Research Article

Human Multidrug Resistance Protein 7 (*ABCC10*) Is a Resistance Factor for Nucleoside Analogues and Epothilone B

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Abstract

Multidrug resistance protein 7 (MRP7; *ABCC10*) is an ATP-binding cassette transporter which is able to transport amphipathic anions and confer resistance to docetaxel and, to a lesser extent, vincristine and paclitaxel. Whereas some detail on the resistance profile of MRP7 is known, the activities of the pump have not been completely determined. Here, it is shown by the analysis of MRP7-transfected HEK293 cells that, in addition to natural product agents, MRP7 is also able to confer resistance to nucleoside-based agents, such as the anticancer agents cytarabine (Ara-C) and gemcitabine, and the antiviral agents 2′,3′-dideoxycytidine and PMEA. Consistent with the operation of an efflux pump, expression of MRP7 reduced the accumulation of Ara-C and PMEA. In addition, MRP7 is also able to confer resistance to the microtubule-stabilizing agent epothilone B. Ectopic expression of MRP7 in mouse embryo fibroblasts deficient in P-glycoprotein and Mrp1 revealed that MRP7 has a broad resistance profile for natural product agents. In this drug-sensitive cellular background, MRP7 conferred high levels of resistance to docetaxel (46-fold), paclitaxel (116-fold), SN-38 (65-fold), daunorubicin (7.5-fold), etoposide (11-fold), and vincristine (56-fold). Buthionine sulfoximine did not attenuate MRP7-conferred resistance to docetaxel or Ara-C. These experiments indicate that the resistance capabilities of MRP7 include nucleoside-based agents and a range of natural product anticancer agents that includes nontaxane antimicrotubule agents that are not susceptible to P-glycoprotein–mediated transport and that, unlike MRP1 and MRP2, MRP7-mediated drug transport does not involve glutathione. [Cancer Res 2009;69(1):178–84]

Introduction

Cellular resistance to chemotherapeutic agents is a major obstacle to cancer treatment. ATP-binding cassette (ABC) transporters are involved in resistance by virtue of their ability to extrude drugs from the cell (1, 2). Many of the ABC transporters that function as drug efflux pumps are termed multidrug resistance proteins (MRP) and reside in the C family of ABC transporters—one of the largest families of ABC transporters (3, 4). The MRP family is composed of nine members, eight of which have been characterized and established as having the facility for transporting amphipathic anions. Notable substrates of MRPs include conjugates of glutathione, glucuronic acid, and sulfate, and members of this family are, therefore, considered to be transporters of the products of phase II of xenobiotic detoxification. MRPs are grouped according to whether they possess two or three transmembrane-spanning domains (5). With respect to drug transport, the most extensively characterized MRPs are MRP1, MRP2, and MRP3 (three membrane-spanning domains) and MRP4, MRP5, and MRP8 (two membrane-spanning domains). This structural classification also seems to be relevant with respect to certain functional properties. MRPs in the former group are able to confer resistance to varying extents to natural product anticancer agents, whereas the shorter MRPs are distinguished by their ability to confer resistance to nucleoside-based agents. In addition, MRP4, MRP5, and MRP8 are able to transport cyclic nucleotides—a feature that is not shared by the larger MRPs (6, 7). Pharmacologic studies on gene-disrupted mice have established that MRP1 and MRP4 are able to function *in vivo* as resistance factors for normal tissues (8–10) and that the latter pump, along with MRP2 and MRP3, are involved in various aspects of drug disposition (11). Studies on knockout mice have also suggested physiologic functions. Mrp1 has been implicated in dendritic cell function and inflammation by virtue of its ability to efflux leukotriene C4, Mrp3, and Mrp4 protect cholestatic liver from endogenous compounds, such as bile acids, and Mrp4 has been implicated in PGE2-mediated inflammatory responses (12–16).

Recently, our laboratory reported initial functional characterizations of MRP7, an MRP family member that possesses three membrane-spanning domains (7, 17). We found that, although MRP7 is about equally related to C family ABC transporters involved in the regulation of ion transport as it is to MRPs, it nevertheless possesses features that are characteristic of MRPs. MRP7 is competent in the transport of the canonical MRP transport substrate $E_2 17\beta G$ but possesses little or no activity toward a range of other substrates handled by other MRPs, including glutathione and sulfate conjugates, bile acids, and cyclic nucleotides (18). In addition, MRP7 is able to confer resistance to certain natural product agents, a property that is shared by other MRPs that have three membrane-spanning domains (19). However, a distinctive feature of MRP7 is that it is able to confer sizable levels of resistance to docetaxel. In addition, it is able to confer lower levels of resistance to paclitaxel and *Vinca* alkaloids. Whereas these reports provided some detail on the functional characteristics of MRP7, the drug resistance properties of the pump have not been completely analyzed. Here, we use a combination of MRP7-transfected HEK293 and Pgp/Mrp1-deficient fibroblasts to more precisely define the MRP7 drug resistance profile. It is shown that, unlike other large members of the MRP family, MRP7 is able to confer resistance to nucleoside-based agents. In addition, the resistance activity of the pump extends to a wider range of natural

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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products agents than previously suspected. Notably, MRP7 is also able to confer resistance to epothilone B, a class of natural product agents that are not known to be susceptible to transport by other drug efflux pumps.

Materials and Methods

Cell lines, plasmids and transfection. The generation of HEK293 clones stably transfected with MRP7 expression vector (HEK-MRP7-C17 and HEK-MRP7-C18) and parental vector-transfected control cells (HEK293-pcDNA) was previously described (18). Triple knockout (TKO) *Mrp1*$^{-/-}$,*Mdr1a/b*$^{-/-}$ fibroblasts (MEF3.8 cells) were kindly provided by Dr. Alfred Schinkel (20, 21). HEK293 cells, NIH/3T3 cells, and MEF3.8 cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mmol/L glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. The MEF3.8 cells required the same medium with the addition of 2.5 μg/mL puromycin to maintain MRP7 expression. The MDCKII cells were grown in low glucose DMEM supplemented with 10% FBS, 2 mmol/L glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. A vector for expression of MRP7 in *Mrp1*$^{-/-}$,*Mdr1a,b*$^{-/-}$ mouse embryo fibroblasts was prepared by excising the cDNA insert encoding MRP7 from pcDNA 3.1 MRP7 (18) using SnaB and inserting it into the SnaB site of retroviral vector pBabe(puro) to create pBabe(puro)-MRP7. Phoenix retroviral packaging cells were transfected with pBabe(puro)-MRP7 or parental vector and harvested retroviral particles were used to transduce *Mrp1*$^{-/-}$, *Mdr1a,b*$^{-/-}$ fibroblasts. After 48 h, the cells were passaged, and stable clones were selected in 2.5 μg/mL puromycin. MRP7 overexpressing clones were identified by immunoblotting. A vector for expression of MRP7 in baculovirus was prepared by excising the MRP7 cDNA insert from pcDNA 3.1 MRP7 (18) and inserting it into the Not1 and EcoR1 sites of PVL1392 (Pharmingen). MRP7 baculovirus was generated using BacVector 3000 (Invitrogen).

Immunoblot analysis. Monolayers were washed with ice-cold PBS and incubated on ice for 20 min in radioimmunoprecipitation assay buffer containing 10 μg/mL aprotinin, 5 μg/mL leupeptin, and 1 mmol/L phenylmethyl sulfonyl fluoride. Lysates were passed through a syringe and then centrifuged in a cold microfuge for 15 min at 15,000 rpm. Proteins were separated by SDS-PAGE on 4% to 12% Bis-Tris gels and transferred to nitrocellulose filters using a wet transfer system, as previously described (22, 23). MRP7 was detected using anti-MRP7 monoclonal antibody (1:10) in conjunction with horseradish peroxidase (HRP)–labeled goat anti-mouse IgG (NEN) used at a dilution of 1:2,500. β-Actin–HRP conjugated antibody was used at a dilution of 1:5,000 (Abcam) Membrane vesicles were prepared by the nitrogen cavitation method as described previously (24).

Drug sensitivity assays. Drug sensitivity was analyzed using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt/phenazine methosulfate (MTS/PMS) microtiter plate assay (CellTiter 96 Cell Proliferation Assay, Promega). HEK-pcDNA3, HEK-MRP7-C17, and HEK293-MRP7-C18 were seeded in triplicate at 3,000 cells per well in 96-well plates in DMEM containing 10% FBS. Parental vector-transduced MEF3.8 cells (TKO-pBabe) and MRP7-transduced MEF3.8 cells (TKO-MRP7-7-21) were seeded at 1,500 cells per well in 10% DMEM. All other cell lines were seeded at 3,000 cells per well in 10% DMEM. The following day, drugs were added at various concentrations to the growth medium. Cellular proliferation assays were performed after 72 h of incubation in the presence of drug. Vincristine, vinblastine, paclitaxel, daunorubicin, cisplatin, 5-fluoro-2′-deoxyuridine, 5-fluoro-5′-deoxyuridine, 5-azacytidine, 5-fluorouracil, 6-thioguanine, 2′-chloro-2′ deoxythymidine, 2′,3′-dideoxycytidine, cytabarine, 6-mercaptopurine and buthionine sulfoximine (BSO) were purchased from

Table 1. Drug sensitivity of MRP7-transfected HEK293 cells

Drug	IC_{50}* (μmol/L)			Fold resistance †	
	HEK-pcDNA3	HEK-MRP7-C17	HEK-MRP7-C18	HEK-MRP7-C17	HEK-MRP7-C18
GEM	2.6 ± 0.4	7.7 ± 0.9	7.2 ± 0.9	3.0 ‡	2.8 ‡
5-FU	9.6 ± 2.5	12 ± 1.7	12 ± 2.0	1.3	1.3
5-dFUrd	0.011 ± 0.002	0.014 ± 0.002	0.018 ± 0.002	1.3	1.6 ‡
5-FdUrd	0.024 ± 0.007	0.020 ± 0.005	0.020 ± 0.003	0.8	0.8
5-AZ	2.2 ± 0.6	4.3 ± 0.9	5.1 ± 1.5	2.0§	2.3
Ara-C	0.20 ± 0.3	0.91 ± 0.4	1.7 ± 0.9	4.6 ‡	8.5§
CdA	1.0 ± 0.2	1.4 ± 0.2	1.2 ± 0.3	1.4	1.2
6-TG	1.2 ± 0.6	1.5 ± 0.8	1.6 ± 0.6	1.3	1.3
6-MP	2.8 ± 0.7	1.6 ± 0.7	2.6 ± 0.7	0.6	0.9
ddC	76 ± 20	375 ± 150	562 ± 230	4.9 ‡	7.4 ‡
PMEA	24 ± 7.3	66 ± 11	67 ± 12	2.8 ‡	2.8 ‡
MX	0.41 ± 0.5	0.20 ± 0.4	0.25 ± 0.8	0.5	0.6
EpoA (nmol/L)	5.4 ± 1.7	6.2 ± 2.2	4.4 ± 1.1	1.2	0.8
EpoB (nmol/L)	0.99 ± 0.2	5.2 ± 1.9	6.7 ± 2.1	5.3 ‡	6.8 ‡
PHOM (nmol/L)	1000 ± 300	1130 ± 370	1190 ± 480	1.1	1.2
MAC321 (nmol/L)	63 ± 9.8	91 ± 10	105 ± 21	1.4	1.7 ‡
MST997 (nmol/L)	40 ± 8.4	48 ± 13	60 ± 10	1.2	1.5 ‡
HTI286 (nmol/L)	43 ± 18	77 ± 40	122 ± 54	1.8	2.8 ‡

Abbreviations: GEM, gemcitabine; 5-FdURd, 5-fluoro-5′-deoxyuridine; 5-dFUrd, 5′-fluoro-2′-deoxyuridine; 5-AZ, 5-azacytidine; CdA, 2′-chloro-2′deoxyadenosine; ddC, 2′,3′-dideoxycytidine; MX, mitoxantrone; EpoA, epothilone A; EpoB, epothilone B; PHOM, phomopsin A.

*Drug sensitivities were analyzed by the use of a 3-d colorimetric assay in which cells were continuously exposed to the indicated agents. IC_{50} is the concentration that inhibited cell survival by 50% (means ± SE). n = 6-11 for Ara-C, GEM, 5-FU, 5-dFUrd, CdA, PMEA, MAC321, 5-FdUrd, 5-AZ, HTI286, ddC, EpoB, and PHOM; n = 3-5 for MX, MST997, 6-TG, 6-MP, and EpoA.

†IC_{50} of HEK-MRP7-C17 or HEK-MRP7-C18 divided by IC_{50} of control HEK-pcDNA3 cells.

‡Significantly different from the control transfectant as assessed by the nonparametric two-tailed Wilcoxon test ($P < 0.05$).

§$P < 0.01$.

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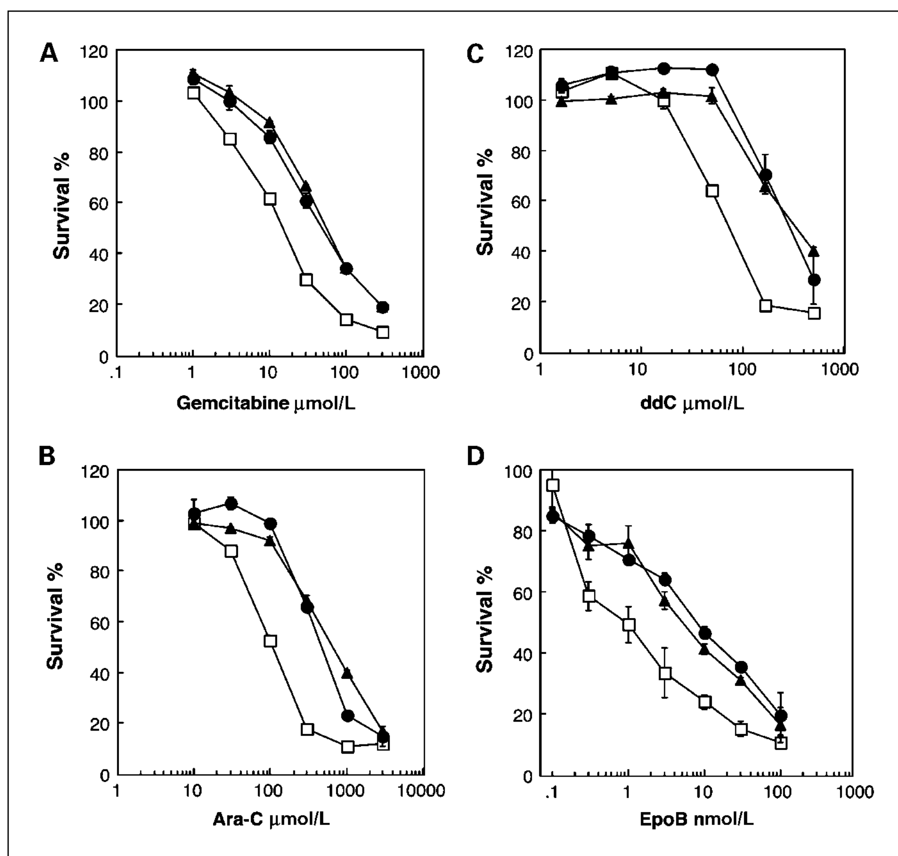

Figure 1. Sensitivity of control and MRP7-transfected HEK293 cells to gemcitabine, Ara-C, ddC, and epothilone B. The drug sensitivities of parental vector-transfected HEK293 cells (□, HEK-pcDNA 3) and MRP7-transfected HEK 293 cells (▲, HEK-MRP7-C17; ●, HEK-MRP7-C18) were analyzed toward gemcitabine (*A*), Ara-C (*B*), ddC (*C*), and epothilone B (*D*) using the MTS/PMS assay, as described in Materials and Methods. *Points,* means of triplicate determinations; *bars,* SD. Representative experiments are shown.

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Sigma Chemical Company. SN-38 was generously provided by Pharmacia Corporation. Etoposide (Bristol Meyers Squibb), gemcitabine (Eli Lilly), and docetaxel (Aventis Pharmaceuticals) were obtained from the pharmacy of the Fox Chase Cancer Center. MAC231, MST997, and HTI286 were kindly provided by Wyeth Research. Epothilone B, epothilone A, and phomopsin A were obtained from Calbiochem. PMEA [9-(2-phosphonylmethoxynyl)-adenine] was kindly provided by Gilead.

Drug accumulation assays. For PMEA accumulation, control HEK-pcDNA cells and HEK-MRP7-C18 cells were seeded in triplicate at 3×10^5 cells per well in 24-well dishes. On the next day, [^{3}H]bis-pom PMEA (3.0 Ci/mmol, Moravek) was added to a final concentration of 0.1 μmol/L, and the cells were incubated at 37°C. At various time points, the cells were washed with ice-cold PBS and trypsinized. An aliquot of cells was used to determine the cell number, and the remaining cells were pelleted at 4°C and washed twice with ice-cold PBS. Radioactivity was measured by the use of a liquid scintillation counter (Packard Instrument Company, Inc.). For cytarabine (Ara-C) accumulation, TKO-pBabe and TKO-MRP7-7-21 cells were seeded in triplicate in 24-well plates at 3×10^5 per well, whereas HEK-pcDNA and HEK-MRP7-C18 cells were seeded in 24-well plates at 5×10^5 per well. After overnight incubation, [^{3}H]Ara-C (24 Ci/mmol; Moravek) was added to a final concentration of 0.1 μmol/L, and the cells were incubated at 37°C for various times. The cells were trypsinized, collected by centrifugation at 2,000 × rpm for 5 min, washed thrice with ice-cold PBS, and lysed in 10 mmol/L lysis buffer [1% Triton X-100, 0.2% SDS (pH 7.4)]. Lysates were placed in scintillation fluid, and radioactivity was measured in a Packard TRI-CARB 1900CA liquid scintillation analyzer.

Generation of MRP7 monoclonal antibody. A cDNA fragment encoding amino acids 890-894 of MRP7 (17) was inserted downstream of the glutathione *S*-transferase coding sequence in the pGEX-2T prokaryotic expression vector (Pharmacia Biotech, Inc.). The fusion protein was isolated by electroelution from a preparative SDS-PAGE gel. Immunization of five BALB/c mice, splenic fusion, and enzyme-linked immunoabsorbent assays were performed, as described previously (25). For enzyme-linked immunoadsorbent assays of hybridoma supernatants, lysates from insect cells infected with MRP7 baculovirus were used to coat 96-well dishes. Hybridoma preparation was accomplished by the Fox Chase Cancer Center hybridoma facility.

Measurement of cellular glutathione concentrations. Subconfluent cells grown in 25-cm^2 flasks were harvested by trypsinization, pelleted, and washed with PBS. The cells were resuspended in 5% metaphosphoric acid and sonicated. The suspensions were then pelleted to remove cellular debris, and 200 μL of the supernatant were removed for the determination of glutathione levels. Analysis of glutathione was accomplished using a BIOXYTECH GSH-400 kit (Oxis International), according to the manufacturer's instructions. To determine the effect of BSO on intracellular glutathione concentrations, the cells were grown overnight with 50 μmol/L BSO before harvesting.

Results

Having determined in a previous study involving natural product agents that MRP7 is able to confer resistance to microtubule active agents, such as taxanes and *Vinca* alkaloids (19), we sought to extend our characterization of the pump by defining its capabilities with respect to another important class of anticancer agents—nucleoside analogues. To investigate this, the sensitivities of two previously described clones of MRP7-transfected HEK293 cells and parental vector-transfected control cells were analyzed. A range of anticancer nucleoside analogues (gemcitabine, fluoropyrimidines, 5 Aza, Ara-C, and CDA) and nucleobase analogues [6-thioguanine (6-TG), 6-mercaptopurine (6-MP)] were examined. As shown in Table 1, increased resistance was observed for gemcitabine and Ara-C, for which the MRP7-transfected cells exhibited 2.8-fold to 3.0-fold and 4.6-fold to 8.5-fold levels of resistance, respectively.

Low levels of resistance toward 5-Aza (2.0-fold) and 5-fluoro-2′-deoxyuridine (5-FdUrd; 1.6-fold) were observed for HEK-MRP7-C17 and HEK-MRP7-C18, respectively. In addition, resistance toward two antiviral agents, the nucleoside analogue 2′,3′-dideoxycytidine (ddC) and the nucleotide analogue PMEA, was analyzed. The MRP7-transfected cells exhibited resistance to each of these agents, with fold resistance levels of 4.9 to 7.4 and 2.8 toward ddC and PMEA, respectively. Representative dose-response curves for nucleoside analogues are shown in Fig. 1*A-C*.

Next, the activity of MRP7 toward several microtubule active agents that are currently in development was analyzed (Table 1). These agents included the macrolides epothilones A and B (26), the macrocyclic heptapeptide phomopsin (27), the docetaxel analogues MAC-321 and MST-997 (28, 29), and HTI-286, an analogue of the tripeptide hemiasterlin (30). Notably, the MRP7-transfected cells exhibited resistance toward epothilone B (5.3-fold to 6.8-fold; Fig. 1*D*). In addition, low levels of resistance (1.5-fold to 2.8-fold) were observed for HEK-MRP7-C18 toward MAC-321, MST-997, and HTI-286.

To confirm that MRP7 affects sensitivity to nucleoside analogues by reducing cellular accumulation, the cellular kinetics of radiolabeled PMEA and Ara-C was analyzed. As shown in Fig. 2, HEK-MRP7-C18 exhibited reduced accumulation for each of these agents. At 30 minutes, accumulation of PMEA was reduced by 34% compared with the control cells, and this deficit was maintained at the 60-minute time point (Fig. 2*A*). At 90 minutes, the accumulation of Ara-C was reduced by 43% in MRP7-transfected cells (Fig. 2*B*).

To more precisely characterize the resistance activity of MRP7, the pump was expressed in mouse embryonic fibroblasts that are genetically deficient in Mdr1a/1b and Mrp1. The absence of these two pumps renders the cells sensitive to drug substrates of the latter pumps (21, 31) and, thus, provides a cellular background that affords a clearer picture of the activity of ectopically expressed resistance factors. Figure 3*A* shows the ectopic expression of MRP7 in Mdr1a/1b, Mrp1-deficient MEF3.8 mouse embryo fibroblasts. In this cellular background, MRP7 exhibited robust activity toward almost all of the natural product agents tested. Notably, resistance was observed toward agents for which activity was not apparent in the HEK293 cellular background, such as SN-38, daunorubicin, and etoposide (Table 2; for comparison, our previously reported results using MRP7-transfected HEK293 cells are provided in parentheses in the last column). Particularly high resistance levels were exhibited by TKO-MRP7-7-21 for paclitaxel, docetaxel, SN-38 (the active metabolite of irinotecan), and vincristine (116-fold, 46-fold, 65-fold, and 56-fold resistance, respectively). TKO-MRP7-7-21 was also resistant to etoposide (11-fold) and daunorubicin (7.5-fold). By contrast with these agents, the levels of resistance observed for epothilone B, gemcitabine, 5-fluorouracil (5-FU), Ara-C, and cisplatin (CDDP) were roughly comparable with the levels observed for MRP7-transfected HEK293 cells (Table 2). Analysis of a second MEF3.8 transfectant, which expressed lower levels of MRP7 compared with TKO-MRP7-7-21, revealed resistance levels of 14-fold to paclitaxel, 12-fold to docetaxel, 23-fold to vincristine, 29-fold to SN-38, and 6.4-fold to epothilone B (data not shown). As expected, when cellular kinetics was analyzed in TKO-MRP7-7-21 cells using radiolabeled Ara-C as a probe, reduced accumulation was observed (Fig. 2*C*).

Glutathione has been implicated in the transport of natural product agents by MRP1 and MRP2 (32–34). To evaluate this feature of MRP7, the ability of BSO, an agent that depresses cellular glutathione levels by inhibiting the rate-limiting step in glutathione synthesis, to inhibit resistance was assessed. As shown in Supplementary Table S1, treatment of MRP7-transfected HEK293 cells with 50 μmol/L BSO did not affect resistance toward either docetaxel or Ara-C. Under these conditions, cellular glutathione levels were depressed by ~84%, a marked reduction (2.06 ± 0.04

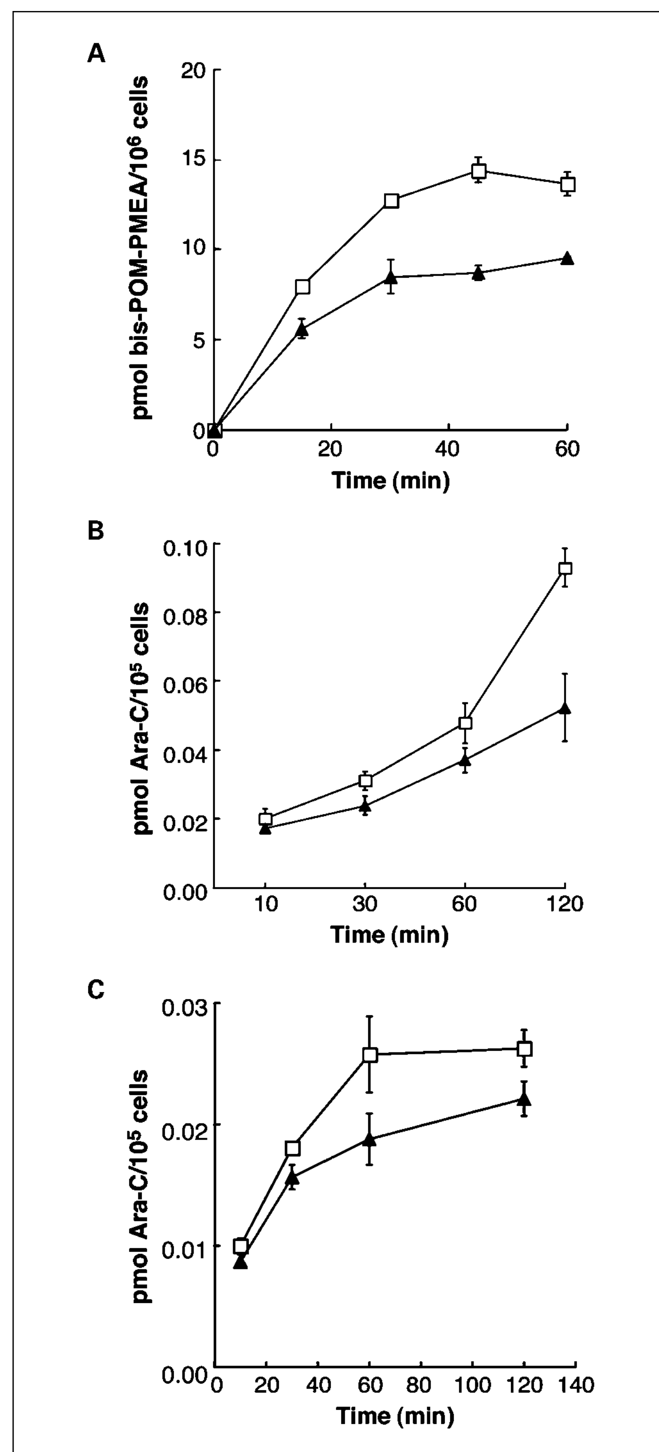

Figure 2. Accumulation of nucleoside analogue-based agents in MRP7-transfected HEK293 cells and MEF3.8 cells. *A,* accumulation of PMEA and Ara-C in control HEK-pcDNA 3.1 (□) and MRP7-transfected HEK-MRP7-C18 (▲) cells. *B,* accumulation of Ara-C in control TKO-pBabe (Δ) and MRP7-transduced TKO-MRP7-7-21 (♦) cells. Cells were incubated in the presence of 0.1 μmol/L [^{3}H]bis-POM-PMEA or 0.1 μmol/L [^{3}H]Ara-C, and accumulation was measured at various time points, as described in Materials and Methods. *Points,* means; *bars,* SD.

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and 1.73 ± 0.28 nmol/10^6 cells in the presence of BSO glutathione levels compared with 12.4 ± 0.39 and 11.4 ± 0.16 nmol/10^6 cells in the absence of BSO glutathione levels, respectively, for HEK-pcDNA3 and HEK-MRP7-C18 cells). Activity toward docetaxel is a prominent feature of the MRP7 resistance profile. To determine whether other MRP family members that confer resistance to natural product drugs could also transport docetaxel, MRP1, MRP2, and MRP3-transfected cells were tested. Resistance activity toward docetaxel was not detected for these efflux pumps (Supplementary Table S2).

Discussion

The present analysis of MRP7 activity provides surprising detail on the resistance profile of the pump. In a previous study, we focused our analysis on natural product anticancer agents because resistance toward at least some agents of this family is characteristic of MRP family members that possess three membrane-spanning domains. However, the relatively low degree of similarity between MRP7 and other MRP family members raised the possibility that its activity might not be restricted to natural product agents. By investigating this conjecture, we determined that the MRP7 resistance profile includes nucleoside-based agents, a class of compounds which are not known to be components of the resistance profiles of other large members of the MRP family but instead are agents toward which the small members, such as MRP4, MRP5, and MRP8, have activity (35–38). The nucleoside-based agents toward which MRP7 is able to confer resistance included anticancer agents, such as Ara-C, a mainstay in the treatment of acute myelogenous leukemia, and gemcitabine, an agent with utility in pancreatic and lung cancers. This is the first example of an MRP family member that is able to confer resistance to either of these widely used agents. In addition, MRP7 is also able to confer resistance to antiviral agents, such as ddC, similar to MRP8 and PMEA, as observed with MRP4, MRP5, and MRP8. It is likely that MRP7, which we have established as being competent in the transport of amphipathic anions (18), effluxes the negatively charged monophosphate metabolites of Ara-C, gemcitabine, and ddC as opposed to the uncharged parent nucleosides. This mechanism of resistance would be in accord with the ability, for example, of MRP8 to transport the monophosphate of 5-FU but not the uncharged parent nucleoside analogue (38). By contrast with the former nucleoside analogues, PMEA is a charged nucleotide analogue, which is likely to be a direct substrate of MRP7.

The present study also revealed that the range of MRP7 toward natural product agents is much broader than had been previously inferred from our prior analysis of MRP7-transfected HEK293 cells (17). Confirming our previous reported findings, we show that expression of MRP7 in the context of Pgp/Mrp1-deficient fibroblasts confers resistance to taxanes and *Vinca* alkaloids. However, in this genetically deficient context, MRP7 also protected cells from anthracyclines (daunorubicin), camptothecins (SN-38), and epipodophyllotoxins (etoposide). The ability to detect the increased activity of MRP7 toward the former agents and the emergence of activity toward the latter agents when the pump is expressed in Pgp/Mrp1-deficient fibroblasts compared with HEK293 cells (Table 2) is consistent with the phenotype of MEF3.8 cells. This cell line is reported to be 11-fold to 51-fold, 2-fold to 12-fold, 4-fold to 6-fold, 5-fold to 9-fold, and 19-fold to 41-fold more sensitive to paclitaxel,

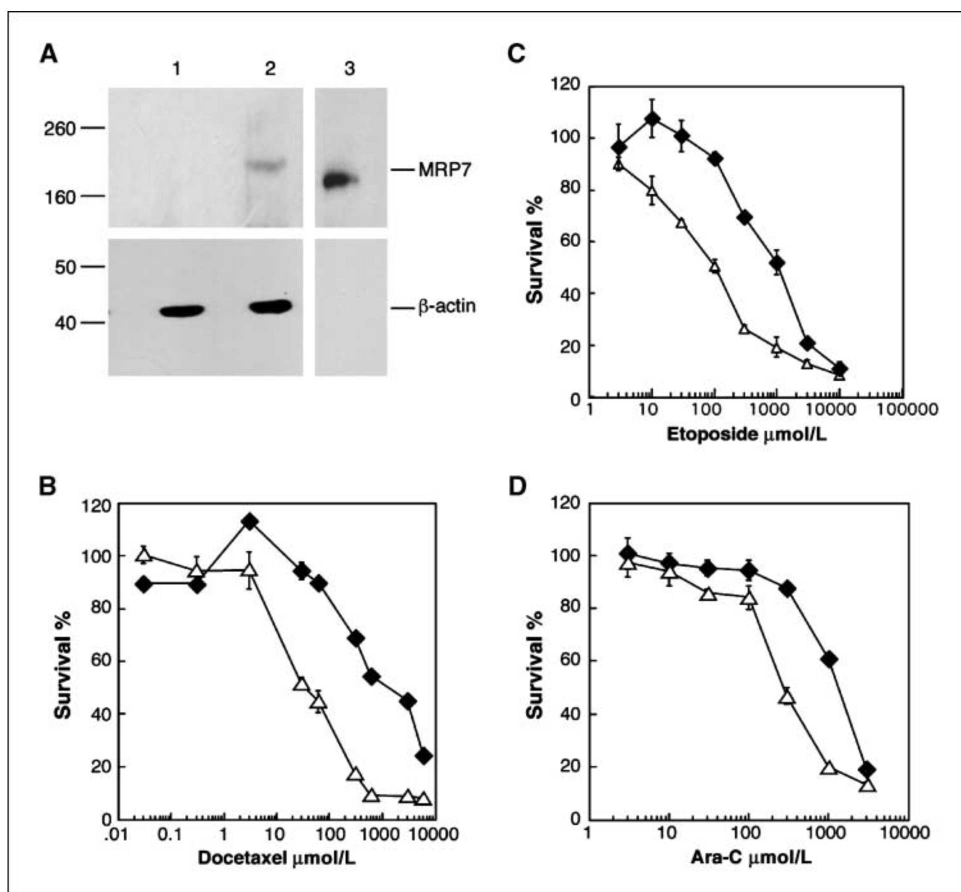

Figure 3. Immunoblot detection of MRP7 in MEF3.8 cells and drug sensitivity analysis. *A,* immunoblot detection of MRP7 expressed in MEF3.8 cells. Total cellular lysates were prepared from MRP7-transduced (TKO-MRP7-7-21) and parental vector-transduced MEF3.8 (TKO-pBabe) cells, and proteins (90 μg) were separated by SDS-PAGE. *Lane 1,* TKO-pBabe.; *lane 2,* TKO-MRP7-21; *lane 3,* membrane vesicles prepared from MRP7 baculovirus-infected Sf9 cells (positive control). MRP7 was detected using anti-MRP7 monoclonal antibody. MRP7 expressed in glycosylation-impaired insect cells runs slightly faster than in MEF3.8 cells. Molecular weight markers are shown (*left*). *B* and *C,* sensitivity of TKO-pBabe (Δ) and TKO-MRP7-21 cells (♦) to docetaxel, etoposide, and Ara-C. Drug sensitivity was analyzed using the MTS/PMS assay, as described in Materials and Methods. *Points,* means of triplicate determinations; *bars,* SD. Representative experiments are shown.

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Table 2. Drug sensitivity of MRP7-transfected *Mrp1*$^{-/-}$,*Mdr1a,b*$^{-/-}$ MEF3.8 (TKO) cells

Drug	IC_{50}* nmol/L		Fold resistance †	
	TKO-pBabe	TKO-MRP7-7-21	TKO-MRP7-7-21	HEK293-MRP7 ‡
PAC	7.0 ± 2.2	811 ± 380	116§	(3.3–3.4)
DOC	23 ± 14	1050 ± 490	46§	(8.7–12.7)
SN-38	16 ± 4.7	1040 ± 400	65§	(0.24–0.25)
DNR	10 ± 1.4	75 ± 8.6	7.5§	(1.0–1.1)
ETOP (μmol/L)	173 ± 61	1920 ± 840	11§	(1.1–1.0)
VCR	1.5 ± 0.3	84 ± 31	56§	(3.3–3.4)
EpoB	9.3 ± 3.3	31 ± 14	3.3‖	5.3–6.8
GEM	14 ± 2.2	32 ± 5.0	2.3	2.8–3.0
5-FU (μmol/L)	901 ± 180	1440 ± 110	1.6§	1.3
Ara-C	239 ± 54	1230 ± 310	5.1§	4.6-8.5
CDDP (μmol/L)	9.8 ± 4.4	18 ± 4.2	1.8§	(1.1–1.4)

Abbreaviations: PAC, paclitaxel; DOC, docetaxel; DNR, daunorubucin; ETOP, etoposide; VCR, vincristine; EpoB, epothilone B; GEM, gemcitabine.
* Drug sensitivities were analyzed by the use of a 3-d colorimetric assay in which cells were continuously exposed to the indicated agents. IC_{50} is the concentration that inhibited cell survival by 50% (means ± SE). n = 8 for ETOP, EpoB; n = 7 for DOC, SN-38, DNR, VCR, CDDP; n = 6 for PAC, 5-FU, Ara-C; n = 5 for GEM.
† IC_{50} of TKO-7-21 divided by IC_{50} of TKO-pBabe.
‡ Values in parentheses are from ref. 19; other values are from Table 1.
§ Significantly different from the control transfectant as assessed by the nonparametric two-tailed Wilcoxon test ($P < 0.05$).
‖ $P < 0.01$.

docetaxel, SN-38, daunorubucin, etoposide, and vincristine, respectively, compared with wild-type fibroblasts (21). The lack of significant enhancement of MRP7 activity for nucleoside analogues, such as Ara-C, and the alkylating agent cisplatin in Pgp/Mrp1-deficient cells compared with HEK293 cells is also consistent with the absence of sensitization of MEF3.8 cells toward these two agents (0.8-fold to 1.5-fold and 0.6-fold to 1.3-fold, respectively). That MRP7 activity (e.g., docetaxel) is enhanced in MEF3.8 cells toward agents for which this cell line is sensitized whereas its activity (e.g., Ara-C) is not increased toward agents for which MEF3.8 cells are not sensitized tends to support the validity of the MRP7 phenotype we describe here.

Our previous study disclosed that MRP7 is able to confer resistance to two classes of agents that target microtubules (*Vinca* alkaloids and taxanes). It was, therefore, of interest to determine if MRP7 might also confer resistance to newer antimicrotubule agents. A notable feature of MRP7 that emerged from this line of investigation is that it is able to confer resistance to epothilone B. Of the nontaxane microtubule-stabilizing agents that are in clinical development, epothilones are the most advanced (39–41). Epothilones, which have broad antitumor activity, are considered to be particularly attractive agents because they are not susceptible *in vitro* or *in vivo* to transport by P-glycoprotein or any previously tested drug efflux pumps (39). In accord with this situation, the absence of enhanced levels of MRP7-conferred resistance toward epothilone B in the MEF3.8 system compared with HEK293 cells is expected, in that MEFs that are deficient in P-glycoprotein and MRP1 should not be rendered sensitive to this agent. The finding that MRP7-transfected HEK293 cells are resistant to epothilone B indicates that there is at least one pump capable of effluxing this agent. By contrast with epothilone B, we did not detect resistance toward epothilone A, which differs from the former compound by the absence of a methyl group at C12. In addition, MRP7-transfected HEK293 cells exhibited either relatively modest or no resistance to the taxane analogues MAC-321 and MST-997, and the microtubule-destabilizing agents phomoposin A and HTI-286.

Another feature of MRP7 that was investigated in this study is the involvement of glutathione in MRP7-mediated resistance. The finding that BSO had no effect on resistance to docetaxel or Ara-C suggests that transport of anticancer agents by MRP7 does not require glutathione. This finding, in combination with the observations that glutathione levels are not significantly decreased in MRP7-transfected HEK293 cells (19) or MRP7-transfected MEF3.8 cells (present study), suggests that glutathione is not a substrate of MRP7. In this regard, MRP7 is distinct from MRP1 and MRP2, both of which require glutathione for transport of natural product agents and are competent in the transport of this tripeptide (3). Instead, MRP7 is similar to MRP3, which seems to transport etoposide in a glutathione-independent fashion (42). We also show in the present study that, in apparent contrast to MRP7, all three of the latter pumps are unable to confer resistance to docetaxel. However, with respect to MRP3, in contrast to studies using MRP3-transfected HEK293 cells, ovarian cancer 2008 cells, and P-glycoprotein and Mrp1-deficient MEF cells (42–44), a recent report found that when expressed in a breast cancer cell line MRP3 is able to confer resistance to paclitaxel (45). This suggests that the activity of MRP3 may depend on cell context. Therefore, with regard to the activity of MRP3 toward docetaxel, further studies are warranted using additional cell lines to the HEK293 cells used here.

To better understand the potential effect of MRP7 on the sensitivity of normal tissues and tumors, detailed information will be needed on the protein expression pattern of the pump. Our laboratory and others have detected MRP7 transcript in a variety of tissues, including pancreas, liver, placenta, kidney, brain, ovary, spleen, heart, skeletal muscle, testis, intestine, prostate, and WBC, as well as in various fetal tissues (refs. 17, 46; Supplementary Fig. S1). Transcript has also been detected in several adenocarcinomas, including tumors, such as breast, ovary, and lung (46, 47). This is of

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potential interest in that the latter tumors are treated with taxanes. In addition, MRP7 may be induced by anticancer agents toward which the pump confers resistance. MRP7 transcript and protein were reported to be induced by vincristine exposure in two salivary gland adenocarcinoma cell lines that are cross-resistant to docetaxel (48), and transcript was reported to be increased in MCF7 cells by exposure to doxorubicin (46). In addition, it was recently reported that MRP7 is induced by paclitaxel in a non–small cell lung cancer cell line (49). MRP7 antibodies and an *Mrp7* gene-disrupted mouse we have generated[5] should be valuable tools for understanding the potential effect of MRP7 on drug sensitivity.

[5] E.H-B. and G.D.K, unpublished data.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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