Jpn. J. Cancer Res. **90**, 439–447, April 1999

Differences in Substrate Specificity among Glutathione Conjugates (GS-X) Pump Family Members: Comparison between Multidrug Resistance-associated Protein and a Novel Transporter Expressed on a Cisplatin-resistant Cell Line (KCP-4)

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The substrate specificity of primary active transporters expressed on two kinds of human epidermoid KB-3-1 derived cell lines, C-A500 and KCP-4, was examined; the former expresses multidrug resistance-associated protein (MRP1), whereas the latter is resistant to *cis*-diamminedichloroplatinum (II) (cisplatin). Northern blot analysis indicated that neither P-glycoprotein, MRP1, MRP2 (canalicular multispecific organic anion transporter; cMOAT) nor MRP3 was overexpressed on KCP-4. Membrane vesicles isolated from C-A500 and KCP-4, but not from KB-3-1, exhibited the ATP-dependent uptake of glutathione conjugates (GS-X) such as leukotriene C_4 and 2,4-dinitrophenyl-*S*-glutathione (DNP-SG), indicating the presence of GS-X pumps on these cells. The uptake of these GS-X by membrane vesicles from C-A500 was approximately twice that in the case of KCP-4. Kinetic analysis indicated that the K_m and V_{max} values for DNP-SG uptake were 2.56 and 1.43 μM, and 570 and 160 pmol/min/mg protein for C-A500 and KCP-4, respectively. In marked contrast, significant ATP-dependent uptake of glutathione-platinum complex was observed only in membrane vesicles from KCP-4, but not those from KB-3-1 and C-A500. The transport properties of estradiol-17β-D-glucuronide ($E_2$17βG) were also different between the two cell lines. This was reflected in the findings that the ATP-dependent uptake of this conjugated metabolite in membrane vesicles from C-A500 (K_m=2.33 μM, V_{max}=34 pmol/min/mg protein) was much more extensive than that in the case of KCP-4 (K_m=5.5 μM, V_{max}=35 pmol/min/mg protein), and that comparable uptake was observed between KCP-4 and KB-3-1. Overall, a clear difference in substrate specificity among GS-X pump family members expressed on resistant tumor cells was demonstrated.

Key words: ABC-transporter — Cisplatin resistance — GS-X pump — MRP — Substrate specificity

Multidrug resistance of tumor cells is often acquired by the overexpression of P-glycoprotein (P-gp) and/or multidrug resistance-associated protein (MRP1).[1–3] The drug resistance spectrum of cells overexpressing MRP1 resembles that of cells overexpressing P-gp; indeed, reduced intracellular concentrations of certain antitumor drugs (such as vincristine and adriamycin) are observed both in P-gp- and MRP1-transfected cells.[1–3] The mechanisms of extrusion of antitumor drugs, however, differ between the two transporters; P-gp, but not MRP1, can extrude these antitumor drugs in their unmodified forms.[1–3] In contrast, it is well established that MRP1 can extrude several organic anions, including glutathione conjugates (GS-X).[2, 3] Moreover, the role of glutathione (GSH) in the resistance imparted by MRP1 has also been a topic of investigation.[2–4] The role of the GS-X pump in the detoxification of electrophiles has been highlighted by Ishikawa.[5] He proposed that the cells eliminate a broad range of lipophilic toxins from the cytosol after their conjugation with GSH, followed by extrusion via the GS-X pump.[5] The detoxification of aflatoxin B_1,[6] chlorambucil and melphalan[7] may be accounted for by this hypothesis.

Although MRP1 is an important member of the GS-X pump family, other members are expressed on mammalian cells; these include MRP2 (also referred to as canalicular multispecific organic anion transporter (cMOAT)), a recently cloned primary active transporter for the biliary excretion of anionic compounds,[8–14] including conjugated metabolites and anionic antitumor drugs such as methotrexate[15] and CPT-11 and related metabolites.[16–19] It has been suggested that MRP2/cMOAT is overexpressed in some cell lines and confers resistance to a number of antitumor drugs.[13, 20–22] Moreover, at least four additional kinds of MRP homologues have been identified.[21, 23–26]

Previously, we found a novel transporter which exhibited GS-X pump activity in KCP-4, a *cis*-diamminedichloroplatinum (II) (cisplatin)-resistant cell line derived from human epidermoid carcinoma (KB-3-1).[27, 28] Although northern and western blot analyses indicated that neither

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P-gp nor MRP1 is overexpressed in KCP-4,[28] the intracellular accumulation of cisplatin was reduced compared with that in KB-3-1.[29] Moreover, since ATP-dependent uptake of a typical GS-X (leukotriene C_4; LTC_4) was observed in membrane vesicles from KCP-4, but not from KB-3-1,[27] it was concluded that a novel GS-X pump, other than MRP1, is expressed on KCP-4.[28] Due to the elevated intracellular concentration of GSH,[21] it is plausible that cisplatin is converted to bis(glutathionato)platinum (II) (GS-platinum complex) and thereafter extruded from the cells with the aid of the GS-X pump.[30]

The purpose of the present study is to characterize the transport properties of the novel GS-X pump expressed on KCP-4 and to compare them with those of MRP1 expressed on C-A500, a cell line also established from KB-3-1.[31] Although the substrate specificities of the GS-X pumps have been shown to be relatively similar, in that both MRP1 and MRP2/cMOAT accept typical GS-X (such as LTC_4 and 2,4-dinitrophenyl-*S*-glutathione (DNP-SG)) and glucuronide conjugates (such as estradiol-17β-D-glucuronide; $E_2$17βG),[2, 3, 32] the results of the present study provide an example of a difference in specificity among GS-X pump family members expressed on resistant tumor cells.

MATERIALS AND METHODS

Materials [^{3}H]LTC_4 (52.0 μCi/nmol), [^{3}H]GSH (44.8 μCi/nmol) and [^{3}H]$E_2$17βG (51.0 μCi/nmol) were purchased from New England Nuclear (Boston, MA). Unlabeled and ^{3}H-labeled DNP-SG (44.8 μCi/nmol) were synthesized by the method of Kobayashi *et al.*[33] Unlabeled and ^{3}H-labeled GS-platinum complex (89.6 μCi/nmol) were synthesized by the method of Ishikawa and Ali-Osman.[30] ATP, AMP, creatine phosphate, and creatine phosphokinase were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were commercial products of reagent grade.

Cells KB-3-1 cells were cultured in minimal essential medium, supplemented with 10% fetal bovine serum. The other KB-3-1-derived cells were selected from KB-3-1 cells and cultured in selection medium containing colchicine (2 μg/ml) for KB-C2,[34] cepharanthine (1 μg/ml), adriamycin (0.5 μg/ml) and mezerein (0.065 μg/ml) for C-A500[31] and cisplatin (7 μg/ml) for KCP-4.[27, 29]

MTT assay Chemosensitivity *in vitro* was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay performed in 96-well plates.[35] The assay is dependent on the reduction of MTT by the mitochondrial dehydrogenase of viable cells to a blue formazan product that can be measured spectrophotometrically. Cells (2×10^3 for KB-3-1 and KB-C2; 5×10^3 for C-A500 and KCP-4) were inoculated into each well with 50 μl of phenol red-free culture medium. After overnight incubation (at 37°C, 5% CO_2), 50 μl of drug solution was added to the cells and incubation was continued for 4 days. Thereafter, 50 μl of MTT solution was added to each well and incubation was continued for 4 h. The resulting formazan was dissolved in 100 μl of dimethyl sulfoxide. Plates were placed on a plate shaker for 5 min and read immediately at 595 nm using a Micro Plate Reader 3550 (BIO-RAD, Hercules, CA). The 50% inhibitory concentration (IC_{50}) was calculated from the following equation:

$$S = S_{max} \times [1 - C^{\delta} / (C^{\delta} + IC_{50})] \quad (1)$$

where S and C represent the surviving fraction and the drug concentration (μM), respectively. The relative resistance was defined as the IC_{50} of the derived cell lines divided by that of KB-3-1.

Northern blot Total cellular RNA was extracted from the cells in a single step using ISOGEN (Nippon Gene Co., Tokyo) and poly A^+ RNA was purified from the total RNA using Oligo(dT)-Latex (Takara Shuzo Co., Otsu). Five micrograms of poly A^+ RNA was separated by electrophoresis on a formaldehyde-agarose denaturing gel. The mRNA was transferred to a nitrocellulose membrane (Nippon Gene Co.), prehybridized in 50% formamide, 5× SSC (1× SSC contains 0.15 *M* NaCl and 0.015 *M* sodium citrate, pH 7.0)-5× Denhardt's solution, 1% sodium dodecyl sulfate (SDS), and sheared, denatured salmon sperm DNA (100 μg/ml) for 4 h at 42°C. The blot was hybridized with a 400 bp cDNA fragment of the carboxyl-terminal ATP binding cassette region of each transporter labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham Pharmacia Biotech, Uppsala, Sweden) by the random priming method.[36] Our preliminary experiments indicated that the expression levels of P-gp, MRP1, MRP2/cMOAT and MRP3 can be discriminated by using these probes. Hybridization was carried out for 12–16 h at 42°C. Membranes were washed twice in 2× SSC-0.1% SDS for 10 min at room temperature, then twice in 0.1× SSC-0.1% SDS for 10 min at 55°C, followed by autoradiography. To estimate the variation in the amount of RNA loaded on the gel, the blots were rehybridized with a ^{32}P-labeled cDNA for G3PDH.

Preparation of membrane vesicles Membrane vesicles were prepared by the nitrogen cavitation method.[37, 38] Cell monolayers (10^7–10^8 cells) were washed once and scraped into phosphate buffer (PBS). The cells were washed by centrifugation (4,000*g*, 10 min) at 4°C in PBS and then in buffer A (0.01 *M*-Tris-HCl containing 0.25 *M* sucrose and 0.2 m*M* $CaCl_2$, pH 7.4) and equilibrated at 4°C under a nitrogen pressure of 900 psi for 15 min. EDTA was added to the cell lysate to give a final concentration of 1 m*M*. The lysed cell suspension was then diluted 1:4 with buffer B (0.01 *M* Tris-HCl containing 0.25 *M* sucrose, pH 7.4) and centrifuged at 1,000*g* for 10 min at 4°C to remove

nuclei and unlysed cells. The supernatant was layered onto a 35% sucrose cushion (0.01 *M* Tris-HCl containing 35% sucrose and 1 m*M* EDTA, pH 7.4) and centrifuged for 30 min at 16,000*g* at 4°C. The interface was collected, diluted 1:5 in buffer B, and then centrifuged for 45 min at 100,000*g*. The vesicle pellet was resuspended in buffer B using a 25-G needle. Vesicles were stored at −100°C prior to use. The enrichment and sidedness were determined by measuring the alkaline phosphatase activity using *p*-nitrophenyl phosphate as a substrate in the presence and absence of 0.2% (v/v) Triton X-100.[39]

Transport studies The uptake of [^{3}H]GS-platinum complex (100 μM), [^{3}H]LTC_4 (1.3 n*M*), [^{3}H]DNP-SG (0.5 μM) and [^{3}H]$E_2$17βG (0.05 μM) into membrane vesicles was determined as reported previously.[30, 40] The transport medium (10 m*M* Tris-HCl, 250 m*M* sucrose and 10 m*M* $MgCl_2$, pH 7.4) contained the isotopically labeled ligands, 5 m*M* ATP (or AMP) and an ATP-regenerating system (10 m*M* creatine phosphate and 100 μg/ml creatine phosphokinase). An aliquot of transport medium (17–18 μl) was mixed rapidly with the vesicle suspension (5 μg protein in 2–3 μl). The transport reaction was stopped by the addition of 1 ml of ice-cold buffer containing 250 m*M* sucrose, 0.1 *M* NaCl and 10 m*M* Tris-HCl (pH 7.4). The stopped reaction mixture was filtered through a 0.45 μM HA filter (Millipore Corp., Bedford, MA), and then washed twice with 5 ml of stop solution. The radioactivity retained on the filter and that present in the reaction mixture were determined in a liquid scintillation counter (LS6000SE, Beckman Instruments, Fullerton, CA). The uptake of ligands was normalized with respect to the amount of membrane protein.

Determination of kinetic parameters The kinetic parameters for ATP-dependent uptake, defined as the difference in uptake in the presence and absence of ATP, were estimated from the following equation:

$$v_0/S = V_{max} / (K_m + S) + P_{dif} \quad (2)$$

where v_0 is the initial uptake rate of ligand by membrane vesicles (pmol/min/mg protein), S is the ligand concentration in the medium (μM), K_m is the Michaelis constant (μM), V_{max} is the maximum uptake velocity (pmol/min/mg protein), and P_{dif} is the non-specific uptake clearance (μl/min/mg protein). The equation was fitted to the uptake data sets by an iterative nonlinear least-squares method using the MULTI program[41] to obtain estimates of kinetic parameters. The input data were weighted as the reciprocal of the observed values and the algorithm used for fitting was the Damping Gauss Newton Method.[41]

RESULTS

Resistance to cisplatin Table I shows the resistance to cisplatin measured by MTT assay in KB-derived cell lines. These values are also indicated as the relative resistance with respect to the parental cell line, KB-3-1. The resistance to cisplatin was 16- and 78-fold higher in C-A500 and KCP-4 over the parental cell line, respectively. In particular, KCP-4 acquired high resistance to cisplatin. In contrast, no resistance to cisplatin was acquired in KB-C2 cells.

Northern blot analysis Fig. 1 shows the expression of previously reported efflux transporters in KB-derived cell lines. The expression of P-gp and MRP1 was marked in

Table I. Resistance to Cisplatin of KB-derived Cell Lines

Cell line	IC_{50} (μM) mean±SD	Relative resistance mean±SD
KB-3-1	0.70±0.17	1.00±0.33
KB-C2	1.78±0.52	2.53±0.95
C-A500	11.0±5.2	15.6±8.3
KCP-4	54.5±11.9	77.5±24.9

Resistance to cisplatin was determined by MTT assay. IC_{50} was calculated from Eq. (1) in the text. Relative resistance is expressed as the IC_{50} of the resistant cells divided by the IC_{50} of their parental cells (KB-3-1).

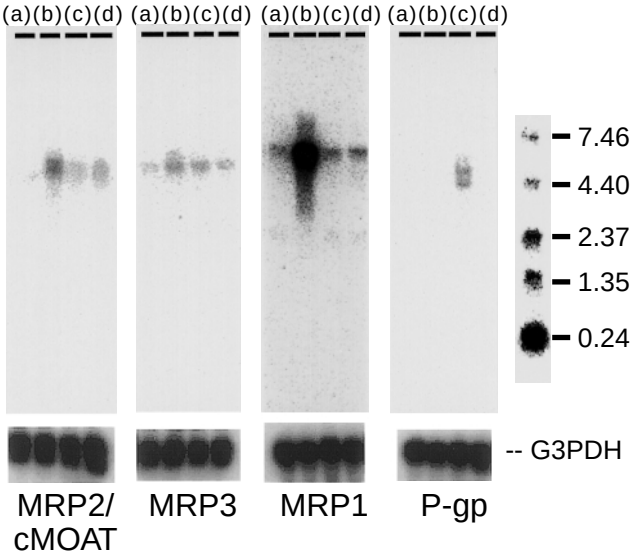

Fig. 1. Northern blot analysis of poly A^+ RNA from KB-derived cell lines. Five micrograms of poly A^+ RNA from KCP-4 (lane a), C-A500 (lane b), KB-C2 (lane c) or KB-3-1 (lane d) was used for the analysis. The autoradiograph of the blot, probed with ^{32}P-labeled cDNA encoding the carboxyl-terminal ATP binding cassette region of each transporter, was obtained with a 60 h exposure at −100°C with an intensifying screen. Rehybridization of the same membrane with ^{32}P-labeled G3PDH cDNA indicated the presence of the same amount of mRNA in

KB-C2 and C-A500, respectively, whereas these transporters were not highly expressed in KB-3-1 and KCP-4. Although overexpression of MRP3 was observed in KB-C2 and C-A500, its expression was almost identical in KB-3-1 and KCP-4. The expression level of human MRP2/cMOAT was somewhat reduced in KCP-4, whereas in C-A500, its expression was enhanced.

Table II. Sidedness and Enrichment of Membrane Vesicles Prepared from KB-derived Cell Lines

Cell line	Sidedness (inside-out %) mean±SE	Enrichment mean±SE
KB-3-1	42.9±2.3	4.44±0.26
KB-C2	39.2±3.3	5.55±0.24
C-A500	37.4±5.9	5.12±0.35
KCP-4	43.9±4.1	5.24±0.40

Sidedness and enrichment of membrane vesicles were calculated from the alkaline phosphatase activity, with or without detergent [0.2% (v/v) Triton X-100]. Each value represent the mean±SE of 4 determinations from 2 independent preparations.

Transport studies To clarify the substrate specificity of transporters expressed on resistant cells, we performed an uptake study with membrane vesicles. The sidedness and enrichment of prepared vesicles were calculated from the alkaline phosphatase activity (Table II). In all vesicles the content of inside-out vesicles was approximately 40%, and the enrichment was 4- to 5-fold over cell lysate. Since there was no significant difference in enzyme activity among membrane vesicles isolated from four kinds of cell lines, transport properties can be compared using isolated membrane vesicles.

ATP-dependent uptake of LTC_4 and DNP-SG was observed in membrane vesicles from both C-A500 and KCP-4 (Fig. 2, B and C). The K_m values for the uptake of

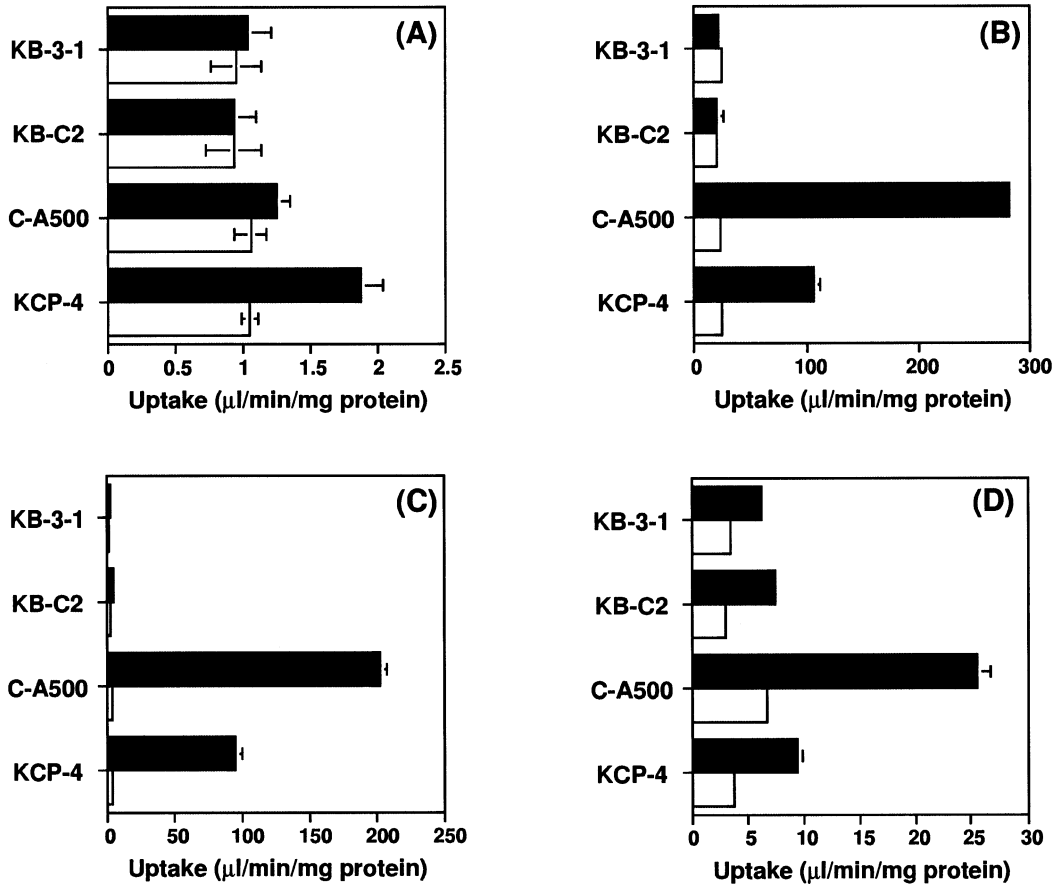

Fig. 2. ATP-dependent uptake of organic anions by membrane vesicles prepared from KB-derived cell lines. The uptake of [^{3}H]GS-platinum complex (100 μM; panel A), [^{3}H]LTC_4 (1.34 nM; panel B), [^{3}H]DNP-SG (0.5 μM; panel C) and [^{3}H]$E_2$17βG (0.05 μM; panel D) was studied in medium containing 5 mM ATP (closed squares) or AMP (open squares) and an ATP-regenerating system (10 mM creatine phosphate and 100 μg/ml creatine phosphokinase). ■ ATP(+), □ ATP(−).

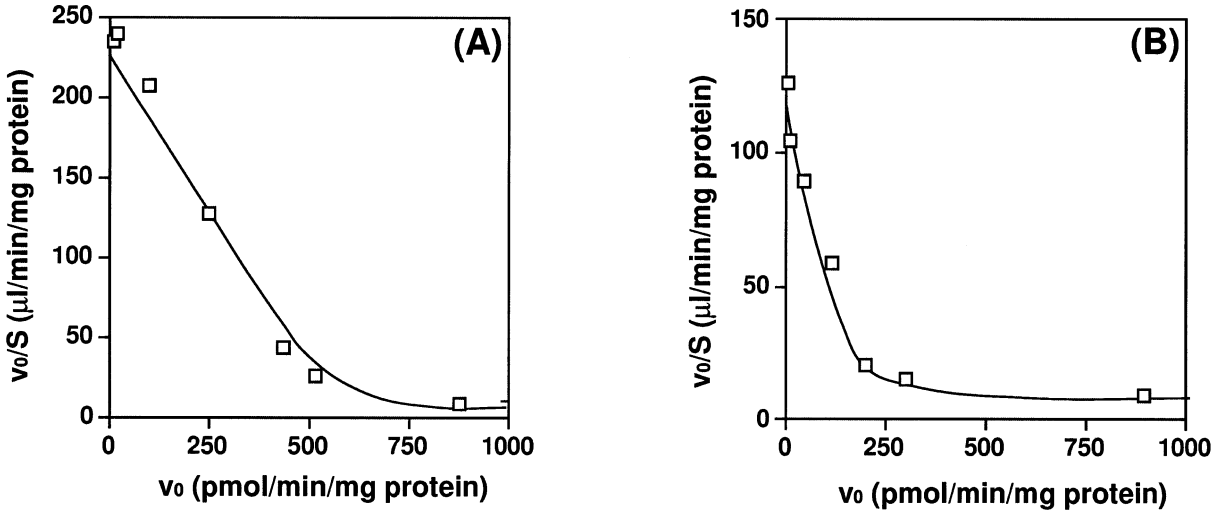

Fig. 3. Eadie-Hofstee plots for the ATP-dependent uptake of DNP-SG by membrane vesicles. ATP-dependent uptake was obtained as the difference in uptake in the presence and absence of ATP. Each point represents the mean of 3 determinations. The saturable uptake of [^{3}H]DNP-SG was studied in membrane vesicles isolated from C-A500 (panel A) and KCP-4 (panel B). The K_m, V_{max} and P_{dif} values in C-A500 and KCP-4 were 2.56±0.34 and 1.43±0.21 μM, 569±17 and 161±19 pmol/min/mg protein and 4.42±9.42 and 7.28±0.77 μl/min/mg protein, respectively.

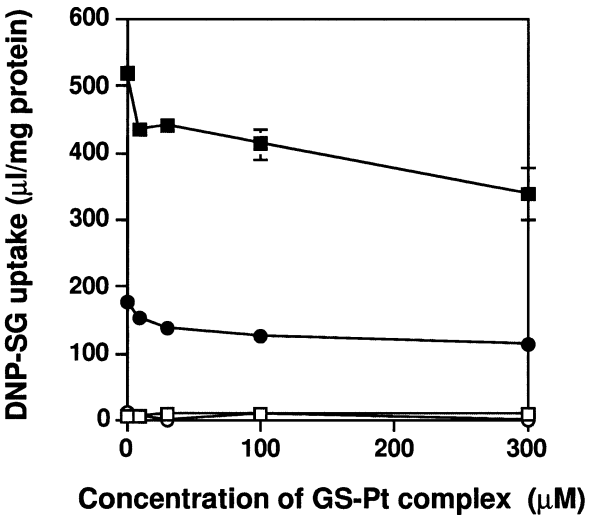

Fig. 4. Effect of GS-platinum complex on the uptake of DNP-SG by membrane vesicles. Each point and vertical bar represent the mean±SE of 3 determinations. Membrane vesicles prepared from C-A500 (squares) and KCP-4 (circles) were incubated with [^{3}H]DNP-SG (0.5 μM), with or without unlabeled GS-platinum complex, in the presence and absence of ATP for 2 min. ■ C-A500 (ATP+), □ C-A500 (ATP–), ● KCP-4 (ATP+) and ○ KCP-4 (ATP–).

DNP-SG were 2.6 and 1.4 μM in C-A500 and KCP-4, respectively (Fig. 3). The reason why the apparent transport activity was higher in C-A500 than in KCP-4 was the difference in V_{max} (569 and 161 pmol/min/mg protein in C-A500 and KCP-4, respectively). Moreover, the effect of GS-platinum complex on the transport of DNP-SG was examined in C-A500 and KCP-4 (Fig. 4). It was found that the GS-platinum complex almost equally inhibits DNP-SG uptake in membrane vesicles from both cell lines; the addition of 300 μM GS-platinum complex reduced the uptake to 60% for these specimens. In contrast, it was shown that the GS-platinum complex itself was recognized as a substrate, but only in KCP-4 (Fig. 2A). Then, we examined the transport of $E_2$17βG, another typical substrate for both MRP1 and MRP2/cMOAT (Fig. 2D). Although ATP stimulated the uptake of $E_2$17βG into membrane vesicles from all cell lines, marked ATP-dependent transport was observed only in C-A500. The K_m and V_{max} values for the uptake of $E_2$17βG in C-A500 and KCP-4 were 2.3 and 5.5 μM, and 34.4 and 35.3 pmol/min/mg protein, respectively.

DISCUSSION

One of the most important mechanisms for the acquisition of resistance to cisplatin is reduction of the intracellular concentration of the drug.[30] Two consecutive pathways have been proposed for the extrusion of cisplatin from resistant cells: cisplatin is conjugated with GSH to form GS-platinum complex and then this conjugate is extruded from the cells with the aid of the GS-X pump.[30] Indeed, in cisplatin-resistant KCP-4, we and others have observed reduced intracellular accumulation of cisplatin,[29] along

with an increased concentration of intracellular GSH[21] and increased GS-X pump activity on the plasma membrane.[27, 28]

In the present study, the drug resistance of three kinds of KB-3-1-derived cell lines was analyzed in relation to the transporters located on the plasma membrane. Marked resistance to cisplatin was not observed for KB-C2 or C-A500 in which P-gp or MRP, respectively, is overexpressed (Table I), whereas significant resistance of these cell lines to vinca alkaloids and adriamycin has been reported.[31] These observations are in good agreement with the hypothesis that overexpression of these two kinds of transporters does not confer resistance to cisplatin.[28, 29] Cells overexpressing P-gp or MRP1, obtained by either cDNA transfection or by stepwise selection with substrates, are sensitive to cisplatin.[1, 4, 42] Since no significant ATP-dependent transport of GS-platinum complex was observed in membrane vesicles isolated from C-A500 (Fig. 2A), it was suggested that the resistance of this cell line should be ascribed to mechanism(s) other than the overexpression of GS-X pump (such as increased intracellular metallothioneins and enhanced DNA repair activity).[43]

For the purpose of identifying the transporter responsible for increased GS-X pump activity in KCP-4, the expression levels of previously identified MRP-family members were examined. Although Kauffman *et al.*[44] indicated that cMOAT expression is induced by cisplatin in cultured rat hepatocytes and Taniguchi *et al.*[13] found that MRP2/cMOAT is overexpressed in KCP-4, we found a reduction in MRP2/cMOAT mRNA in this cell line, which is consistent with the recent finding by Kool *et al.*[21] Moreover, expression of MRP1 was comparable between KB-3-1 and KCP-4 (Fig. 1). As with MRP2/cMOAT, the expression of MRP3 was also somewhat reduced in KCP-4 (Fig. 1). These results indicate that KCP-4 does not overexpress previously identified GS-X pumps.

The transport properties of this pump were characterized by comparison with those of MRP1 (Fig. 2). In membrane vesicles from C-A500, in which MRP1 is overexpressed, kinetic parameters were determined for the ATP-dependent uptake of DNP-SG and $E_2$17βG (Fig. 3). The K_m and V_{max} values for DNP-SG were 2.56 μM and 570 pmol/min/mg protein, respectively (Fig. 3), which are comparable with those determined in membrane vesicles from MRP1-transfected HeLa cells (K_m = 3.6 μM and V_{max} = 410 pmol/min/mg protein),[45] indicating almost identical expression of MRP1 in the two cell lines. Similarly, the kinetic parameters for the uptake of $E_2$17βG were also comparable; the K_m and V_{max} values were 2.33 and 1.5 μM and 34 and 42 pmol/min/mg protein for C-A500 (Fig. 5) and MRP1-transfected HeLa cells,[45] respectively. The transport activity for LTC_4 in C-A500 (Fig. 2), however, was much less than that in MRP1-transfected HeLa.[45] Consequently, the clearance for ATP-dependent uptake of these substrates was in the order, LTC_4 (1,030 μl/min/mg protein) >> DNP-SG (114 μl/min/mg protein) > $E_2$17βG (42 μl/min/mg protein) for MRP1-transfected HeLa,[45]

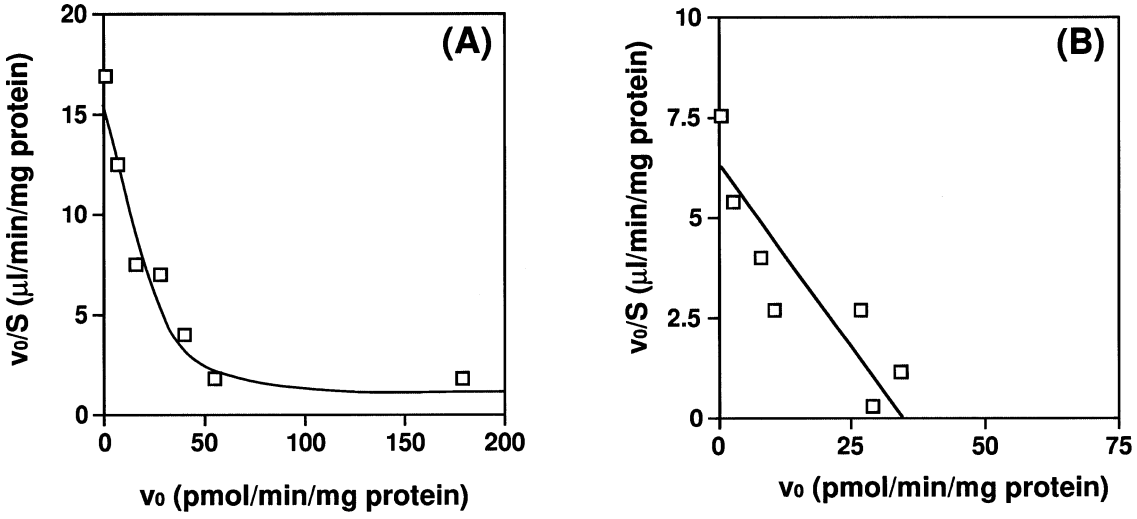

Fig. 5. Eadie-Hofstee plots for the ATP-dependent uptake of estradiol-17β-glucuronide by membrane vesicles. ATP-dependent uptake was obtained as the difference in uptake in the presence and absence of ATP. Each point represents the mean of 3 determinations. The saturable uptake of [^{3}H]$E_2$17βG was studied in membrane vesicles isolated from C-A500 (panel A) and KCP-4 (panel B). The K_m, V_{max} and P_{dif} values in C-A500 and KCP-4 were 2.33±0.82 and 5.50±3.08 μM, 34.4±9.9 and 35.2±4.4 pmol/min/mg protein and 0.81±0.44 and 0.00±3.56 μl/min/mg protein, respectively.

whereas the order was LTC_4 (280 μl/min/mg protein) = DNP-SG (200 μl/min/mg protein) > $E_2$17βG (25 μl/min/mg protein) for C-A500 (Figs. 2, 3 and 5). The reason for the poorer transport of LTC_4 in C-A500 remains to be clarified.

In KCP-4, the K_m and V_{max} values for the ATP-dependent transport of DNP-SG were determined as 1.43 μM and 160 pmol/min/mg protein, respectively (Fig. 3), indicating that the affinity of this glutathione conjugate for the transporter on KCP-4 is higher than for MRP1. The ratio of the clearance for the ATP-dependent uptake of LTC_4 by KCP-4 to that by C-A500 was approximately 0.4, which is comparable with the ratio for the uptake of DNP-SG by these cell lines (0.5) (Fig. 2). Moreover, the uptake of DNP-SG was reduced by GS-platinum complex in a concentration-dependent manner (Fig. 4). Although there is a difference in the absolute values of the kinetic parameters, this is consistent with the previous finding by Ishikawa *et al*.,[46] who reported that the K_m for the uptake of GS-platinum complex by membrane vesicles from cisplatin-resistant HL-60 cells (HL-60/R-CP) is 130 μM. They also reported that the uptake of LTC_4 was reduced by GS-platinum complex with an IC_{50} of ca. 150 μM.[47]

A marked difference, however, was observed between C-A500 and KCP-4 in the ATP-dependent transport of GS-platinum complex. Significant ATP-dependent uptake of GS-platinum complex was observed in membrane vesicles from KCP-4, but not from C-A500, suggesting that the GS-platinum complex is an inhibitor, but not a good substrate for MRP1 (Fig. 2). This suggestion is in marked contrast to the previous findings by Ishikawa *et al*., who demonstrated that MRP1, along with γ-glutamylcysteine synthetase (γ-GCS), a rate-determining enzyme for GSH synthesis, was overexpressed in HL-60/R-CP compared with its host cell line (HL-60), and that the ATP-dependent uptake of GS-platinum complex was increased in HL-60/R-CP compared with HL-60.[30, 47] One of the possible reasons for this discrepancy is that a GS-X pump, other than MRP1, is also expressed on HL-60/R-CP. Previously, it has been shown that transfection of MRP1 confers resistance to some heavy metal anions, including arsenite and antimony, but not to cisplatin.[4, 42] Zaman *et al*.[4] have reported that arsenite efflux from MRP1-transfected cells was accompanied by a significant increase in GSH efflux, suggesting that the intracellularly formed GSH conjugate of arsenite is extruded from the cells via MRP1. The difference in the resistance to cisplatin and arsenite has been ascribed to a difference in the rate-determining process of detoxification. Acquisition of resistance to arsenite by MRP1 transfection may be accounted for by considering the rapid conjugation of this heavy metal with GSH.[47] In contrast, due to the slow conjugation of cisplatin with GSH, a coordinate expression of γ-GCS and MRP is required for the acquisition of resistance to cisplatin.[47] However, these results may also be accounted for by the hypothesis that the GSH conjugate of arsenite, but not the GS-platinum complex, can be a substrate for MRP. An alternative transporter, whose substrate specificity resembles that of the transporter expressed on KCP-4, may be responsible for the extrusion of GS-platinum complex from the previously described resistant cells.

Moreover, a difference in substrate specificity was observed between C-A500 and KCP-4 as far as the uptake of glucuronide was concerned (Fig. 2). Although the transporter on KCP-4 accepts these two kinds of GS-X as substrates, transport of $E_2$17βG by this transporter was minimal (Fig. 2). The previously described ratio for the uptake of $E_2$17βG was as small as 0.15, much lower than that for DNP-SG (0.5) and LTC_4 (0.4), suggesting that this transporter accepts glutathione, but not glucuronide conjugates, as substrates. Since it has been shown that the substrate specificities of MRP1 and MRP2/cMOAT, both of which belong to the GS-X pump family, are similar in that both accept glutathione and glucuronide conjugates as substrates,[2, 3, 32] this is a clear demonstration of marked substrate specificity among GS-X pumps expressed on resistant cell lines. It should be mentioned that Keppler and his collaborators have shown that there is preferential uptake of glucuronide in MRP2/cMOAT compared with MRP1 and that the inhibitory effect of MK571, a leukotriene D_4 antagonist, is more potent for MRP2/cMOAT than for MRP1.[48]

In conclusion, the results of the present study suggest that a GS-X pump different from MRP1, MRP2/cMOAT, or MRP3 is responsible for the transport of GS-platinum complex from KCP-4. Transport studies with isolated membrane vesicles indicated that the substrate specificity of this GS-X pump is quite different from that of MRP1, in that $E_2$17βG and GS-platinum complex are not good substrates for this GS-X pump and MRP1, respectively. There is a clear difference in substrate specificity among GS-X pump family members expressed on resistant tumor cells.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas “ABC proteins” (# 10044243) from the Ministry of Education, Science, Sports and Culture of Japan.

(Received October 17, 1998/Revised January 19, 1999/Accepted January 29, 1999)

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