absence of vesicle protein (usually <5% of the total radioactivity).

Inhibition of Photoaffinity Labeling of MRP with [^{3}H]LTC_4 by Conjugated Steroids—Vesicle membrane proteins (150 μg) were photoaffinity labeled with [^{3}H]LTC_4 (0.5 μCi; 78 nM) as described (55) in the presence of various concentrations of $E_2$17βG, or other steroid derivatives. Radiolabeled vesicles (100 μg of membrane protein) were solubilized in Laemmli's buffer, analyzed on a 7% gel by SDS-PAGE, and subjected to fluorography.

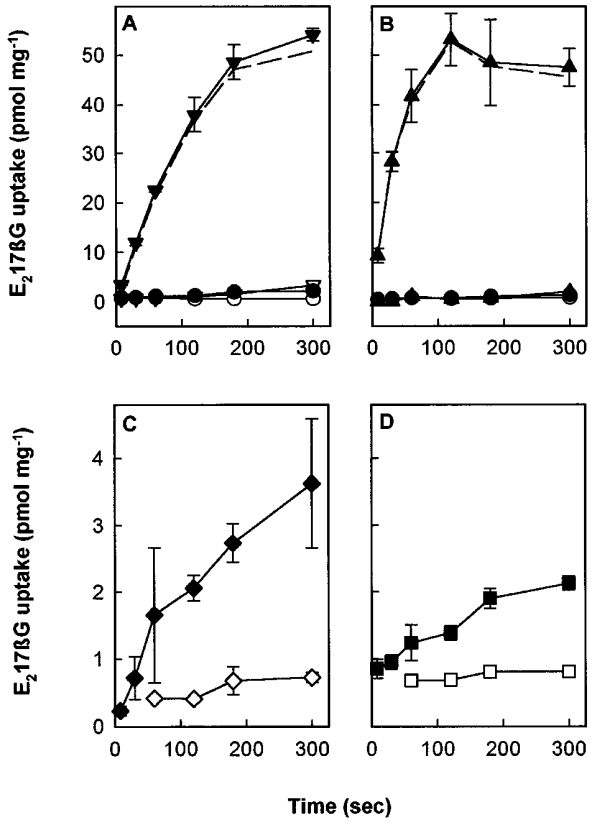

FIG. 1. **Time course of [^{3}H]$E_2$17βG uptake by membrane vesicles from MRP-transfected HeLa cells (T14), control HeLa (C6) cells, drug-sensitive H69 cells, multidrug-resistant H69AR cells, revertant H69PR cells, and P-glycoprotein overexpressing 8226/Dox40 cells.** Membrane vesicles were incubated at 37 °C in transport buffer containing [^{3}H]$E_2$17βG (50 nM, specific activity 13 Ci mmol^{-1}) and ATP (4 mM) (*closed symbols*) or AMP (4 mM) (*open symbols*) for the times indicated. Vesicles were derived from the following cells, as described under "Experimental Procedures:" *Panel A,* HeLa C6 (○, ●) and T14 (▽, ▼); *Panel B*, H69 (○, ●) and H69AR (△,▲); *Panel C*, H69PR (◇, ♦); *Panel D*, 8226/Dox40 (□, ■). The *broken curves* in *Panels A* and *B* indicate ATP-dependent uptake for T14 and H69AR cells, respectively. Data points represent the means (± S.E.) of triplicate determinations in a typical experiment.

RESULTS

*ATP-dependent Transport of $E_2$17βG*—The kinetics and ATP dependence of [^{3}H]$E_2$17βG accumulation by vesicles prepared from MRP-transfected HeLa T14 cells are shown in Fig. 1*A*. ATP-dependent uptake was linear up to 60 s and approached steady-state after 180 s. During the linear phase, the rate of uptake at 37 °C was approximately 22 pmol mg protein^{-1} min^{-1} at an initial concentration of 50 nM [^{3}H]$E_2$17βG and the rate increased linearly with the amount of membrane protein up to 40 μg (data not shown). [^{3}H]$E_2$17βG uptake in the presence of AMP rather than ATP was approximately 1 pmol mg^{-1} min^{-1}. At the same initial substrate concentration, the rate of [^{3}H]LTC_4 uptake by T14 vesicles was 150–200 pmol mg^{-1} min^{-1} at 23 °C. No ATP dependence of [^{3}H]$E_2$17βG uptake could be demonstrated with vesicles from control C6 cells.

Correlation between ATP-dependent [^{3}H]$E_2$17βG transport rates and levels of MRP expression was examined using vesicles isolated from drug-sensitive H69, drug-resistant H69AR, and revertant H69PR cells. The rate of ATP-dependent [^{3}H]$E_2$17βG uptake in H69AR vesicles (60 pmol mg^{-1} min^{-1}) was approximately 3-fold higher than for T14 vesicles (Fig. 1*B*), consistent with the 3–4-fold higher levels of MRP in H69AR

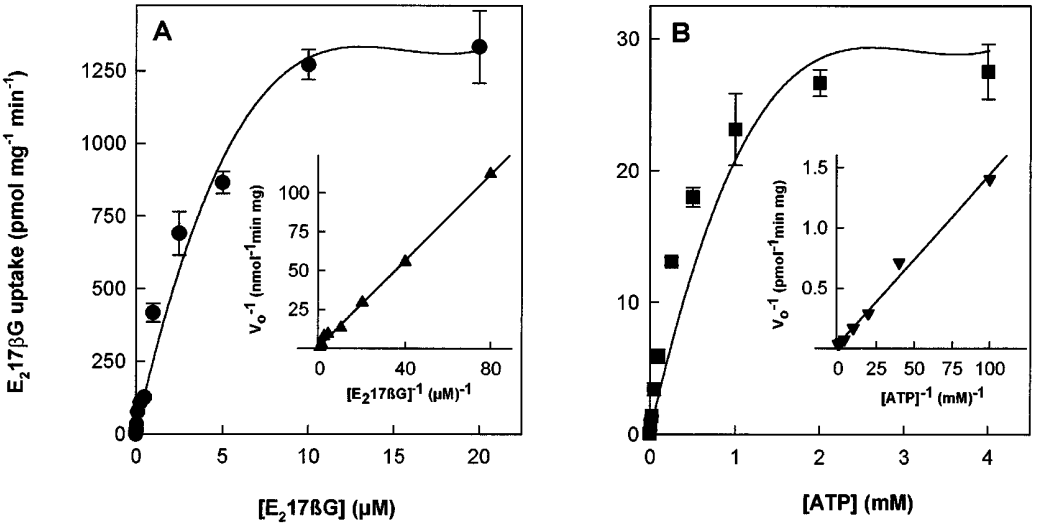

FIG. 2. **Effect of [^{3}H]$E_2$17βG and ATP concentration on [^{3}H]$E_2$17βG uptake by T14 vesicles.** *Panel A*, the rate of ATP dependent [^{3}H]$E_2$17βG uptake by T14 membrane vesicles was measured at various $E_2$17βG concentrations (12.5 nM to 25 µM) for up to 60 s at 37 °C in the presence of a fixed concentration of nucleotide (4 mM), as described under "Experimental Procedures." Data were plotted as V_o *versus* [S] to confirm that the concentration range selected was appropriate to observe both zero-order and first-order rate kinetics. Kinetic parameters (K_m 2.5 µM and V_{max} 1.4 nmol mg^{-1} min^{-1}) were determined from regression analysis of the Lineweaver-Burk transformation of the data (*inset*). *Panel B*, ATP-dependent uptake of [^{3}H]$E_2$17βG was measured as described for *Panel A* at various concentrations of nucleotide (1 to 4000 µM) in the presence of a fixed concentration of [^{3}H]$E_2$17βG (50 nM). An apparent K_m of 390 µM for ATP was determined from regression analysis of the Lineweaver-Burk data transformation (*inset*).

cells (12) and vesicle preparations (data not shown). No ATP dependence of [^{3}H]$E_2$17βG uptake could be demonstrated with H69 vesicles. The rate of ATP-dependent [^{3}H]$E_2$17βG uptake in H69PR membrane vesicles was approximately 1% that of H69AR cells (Fig. 1*C*). This is consistent with the low, but detectable levels of MRP mRNA (1) and protein (34) in these cells. To examine the ability of P-glycoprotein to transport $E_2$17βG, we used vesicles from the multidrug-resistant myeloma cell line, 8226/Dox40, which overexpresses P-glycoprotein (37) and has MRP levels comparable to those in H69PR cells (data not shown). Vesicles from these cells exhibited levels of [^{3}H]$E_2$17βG transport no higher than those of H69PR-derived vesicles (Fig. 1*D*). Thus the data are consistent with $E_2$17βG transport by 8226/Dox40 vesicles being attributable to their low levels of MRP. They also indicate that there is little if any transport of $E_2$17βG by P-glycoprotein.

Kinetic Parameters of [^{3}H]$E_2$17βG Transport in HeLa T14 Vesicles—Rates of ATP-dependent uptake were determined at several concentrations of $E_2$17βG (12.5 nM to 25 µM) and used to calculate K_m and V_{max} for transport by T14 membrane vesicles. A Lineweaver-Burk plot of the data yielded an apparent K_m of 2.5 µM for $E_2$17βG and a V_{max} of 1.4 nmol mg^{-1} min^{-1} (Fig. 2*A*, *inset*). An apparent K_m for ATP of 390 µM was also determined by measuring initial rates of [^{3}H]$E_2$17βG uptake at 60 s in the presence of different concentrations of nucleotide (1–4000 µM) (Fig. 2*B, inset*).

*Osmotic Sensitivity and Nucleotide Specificity of [^{3}H]$E_2$17βG Transport by T14 Membrane Vesicles*—The rate of [^{3}H]$E_2$17βG uptake in T14 vesicles was osmotically-sensitive and decreased linearly with increasing extravesicular sucrose concentration between 0.25 and 1.0 M (Fig. 3*A*). Steady-state levels of accumulation showed similar osmotic sensitivity (data not shown) confirming that the increase in vesicle associated [^{3}H]$E_2$17βG reflected transport into the vesicle lumen rather than surface binding.

Nucleotide dependent transport was not detectable when AMP-PNP, AMP-PCP, or ATPγS (4 mM) were substituted for ATP, thus indicating a requirement for ATP hydrolysis (Fig. 3*B*). As expected when compared with other nucleotide triphosphates, ATP supported the highest rate of [^{3}H]$E_2$17βG transport. However, the rate of transport in the presence of GTP was substantial and approached approximately 70% of that achievable in the presence of ATP.

Inhibition of Transport by MRP Specific mAbs—To obtain additional evidence of a direct involvement of MRP in the transport of $E_2$17βG, we examined the ability of two MRP specific mAbs to inhibit this process. mAb QCRL-3, which is specific for an intracellular conformation-dependent epitope of MRP (34), completely inhibited [^{3}H]$E_2$17βG uptake at a concentration of 20 µg ml^{-1}. In contrast, the same concentration of mAb QCRL-1, which recognizes a linear intracellular epitope of MRP (34), had no significant effect. Isotype control immunoglobulins (mouse IgG_1, IgG_{2a}, and total IgG) were also without effect at concentrations of 70 µg ml^{-1}.

Inhibition of [^{3}H]LTC_4 Transport by Estradiol Glucuronides—To determine whether $E_2$17βG and LTC_4 interact with the same or overlapping sites on the protein, we examined the ability of $E_2$17βG, and its structural isomer, $E_2$3βG, to inhibit ATP-dependent [^{3}H]LTC_4 transport by T14 vesicles. $E_2$17βG acted as a competitive inhibitor, with an apparent K_i of 22 µM (Fig. 4, *upper panel*). In contrast, the non-cholestatic estrogen conjugate, $E_2$3βG, did not inhibit [^{3}H]$E_2$17βG transport at concentrations up to 100 µM (Fig. 4, *lower panel*). These data suggest that LTC_4 and $E_2$17βG interact with the same or overlapping sites on MRP. They also demonstrate that interaction of the estrogen glucuronides with MRP displays specificity with respect to the position of the glucuronide moiety on the steroid nucleus.

*Inhibition of [^{3}H]$E_2$17βG Transport by Various Steroid Glucuronides and Bile Salt Derivatives*—$E_2$17βG and other steroid D-ring glucuronide conjugates have been shown to inhibit bile flow and to cause a reversible type of cholestasis (31–33). In contrast, steroid glucuronide conjugates of the A ring such as $E_2$3βG are not cholestatic (40, and references cited therein). Consequently, we compared the cholestatic potency of various estriol, estradiol, and bile salt derivatives with their ability to inhibit [^{3}H]$E_2$17βG transport by T14 membrane vesicles. Glucuronic acid itself was not inhibitory up to 5 mM and did not augment the modest inhibition (20%) of [^{3}H]$E_2$17βG transport by 17β-estradiol (10 µM) (Table I). Of the estrogen conjugates tested, 17-(β-D-glucuronides) of estradiol and estriol and the

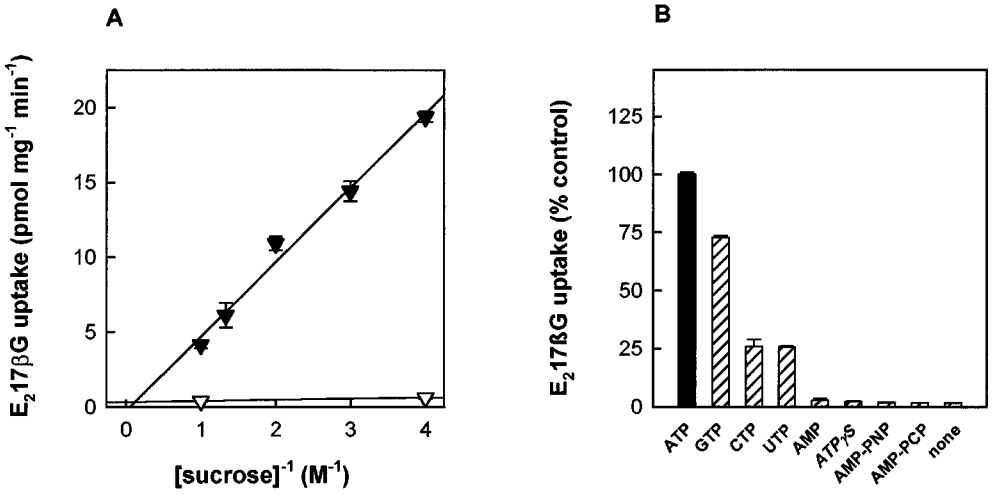

FIG. 3. **Osmotic sensitivity and nucleotide specificity of [^{3}H]$E_2$17βG transport by T14 membrane vesicles.** *Panel A*, T14 membrane vesicles were preincubated for 10 min in transport buffer containing sucrose at concentrations ranging from 250 to 1000 mM. Rates of [^{3}H]$E_2$17βG uptake at 37 °C under various conditions of osmolarity were measured at a substrate concentration of 50 nM in the presence of 4 mM AMP (▽) or ATP (▼), as described under "Experimental Procedures." *Panel B*, rates of [^{3}H]$E_2$17βG uptake were determined at 37 °C, in the presence of various hydrolyzable and non-hydrolyzable nucleotides (4 mM), as described under "Experimental Procedures" except that the nucleoside triphosphate regenerating system was omitted. The rate observed in the presence of 4 mM ATP was not affected by omission of the regenerating system (data not shown). The rates of uptake supported by various nucleotides have been expressed as a percentage of that obtained with ATP. The results shown are the means (± S.E.) of triplicate determinations in a single experiment. The rate of ATP-dependent uptake in the control was 31.7 pmol mg^{-1} min^{-1}.

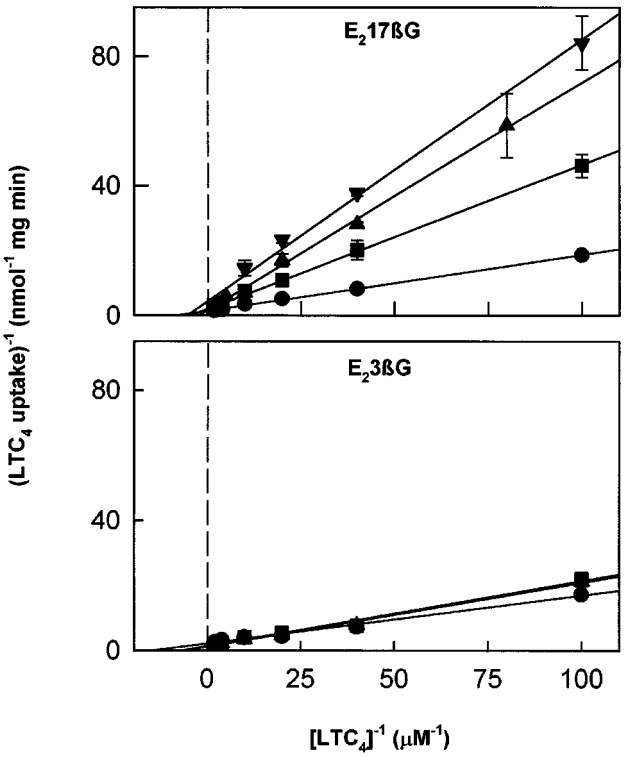

FIG. 4. **Effect of glucuronidated estradiols on [^{3}H]LTC_4 uptake by T14 membrane vesicles.** The rates of [^{3}H]LTC_4 uptake by T14 vesicles were determined at 23 °C, at various substrate concentrations (25 to 1000 nM) in the absence (●) or presence (■, 20 μM; ▲, 40 μM; ▼, 100 μM) of $E_2$17βG (*upper panel*) or $E_2$3βG (*lower panel*). Double-reciprocal plots were generated for each concentration of potential inhibitor and used to determine K_i. The results shown are the means (±S.E.) of triplicate determinations at each substrate and inhibitor concentration.

3-sulfate derivative of $E_2$17βG were the most potent inhibitors of transport (approximately 90% inhibition or greater at 10 μM). In contrast, the 3-(β-D-glucuronides) of estradiol and estriol were not inhibitory and only modest inhibition was observed with $E_3$16αG. However, the cholestatic bile salt glycolithocholate-3-sulfate was a potent inhibitor of transport (94% inhibition at 10 μM) while glycocholate and taurocholate (which are not derivatized at the 3-position of the A ring) were far less inhibitory (30 and 35% at 10 μM, respectively). The 17α-substituted synthetic estrogen, 17α-ethinyl-17β-estradiol, was not inhibitory.

TABLE I

Inhibition of [^{3}H]$E_2$17βG transport by various steroid glucuronides and bile salt derivatives

T14 membrane vesicles were incubated with 50 nM [^{3}H]$E_2$17βG in transport buffer for 60 s at 37 °C in the presence of glucuronic acid (1–5 mM), 17β-estradiol ± glucuronic acid (1 and 5 mM), LTC_4, or various steroid conjugates at 10 μM as shown. Transport was measured as described and ATP-dependent [^{3}H]$E_2$17βG uptake was determined and plotted as % control uptake in the absence of inhibitors. Data represent means of triplicate determinations (± S.E.) for a single experiment. The control rate of [^{3}H]$E_2$17βG uptake in this experiment was 22.5 pmol mg^{-1} min^{-1}.

Compound	Concentration	$E_2$17βG uptake
	μM	*% control*
None (control)		100
Glucuronic acid	1000	98 ± 7
	5000	108 ± 1
17β-Estradiol	10	80 ± 3
+1 mM glucuronate	10	96 ± 1
+5 mM glucuronate	10	114 ± 7
Taurocholic acid	10	65 ± 2
	100	22 ± 1
Glycocholic acid	10	70 ± 2
	100	13 ± 1
Leukotriene C_4	0.1	71 ± 1
	1	7 ± 0.1
17α-Ethinyl-17β-estradiol	10	105 ± 8
17β-Estradiol 3-(β-D-glucuronide)	10	97 ± 1
16α,17β-Estriol 3-(β-D-glucuronide)	10	102 ± 5
16α,17β-Estriol 16-(β-D-glucuronide)	10	80 ± 1
16α,17β-Estriol 17-(β-D-glucuronide)	10	12 ± 0.2
17β-Estradiol 3-sulfato-17-(β-D-glucuronide)	10	4 ± 0.3
Glycolithocholate 3-sulfate	10	6 ± 0.3

Lineweaver-Burk plots of [^{3}H]$E_2$17βG uptake by T14 membrane vesicles indicated that LTC_4 behaved as a competitive inhibitor of [^{3}H]$E_2$17βG transport, with a $K_{i(app)}$ of 0.53 μM (Fig. 5). Inhibition by $E_3$17βG ($K_{i(app)}$ 1.4 μM) and the choles-

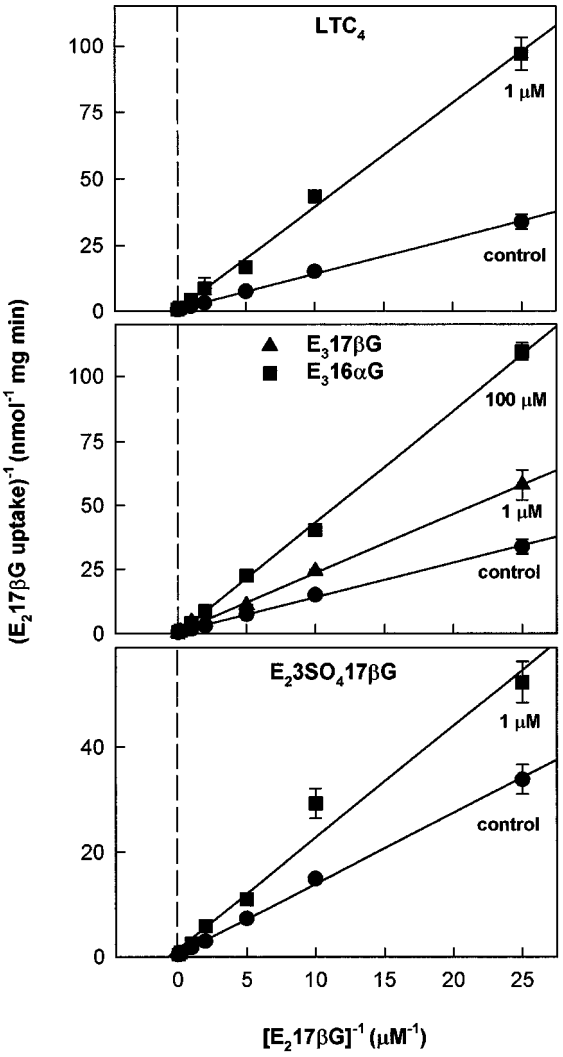

FIG. 5. **Effect of LTC_4, steroid glucuronides, and bile salt derivatives on transport of [^{3}H]$E_2$17βG by T14 membranes.** The rates of uptake of [^{3}H]$E_2$17βG by T14 vesicles were determined at 37 °C, at various substrate concentrations (0.05–25 μM) in the absence or presence of the indicated concentration of LTC_4 (1 μM) (*upper panel*), $E_3$17βG (1 μM) or $E_3$16αG (100 μM) (*middle panel*), and $E_2$3$SO_4$17βG (1 μM) (*lower panel*). Double reciprocal plots were generated for each inhibitor and used to determine a K_i. Results shown are the means (±S.E.) of triplicates determinations at each substrate and inhibitor concentration.

tatic bile salt glycolithocholate-3-sulfate ($K_{i(app)}$ 1.4 μM) (data not shown) was also competitive. $E_3$16αG, which is of intermediate cholestatic potency (40), was a less effective competitive inhibitor of transport, with a $K_{i(app)}$ of 45 μM. Although non-cholestatic in rodents, $E_2$3$SO_4$17βG, was also an effective competitive inhibitor of $E_2$17βG transport by MRP ($K_{i(app)}$ 1.7 μM).

Photolabeling of Membrane Proteins with [^{3}H]LTC_4 and Inhibition of Labeling by Steroid Glucuronides—To confirm the results of inhibition studies, we determined the ability of various steroid derivatives to compete for [^{3}H]LTC_4 binding to MRP (55). $E_2$17βG inhibited photolabeling of MRP by [^{3}H]LTC_4 in a concentration-dependent manner, with a IC_{50} of approximately 20 μM (Fig. 6*A*). We also tested the ability of a single concentration (100 μM) of cholestatic and non-cholestatic steroid glucuronides and bile salts to inhibit [^{3}H]LTC_4 binding. Consistent with the results of transport studies, photolabeling was not inhibited by non-cholestatic $E_2$3βG and $E_3$3βG, but was abolished by the cholestatic steroids, glycolithocholate-3-sulfate, $E_2$17βG, $E_2$3$SO_4$17βG, and $E_3$17βG (Fig. 6*B*).

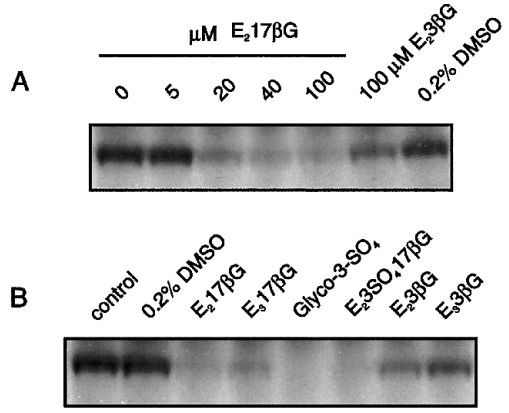

FIG. 6. **Photoaffinity labeling of T14 membrane vesicles by [^{3}H]LTC_4 and inhibition of labeling by $E_2$17βG and other steroid glucuronides.** *Panel A*, T14 vesicles (150 μg of membrane protein) were incubated with [^{3}H]LTC_4 (50 nM) alone or in the presence of various concentrations of $E_2$17βG (5–100 μM) or 100 μM $E_2$3βG, as indicated. Samples were irradiated at 312 nm prior to being subjected to SDS-PAGE and fluorography, as described (55). *Panel B*, photoaffinity labeling of T14 membrane vesicles with [^{3}H]LTC_4 was carried out as above in the presence of various glucuronides and bile salt derivatives (100 μM), as indicated. Photolabeled membranes were analyzed by SDS-PAGE and fluorography. *DMSO*, dimethyl sulfoxide.

*Inhibition of [^{3}H]$E_2$17βG Transport by Chemotherapeutic Agents*—Examples of each of the three major classes of natural product type drugs to which MRP confers resistance were compared for their ability to inhibit [^{3}H]$E_2$17βG transport by T14 vesicles (Fig. 7, *upper panel*). VP-16 and vincristine (100 μM) inhibited transport by 50–60%. Vinblastine and the anthracyclines, daunorubicin and doxorubicin, inhibited transport by 80–90% at the same concentration. Determination of the concentration of drug (in the absence of GSH) required to inhibit [^{3}H]$E_2$17βG transport by 50% indicated that daunorubicin (IC_{50} approximately 8 μM) was more potent than doxorubicin (IC_{50} approximately 50 μM), vinblastine (IC_{50} approximately 30 μM), and vincristine (IC_{50} approximately 70 μM) (Fig. 7, *lower panel*). Physiological concentrations of GSH enhance the inhibitory effect of vincristine and vinblastine (and to a lesser extent VP-16) on MRP dependent transport of [^{3}H]LTC_4 (55). Consequently, we examined whether a similar effect was observed with [^{3}H]$E_2$17βG. GSH (1 mM) increased inhibition from approximately 50 to 75% and from 60 to 90%, for VP-16 and vincristine, respectively, but it did not enhance transport inhibition by vinblastine or the anthracyclines (Fig. 7, *upper panel*). In contrast, glucuronic acid (5 mM) could not substitute for GSH and did not enhance inhibition by any of the drugs tested (Fig. 7, *upper panel*).

DISCUSSION

MRP is expressed in a wide range of normal tissues (1, 28–30) and is overexpressed in many multidrug-resistant cell lines (1, 3, 5, 6, 41). Elevated levels of MRP in membrane vesicles from drug-selected cell lines and MRP transfectants have been correlated with increased ATP-dependent transport of cysteinyl leukotrienes and other glutathione *S*-conjugates (13–15). It has been suggested on the basis of these data, that one of the physiological roles of MRP is that of a GSH conjugate or GS-X pump (42, 43). However, the ability to transport certain glutathione conjugates does not explain the drug cross-resistance profile of MRP-transfected or selected cells (1, 5, 12). Current data indicate that the major classes of drugs to which MRP confers resistance are not metabolized via GSH conjugation (44), although a GSH-dependent transport mechanism may exist for some compounds (55). Other conjugation pathways, such as glucuronidation, appear more important for the

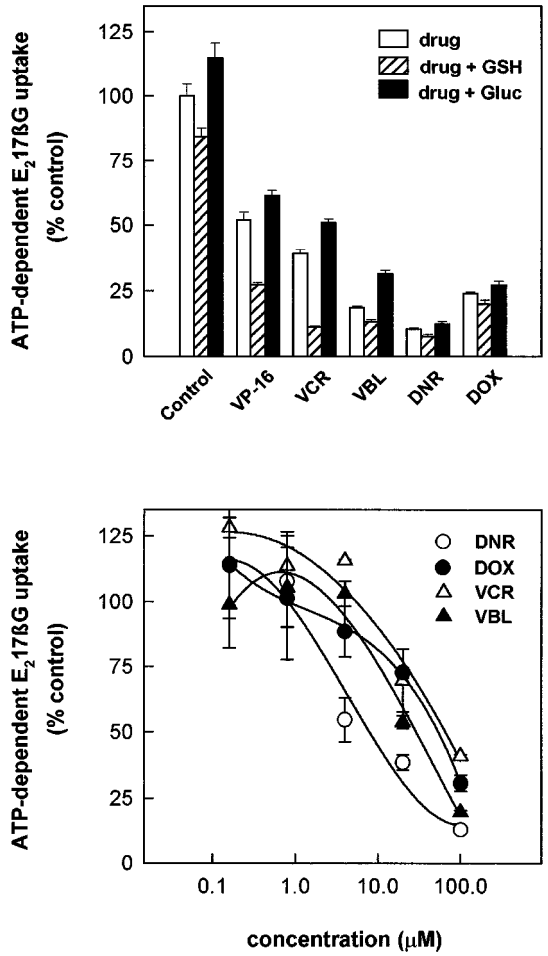

FIG. 7. **Effects of chemotherapeutic agents on [^{3}H]$E_2$17βG uptake by T14 vesicles in the absence or presence of GSH or glucuronic acid.** *Upper panel*, the rate of uptake of [^{3}H]$E_2$17βG (50 nM) by T14 vesicles was determined at 37 °C in the presence of various natural product drugs (100 μM) alone (*open bars*) and in combination with GSH (1 mM) (*hatched bars*) or glucuronic acid (5 mM) (*solid bars*). Results are expressed as a percentage of the control value obtained in the absence of drug and anion. The drug vehicle (dimethyl sulfoxide) was present at a concentration of 0.5% in control incubations lacking drug and had no effect on uptake under the conditions of the assay. The results are the means (±S.E.) of triplicate determinations in a single experiment. The control uptake rate in this experiment was 19.2 pmol mg^{-1} min^{-1}. *DOX*, doxorubicin; *DNR*, daunorubicin; *VBL*, vinblastine; *VCR*, vincristine. *Lower panel*, inhibition of [^{3}H]$E_2$17βG uptake by T14 membrane vesicles by various concentrations of DNR (○), DOX (●), VCR (△), or VBL (▲). Data points represent means of duplicate determinations in a single experiment and are plotted as a percentage of control values obtained in the absence of drug, but in the presence of 0.5% (v/v) dimethyl sulfoxide. The control uptake rate in the experiment shown was 25.8 pmol mg^{-1} min^{-1}.

detoxification of natural product drugs such as VP-16 (45), at least in the liver, where glucuronidation is a major biotransformation pathway for steroid hormones and bile salts. Our data demonstrate that some glucuronide conjugates are potential substrates for MRP.

The rates of ATP-dependent transport of $E_2$17βG by membrane vesicles from MRP-transfected HeLa T14 cells were more than 20-fold higher than vesicles from cells transfected with vector alone. In addition, the rates of $E_2$17βG transport obtained with vesicles from previously characterized H69, H69AR, and H69PR small cell lung cancer cell lines correlate well with their levels of MRP (1, 34). Several other lines of evidence confirm that MRP is the primary active transporter involved. 1) A conformation dependent, MRP-specific mAb that inhibits LTC_4 transport by MRP (55), also inhibits $E_2$17βG transport; 2) $E_2$17βG and LTC_4 compete for ATP-dependent transport by MRP enriched vesicles; and 3) $E_2$17βG blocks the photolabeling of MRP by [^{3}H]LTC_4 in a concentration-dependent manner. Thus LTC_4 and $E_2$17βG appear to interact with similar or at least mutually exclusive sites on MRP.

Despite the ability of MRP to transport anionic compounds with no apparent structural similarity, studies with steroid conjugates reported here indicate that substrate affinity can be markedly influenced by the site of anionic conjugation. In human liver, $E_2$17βG and $E_2$3βG are formed in approximately equal amounts (46, 47). However, only the former is cholestatic. It has been suggested that one mechanism by which some estrogen D-ring conjugates may diminish bile flow is by competing for transport by one or more of the ATP-dependent hepatocanalicular transport proteins. Whether or not MRP is the transporter involved remains to be firmly established. However, the substrate specificity of MRP correlates well with the cholestatic potential of A-ring and D-ring steroid glucuronides. Non-cholestatic $E_2$3βG does not inhibit [^{3}H]LTC_4 transport by MRP nor does it inhibit photolabeling of the protein. Similarly, $E_2$3βG and $E_3$3βG do not compete for [^{3}H]$E_2$17βG transport. Thus A-ring glucuronidation is insufficient to result in detectable interaction with MRP. Alternative forms of anionic modification of the A-ring, such as sulfation, also have little effect on the inhibitory potency of 17-(β-D-glucuronides) (*e.g.* $E_2$3$SO_4$17βG has a K_i of 1.7 μM with respect to $E_2$17βG transport). In contrast, sulfation of the A-ring of bile salts may enhance inhibitory potency, since glycocholic acid itself is a relatively weak inhibitor of $E_2$17βG transport (30% inhibition at 10 μM) compared with the cholestatic bile salt glycolithocholate-3-sulfate (approximately 95% inhibition at 10 μM). More surprising, given the major structural differences between some MRP substrates, is the finding that inhibitory potency is sensitive to the position of glucuronidation within the D-ring itself. The K_i of the moderately cholestatic glucuronide, $E_3$16αG, is more than 20-fold higher than that of $E_3$17βG. These studies demonstrate that the protein can be highly selective with respect to the site of anionic conjugation of some substrates and provide information of potential use in designing agents capable of blocking MRP function. The behavior of the synthetic estrogen, 17α-ethinyl-17β-estradiol is an exception to the correlation between cholestatic potential and the ability to inhibit MRP-dependent transport. This compound does not inhibit transport of $E_2$17βG *in vitro* but is cholestatic. However, it is thought that the glucuronide of this synthetic estrogen, produced in the liver, rather than the parent compound is responsible for the cholestasis (33, 48).

It has been suggested previously that ATP-dependent hepatocanalicular transport of $E_2$17βG is attributable to P-glycoprotein (32). This suggestion stems from studies with two drug-selected cell lines known to overexpress P-glycoprotein, which displayed approximately 5-fold increased resistance to cytotoxic concentrations of $E_2$17βG and a 2–3-fold decrease in accumulation of the compound. We have found that $E_2$17βG transport in vesicles from the P-glycoprotein overexpressing myeloma cell line, 8226/Dox40, is approximately 20- and 100-fold lower than in vesicles from MRP transfected T14 and drug-selected H69AR cells, respectively. Moreover, the drug-resistant myeloma cells also express low but sufficient levels of MRP to account for the low level of $E_2$17βG transport observed. These data combined with recent reports of multidrug-resistant cell lines that express elevated levels of both P-glycoprotein and MRP (8, 49, 50) suggest that MRP rather than P-glycoprotein is responsible for the previously observed transport of $E_2$17βG (32).

Direct binding and transport of chemotherapeutic drugs by

MRP has not been demonstrated, but 30–60% decreases in MRP-dependent LTC_4 transport have been observed in high concentrations (approximately 100 μM) of certain chemotherapeutic agents (15, 55). With some drugs (*e.g.* vincristine and vinblastine), we have shown that inhibition is potentiated by physiological concentrations of GSH and have demonstrated ATP/GSH-dependent transport of vincristine using T14 vesicles (55). Data presented here demonstrate similar inhibition of $E_2$17βG transport with several chemotherapeutic agents and potentiation of inhibition by vincristine in the presence of GSH. A possible explanation of this behavior is that inhibition and/or transport is enhanced as a result of independent interactions of GSH and the *Vinca* alkaloids with distinct regions of a composite binding site on MRP. Consequently, we determined whether the anionic substituents of known MRP substrates, other than GSH, might act in a similar fashion. However, no potentiation of inhibition was observed in the presence of glucuronate with any of the drugs tested and the anion also failed to enhance inhibition $E_2$17βG transport by 17β-estradiol. Inorganic sulfate was also without effect (data not shown). Thus the ability to enhance inhibition and/or transport appears to be GSH-specific, rather than a general property of the known anionic substituents of MRP substrates. In addition, although all drugs tested inhibited $E_2$17βG and LTC_4 transport, their relative potencies differ. For example, daunorubicin is the most potent inhibitor of $E_2$17βG transport while it is the least potent inhibitor of LTC_4 transport (51, 55). Similar differences in the relative potency of various inhibitors have been observed previously in the transport and/or binding of different substrates by P-glycoprotein (52, 53). One interpretation is that interaction of potential substrates or inhibitors with both proteins may occur via multiple, overlapping but not identical sites.

Data presented here combined with those of previous studies demonstrate that the substrate specificities of MRP and MOAT overlap extensively (27, 21, 40, 54). Our studies on the transport of steroid glucuronides are also consistent with a potential role for MRP in bile formation and in the development of cholestasis. However, it remains to be established whether MOAT is a single protein or whether functionally similar but structurally distinct tissue-specific forms exist. With detailed knowledge of the substrate specificities of MRP and the availability of mAbs capable of specifically inhibiting its function, it should soon be possible to determine whether MRP and MOAT are the same protein or different functionally related transporters.

Acknowledgments—We thank D. R. Hipfner for providing MRP-specific monoclonal antibodies and Dr. C. E. Grant for providing HeLa C6 and T14 transfected cells. We also thank Drs. D. Keppler and I. Leier for helpful discussions. The technical assistance of K. Sparks and K. Fraser is gratefully acknowledged.

REFERENCES

1. Cole, S. P. C., Bhardwaj, G., Gerlach, J. H., Mackie, J. E., Grant, C. E., Almquist, K. C., Stewart, A. J., Kurz, E. U., Duncan, A. M. V., and Deeley, R. G. (1992) *Science* **258,** 1650–1654
2. Schneider, E., Cowan, K. H., Bader, H., Toomey, S., Schwartz, G. N., Karp, J. E., Burke, P. J., and Kaufmann, S. H. (1995) *Blood* **85,** 186–193
3. Krishnamachary, N., Ma, L., Zheng, L., Safa, A. R., and Center, M. S. (1994) *Oncol. Res.* **6,** 119–127
4. Barrand, M. A., Heppell-Parton, A. C., Wright, K. A., Rabbitts, P. H., and Twentyman, P. R. (1994) *J. Natl. Cancer Inst.* **86,** 110–117
5. Cole, S. P. C., and Deeley, R. G. (1996) in *Drug Resistance* (Hait, W. N., ed) Kluwer Academic, in press
6. Slovak, M. L., Ho, J. P., Bhardwaj, G., Kurz, E. U., Deeley, R. G., and Cole, S. P. C. (1993) *Cancer Res.* **53,** 3221–3225
7. Eijdems, E. W. H. M., De Haas, M., Coco-Martin, J. M., Ottenheim, C. P. E., Zaman, G. J. R., Dauwerse, H. G., Breuning, M. H., Twentyman, P. R., Borst, P., and Baas, F. (1995) *Int. J. Cancer* **60,** 676–684
8. Brock, I., Hipfner, D. R., Nielsen, B. S., Jensen, P. B., Deeley, R. G., Cole, S. P. C., and Sehested, M. (1995) *Cancer Res.* **55,** 459–462
9. Grant, C. E., Valdimarsson, G., Hipfner, D. R., Almquist, K. C., Cole, S. P. C., and Deeley, R. G. (1994) *Cancer Res.* **54,** 357–361
10. Zaman, G. J. R., Flens, M. J., van Leusden, M. R., De Haas, M., Mulder, H. S., Lankelma, J., Pinedo, H. M., Scheper, R. J., Baas, F., Broxterman, H. J., and Borst, P. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 8822–8826
11. McGrath, T., Latoud, C., Arnold, S. T., Safa, A. R., Felsted, R. L., and Center, M. S. (1989) *Biochem. Pharmacol.* **38,** 3611–3619
12. Cole, S. P. C., Sparks, K. E., Fraser, K., Loe, D. W., Grant, C. E., Wilson, G. M., and Deeley, R. G. (1994) *Cancer Res.* **54,** 5902–5910
13. Leier, I., Jedlitschky, G., Buchholz, U., Cole, S. P. C., Deeley, R. G., and Keppler, D. (1994) *J. Biol. Chem.* **269,** 27807–27810
14. Jedlitschky, G., Leier, I., Buchholz, U., Center, M., and Keppler, D. (1994) *Cancer Res.* **54,** 4833–4836
15. Muller, M., Meijer, C., Zaman, G. J. R., Borst, P., Scheper, R. J., Mulder, N. H., DeVries, E. G. E., and Jansen, P. L. M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 13033–13037
16. Leier, I., Jedlitschky, G., Buchholz, U., Center, M., Cole, S. P. C., Deeley, R. G., and Keppler, D. (1996) *Biochem. J.* **314,** 433–437
17. Kobayashi, K., Sogame, Y., Hara, H., and Hayashi, K. (1990) *J. Biol. Chem.* **265,** 7737–7741
18. Oude Elferink, R. P. J., Ottenhoff, R., Liefting, W. G. M., Schoemaker, B., Groen, A. K., and Jansen, P. L. M. (1990) *Am. J. Physiol.* **258,** G699–G706
19. Steen, H., Merema, M., and Meijer, D. K. F. (1992) *Biochem. Pharmacol.* **44,** 2323–2331
20. Sathirakul, K., Suzuki, H., Yamada, T., Hanano, M., and Sugiyama, Y. (1994) *J. Pharmacol. Exp. Ther.* **268,** 65–73
21. Oude Elferink, R. P. J., and Jansen, P. L. M. (1994) *Pharmacol. Ther.* **64,** 77–97
22. Nishida, T., Gatmaitan, Z., Roy-Chowdhry, J., and Arias, I. M. (1992) *J. Clin. Invest.* **90,** 2130–2135
23. Kitamura, T., Jansen, P., Hardenbrook, C., Kamimoto, Y., Gatmaitan, Z., and Arias, I. M. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 3557–3561
24. Nishida, T., Hardenbrook, C., Gatmaitan, Z., and Arias, I. M. (1992) *Am. J. Physiol.* **262,** G629–G635
25. Kitamura, T., Alroy, J., Gatmaitan, Z., Inoue, M., Mikami, T., Jansen, P., and Arias, I. M. (1992) *Hepatology* **15,** 1154–1159
26. Board, P., Nishida, T., Gatmaitan, Z., Che, M., and Arias, I. M. (1992) *Hepatology* **15,** 722–725
27. Mayer, R., Kartenbeck, J., Buchler, M., Jedlitschky, G., Leier, I., and Keppler, D. (1995) *J. Cell Biol.* **131,** 137–150
28. Stride, B. D., Valdimarsson, G., Gerlach, J. H., Cole, S. P. C., and Deeley, R. G. (1996) *Mol. Pharmacol.*, in press
29. Kruh, G. D., Gaughan, K. T., Godwin, A., and Chan, A. (1995) *J. Natl. Cancer Inst.* **87,** 1256–1258
30. Zaman, G. J. R., Versantvoort, C. H. M., Smit, J. J. M., Eijdems, E. W. H. M., De Haas, M., Smith, A. J., Broxterman, H. J., Mulder, N. H., de Vries, E. G. E., Baas, F., and Borst, P. (1993) *Cancer Res.* **53,** 1747–1750
31. Vore, M. (1993) *Toxicol. Appl. Pharmacol.* **118,** 2–7
32. Gosland, M., Tsuboi, C., Hoffman, T., Goodin, S., and Vore, M. (1993) *Cancer Res.* **53,** 5382–5385
33. Vore, M., and Slikker, W. (1985) *Trends Pharmacol. Sci.* **6,** 256–259
34. Hipfner, D. R., Gauldie, S. D., Deeley, R. G., and Cole, S. P. C. (1994) *Cancer Res.* **54,** 5788–5792
35. Mirski, S. E. L., Gerlach, J. H., and Cole, S. P. C. (1987) *Cancer Res.* **47,** 2594–2598
36. Cole, S. P. C., Pinkoski, M. J., Bhardwaj, G., and Deeley, R. G. (1992) *Br. J. Cancer* **65,** 498–502
37. Dalton, W. S., Durie, B. G. M., Alberts, D. S., Gerlach, J. H., and Cress, A. E. (1986) *Cancer Res.* **46,** 5125–5130
38. Doige, C. A., and Sharom, F. J. (1992) *Biochim. Biophys. Acta* **1109,** 161–171
39. Almquist, K. C., Loe, D. W., Hipfner, D. R., Mackie, J. E., Cole, S. P. C., and Deeley, R. G. (1995) *Cancer Res.* **55,** 102–110
40. Oude Elferink, R. P. J., Meijer, D. K. F., Kuipers, F., Jansen, P. L. M., Groen, A. K., and Groothuis, G. M. M. (1995) *Biochim. Biophys. Acta* **1241,** 215–268
41. Slapak, C. A., Fracasso, P. M., Martell, R. L., Toppmeyer, D. L., Lecerf, J.-M., and Levy, S. B. (1994) *Cancer Res.* **54,** 5607–5613
42. Ishikawa, T., Wright, C. D., and Ishizuka, H. (1994) *J. Biol. Chem.* **269,** 29085–29093
43. Ishikawa, T. (1992) *Trends Biochem. Sci.* **17,** 463–468
44. Tew, K. D. (1994) *Cancer Res.* **54,** 4313–4320
45. Hande, K., Anthony, L., Hamilton, R., Bennett, R., Sweetman, B., and Branch, R. (1988) *Cancer Res.* **48,** 1829–1834
46. Lucier, G. W., and McDaniel, O. S. (1977) *J. Steroid Biochem.* **8,** 867–872
47. Vore, M. (1987) *Gastroenterology* **93,** 643–649
48. Ebner, T., Remmel, R. P., and Burchell, B. (1993) *Mol. Pharmacol.* **43,** 649–654
49. Slapak, C. A., Mizunuma, N., and Kufe, D. W. (1994) *Blood* **84,** 3113–3121
50. Hasegawa, S., Abe, T., Naito, S., Kotoh, S., Kumazawa, J., Hipfner, D. R., Deeley, R. G., Cole, S. P. C., and Kuwano, M. (1995) *Br. J. Cancer* **71,** 907–913
51. Ishikawa, T., Muller, M., Klunemann, C., Schaub, T., and Keppler, D. (1990) *J. Biol. Chem.* **265,** 19279–19286
52. Tamai, I., and Safa, A. R. (1991) *J. Biol. Chem.* **266,** 16796–16800
53. Tamai, I., and Safa, A. R. (1990) *J. Biol. Chem.* **265,** 16509–16513
54. Oude Elferink, R. P. J., Ottenhoff, R., Liefting, W., de Haan, J., and Jansen, P. L. M. (1989) *J. Clin. Invest.* **84,** 476–483
55. Loe, D. W., Almquist, K. C., Deeley, R. G., and Cole, S. P. C., (1996) *J. Biol. Chem.* **271,** 9675–9682