ATP-Dependent Transport of Lipophilic Cytotoxic Drugs by Membrane Vesicles Prepared from MRP-Overexpressing HL60/ADR Cells†

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ABSTRACT: MRP is an ATP-binding cassette family transporter that confers cellular resistance to a variety of natural product cytotoxic agents. However, the biochemical mechanism by which MRP confers resistance has not been established. To gain insight into its mechanism of action, the *in vitro* substrate specificity of MRP was examined by analyzing drug uptake into membrane vesicles prepared from MRP-overexpressing HL60/ADR cells. Compared to control HL60 membrane vesicles, HL60/ADR membrane vesicles exhibited markedly enhanced ATP-dependent transport of daunorubicin, etoposide, and vincristine. In contrast, little or no increased uptake was observed for vinblastine and Taxol. This pattern of *in vitro* substrate specificity was consistent with the analysis of the HL60/ADR drug resistance phenotype, which revealed substantial levels of resistance to anthracyclines, etoposide, and vincristine, but only slightly increased resistance to vinblastine and Taxol. Drug transport into HL60/ADR membrane vesicles was osmotically sensitive and dependent on ATP concentration, with a K_m value of 45 μM for ATP. Lineweaver−Burk analysis indicated that substrate transport was concentration-dependent, with apparent K_m values of 6.1, 5.7, and 5.5 μM for daunorubicin, etoposide, and vincristine, respectively. The P-glycoprotein-modulating agents cyclosporin A, PSC833, and verapamil, which have modest reversing effects on MRP-overexpressing cell lines, were weak competitive inhibitors of daunorubicin transport, with K_i values of 35, 84, and 95 μM, respectively. In addition, the glutathione analog azidophenacyl-glutathione, oxidized glutathione, and the LTD_4 antagonist MK571 were competitive inhibitors of daunorubicin transport, with K_i values of 69, 31, and 3.0 μM, respectively. Genistein, an MRP-specific modulating agent, and arsenate, a compound for which MRP has previously been reported to confer resistance, were also competitive inhibitors, with K_i values of 17 and 29 μM, respectively. These results are consistent with a previous report in which we demonstrated that HL60/ADR membrane vesicles transport azidophenacylglutathione and that transport of this agent is competitively inhibited by daunorubicin, vincristine, and etoposide [Shen et al., (1966) *Biochemistry 35*, 5719−5725]. Together, these uptake studies performed with HL60/ADR membrane vesicles constitute a consistent body of evidence that indicates that MRP transports both glutathione S conjugates and unaltered natural product drugs and support the idea that the direct transport of unaltered lipophilic cytotoxic drugs is the predominant biochemical mechanism whereby MRP confers multidrug resistance.

Drug resistant cancer cells can display a multidrug resistant phenotype that involves simultaneous resistance to a spectrum of structurally and functionally distinct natural product agents, including anthracyclines, vinca alkaloids, epipodophyllotoxins, and dactinomycin. One form of multidrug resistance results from expression of P-glycoprotein, the 170-kDa product of the MDR1 gene. P-Glycoprotein, an ATP-binding cassette (ABC)[1] family transporter, functions as an ATP-dependent efflux pump that reduces the intracellular concentrations of chemotherapeutic agents by transporting them across the plasma membrane (Endicott & Ling, 1989; Gottesman & Pastan, 1993). Recently, MRP, another ABC transporter, was isolated from a drug resistant cell line (Cole et al., 1992). We and others have reported transfection studies that have established that MRP confers a multidrug resistant phenotype (Grant et al., 1994; Kruh et al., 1994), the drug resistance profile of which is similar but not identical to that conferred by P-glycoprotein (Breuninger et al., 1995; Cole et al., 1994; Zaman et al., 1994). However, the biochemical mechanism by which MRP confers resistance has not been established. The detection of increased drug efflux in MRP transfectants (Breuninger et al., 1995; Cole et al., 1994; Zaman et al., 1994) and drug-selected MRP-overexpressing cell lines (Coley et al., 1991; McGrath & Center, 1988; Slovak et al., 1988; Versantvoort et al., 1992) suggests that MRP may function as a pump for unaltered natural product cytotoxic drugs. However, the possibility that MRP confers resistance by transporting either the anionic metabolites or glutathione conjugates of lipophilic drugs, as opposed to transporting unaltered drugs, has been considered on the basis of recent reports that MRP transports drug−glutathione conjugates such as azidophenacylglutathione (APA-SG), DNP-SG, and the endogenous glutathione-

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[1] Abbreviations: APA-SG, azidophenacylglutathione; DNP-SG, dinitrophenacylglutathione; LTC_4, leukotriene C_4; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; ABC, ATP-binding cassette.

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conjugated leukotriene LTC_4 (Jedlitschky et al., 1994; Leier et al., 1994a; Muller et al., 1994; Shen et al., 1996).

We previously examined the effect of MRP expression on the cellular and *in vitro* transport of glutathione conjugates in MRP-overexpressing cells and membrane vesicle preparations (Shen et al., 1996). In that study, we reported that MRP confers enhanced cellular efflux of glutathione conjugates and that membrane vesicles prepared from MRP-overexpressing HL60/ADR cells display increased ATP-dependent transport of the glutathione analog azidophenacylglutathione. In addition, we found that transport of APA-SG in MRP-enriched membrane vesicles was competitively inhibited by several natural product cytotoxic drugs. The later observation suggested that the substrate specificity of MRP might include lipophilic cytotoxic drugs as well as glutathione conjugates. In the present study, we directly examined whether MRP transports natural product cytotoxic drugs. We demonstrate that MRP-enriched membrane vesicles prepared from HL60/ADR cells transport a spectrum of natural product cytotoxic drugs, including anthracyclines, vinca alkaloids, and epipodophyllotoxins. The present study thus supports the idea that the direct transport of unaltered lipophilic cytotoxic drugs is the predominant biochemical mechanism whereby MRP confers multidrug resistance.

EXPERIMENTAL PROCEDURES

Materials. Antipain, creatine phosphate, ATP, 5′-AMP, pepstatin A, benzamidine, RNase A, DNase I, verapamil, genistein, oxidized glutathione (GSSG), and *S*-(*p*-azidophenylacyl)glutathione (APA-SG) were purchased from Sigma. Creatine kinase, leupeptin A, and aprotinin were purchased from Boehringer Mannheim. [^{3}H(G)]Daunomycin (3.7 Ci/mmol) was purchased from DuPont-NEN. [^{3}H]Etoposide (900 mCi/mmol), [^{3}H]vinblastine sulfate (9 Ci/mmol), and [^{3}H]Taxol (7.4 Ci/mmol) were purchased from Moravek Biochemicals, and [^{3}H]vincristine sulfate (10.6 Ci/mmol) was purchased from Amersham. Arsenate was purchased from J. T. Baker. Cyclosporin A and PSC833 were generously provided by Sandoz Pharmaceuticals. MK571 was a generous gift of A. W. Ford-Hutchinson (Merck-Frost Center for Therapeutic Research, Quebec, Canada).

Preparation of Membrane Vesicles. Membrane vesicles were prepared as described (Ishikawa et al., 1990; Leier et al., 1994b). Briefly, cells were pelleted by centrifugation at 1200*g* for 10 min and washed twice in ice-cold phosphate-buffered saline (PBS). The cell pellet was diluted 40-fold with hypotonic buffer [0.5 mM sodium phosphate (pH 7.0) and 0.1 mM EGTA] supplemented with protease inhibitors (2 mM PMSF, 0.5 μg/mL leupeptin, 2 μg/mL aprotinin, 200 μg/mL benzamidine, 1 μg/mL pepstatin A, and 50 μg/mL antipain), 25 μg/mL RNase A, and 50 μg/mL DNase I and gently stirred on ice for 2 h. The cell lysate was centrifuged at 100000*g* for 40 min, and the pellet was resuspended in 20 mL of hypotonic buffer and homogenized with a Potter-Elvehjem homogenizer. The resulting homogenate was diluted with incubation buffer (250 mM sucrose/10 mM Tris-HCl at pH 7.4) supplemented with protease inhibitors and centrifuged at 12000*g* for 10 min. The postnuclear supernatant was kept on ice, and the pellet was resuspended in 20 mL of incubation buffer supplemented with protease inhibitors and homogenized and centrifuged at 12000*g* as described above. The two post nuclear supernatants were combined and centrifuged at 100000*g* for 40 min. The pellets were resuspended in 20 mL of incubation buffer and manually homogenized with a tight-fitting Dounce homogenizer. After dilution with 10 mL of incubation buffer, the suspension was layered onto 38% sucrose/5 mM Hepes-KOH (pH 7.4), and centrifuged in a Beckman SW41 swinging bucket rotor at 280000*g* for 2 h. The interphase was collected, diluted in 20 mL of incubation buffer and homogenized with a Dounce homogenizer. The resulting suspension was centrifuged at 100000*g* for 40 min, and the pellet was resuspended in 1 mL of incubation buffer. All operations were carried out at 4 °C.

Membrane vesicles were formed by passing the final membrane suspension through a 27 gauge needle 20 times. Ten milliliters of the vesicle suspension was mixed with 2.5 mL of Con-A Sepharose equilibrated with 160 mM Tris-HCl (pH 7.4) and incubated at 4 °C for 30 min with gentle shaking. The Con-A Sepharose was removed by centrifugation at 1400*g* for 5 min, and the inside-out membrane vesicles in the supernatant were collected by centrifugation at 100000*g* for 30 min. The pellet was suspended in incubation buffer and stored at −80 °C. The purity of membrane vesicles was assayed using marker enzyme activities as described (Leier et al., 1994b).

Analysis of Drug Uptake by Membrane Vesicles. Drug uptake by membrane vesicles was measured by the rapid filtration technique (Horio et al., 1988). Inverted membrane vesicles were quickly thawed at 37 °C shortly before use and kept on ice. Radiolabeled drugs were mixed with ATP reaction buffer [10 mM Tris-HCl (pH 7.5)/4 mM ATP/10 mM $MgCl_2$/10 mM creatine phosphate/0.25 M sucrose/100 μg/mL creatine kinase], and membrane vesicles (20 μg of protein) were added. The suspension was incubated at 37 °C and 20 μL aliquots were removed at various time points and diluted in 1.0 mL of ice-cold incubation buffer. The suspensions were immediately applied to 0.45 μM Millipore HAWP nitrocellulose filters (presoaked in incubation buffer) under suction and washed twice with 5.0 mL of ice-cold incubation buffer. The filters were dissolved in scintillation fluid, and the radioactivity was measured in a Beckman liquid scintillation counter. In parallel control experiments, ATP was replaced with 4 mM 5′-AMP. ATP-dependent transport was calculated by subtracting the uptake values obtained with 5′-AMP from those obtained with ATP. Osmotic sensitivity experiments were performed by incubating the membrane vesicles in incubation buffer containing various concentrations of sucrose for 15 min at 25 °C. Drug uptake was then measured in the presence of either ATP or 5′-AMP as described above, except that the sucrose concentration of the ATP reaction buffer was adjusted to that of the incubation buffer. For Lineweaver−Burk analyses and sucrose inhibition studies, initial reaction rates measured at 1 min were used. All experiments were performed in triplicate. A simple regression curve fit program (Cricket Graph) was used to obtain the K_m values for various substrates.

Immunoblot Analysis. Fifty microgram protein samples were separated on 6% SDS−polyacrylamide gels and transferred to nitrocellulose membranes (Millipore, Bedford, MA). Filters were blocked in PBS containing 0.1% Tween-20 (PBST) and 5% bovine serum albumin, and MRP was detected using MRPmAb-1, a monoclonal antibody generated against amino acids 609−761 of the protein (Paul et al., 1996) and a secondary anti-mouse antibody. P-Glycoprotein

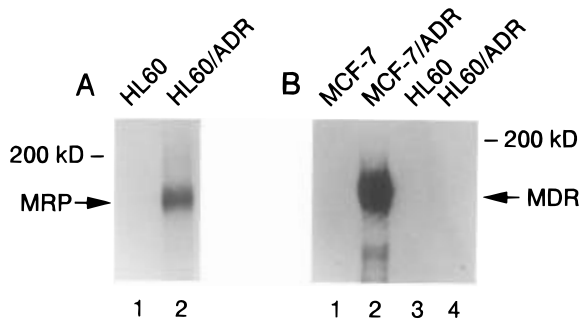

FIGURE 1: Immunoblot analysis of MRP in inside-out membrane vesicles prepared from HL60 and HL60/ADR cells. (A) Detection of MRP in inside-out membrane vesicles prepared from HL60 (1) and HL60/ADR (2) cells. (B) Detection of P-glycoprotein in inside-out membrane vesicles prepared from HL60 (3) and HL60/ADR (4) cells. Cell lysates prepared from MCF-7 (1) and P-glycoprotein-overexpressing MCF-7/ADR cells (2) are included as controls. Fifty microgram samples of membrane vesicles or lysates were separated by 6% SDS−PAGE. MRP and P-glycoprotein were detected with anti-MRP or anti-P-glycoprotein monoclonal antibodies, respectively, as described in Experimental Procedures. The position of the 200 kDa molecular mass marker is shown. The products of the MRP and MDR1 genes are indicated by arrows.

was detected using monoclonal antibody C219 (Signet Laboratories). Blots were washed several times with PBST before and after the secondary antibody treatment and three times in PBS alone before development with an Amersham ECL chemiluminescence kit.

Drug Sensitivity Analysis. The drug sensitivities of HL60 and HL60/ADR cells were analyzed using the MTT colorimetric assay, as previously described (Campling et al., 1991). Cells were plated in triplicate in 96-well dishes and after overnight growth were treated with various concentrations of cytotoxic drugs. The colorimetric assay was performed after growth in drug-containing medium for 48 h.

RESULTS

ATP-Dependent Uptake of Daunorubicin by Inside-Out Membrane Vesicles Prepared from HL60/ADR Cells. To examine the substrate specificity of MRP, inside-out membrane vesicles were prepared from HL60/ADR cells, a well-characterized doxorubicin-selected cell line that overexpresses MRP (Krishnamachary & Center, 1993; Kruh et al., 1994; Marquardt & Center, 1992; Marsh et al., 1986; McGrath & Center, 1987, 1988), and used in drug uptake studies. Figure 1A shows the high levels of MRP protein expression in membrane vesicles prepared from HL60/ADR cells, compared to control HL60 vesicles, in which MRP is undetectable. P-Glycoprotein expression has previously been reported to be undetectable in HL60/ADR cells (Marsh & Center, 1987; McGrath & Center, 1987), and this cell line has been maintained in the absence of chemotherapeutic agents. The absence of detectable P-glycoprotein expression was confirmed by immunoblotting with a P-glycoprotein monoclonal antibody (Figure 1B). We first analyzed daunorubicin uptake by the membrane vesicles. Figure 2A shows the time course of daunorubicin uptake over 3 min. To distinguish between ATP-dependent transport of drug into membrane vesicles and nonspecific binding, uptake was measured in the presence of ATP or 5′-AMP. Consistent with previous reports of vesicle transport studies using lipophilic cytotoxic drugs (Horio et al., 1988; Kamimoto et al., 1989; Lelong et al., 1992), we observed appreciable levels of nonspecific binding (AMP controls). However, substantial

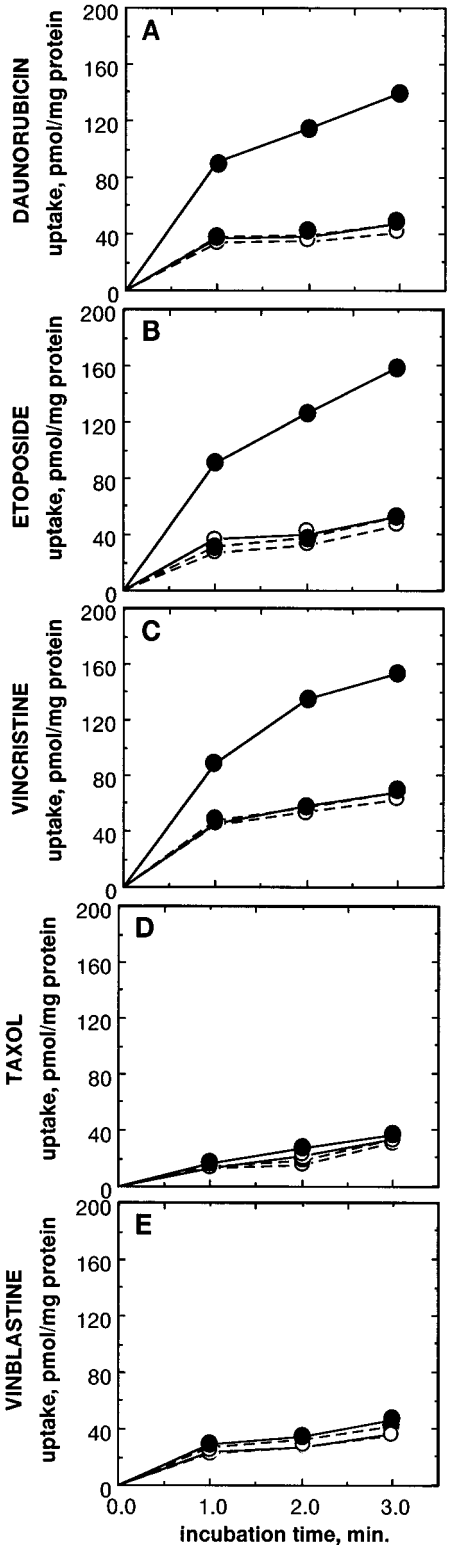

FIGURE 2: ATP-dependent uptake of natural product cytotoxic drugs by inside-out membrane vesicles prepared from HL60/ADR and HL60 cells. ATP-dependent uptake of daunorubicin (A), etoposide (B), vincristine (C), Taxol (D), and vinblastine (E) by inside-out membrane vesicles prepared from HL60/ADR (solid lines) and HL60 cells (broken lines) was measured by the rapid filtration technique. Uptake was measured in the presence of 1.2 mM ATP (closed circles) or 1.2 mM 5′-AMP (open circles). The concentration of radiolabeled drugs was 5 μM. Each data point represents the average of triplicate determinations. Experiments were performed at least three times with similar results, and typical experiments are shown.

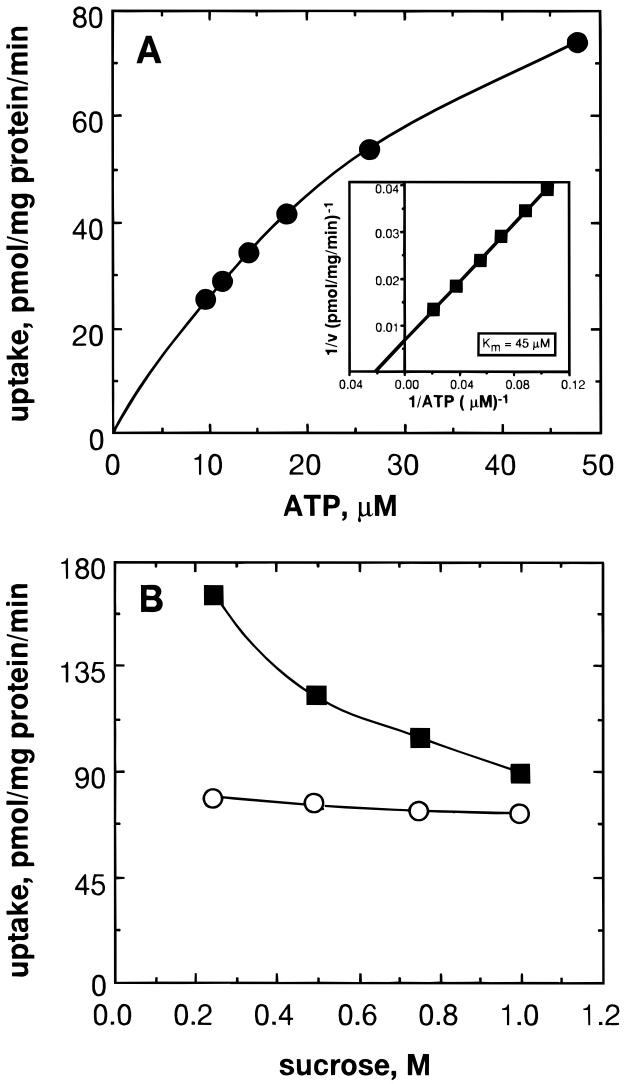

FIGURE 3: ATP dependence and osmotic sensitivity of daunorubicin uptake. (A) ATP concentration dependence of daunorubicin uptake. ATP-dependent uptake of daunorubicin by inside-out membrane vesicles prepared from HL60/ADR cells was measured at various concentrations of ATP. ATP-dependent uptake was calculated by subtracting uptake values in the presence of 5′-AMP from values obtained with ATP. Initial reaction rates were measured at 1 min. The concentration of ATP was 9.5−47.5 μM, and the concentration of daunorubicin was 5 μM. (Inset): Lineweaver−Burk plot of the data. The K_m of ATP was 45 μM. (B) Osmotic sensitivity of daunorubicin uptake. Daunorubicin uptake was measured in the presence of 1.2 mM ATP (closed boxes) or 1.2 mM 5′-AMP (open circles) after prior incubation of the vesicles in buffer containing various sucrose concentrations. Initial reaction rates were measured at 1 min.

ATP-dependent drug uptake was detected for the HL60/ADR vesicles but not for the control vesicles. When 5′-AMP values (i.e. nonspecific binding) were subtracted from the ATP values at 3 min, net daunorubicin uptake by the HL60/ADR vesicles was ~16-fold greater than that by the control vesicles.

ATP Concentration Dependence and Osmotic Sensitivity of Daunorubicin Transport. To confirm that daunorubicin uptake was the result of ATP-dependent transport into membrane vesicles, the ATP concentration dependence and osmotic sensitivity of uptake were analyzed. Increasing the ATP concentration in the incubation mixture should result in increased drug uptake if transport is ATP-dependent. As shown in Figure 3A, daunorubicin uptake by HL60/ADR vesicles increased with increasing ATP concentrations. Drug transport was saturable with respect to ATP concentration, with a K_m of 45 μM for ATP (Figure 3A, inset). A biochemical hallmark of transport into membrane vesicles, as opposed to nonspecific binding, is inhibition at high osmotic pressures (Horio et al., 1988; Kamimoto et al., 1989; Lelong et al., 1992). High osmotic pressure shrinks membrane vesicles, thereby reducing the intravesicular space available for substrate accumulation. Osmotic sensitivity was examined by analyzing daunorubicin uptake into membrane vesicles after the vesicles were incubated in buffer containing various concentrations of sucrose. As expected for transport into membrane vesicles, ATP-dependent drug uptake dramatically decreased as sucrose concentrations were increased above the standard assay conditions of 0.25 M (Figure 3B), whereas the AMP control values were unchanged. At 1.0 M sucrose, the level of vesicle-associated drug in the presence of ATP was comparable to that of the AMP control.

Specificity of Natural Product Drug Transport. We next examined the *in vitro* substrate specificity of MRP by performing membrane vesicle uptake assays with several lipophilic cytotoxic drugs. In addition to being seen for daunorubicin, markedly enhanced ATP-dependent uptake was observed for etoposide and vincristine (Figure 2). When AMP control values were subtracted, the HL60/ADR membrane vesicles exhibited ~15- and ~18-fold increased ATP-dependent uptake of etoposide and vincristine, respectively, compared to that of the control HL60 vesicles. Similar levels of ATP-dependent uptake were also observed for doxorubicin (data not shown). In contrast, when AMP control values were subtracted, only ~1.5-fold increases in ATP-dependent uptake of Taxol and vinblastine transport were observed. Although the modest increases in uptake of the latter two drugs were consistently observed, they were not statistically significant.

Drug Resistance Phenotype of HL60/ADR Cells. The observed pattern of natural product drug uptake suggested that MRP should confer resistance to anthracyclines, vincristine, and etoposide but that MRP overexpression should be accompanied by little or no increase in resistance to vinblastine and Taxol. A previous report found that HL60/ADR cells, which were isolated by growth in doxorubicin (100-fold resistant), are also resistant to vincristine (40-fold) (McGrath & Center, 1988). We analyzed the extended drug sensitivity pattern of HL60/ADR cells to determine if the *in vitro* analysis of drug uptake was consistent with the phenotype of the cells from which the membranes were prepared. In view of the fact that resistance in cell lines obtained by stepwise selection in drug is multifactorial (Cole et al., 1991; Zijlstra et al., 1987), the phenotype of HL60/ADR cells was in reasonably good agreement with the membrane vesicle transport analysis (Table 1). High levels of resistance were observed for etoposide, doxorubicin, daunorubicin, and vincristine, whereas vinblastine and Taxol sensitivity was only slightly decreased compared to that of alkylating agents, a class of drugs for which MRP does not confer resistance (Breuninger et al., 1995; Cole et al., 1994).

Concentration Dependence of Natural Product Drug Transport. To examine the affinity of MRP for lipophilic cytotoxic drugs, the drug concentration dependence of transport was analyzed for daunorubicin, etoposide, and vincristine. Typical Lineweaver−Burk plots of the relationship between drug transport and drug concentration are

Table 1: Drug Sensitivity Analysis of HL60/ADR

drug	IC_{50}[a] (nM) HL60	HL60/ADR	fold resistance[b]
etoposide	4.3×10^1	2.2×10^4	458
doxorubicin	1.0×10^1	1.1×10^3	110
daunorubicin	9.5	9.0×10^2	95
vincristine	1.1	4.2×10^1	38
vinblastine	1.8	4.0	2.2
taxol	2.8×10^1	7.5×10^1	2.7
cisplatin	1.6×10^4	2.0×10^4	1.2
cytoxan	1.2×10^1	1.4×10^1	1.2

[a] The concentration of drug that inhibited cell growth by 50%. The values represent the results of an experiment performed in triplicate and are representative of several independent experiments. [b] IC_{50} of HL60/ADR divided by the IC_{50} of HL60.

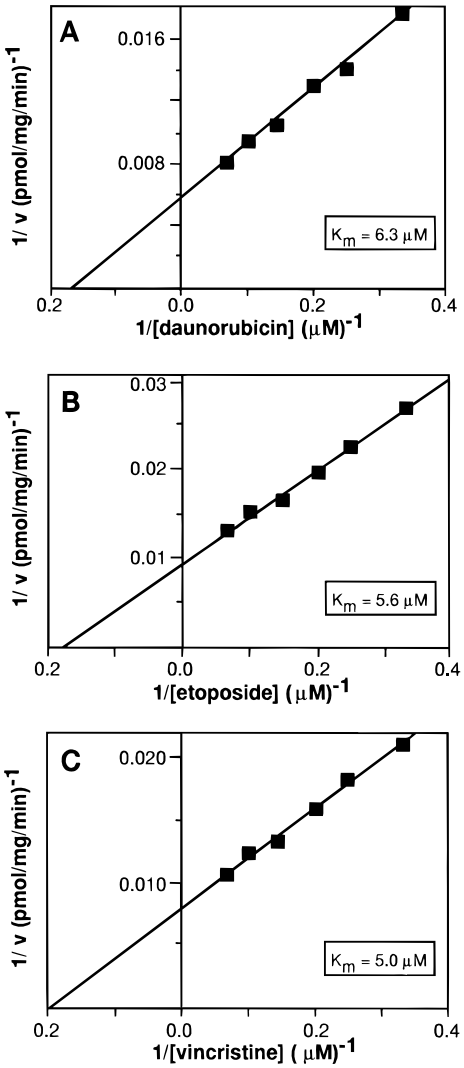

FIGURE 4: Kinetic analysis of natural product cytotoxic drug transport by inside-out membrane vesicles prepared from HL60/ADR cells. Lineweaver−Burk plots of the concentration dependence of daunorubicin (A), etoposide (B), and vincristine (C) transport. The K_m values of daunorubicin, etoposide, and vincristine were 6.3, 5.6, and 5.0 μM, respectively, and the V_{max} values were 174, 125, and 105 pmol mg^{-1} min^{-1}, respectively, in these experiments. The concentration of ATP was 1.2 mM, and the concentration of cytotoxic drugs was 3.0−15 μM. Initial reaction rates were measured at 1 min. Independent experiments were performed at least three times with similar results.

shown in Figure 4. Drug transport was saturable with respect to drug concentration, with K_m values of 6.1 ± 0.6, 5.7 ± 0.6, and 5.5 ± 0.4 μM for daunorubicin, etoposide, and vincristine, respectively (K_m values are the averages of at least three independent determinations).

Inhibition of Daunorubicin Transport by APA-SG and Other Compounds. In addition to the transport of natural product drugs, we have also observed MRP transport of the glutathione conjugate APA-SG (Shen et al., 1996), and MRP has also been reported to transport DNP-SG and the glutathione-conjugated leukotriene LTC_4 (Jedlitschky et al., 1994; Leier et al., 1994a; Muller et al., 1994). To confirm that MRP has broad substrate specificity, we examined the ability of APA-SG and oxidized glutathione to inhibit daunorubicin transport. As shown in Figure 5 (A and B), APA-SG and oxidized glutathione were competitive inhibitors of daunorubicin transport, with K_i values of 69 and 31 μM, respectively. (The increase in the apparent K_m of daunorubicin with no alterations in V_{max} indicated competitive inhibition.) MRP has been reported to confer resistance to arsenate (Cole et al., 1994), and the LTD_4 receptor antagonist MK571 (Jones et al., 1989), an anionic quinolone derivative, has been reported to inhibit LTC_4 transport by MRP (Gekeler et al., 1995). Both arsenate and MK571 were competitive inhibitors of daunorubicin transport, with K_i values of 29 and 3.0 μM (C and D). Previous studies have shown that potent P-glycoprotein-modulating agents such as cyclosporin A, PSC833, and verapamil are weak multidrug resistance-reversing agents for MRP-overexpressing cell lines (Barrand et al., 1993; Breuninger et al., 1995; Cole et al., 1989; Coley et al., 1991; Twentyman et al., 1986; Versantvoort et al., 1993). These three agents also inhibited daunorubicin transport in a dose-dependent fashion, with K_i values of 35, 84, and 95 μM, respectively (E−G). The relatively high K_i values for the P-glycoprotein-reversing agents were consistent with the modest effects of these agents on MRP-overexpressing cell lines. Genistein, an isoflavonoid inhibitor of protein tyrosine kinases, has been reported to be a specific modulator of multidrug resistance in MRP-overexpressing cell lines (Versantvoort et al., 1993). This agent was also a competitive inhibitor of daunorubicin transport (Figure 5H), and its relatively low K_i of 17 μM, compared to K_i values of the P-glycoprotein-modulating agents, is consistent with its greater potency on MRP-overexpressing cell lines. Competitive inhibition of daunorubicin uptake by these diverse compounds suggests the possibility that they share a common or overlapping binding site on the transporter with lipophilic drugs.

DISCUSSION

In the present study, we examined the biochemical substrate specificity of MRP in order to gain insight into the mechanism by which the transporter confers resistance to natural product cytotoxic drugs. We found that MRP in membrane vesicles prepared from HL60/ADR cells transports the natural product cytotoxic drugs daunorubicin, vincristine, and etoposide in an ATP-dependent, sucrose inhibitable fashion. These experiments support the idea that the direct transport of unaltered lipophilic cytotoxic drugs is the predominant mechanism whereby MRP confers multidrug resistance. The low micromolar K_m values we observed for lipophilic cytotoxic drugs indicate that the drug transport activity of MRP is pharmacologically significant, and the comparability of these values to the K_m of 2.0 μM reported for vinblastine transport by P-glycoprotein (Horio et al., 1988) supports the mechanism of MRP-conferred resistance

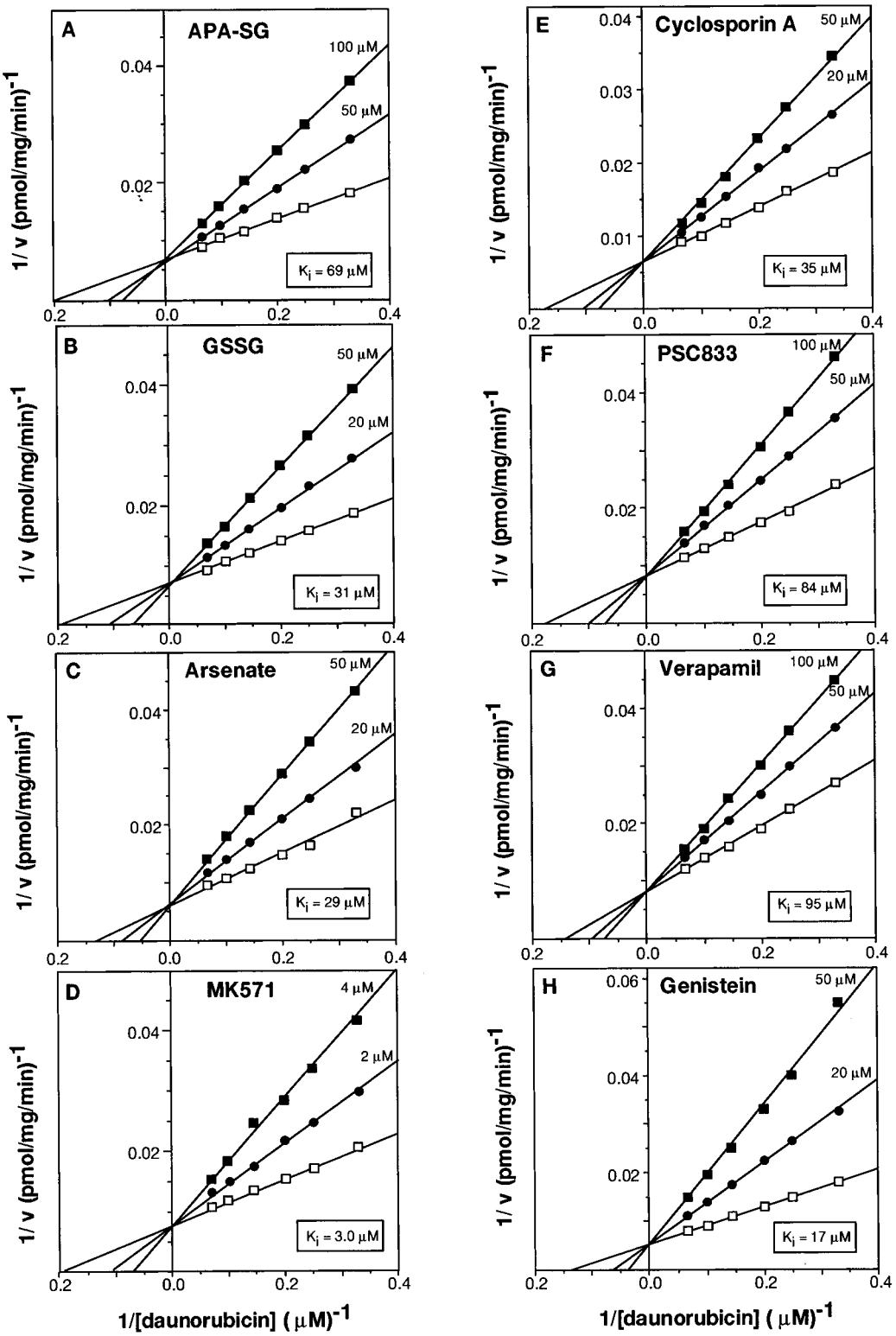

FIGURE 5: Inhibition of daunorubicin transport by diverse compounds. Lineweaver−Burk plots of the concentration dependence of daunorubicin uptake in the presence or absence of APA-SG (A), oxidized glutathione (B), arsenate (C), MK571 (D), cyclosporin A (E), PSC833 (F), verapamil (G), and genistein (H). Transport in the absence of inhibitors is indicated by open squares. The concentrations of inhibitors were as follows: APA-SG, 50 μM (closed circles) and 100 μM (closed squares); GSSG, 20 μM (closed circles) and 50 μM (closed squares); arsenate, 20 μM (closed circles) and 50 μM (closed squares); MK571, 2 μM (closed circles) and 4 μM (closed squares); cyclosporin A, 20 μM (closed circles) and 50 μM (closed squares); PSC833, 50 μM (closed circles) and 100 μM (closed squares); verapamil, 50 μM (closed circles) and 100 μM (closed squares); and genistein, 20 μM (closed circles) and 50 μM (closed squares). The plots in the presence and absence of each inhibitor gave lines that intersected at the ordinate, indicating that V_{max} was unchanged and inhibition was competitive. K_i values were determined from the Lineweaver−Burk plots using plots of K_m/V_{max} versus inhibitor concentration. The K_i values were as follows: APA-SG, 69 μM; GSSG, 31 μM; arsenate, 29 μM; MK571, 3.0 μM; cyclosporin A, 35 μM; PSC833, 84 μM; verapamil, 95 μM, and genistein, 17 μM. The concentration of ATP and AMP was 1.2 mM, and the concentration of daunorubicin was 3−15 μM.

we propose. Transport of natural product drugs by HL60/ADR membrane vesicles and inhibition of drug transport by the glutathione analog APA-SG are consistent with a previous study in which we found that the HL60/ADR

membrane vesicles transport APA-SG and that APA-SG transport is competitively inhibited by daunorubicin, vincristine and etoposide (Shen et al., 1996). *In vitro* transport of natural product drugs is consistent with the detection of decreased cellular accumulation and increased efflux of drug in HL60/ADR cells (Marsh et al., 1986; McGrath & Center, 1988); and the *in vitro* drug specificity we observed is in reasonably good agreement with the drug resistance profile of HL60/ADR cells. We found that this cell line exhibits high levels of resistance to the drugs for which we detected *in vitro* transport, including anthracyclines, vincristine, and etoposide, but only modestly reduced sensitivity to vinblastine and Taxol, drugs for which we did not observe substantial levels of membrane vesicle transport. The disproportionately higher levels of etoposide resistance, compared to anthracycline and vincristine resistance levels, probably relate to collateral resistance mechanisms, such as altered topoisomerase II activity, that are known to exist in anthracycline resistant cell lines obtained by step wise selection in drug (Cole et al., 1991; Zijlstra et al., 1987). Transport assays performed using membrane vesicles prepared from NIH3T3 cells transfected with an expression vector harboring the MRP cDNA isolated from HL60/ADR cells are completely consistent with the results of the present study (Paul et al., 1996). Membrane vesicles prepared from the MRP-transfected NIH3T3 cells transport daunorubicin, vincristine, and etoposide in an ATP-dependent, sucrose inhibitable fashion but display little or no uptake of vinblastine or Taxol (Paul et al., 1996). The pattern of drug resistance observed in the MRP transfectants is also similar to that of HL60/ADR and in good agreement with the membrane vesicle transport studies. The MRP-transfected NIH3T3 cells are resistant to etoposide, anthracyclines, and vincristine but display only marginally reduced sensitivity to Taxol and vinblastine (Breuninger et al., 1995). These *in vitro* studies of natural product drug and glutathione S conjugate transport using membrane vesicles prepared from HL60/ADR and NIH3T3 cells transfected with the HL60/ADR MRP cDNA constitute a consistent body of evidence supporting the idea that MRP transports unaltered natural product drugs.

A recent report describing transport studies performed with membrane vesicles prepared from HeLa cells transfected with an MRP cDNA isolated from MRP-overexpressing H69AR cells is not consistent with the present study in that transport of unaltered lipophilic cytotoxic drugs was not detected (Jedlitschky et al., 1996). The reason for the lack of agreement between the analysis of MRP-transfected HeLa cells and our experiments using vesicles prepared from two different MRP-overexpressing cell lines is unclear. Since the rapid filtration assays used in these studies are similar, it seems unlikely that methodological differences account for the different observations. One possible explanation is that, unlike that of P-glycoprotein, natural product drug transport by MRP may be influenced by cellular components that may be cell type-specific. HL60, a human myeloid tumor cell line, and NIH3T3, a murine fibroblast cell line, are derived from mesodermal cellular elements, whereas H69, a human small cell lung cancer cell line, and HeLa, a human adenocarcinoma cell line, are derived from epithelial cellular elements. An alternative explanation for the difference in lipophilic cytotoxic drug transport is that the activities of the proteins encoded by the two MRP cDNAs are different. Consistent with this possibility, the reported H69R MRP cDNA (Cole et al., 1992) differs in three amino acid positions when compared to the HL60/ADR MRP cDNA (Breuninger et al., 1995). Since single amino acid alterations in P-glycoprotein are known to affect transport activity (Gottesman & Pastan, 1993), it is possible that these differences in MRP are functionally significant. Whether due to cell type specificity, differences in MRP primary structure, or other factors, differences in the characteristics of the MRP-overexpressing cell lines used in the membrane vesicle studies support the possibility that there is variation in the properties of the transporter. For example, although MRP is dramatically overexpressed in H69AR (Cole et al., 1992), cellular accumulation of natural product drugs, including daunomycin and etoposide, has been reported to be unaltered compared to that in the parental drug sensitive cell line (Cole et al., 1991). In contrast, decreased drug accumulation in HL60/ADR is prominent (Marsh et al., 1986). Cellular drug kinetics in MRP-transfected HeLa and NIH3T3 cells also differ. HeLa cells transfected with the H69AR MRP cDNA display altered drug accumulation but only slight differences in drug efflux (Cole et al., 1994), whereas substantial reductions in drug accumulation and efflux were observed in NIH3T3 cells transfected with the HL60/ADR MRP cDNA (Breuninger et al., 1995). In addition to distinct cellular drug kinetics, H69AR and MRP-transfected HeLa cells differ from HL60/ADR and MRP-transfected NIH3T3 cells in chemosensitization by verapamil. Anthracycline sensitivity in the former two cell lines has been reported to be unchanged by verapamil (Cole et al., 1989, 1994), whereas MRP-transfected NIH3T3 cells (Breuninger et al., 1995) and HL60/ADR are partially sensitized by this agent.[2] Verapamil has also been reported to reverse the cellular accumulation deficit in HL60/ADR cells (McGrath et al., 1989). Consistent with the effect of verapamil on HL60/ADR and MRP-transfected NIH3T3 cells, we demonstrate in this report that verapamil is a competitive inhibitor of daunorubicin transport by MRP in HL60/ADR membrane vesicles. Although the reason why cytotoxic drug transport can be detected in membrane vesicles prepared from HL60/ADR and MRP-transfected NIH3T3 cells, but not in MRP-transfected HeLa cells, is unclear, this variability raises the possibility that the biochemical mechanism whereby MRP pumps unaltered natural product drugs may be distinct from that of P-glycoprotein. This possibility is also suggested by the low degree of amino acid identity shared by the two transporters (15%) (Cole et al., 1992) and by our observation that vinblastine and Taxol, drugs that are excellent P-glycoprotein substrates, are transported poorly or not at all by MRP.

The observation that MRP in membrane vesicle preparations pumps glutathione S conjugates (Jedlitschky et al., 1994; Leier et al., 1994a; Muller et al., 1994; Shen et al., 1996) has led to consideration of the idea that the transporter confers multidrug resistance by pumping the glutathione conjugates or anionic metabolites of lipophilic cytotoxic drugs (Ishikawa et al., 1995). The present study does not exclude the possibility that this activity contributes to multidrug resistance. However, glutathione conjugates of the natural product drugs for which MRP confers resistance have not been isolated (Tew, 1994), and MRP transfectants do not exhibit increased resistance to alkylating agents

[2] L. M. Breuninger and G. D. Kruh, unpublished observations.

(Breuninger et al., 1995; Grant et al., 1994), the class of drugs for which glutathione conjugation is well established (Tew, 1994). While sulfated and glucuronidated metabolites of anthracyclines and etoposide have been described, and glucuronosyletoposide has been reported to be an *in vitro* substrate of MRP (Jedlitschky et al., 1996), the extent to which formation of these compounds influences cytotoxicity is unknown (Pratt et al., 1994). The observations that the LTD_4 antagonist MK571 is a competitive inhibitor of LTC_4 transport by MRP in membrane vesicles (Leier et al., 1994a) and that MK571 and the glutathione-depleting agent BSO can reverse the resistance phenotype of MRP-overexpressing cell lines (Gekeler et al., 1995; Lutzky et al., 1989; Schneider et al., 1995; Versantvoort et al., 1995) have also contributed to speculation that glutathione conjugate transport is the biochemical mechanism of MRP-conferred resistance. Our observation that MK571 is a potent competitive inhibitor of natural product transport by MRP indicates that the resistance-modulating activity of this compound is not necessarily based on competing with drug glutathione conjugates. BSO might also modulate MRP activity by mechanisms distinct from the inhibition of drug−glutathione conjugate formation. For example, BSO could increase the cellular levels of endogenous metabolites such as oxidized glutathione, a compound that we found to be a competitive inhibitor of natural product transport by MRP. This agent might also be a direct competitive inhibitor of MRP transport. Although we favor the idea that BSO modulation of MRP-conferred multidrug resistance is unrelated to reduced formation of glutathione S conjugates, it is possible that the sensitivity of MRP but not P-glycoprotein to this agent reflects as yet undetermined differences in the biochemical mechanism whereby the two proteins transport unaltered natural product drugs.

Our observation that MRP in HL60/ADR membrane vesicle preparations pumps the glutathione S conjugate APA-SG (Shen et al., 1996), in addition to unaltered natural product cytotoxic drugs, indicates that the substrate specificity of the transporter is surprisingly broad. In addition, the observation that natural product drugs inhibit APA-SG transport (Shen et al., 1996), and that APA-SG inhibits natural product transport (the present study), suggests that these chemically distinct classes of compounds share a common or overlapping binding site on the transporter. In addition to APA-SG, MRP has been reported to transport the glutathione S conjugates LTC_4, DNP-SG, and glutathionylmelphalan (Jedlitschky et al., 1994, 1996; Leier et al., 1994a; Muller et al., 1994). This broad substrate specificity, combined with the widespread expression pattern of MRP transcript in human tissues (Kruh et al., 1995), indicates that the transporter represents a ubiquitous and versatile cellular defense mechanism for both lipophilic xenobiotics and the products of phase II detoxification enzymes. MRP-mediated transport has also been reported for glucuronidated and sulfated compounds, including the steroid conjugate 17β-glucuronosylestradiol, and the bile salt conjugates glucuronosylhydrodeoxycholate and sulfatolithocholyltaurine (Jedlitschky et al., 1996). Additional studies should determine whether the transport of endogenous conjugated compounds such as LTC_4 and metabolized bile salts and steroids is a physiological function of MRP.

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