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Development and Characterization of a Recombinant Madin-Darby Canine Kidney Cell Line That Expresses Rat Multidrug Resistance-Associated Protein 1 (rMRP1)

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ABSTRACT

Multidrug resistance-associated protein 1 (MRP1) is one of the major proteins shown to mediate efflux transport of a broad range of antitumor drugs, glucuronide conjugates, and glutathione, in addition to endogenous substrates. Significant differences in substrate selectivity were reported for murine and human MRP1. As preclinical drug disposition and pharmacokinetics studies are often conducted in rats, we have recently cloned the rat MRP1 (rMRP1) and demonstrated that rMRP1 expressed in transfected cells effluxes calcein, a commonly used fluorescence substrate for human MRP1. To further characterize the rat ortholog of MRP1, we isolated a cell line stably expressing recombinant rMRP1. These cells were tested for their ability to transport calcein and a range of chemotherapeutic drugs. Our results showed that cells expressing rMRP1 consistently efflux calcein at a rate 5-fold greater than control cells. The rMRP1 transfected cells, like their human ortholog, can confer drug resistance to vinca alkaloid (vinblastine and vincristine) and anthracycline drugs (daunorubcin and doxorubicin), and the resistance conferred by the MRP1 can be partially abolished by the MRP-specific inhibitors. The transepithelial permeability due to rMRP1 expression in differentiated Madin-Darby canine kidney cells (MDCK) cells was also investigated. The MRP1 transport activity is directional, as demonstrated by directional vinblastine transport. Collectively, our results demonstrate that the cellular expression of rMRP1, like its human ortholog, could confer resistance to anticancer drugs.

KEYWORDS: rat, MRP1, drug resistance, chemotherapeutic agents, cytotoxicity, transport, ATP-binding cassette, transwell

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INTRODUCTION

The development of cellular resistance to cytotoxic drugs is a major obstacle to the treatment of all kinds of human cancer. In the clinical setting, the overexpression of multidrug resistance proteins such as multidrug resistance protein-1 (MDR1) or multidrug resistance-associated protein 1 (MRP1) is often associated with poor prognosis for cancer patients. There are 2 well-characterized major adenosine triphosphate (ATP) binding cassette (ABC) superfamily members, ABC-B and ABC-C, involved in conferring drug resistance to cancer cells.[1,2] Within these 2 families, 2 protein isoforms, 1 from each family, play a critical role in drug resistance. They are P-glycoprotein (Pgp or MDR1), which belongs to the ABC-B family,[3,4] and MRP1, which belongs to the ABC-C family.[5] Pgp is the most extensively studied ABC transporter, functioning as a biological barrier by extruding toxic substances and xenobiotics out of the cells.

Despite some similarities in drug resistance profiles, these 2 transporters differ somewhat in substrate selectivity, molecular structure, tissue distribution, and membrane location in cells. At the genetic level, MDR1 and MRP1 are only 15% identical in their amino acid sequences.[6] MRP1 is capable of transporting many lipophilic anions and conjugated substances. The MRP1 substrate includes a variety of structurally diverse anticancer drugs, GSH-conjugates, glucuronides, leukotriene C4 (LTC4), unmodified drugs, and some drugs that are multivalent organic anions,[7-9] while Pgp substrates are mostly natural or mildly cationic molecules. A few previous studies[10-12] suggest that there are differences in substrate recognition and transport activity between the mouse and human ortholog of MRP1, indicating that significant interspecies differences exist for MRP1.

In addition to MRP1, 8 other MRPs isoforms (MRP2 to MRP9) have been identified to date.[13] Most of them were originally found and studied in humans. In rat, only a few reports are available for MRPs. It has been shown

that rat MRP1 expression occurs in almost all tissues and its overexpression could confer multidrug resistance in cancer cells.[14] Expression of rMRP1 in the blood-brain barrier (BBB) capillary system and placenta has also been reported,[15-18] suggesting that MRP1 plays certain roles in drug distribution into the central nervous system and in drug exchanges between the mother and fetus. In rats, rMRP2 is expressed in the liver and rMRP3 is expressed in intestine, where they mainly contribute to the secretion of conjugated substances into bile or blood, respectively.[14,19] The expression and functions of other rat isoforms of MRP are not yet clear.

Recently, we and others have cloned and characterized the rat MRP1 and found that it exhibits 94.3% and 83.0% nucleotide sequence homology to that of mice and human, respectively.[20,21] The ability of rMRP1 to efflux the fluorescent MRP1 substrate calcein is similar to that of human. Detailed studies on the rMRP1-induced drug resistance profile, compared with that of human MRP1, have not yet been determined in a consistent manner. Since most preclinical drug disposition and toxicology studies are often conducted in rats, information on interspecies variations between the rat and human homolog of MRP1 is needed to predict which drug candidates are likely to be substrates of this efflux transporter.

Therefore, the aim of this study is to develop a stable cell line expressing recombinant rMRP1 that is suitable for transepithelial transport study. We seek to elucidate the role of rMPR1 in mediating resistance to and modifying the drug disposition profile of commonly prescribed antitumor drugs.

MATERIALS AND METHODS

Chemicals and Drugs

The most commonly used chemotherapeutic agents, vinblastine sulfate (VBL), vincristine sulfate (VCR), doxorubicine HCl (Dox), and daunorubicin HCl were obtained through the University of Washington Medical Center, Seattle, WA. Each drug formulation was diluted with culture medium, yielding working solutions over a wide range of concentrations. MRP1-specific inhibitor, MK-571 (3-[[[3-[2-(7-chloro-2-quino-linyl)ethenyl]-phenyl[[3-(dimethylamino)-3-oxopropyl]thio]-methyl]thio] propionic acid], was kindly provided by Dr AW Ford-Hutchinson at Merck-Frosst Center Canada and Co (Quebec, Canada). Indomethacin was purchased from Sigma-Aldrich Co (St Louis, MO). Methy-[^{3}H]-thymidine (1.1 Ci/mmol) was obtained from ICN Biochemical Corp (Aurora, OH) and [^{3}H]-vinblastine (5.9 Ci/mmol) was supplied by Moravek Biochemical Inc (Brea, CA). Calcein acetoxymethyl ester (calcein-AM) was purchased from Molecular Probes Inc (Eugene, OR). Tissue culture media DMEM, trypsin-EDTA, and antibiotic solutions were supplied by GIBCO Invitrogen Corp (Carlsbad, CA). Fetal bovine serum was obtained from Hyclone (Logan, UT). MRP1 antibody (MRPr1) was purchased from Alexis Biochemicals (San Diego, CA). Other reagents were of analytical grades or higher.

Cell Culture and Transfection of Rat Multidrug Resistance-Associated Protein 1 cDNA Expression Plasmids into Madin-Darby Canine Kidney Cells

To assess the function of the cloned *rMRP1*, the Madin-Darby canine kidney (MDCK) type II cells, a well-characterized polarized renal epithelial cell line purchased from ATCC (Manassas, VA), was selected as host for establishing stable MRP1 expression. MDCK cells were transfected with the *pCR3.1* vector or *pCR3.1-rMRP1* plasmid[20] (herein referred to as *prMRP1*) using the Lipofectamine2000 reagent (Invitrogen). Briefly, MDCK cells were allowed to grow for 24 hours at 80% to 90% confluence on 12-well plates. The growth medium of the cells was replaced with serum-free medium Opti-MEM (Invitrogen) for 30 minutes. Two μg vector DNA or *prMRP1* plasmid and 6 μL of Lipofectamine2000 were each placed in 100 μL of Opti-MEM medium, mixed, and incubated at 20°C for 20 minutes. The DNA-Lipofectamine mixture was then added to each well of MDCK cells and incubated at 37°C in a 5% CO_2 humidified incubator for 6 to 8 hours. The cells were then allowed to grow in growth medium containing serum. At 24 hours after transfection, cells from 1 or 2 wells were trypsinized and plated into a T75 flask or a 12-well culture plate at a dilution of ~1:20 in Dulbecco's modified eagle's medium (DMEM). At 48 hours posttransfection, G418 at 500 μg/mL was added. Medium containing G418 was refreshed every 3 to 4 days for 3 weeks. Cells that survived under G418 selection pressure were used subsequently for single cell colony selection by limited cell dilution in 96-well plates. Each cell subclone was tested for the rMRP1 expression by calcein efflux, Western blotting, and reverse transcription polymerase chain reaction (RT-PCR). Cell clones exhibiting a high degree of resistance to G418 and MRP1 expression were expanded for functional studies. The human MRP1-transfected MDCK cells, selected using a similar approach, were provided by Dr Joanne Wang (University of Washington) and used for comparison with rMRP1.

Calcein-Retention and Efflux in prMRP1-Transfected Madin-Darby Canine Kidney Cells

Calcein-AM, a fluorescence substrate for MRP1, was used to assess the efflux transport activity of *prMRP1*-transfected MDCK cells. The *pCR3.1* vector-transfected MDCK cells served as background control. Steady-state calcein retention studies were conducted in the presence and absence of MRP1 inhibitors. Briefly, cells were plated into 12-well plates at 10^6 per well for 2 days at 37°C. Culture medium was removed from the cells, which were then washed twice with 2 mL of prewarmed Dulbecco's phosphate-buffered saline containing 1 mM D-glucose (DPBSG) at pH 7.3. For the inhibitor experiments, cells were preincubated for 10 minutes with 1 mL of DPBSG containing either 20 μM MK571 or 100 μM indomethacin at 37°C. For the corresponding control incubations, the cells were preincubated with DPBSG or DPBS along with solvent vehicle. The cells were then incubated with 1 mL of 0.5 μM calcein-AM in DPBSG with or without the MRP1 inhibitor. After the 20-minute incubation with calcein-AM, the cells were quickly washed twice with 4 mL of cold DPBSG and lysed with 1.25 mL 1% Triton X-100 (Sigma, St. Louis, MO). The amount of intracellular calcein was measured with a Hatachi-4500 fluorescence spectrophotometer (Hatachi Ltd., Tokyo, Japan) set at λ_{ex} = 485 nm and λ_{em} = 530 nm. For all experiments described above, cellular protein concentrations were measured using a BCA protein assay kit (Pierce, Rockford, IL) in microplate format. Measurements of calcein fluorescence retention were normalized to protein concentration.

FACS Analysis

The distribution of calcein fluorescence intensity (λ_{em}= 530 nm) in the vector and *pMRP1*-transfected cell populations was also compared using a fluorescence-activated cell sorter (FACSAN II, Becton Dickinson, Franklin Lakes, NJ). Cells were incubated with calcein-AM in the absence or presence of inhibitors for 20 minutes at 37°C. After washing twice with cold DPBSG, the cells were trypsinized with 0.25% Trypsin-1 mM ethylenediaminetetra acetic acid (EDTA) at 4°C. The detached cells were then washed once by pelleting at 4°C. Finally, they were resuspended in 1 mL of phosphate buffer solution (PBS) at 4°C and subject to FACS analysis immediately.

Immunofluorescent Staining of MDCK Cells

Control and *rMRP1*-transfected MDCK cells (2×10^3) were plated into Falcon 4-well chamber slides (Becton Dickinson Co) and were incubated for 4 days at 37°C. Slides were washed 3 times with PBS and fixed with a methanol/acetone mixture (1:1) for 15 minutes. After rehydration and blocking with 1% bovine serum albumin (BSA) in PBS for 1 hour, the MRP1 monospecific antibody MRPr1 (Alexis Biochemicals) was diluted at 1:200, added to the slide chambers, and incubated for 1 hour. The slides were washed 3 times with PBS, and the FITC-labeled rabbit antirat IgG was applied to each chamber. After 30-minute incubation, the slides were extensively washed and air dried in the dark. The slides were covered with coverslips with one drop of antifad oil. Slides were viewed under a fluorescence microscope and photographed (K507 Leica confocal microscope, Leica Microsystems Inc, Bannockburn, IL).

Cytotoxicity Tests

The conferred drug resistance of cloned rMRP1 and human MRP1 (hMRP1) to transfected MDCK cells was evaluated by methyl-[^{3}H]-thymidine uptaking measurement in 96-well plate format. Briefly, transfected cells (control, prMRP1, phMRP1) were seeded at 10^4 density per well in 100 μL culture medium. At 4 hours, another 100 μL of media containing a wide range of dilution of selected chemotherapeutic agents, alone or with selected inhibitors, were added to appropriate wells. Each concentration of drug was run in quadruplicate. The culture was maintained at 37°C in a 5% CO_2 humidified incubator for 72 hours. Twenty-five microliters of the methyl-[^{3}H]-thymidine in culture medium at 1 μCi/well were added into each well and incubated for 24 hours. Cellular DNA was harvested using 24-well cell harvest (PHD cell harvester, Cambridge Technologies Inc, Watertown, MA). Glass fiber filters containing tritium-labeled DNA were placed into 5-mL scintillation vials with 2 to 3 mL of scintillation fluids and were counted using a scintillation counter. Average data from each set of quadruplicates were converted to a percentage of their controls. IC_{50} values were estimated by fitting percentage inhibition of [^{3}H]-thymidine uptake vs drug concentration data to the Inhibitory Effect Sigmoid Emax model [effect C = 0 at Emax, C = infinity at 0, E = Emax*(1 - (C**Gamma/(C**Gamma + IC_{50}**Gamma)))] (Pharsight Corp, Mountain View, CA). The relative resistance ratio was calculated by dividing the IC_{50} value of cells transfected with MRP1 by the value of cells transfected with vector.

Transport Studies

The rMRP1 transport activity was further evaluated in a transwell system by [^{3}H]-vinblastine sulfate transport.

Cells (control, phMRP1-MDCK, and prMRP1-MDCK) were cultured for 8 days at an initial density of 7.5×10^5 per transwell permeable growth support (0.4 μm pore size, 1-cm^2 clear polyester filter cup insert from Corning-Costar Corp, Cambridge, MA) in culture media. Culture medium was refreshed every 3 days. The transepithelial electrical resistance (TEER) value for each well was measured using Millicell-ERS (Millipore, Bedford, MA) before the experiment to ensure that the cells had polarized and formed tight junctions (TEER value reached ~300 ohms/cm^2). The assay was performed using serum-free medium Opti-MEM from Invitrogen. [^{3}H]-vinblastine sulfate was diluted with unlabeled vinblastine to give an activity of 0.06 Ci/mmol and a final concentration of 200 nM in the assay. For measurement of [^{3}H]-vinblastine transport from the apical side to the basal side, the basal medium was replaced with 1.5 mL of 200 nM of nonradioactive vinblastine, while the apical medium was replaced with 0.5 mL of radiolabeled vinblastine. In contrast, to measure the vinblastine transport from the basal to the apical side, 1.5 mL of [^{3}H]-vinblastine was added to the basal chamber, and 0.5 mL of cold vinblastine was added to the apical chamber. The cells were incubated at 37°C in 5% CO_2. At time intervals of 1, 2, 3, and 4 hours, 25-μL aliquots of medium from the apical chamber (for B to A) or 50-μL aliquots (for A to B) from the basal chamber were transferred into a scintillation vial with 1.5 mL scintillant. Also, 25-μL aliquots were taken from the basal chamber (for B to A) or the apical side (A to B) to measure the remaining [^{3}H]-vinblastine in reservoir at 4 hours postincubation. The radioactivity of all samples of aliquots was quantified using a scintillation counter. To inhibit the MRP1 transport activity, 20 μM of MK571, an MRP1-specific inhibitor, was mixed with vinblastine solution and applied to appropriate source chambers as stated above. Data were collected from quadruplicate determinations, and the permeability of (B to A) or (A to B) for each cell type was calculated based on the following Papp, apparent permeability formula[22]:

$$Papp = \frac{dC/dt}{(C/V) * A} \qquad (1)$$

where A is the area of membrane surface of filter (A = 1 cm^2); C is the concentration of the vinblastine added into the chamber; V is the volume of the donor chamber; and dC/dt is the slope of plot of concentration vs time (hours). The directional permeability ratio was estimated by taking the Papp of (B to A) and (A to B).

RESULTS AND DISCUSSION

Development of a Stable MDCK Cell Clone That Overexpresses Rat MRP1

The ability of hMRP1 and mMRP1 to confer drug resistance has been studied before.[11,23-25] Little is known about rMRP1 characterization because there is no functional rMRP1 stable cell clone available. Since most pharmacokinetic studies are done in rats, we cloned the full-length *rMRP1* cDNA into an expression vector. We chose MDCK II cells as a mammalian host to isolate a stable clone of epithelial cells that consistently expressed rMRP1, which could be differentiated to form a polarized monolayer for transepithelial flux studies. These cells were transfected with a mammalian expression plasmid, *pCR3.1* vector containing *rMRP1*, which was shown in our previous report to express full-length transcript and functional MRP1 protein.[20] The cells that consistently overexpressed rMRP1 were expanded from a single cell clone and analyzed for MRP1 function.

The activity of rMRP1 in isolated stable MDCK cells was characterized by flow cytometry. We found that most of the cells in the expanded population exhibited much lower intracellular calcein fluorescence, compared with MDCK cells transfected with control plasmid (Figure 1A). Calcein fluorescence intensity increased in the presence of the MRP1 inhibitor MK571 (Figure 1B). These results were consistent with positive immunofluorescence staining of rMRP1 recombinant MDCK cells when exposed to monospecific antibody against human MRP1 (Figure 2), which is known to recognize a linear epitope of 10 amino acids in hMRP1, $_{238}$GSDLWSLNKE, 9 of which are conserved in both mMRP1 and rMRP1.[26]

A stable clone of MDCK cells expressing rMRP1 exhibited consistent efflux transport of an MRP1 fluorescence substrate calcein, as evidenced by 5.2- to 8.7-fold higher efflux activity over 17 weeks in culture. In addition, the efflux transport activity is specifically mediated by MRP1, because addition of MRP1 inhibitors, indomethacin (100 μM) or MK571 (10 μM, an MRP1-specific inhibitor), suppressed efflux transport of calcein back to the control level (Table 1).

Collectively, these data indicate that the isolated clone of MDCK cells expressing rMRP1 is a stable clone and functionally competent. These cells were then used for the additional studies described below.

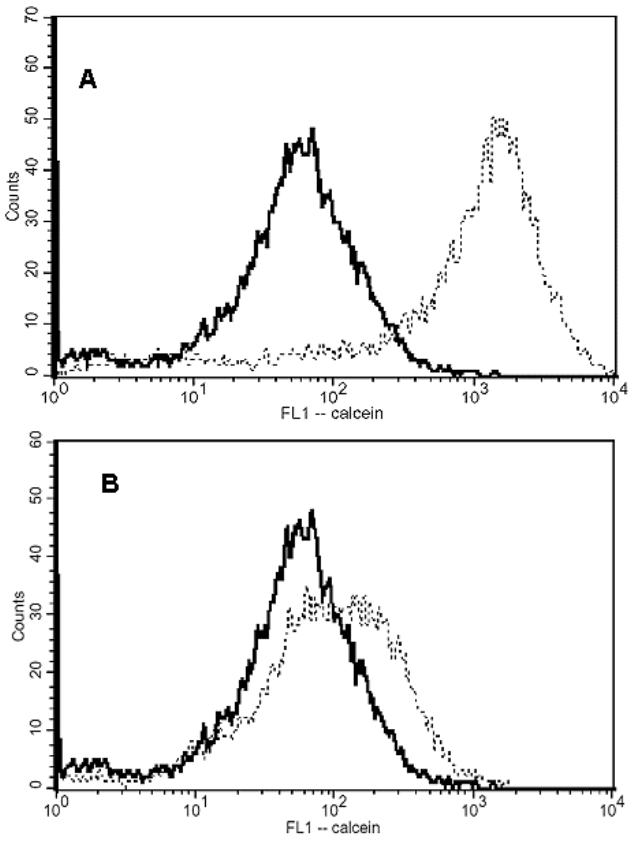

Figure 1. (A) FACS analysis of the ability of recombinant MDCK cells expressing *prMRP1* to reduce intracellular calcein retention compared with MDCK cells expressing control vector. Recombinant MDCK cells expressing *rMRP1 plasmid* (*prMRP1,* thick line —) and control plasmid (*pCR3.1,* dash line ---) were incubated with 0.5 μM calcein-AM for 20 minutes. The distribution of cell counts at varying intracellular calcein fluorescence (FL-1) was analyzed with a fluorescence-activated cell sorter and presented as a semi-log histogram. (B) FACS analysis of the inhibition of rMRP1 efflux function by MRP-specific inhibitor MK571. Recombinant MDCK cells expressing *rMRP1* were incubated with 0.5 μM calcein-AM or calcien-AM/MK571 mixture for 20 minutes. The distribution of cell counts at varying intracellular calcein fluorescence (FL-1) was analyzed with a fluorescence-activated cell sorter and presented as a semi-log histogram. Thick line — shows MDCK cells expressing *prMRP*-without inhibitor, dash line --- shows MDCK cells expressing *prMRP*-with MK571 (10 μM).

Effects of MRP1 Expression on MDCK Cells' Sensitivity to Chemotherapeutic Agents

The stable rMRP1-expressing MDCK cells were evaluated for their ability to exhibit drug resistance to vincristine, vinblastine, daunorubicin, and doxorubicin. For these experiments, MDCK cells expressing human MRP1 were used as comparison; and the same host cells containing plasmid without MRP1 were included as controls. As shown in Table 2, the expression of rat MRP1 significantly increased the concentration of the drug required to produce half-maximum inhibition in MDCK cells for all of the tested drugs listed above. These data were similar to resistance detected with the same host cells expressing hMRP1. Some differences in IC_{50} values, observed for vincristine and doxorubicin between rMRP1 and hMRP1 did not reach a statistically significant level.

Human MRP1 and rMRP1 appeared to exhibit about the same IC_{50} value for vinblastine, vincristine, daunorubicin, and doxorubicin. On the other hand, rat MRP1, in contrast to low resistance to anthracylines as reported for mouse MRP1, exhibits ~1.39-fold more resistance to doxorubicin than the human counterpart. However, the difference is not significant (2 samples *t* test assuming equal variance, $P = .17$, Table 2). The data demonstrated that the cloned rMRP1, unlike mouse MRP1, could confer drug resistance to anthracycline drugs to MDCK cells. Human embryonic kidney (HEK) 293 cells expressing rat MRP1, which have been previously transformed with sheared human adenovirus type 5 (Ad 5) DNA,[27] IC_{50} values for vincristine, daunorubicin, and doxorubicin, were reported to be ~40 nM, 20 nM, and 130 nM, respectively.[21] These values, while not identical, are reasonably consistent with data presented in Table 2.

Our preliminary analysis of the available amino acid sequences reported by Yang et al,[20] and Nunoya et al,[21] indicate that the 2 rMRP1 clones were practically identical. Nunoya and colleagues used HEK 293, the human embryonic kidney cells, to express rMRP1. These cells were previously transformed with adenovirus and expressed adenovirus-transforming gene.[27] The rMRP1 data reported here were expressed in MDCK cells, isolated from an adult female cocker spaniel, and had not been transformed by virus. Whether the ability of MDCK cells to differentiate into polarized epithelial cells, or expression of adenoviral products in undifferentiated HEK 293 could contribute some qualitative differences in drug resistance between human and rat orthologs, is not known and requires further evaluation. In addition, the setting and culture condition differences between these 2 culture systems might produce variations in experimental results. However, no detailed information on culture conditions was discernable in the report of Nunoya et al[21] for direct comparison.

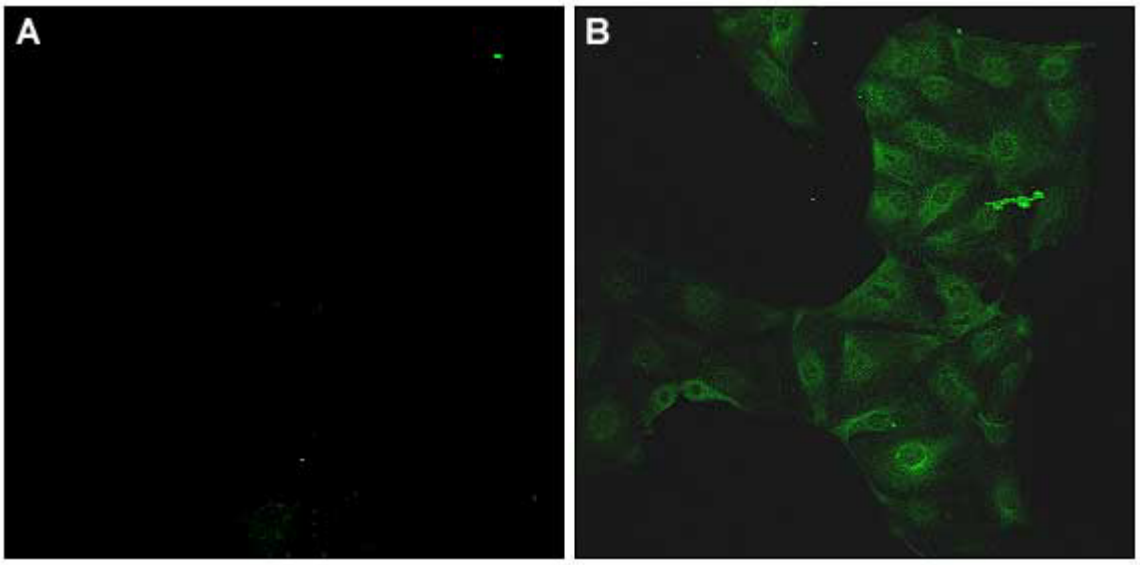

Figure 2. Immunofluorescence analysis of MRP1 expression in MDCK cells. Control (A) and rMRP1 expressed in MDCK (B) cells were exposed to monospecific antibody against rat MRP1, and immunofluorescence was detected after staining with an FITC-labeled secondary antibody.

Table 1. Effect of MRP1 Inhibitors to Reverse the Ability of MDCK Cells Stably Transfected With *prMRP1* to Reverse Calcein Efflux Function*

MRP1 Inhibitor	% Inhibition of *prMRP1*-Mediated Intracellular Calcein Efflux†
Blank Buffer	0.0 ± 13.4
+ Indomethacin (100 μM)	58.5 + 10.36
+ MK 571 (20 μM)	65.33 ± 14.15

*MRP1 indicates multidrug resistance-associated protein 1; MDCK, Madin-Darby canine kidney cells; and *prMRP1,* MDCK cells transfected with the *pCR3.1* vector or *pCR3.1-rMRP1* plasmid. MRP inhibitors at indicated concentrations were co-incubated with 0.5 μM calcein-AM for 20 minutes. The ability of the respective inhibitors to block the calcein efflux in *prMRP1*-expressed MDCK cells was analyzed as percentage of inhibition, after correcting for the effects of buffer control on *prMRP1* plasmid transfected cells using the formula of $1-\frac{C_1-C_o}{C_1}\times 100\%$, where C_I is the calcein amount in cells with inhibitor, and C_0 is the amount of calcein intracellularly in *prMRP1*-transfected cells incubated with buffer.
†Data expressed are mean ± SD of quadruplicates.

The role of MRP1 in mediating drug resistance is further characterized by inhibition studies with an MRP1-specific inhibitor, MK571. As presented in Table 3, in the presence of MK571 (10 μM), ~44% and 40% of vincristine resistance in cells expressing rat and human MRP1 could be reversed. A similar degree of MK571-dependent reversal of drug resistance was detected with vinblastine (Table 3). Taken together, these data suggest that rat MRP1 exhibit drug resistance to the 4 chemotherapeutic drugs that have been shown to be influenced by human MRP1 expression. However, there are some differences which did not reach statistical significance for 2 of the 4 drugs tested.

Effects of rMRP1 on Transepithelial Transport of Vinblastine

We next used vinblastine as a model chemotherapeutic agent to determine whether recombinant MDCK cells expressing MRP1 could be differentiated to form tight junctions suitable to study transepithelial transport. For these studies, we used [^{3}H]-vinblastine as a radiolabeled tracer. The formation of tight junctions by polarized MDCK cells was verified by resistance measurement. The directional transport activity was presented as the ratio of apparent permeability between the B→A over A→B. As shown in Table 4, rat and human MRP1 expressed in MDCK cells exhibit ratios of Papp of 8.42 ± 0.55 and 7.38 ± 0.77, respectively. These efflux ratios were reduced to about half in the presence of an MRP1

Table 2. Effects of MRP1 Expression on MDCK Cells' Sensitivity to Chemotherapeutic Agents*

Drug	IC_{50} (nM)†		
	Control	rMRP1	hMRP1
Vincristine	17.96 ± 3.44	65.91 ± 25.27‡	93.9 ± 21.55‡
Vinblastine	9.96 ± 5.13	40.13± 5.27§	50.75 ± 7.54‡
Daunorubicin	31.96 ± 6.29	75.07 ± 13.38§	87.77 ± 19.68§
Doxorubicin	8.26 ± 3.54	84.33 ± 22.28‡	60.5 ± 17.46‡

*Abbreviations are explained in the footnote to Table 1.
†The IC_{50} values were determined as described in the Materials and Methods section. Student *t* test was used to evaluate the significance of the difference between control vs human MRP1 or control vs rat MRP1.
‡$P < .01$ compared with control.
§$P < .05$ compared with control.

Table 3. Effects of MRP1 Inhibitors to Reverse Drug Resistance in Recombinant MDCK Cells Expressing MPR1*

Drug or Drug ± Inhibitor†	IC_{50} (nM)†		
	Control	rMRP1	hMRP1
Vincristine (VCR)	26.38 ± 12.90	75.35 ± 8.66	107.33 ± 29.72
Plus MK571‡	24.69 ± 4.42	36.25 ± 6.70	57.50 ± 7.00
% Difference§	(6.4)	(53.8)	(37.6)
Vinblastine (VBL)	13.5 ± 3.24	63.50 ± 8.16	73.75 ± 17.29
Plus MK571‡	11.25 ± 2.56	27.74 ± 5.01	35.54 ± 13.77
% Difference§	(14.8)	(53.9)	(28.0)

*Abbreviations are explained in the footnote to Table 1.
†The IC_{50} values were determined as described in the Materials and Methods section. Data are the representatives of at least 3 experiments for each drug.
‡Inhibitor MK571 at 10 μM, indomethacin at 100 μM.
§The mean percentage of inhibition was calculated by [1 − (IC_{50} of cells with inhibitor)/IC_{50} of cells without inhibitor] * 100.

Table 4. Permeability Ratio of MRP1 Expressed in Recombinant MDCK Cells Using [^{3}H]-Vinblastine as a Substrate*

MDCK Cells That Express MRP1 Ortholog	Control (Without Inhibitor)		With 20 μM MK571	
	Mean Ratio	SD	Mean Ratio	SD
Control (*pCR3.1*)	2.88	0.191	1.16	0.064
Rat (*rMRP1*)	8.42	0.546	4.48	0.399
Human (*hMRP1*)	7.38	0.77	3.62	0.614

*Abbreviations are explained in the footnote to Table 1. Directional efflux of ^{3}H-vinblastine across differentiated recombinant MDCK cells grown on transwells in the presence or absence of specific inhibitor MK571 was estimated as described in the Materials and Methods section. Raw data at each time point were converted to the percentage of transported of initial loading dosage for each well and plotted. Permeability from A to B and B to A were calculated based on the formula described in the Materials and Methods section, and mean values from at least 3 replicates were obtained and expressed as mean and SD ratios of Papp (B to A)/(A to B) as described in the Materials and Methods section.

inhibitor, MK571 (20 μM), verifying the role of MRP1 in the directional transport of vinblastine. A background Papp of 2.88 ± 0.191 was recorded for control cells that carried *pCR3.1* plasmid. MK571 also reduced the Papp in control cells, suggesting a baseline level of directional vinblastine transport in MDCK cells. It is possible that the background observed could be contributed by endogenous low-level expression of MDR1, MRP1, or

other isoforms of MRP family members such as MRP5 in MDCK cells,[28] as vinblastine, a known unconjugated drug, has been shown to be a substrate for these transporters.[11,29] Nevertheless, expression of both human and rat MRP1 significantly increased the ratio of Papp to similar degrees.

It is interesting to note that, while there is no apparent difference in transepithelial flux of vinblastine between the human and rat homolog of MRP1, drug resistance mediated by the 2 homologs in the same cells was different. It is possible that intracellular concentrations of vinblastine for the differentiated and polarized cells would be different from that of undifferentiated cells used in the drug-sensitivity studies. On the other hand, some of the MRP1 could be expressed in subcellular organelles that redirect vinblastine from the target sites of actions. These and other possibilities could be evaluated with the described recombinant cells that stably express MRP1.

In summary, we have established and characterized stable recombinant MDCK cells expressing rMRP1. The rMRP1 has a similar substrate selectivity compared with human MRP1 and could confer drug resistance to cells. The differentiated recombinant rMRP1-MDCK cells exhibit directional transepithelial transport of vinblastine. These stable recombinant cells could be used to evaluate drug candidates that are either MRP1 substrates or inhibitors, as well as further elucidate MRP1 structure and function.

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