

Analysis of the Drug Resistance Profile of Multidrug Resistance Protein 7 (ABCC10): Resistance to Docetaxel

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ABSTRACT

The multidrug resistance protein (MRP) family consists of nine members that can be categorized according to whether or not a third (NH₂-terminal) membrane-spanning domain is present. Three (MRP1, MRP2, and MRP3) of the four members that have this structural feature are able to confer resistance to natural product anticancer agents. We previously established that MRP7, the remaining family member that has three membrane-spanning domains, possesses the cardinal biochemical activity of MRPs in that it is able to transport amphipathic anions such as 17 β -estradiol 17-(β -D-glucuronide). However, the drug resistance profile of the pump has not been determined. In this study, the drug resistance capabilities of MRP7 are evaluated by analyzing the resistance profiles of two clones of HEK293 cells in which the pump was ectopically expressed. MRP7-transfected HEK293 cells exhibited the highest levels of resistance toward docetaxel (9–13-fold). In addition, lower levels of resistance were observed for paclitaxel (3-fold), vincristine (3-fold), and vinblastine (3–4-fold). Consistent with the operation of an ATP-dependent efflux pump, MRP7-transfected cells exhibited reduced accumulation of radiolabeled paclitaxel compared with HEK293 cells transfected with parental plasmid. These results indicate that MRP7, unlike other MRPs, is a resistance factor for taxanes.

INTRODUCTION

The multidrug resistance protein (MRP) family is composed of nine related ABC transporters, many of whose substrate selectivities and drug resistance capabilities have now been determined to at least some extent (1). Structurally, members of this family can be classified according to whether or not they possess a third (NH₂-terminal) membrane-spanning domain (2). This topological feature is present in MRP1, MRP2, MRP3, MRP6, and MRP7, whereas it is absent in MRP4, MRP5, MRP8, and MRP9 (2–6). This classification also appears to be useful for categorizing the functional properties of the proteins. For example, although all of the characterized MRPs are able to transport amphipathic anions, such as conjugates of glutathione and glucuronic acid, only MRP4, MRP5, and MRP8 are competent in transporting cyclic nucleotides (7–9). In addition, whereas MRP1, MRP2, MRP3, and MRP6 are able to confer resistance to natural product agents (10–22), MRP4, MRP5, and MRP8 do not appear to have this ability and instead are able to confer resistance to nucleotide analogs (7, 9, 23, 24, 25).

Recently, we analyzed the predicted protein of MRP7 and reported that it has the lowest degree of structural resemblance to other MRPs on the basis of amino acid alignments (~34–36%; Ref. 3). Despite this relatively low degree of amino acid identity, we determined using membrane vesicle transport assays that MRP7 possesses the cardinal biochemical feature of this family, in that it is competent in the

transport of amphipathic anions, such as the prototypical MRP family substrate 17 β -estradiol 17-(β -D-glucuronide) (26). The ability of the pump to transport amphipathic anions such as 17 β -estradiol 17-(β -D-glucuronide), in combination with the presence of a third membrane-spanning domain, suggests that like other MRPs that have this structural feature, MRP7 might have the facility for conferring resistance to natural product anticancer agents. However, the drug resistance capabilities of MRP7 have not been determined to any extent. In this study, we assess the drug resistance activity of the pump. It is shown that MRP7 is able to confer low levels of resistance to agents such as *Vinca* alkaloids and substantial levels of resistance to docetaxel, the latter of which distinguishes MRP7 from other characterized MRP family members.

MATERIALS AND METHODS

Cell Lines. 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 described previously (26). HEK293 cells were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin.

Preparation of Membranes and Immunoblotting. Confluent cells were washed with PBS containing 1% aprotinin, collected by trypsinization, and pelleted. Lysis buffer [10 mM KCl, 1.5 mM MgCl₂, 1 M HEPES (pH 7.4), 1 mM *p*-aminodiphenylmethanesulfonylfluoride, and 2 μ g/ml aprotinin] was added, and after 10 min on ice, homogenization was accomplished by 30 strokes of an electrical homogenizer. Intact cells and nuclei were removed by centrifugation at 5000 \times g for 10 min at 4°C. Crude membranes were pelleted by ultracentrifugation at 35,000 \times g for 40 min at 4°C and resuspended in dilution buffer [10 mM Tris-HCl (pH 7.4) and 1 mM *p*-aminodiphenylmethanesulfonylfluoride].

Membrane proteins were separated by 8% SDS-PAGE and transferred to nitrocellulose filters using a wet transfer system, as described previously (27, 28). MRP7 protein was detected using the previously described anti-MRP7 polyclonal antibody (26) at a dilution of 1:500 and horseradish peroxidase-labeled goat antirabbit IgG (NEN, Boston, MA).

Measurement of Cellular Glutathione Concentrations. Subconfluent cells growing in 25-cm² 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 μ l of the supernatant was removed for determination of glutathione levels. Analysis of glutathione was accomplished using a BIOXYTECH GSH-400 kit (Oxis International, Portland, OR) according to the manufacturer's instructions.

Analysis of Drug Sensitivity. Drug sensitivity was analyzed using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt/phenazine methosulfate microtiter plate assay (Cell-Titer 96 Cell Proliferation Assay; Promega, Madison, WI). HEK293-pcDNA3 and MRP7-transfected cell lines HEK293-MRP7-C17 and HEK293-MRP7-C18 were seeded in triplicate at 5000 cells/well in 96-well dishes in DMEM containing 10% fetal bovine serum. The following day, drugs were added at various concentrations to the growth medium. Growth assays were performed after 72 h of incubation in the presence of drug. Vincristine, vinblastine, paclitaxel, daunorubicin, cisplatin, and doxorubicin were purchased from Sigma Chemical Company (St. Louis, MO). SN38 was generously provided by Pharmacia Corporation (Kalamazoo, MI). Etoposide (Bristol Meyers Squibb, Princeton, NJ) and docetaxel (Aventis Pharmaceuticals, Bridgewater, NJ) were obtained from the Fox Chase Cancer Center pharmacy.

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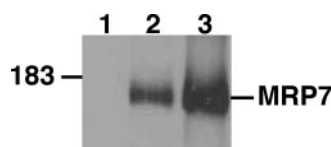


Fig. 1. Immunoblot detection of MRP7 protein in transfected HEK293 cells. Membranes preparations from control HEK-pcDNA3 cells and MRP7-transfected HEK-MRP7-C17 and HEK-MRP7-C18 cells were separated by SDS-PAGE. Proteins were transferred to nitrocellulose filters, and MRP7 was detected by immunoblotting with anti-MRP7 polyclonal antibody. Lane 1, HEK-pcDNA3; Lane 2, HEK-MRP7-C17; Lane 3, HEK-MRP7-C18. The location of a protein M_r marker is shown on the left.

Drug Accumulation. For paclitaxel accumulation experiments, control HEK293-pcDNA cells and HEK-MRP7-C18 cells were seeded in triplicate at 3×10^5 cells/well in 24-well dishes. The next day, [3 H]paclitaxel (3.0 Ci/mmol; Moravex, Brea, CA) was added to a concentration of 0.1 μ M, 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 analyze cell number, and the remaining cells were pelleted at 4°C and washed two more times with ice-cold PBS. Radioactivity was then measured by use of a liquid scintillation counter.

RESULTS

To evaluate the drug resistance capabilities of MRP7, the drug sensitivities of two clones of MRP7-transfected HEK293 cells (HEK-MRP7-C17 and HEK-MRP7-C18) were compared with HEK293 cells transfected with parental vector (HEK-pcDNA3). These stable transfectants were previously used for analyzing the substrate selectivity of MRP7 (26). Ectopic expression of MRP7 in the two MRP7-transfected clones is indicated by the intensely immunoreactive bands that migrated with an apparent $M_r \sim 171,000$, but were not present in membranes prepared from the control cells (Fig. 1). A major degradation product of $M_r \sim 113,000$ was frequently observed in the MRP7-transfected cells (not shown in Fig. 1).

The drug sensitivities of the cell lines were analyzed by the use of a 3-day colorimetric growth assay. As shown in Table 1 and Fig. 2, increased resistance was observed for several natural product anticancer agents. Interestingly, the highest levels of resistance were observed for the taxane docetaxel, for which HEK-MRP7-C17 and HEK-MRP7-C18 exhibited 8.7- and 12.7-fold resistance, respectively. Resistance to paclitaxel was also detected, but at lower levels (3.3- and 3.4-fold for HEK-MRP7-C17 and HEK-MRP7-C18, respectively). Increased resistance was also observed for *Vinca* alkaloids. HEK-MRP7-C17 and HEK-MRP7-C18 exhibited 3.4- and 3.2-fold resistance toward vincristine and 4.0- and 3.3-fold resistance toward vinblastine, respectively. Although MRP7 protein levels were some-

what higher in HEK-MRP7-C18 than in HEK-MRP7-C17, this difference was not consistently reflected in the levels of resistance observed for the two cell lines. The absence of strict concordance with protein expression levels could be attributable to differing amounts of MRP7 protein that is routed to the plasma membrane in the two cell lines. The IC_{50} s obtained for several other natural product agents including etoposide, SN38, daunorubicin, and doxorubicin were not significantly higher than the control cells. However, the IC_{75} s (but not

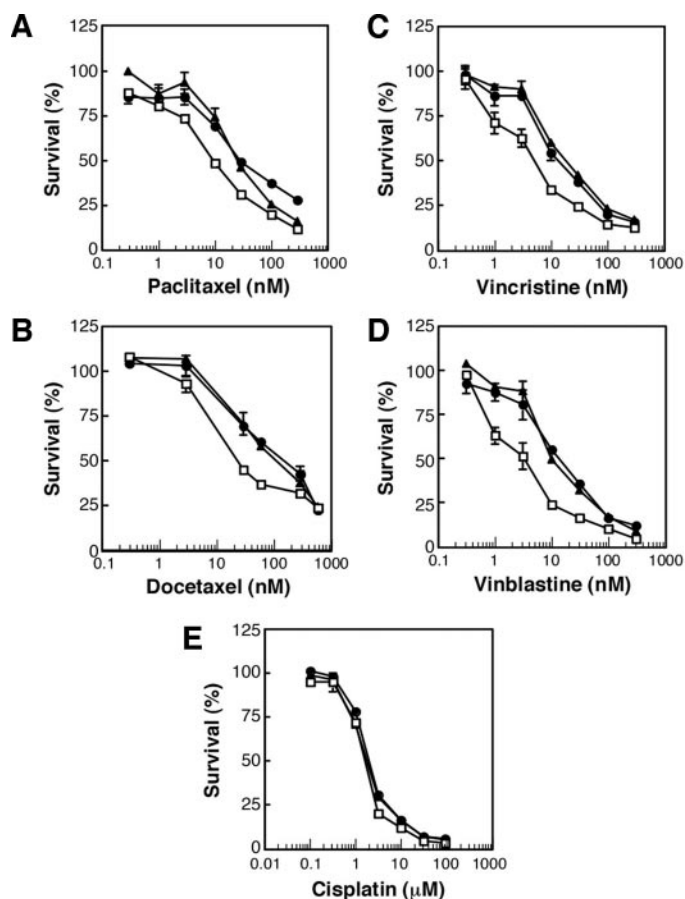


Fig. 2. Sensitivity of control and MRP7-transfected cells to docetaxel (A), paclitaxel (B), vincristine (C), vinblastine (D), and cisplatin (E). The drug sensitivities of parental vector-transfected HEK293 cells (HEK-pcDNA3; \square), HEK-MRP7-C17 (\blacktriangle), and HEK-MRP7-C18 (\bullet) were analyzed by use of the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt/phenazine methosulfate assay, as described in "Materials and Methods." Data points are the means \pm SD of triplicate determinations. Representative experiments are shown.

Table 1 Drug sensitivity of MRP7-transfected HEK293 cells

Drug	IC_{50} ^a nM			Fold resistance ^b	
	HEK-pcDNA3	HEK-MRP7-C17	HEK-MRP7-C18	HEK-MRP7-C17	HEK-MRP7-C18
VCR ^c	5.97 \pm 0.4	20.4 \pm 1.3	19.4 \pm 1.9	3.41 ^d	3.25 ^d
VBL	5.96 \pm 0.42	23.8 \pm 2.3	19.9 \pm 3.9	3.99 ^d	3.33 ^d
PAC	9.25 \pm 0.40	30.6 \pm 2.5	31.7 \pm 2.7	3.31 ^d	3.43 ^d
DOC	11.9 \pm 2.7	103 \pm 40	151 \pm 38	8.66 ^d	12.7 ^d
ETOP	5800 \pm 900	6290 \pm 1700	5930 \pm 1100	1.09	1.02
SN38	1.00 \pm 0.21	0.25 \pm 0.02	0.24 \pm 0.02	0.25	0.24
DNR	12.6 \pm 3.9	13.3 \pm 4.5	12.2 \pm 3.8	1.06	0.97
DOX	5.75 \pm 1.0	6.43 \pm 1.8	6.10 \pm 1.2	1.12	1.06
CDDP	1790 \pm 170	1990 \pm 94	2520 \pm 170	1.11	1.41 ^d

^a Drug sensitivities were analyzed by the use of a 3-day colorimetric assay in which cells were continuously exposed to the indicated agents. IC_{50} is the drug concentration that inhibited cell survival by 50%. $n = 8$ for VCR, VBL, PAC, DOC, and DOX; $n = 7$ for CDDP; and $n = 3$ for ETOP, SN38, and DNR.

^b IC_{50} of HEK-MRP7-17 or HEK-MRP7-18 divided by IC_{50} of control HEK-pcDNA3 cells.

^c VCR, vincristine; VBL, vinblastine; PAC, paclitaxel; DOC, docetaxel; ETOP, etoposide; DNR, daunorubicin; DOX, doxorubicin; CDDP, cisplatin.

^d Significantly different from the control transfectant as assessed by the two-tailed Wilcoxon test. For HEK-MRP7-17, the P values for VCR, VBL, PAC, and DOC were 0.02, 0.02, 0.02, and 0.03, respectively. For HEK-MRP7-18, the P values for VCR, VBL, PAC, DOC, and CDDP were 0.01, 0.02, 0.01, 0.01, and 0.02. The nonparametric two-tailed Wilcoxon test was used to make inferences about the difference between the IC_{50} of the control and MRP7-transfected cells.

the IC_{50} s) obtained for doxorubicin suggested that MRP7 is able to confer low levels of resistance to this agent, in that HEK-MRP7-C17 and HEK-MRP7-C18 exhibited 1.9-fold ($P = 0.0039$) and 1.7-fold resistance ($P = 0.0002$) when their IC_{75} s were compared with that of the control cells. (The IC_{75} s were 17.1 ± 5.8 , 33.2 ± 5.9 , and 28.6 ± 5.9 nM, for HEK-pcDNA3, HEK-MRP7-C17, and HEK-MRP7-C18, respectively; data not shown in Table 1). The differences in doxorubicin sensitivity were not attributable to differences in growth rates; the doubling times of HEK/pDNA3, HEK/MRP7-C17, and HEK/MRP7-C18 were 22.6 ± 3.6 , 21.3 ± 5.3 , and 21.5 ± 3.6 h, respectively. In addition, one of the two clones (HEK-MRP7-C17) exhibited low levels of resistance for the alkylating agent cisplatin (1.4-fold).

The accumulation of radiolabeled paclitaxel was examined to gain insight into the mechanism by which MRP7 confers resistance. As shown in Fig. 3, accumulation of this agent was reduced in HEK-MRP7-C17 cells compared with the control cells, as would be expected were MRP7 operating as a plasma efflux pump. At the 30- and 60-min time points of the assay, HEK-MRP7-C18 accumulated 49 and 54% less drug than control cells. Reduced accumulation of radiolabeled vincristine was also observed for the MRP7-transfected cells (data not shown).

Certain MRPs are able to transport glutathione, a capability which is reflected in reduced levels of this tripeptide in cells in which the pumps are ectopically expressed (1). To assess this feature of MRP7, cellular glutathione levels were analyzed. These measurements showed that the glutathione levels in the MRP7-transfected cells were comparable with the levels in the control cells, suggesting that glutathione is not a transport substrate of the pump. The glutathione levels in HEK-pcDNA3, HEK-MRP7-C17, and HEK-MRP7-C18 were 29.6 ± 2.5 , 28.8 ± 2.5 , and 28.7 ± 2.1 nmol/mg, respectively.

DISCUSSION

The present study shows that MRP7 is able to confer resistance to natural product anticancer agents, including taxanes (docetaxel and paclitaxel), *Vinca* alkaloids (vincristine and vinblastine), and possibly anthracyclines (doxorubicin). This drug resistance profile suggests that natural product drugs are substrates of MRP7, and in combination with our previous determination that MRP7 is competent in the ATP-dependent transport of 17β -estradiol 17-(β -D-glucuronide) (26), indicates that MRP7 is an amphipathic anion transporter whose substrate selectivity extends to uncharged or mildly cationic lipophilic compounds. This conclusion is in accord with our previous determination that MRP7-mediated transport of 17β -estradiol 17-(β -D-glucuronide) is susceptible to inhibition by natural product anticancer agents (26).

With respect to its potency for natural product agents, MRP7 exhibits the highest activity toward docetaxel (9–13-fold), at least as

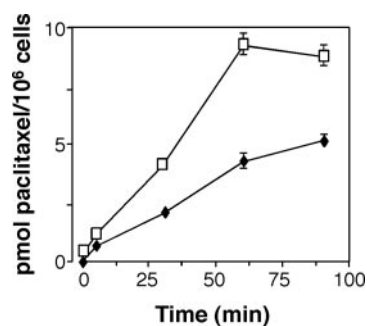


Fig. 3. Accumulation of paclitaxel in control and MRP7-transfected HEK293 cells. Control HEK-pcDNA3 (□) or MRP7-transfected HEK-MRP7-C18 (◆) cells were incubated in the presence of 100 nM [³H]paclitaxel, and accumulation was measured at the indicated time points. Data points are the means \pm SD.

Table 2. Natural product profiles of MRP family members

The data are from Refs. 10–22.

Transporter	Agent ^a								
	DOX	DNR	VCR	VLB	ETOP	DOC	PAC	CPT11	SN38
MRP1	+	+	+	+	+	nr	–	+	+
MRP2	+	nr	+	nr	+	nr	–	+	+
MRP3	–	–	–	–	+	nr	–	–	–
MRP6	+	+	–	–	+	nr	–	–	nr
MRP7	+	–	+	+	–	+	+	nr	–

^a +, >5-fold resistance; –, \leq 5-fold; nr, not reported. The abbreviations for drugs are as indicated in Table 1.

assessed in the cellular model we used. The relatively modest (2–3-fold) activity of MRP7 toward *Vinca* alkaloids and anthracyclines is similar to the resistance levels reported for MRP3 (epidodophyllotoxins) and MRP6 (anthracyclines, *Vinca* alkaloids, and epidodophyllotoxins) but less than that associated with ectopic expression of MRP1 and MRP2 (anthracyclines, *Vinca* alkaloids, epidodophyllotoxins, and camptothecins; Ref. 1). Interestingly, docetaxel, the agent toward which MRP7 exhibits the highest activity, is also the drug that represents the unique aspect of the resistance profile of the pump. In contrast to other characterized MRPs, MRP7 is the only family member that is able to confer resistance to taxanes (the resistance profiles of MRPs are summarized in Table 2). In this regard, MRP7 resembles P-glycoprotein, which is the only other human ABC transporter that has established activity toward taxanes (29). With respect to the biochemical mechanism of MRP7-mediated drug transport, the absence of depressed cellular levels of glutathione suggests that similar to the situation with MRP3 (30) and in contrast to the operation of MRP1 and MRP2 (31–33), MRP7 probably confers resistance to natural product agents in a glutathione-independent fashion.

Although taxanes are active against carcinomas of the breast, lung, ovary, and head and neck, their effectiveness is limited by inherent and acquired resistance mechanisms. Based on investigations of paclitaxel-resistant cell lines, several resistance mechanisms have been proposed for taxanes (29). These include enhanced efflux consequent to increased expression of P-glycoprotein, alterations in microtubule dynamics, point mutations that abrogate drug binding, altered expression of β -tubulin isoforms, and alterations in microtubule-associated signaling pathways. In view of our results, it will be of interest to determine whether increased expression of MRP7 is an additional resistance factor in taxane-resistant cell lines. With respect to the potential for MRP7 to impact cancer treatment, more information is needed on expression of MRP7 in cancers. At the time of writing, expression has only been evaluated in normal tissues. We found using a reverse transcription-PCR assay that MRP7 was expressed in many tissues, but transcript was not readily detected by RNA blot analysis (3). Expression of the mouse MRP7 homologue was reported for heart, liver, skeletal muscle, and kidney (34). In addition to protein expression studies in normal tissues and cancers, it will also be of interest to determine whether MRP7 assumes apical or basolateral subcellular localization in polarized epithelial cells.

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