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Mitoxantrone Permeability in MDCKII Cells Is Influenced by Active Influx Transport†

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Abstract: Mitoxantrone (MXR) is a prototypical substrate for the ABCG2 (bcrp1) efflux transporter and is often used as a positive control in bcrp-mediated transport studies. This study examined processes involved in the cellular accumulation and directional transport of MXR using an *in vitro* wild-type (WT) and *bcrp1*-transfected (bcrp1) MDCKII cell model. Compared to 37 °C, incubation at 4 °C increased MXR accumulation in bcrp1 cells and unexpectedly decreased MXR accumulation in WT MDCKII cells. [^{3}H]-MXR accumulation was concentration dependent in both WT and bcrp1 cells, exhibiting the characteristics of saturable active influx. At tracer concentrations, there was no difference in MXR directional flux between WT and bcrp1 MDCKII cell monolayers, and the A-to-B (apical-to-basolateral) flux was greater than the B-to-A flux in both cell types. However at higher concentration (20 μM), [^{3}H]-MXR directional flux from A to B decreased and B to A increased, revealing the expected efflux process. Therefore, the orientation of the MXR directional flux process is concentration dependent and only at higher concentrations could the difference between WT and bcrp1 MDCKII cells be distinguished. Taken together, these data show that there is a saturable influx transport system on the apical membrane MDCKII cells that is responsible for the active influx of MXR. This is the first report of an active influx transport system for MXR. The expression of the putative MXR influx transporter in selected cell types could lead to misleading results in drug transport assays that screen for bcrp activity. Moreover, the downregulation of the influx transport system could be a heretofore unrecognized mechanism of MXR resistance in tumor cells.

Keywords: MDCKII cell; mitoxantrone; bcrp1; influx transporter; efflux transporter

Introduction

Mitoxantrone (MXR) was developed in the 1970s as an antineoplastic agent. It has a spectrum of activity similar to commonly used anthracyclins, such as doxorubicin and daunorubicin. MXR is efficacious with limited toxicity in a number of malignancies, including breast carcinoma, acute leukemia, and non-Hodgkin's lymphoma.[1] It is most commonly used to treat leukemia and prostate cancer in combination with steroids. Recently, studies examining the use of MXR in combination with various antitumor agents suggest that it has significant activity in the treatment of specific lymphomas.[1,2] Moreover, its immunosuppressant and immunomodulatory properties provide a rationale for use of MXR in active multiple sclerosis.[3]

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Like several other antitumor agents, when MXR was widely employed in the clinic, it was found that MXR treatment may lead to resistance.[4−6] Several efflux transporters may be involved in the MXR resistance process, such as P-gp, MRPs, and BCRP.[7,8] BCRP/Abcg2 (breast cancer resistance protein) is a relatively new member of the ABC transporter family discovered in mitoxantrone-resistant cell lines that do not overexpress P-gp or the MRPs.[9] This efflux protein may play a significant role in MXR absorption and distribution.[9] The motivation for our current study of possible MXR active influx transport resulted from the examination of MXR transport in mouse BCRP/Abcg2 (bcrp1) transfected and parental MDCKII cells. It was observed that MXR cellular accumulation decreased at low temperature (4 °C), which implied the existence of temperature-sensitive MXR influx process in both the WT and bcrp1 MDCKII cells. Our further study confirmed this finding. The existence of a saturable, active influx transporter for MXR may have important implications in the use of MDCKII bcrp1-transfected cells in drug screens and the efficacy and development of resistance to this important antitumor agent.

Materials and Methods

Materials. [^{3}H]-Mitoxantrone was obtained from Moravek Co. (Brea, CA). Unlabeled mitoxantrone (MXR) and all other reagents were obtained from Sigma (St. Louis, MO). All other reagents were analytical grade. The compounds were dissolved in DMSO and diluted to the desired concentrations with phosphate buffer. The final concentration of DMSO in all incubation solutions, including the control group, was less than 0.1%.

Cell Culture. The MDCKII cells, both WT and bcrp1 cell line, were generously provided by Dr. Alfred H. Schinkel from the Netherlands Cancer Institute. Cells at passage 5 to 15 were used in all experiments. Dulbecco's modified Eagle's medium (DMEM) and nonessential amino acids were obtained from Cellgro (Herndon, VA). Fetal bovine serum was purchased from Seracare Life Sciences, Inc. (Milford, MA). Penicillin and streptomycin (10 000 U/mL and 10 000 μg/mL, respectively) were obtained from Sigma. All other cell culture materials were obtained from Costar (Cambridge, MA) and Falcon (Bedford, MA).

Temperature-Dependent Cellular Accumulation. Accumulation experiments were performed with confluent epithelial MDCKII cell monolayers grown on 12-well plates. Cultured cells were washed three times with 1 mL of assay buffer (composed of 122 mM sodium chloride, 25 mM sodium bicarbonate, 10 mM glucose, 10 mM HEPES, 3 mM potassium chloride, 1.2 mM magnesium sulfate, 1.4 mM calcium chloride, and 0.4 mM potassium phosphate dibasic, pH 7.4) and then preincubated in assay buffer for 30 min at 37 °C. Uptake was initiated by adding 1 mL of assay buffer containing [^{3}H]-MXR (specific activity of 3 Ci/mmol, final radioactivity in buffer is 0.167 μCi/mL, yielding a final tracer MXR concentration of 56 nM). In order to investigate temperature influence on MXR accumulation, plates were divided into two groups. Incubations for cellular accumulation were carried out at either 37 °C or 4 °C. After 180 min incubation, assay buffer was removed by suction and discarded. The monolayers were washed three times with 1 mL aliquots of ice-cold PBS, and then 1 mL of 0.1% Triton X-100 solution was added and incubated for 1 h on a rotary shaker at 37 °C. Protein concentration was determined by the Pierce BCA method. Mitoxantrone radioactivity was determined by liquid scintillation counting and expressed as dpm per microgram of cellular protein.

MXR Concentration-Dependent Accumulation. (A) MXR Steady-State Accumulation. Initial accumulation experiments were carried out using a 180 min incubation because MXR cellular accumulation reached a steady state within 3 h (data not shown). Accumulation experimental conditions were the same as depicted above, except accumulation was initiated by adding 1 mL of assay buffer containing [^{3}H]-MXR with different concentrations of unlabeled MXR to examine the concentration dependence of the cellular accumulation. In the steady-state experiments, the concentrations of unlabeled MXR examined were 0, 0.2, 2.0, and 20 μM.

(B) MXR Initial Influx Rate. To determine the initial influx rate, the accumulation experimental conditions were the same as above except for the incubation time. Assay buffer containing [^{3}H]-MXR with different concentrations (0, 1, 2, 5, 10, 20, and 40 μM) of unlabeled MXR was used to examine the concentration dependence of the accumulation process. [^{3}H]-MXR uptake rate was transformed to total MXR uptake rate according to its radioactive specific activity. Uptake of MXR was linear with time for 3 min (data not shown); therefore an uptake period of 2 min was chosen to

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determine initial rates of influx transport, not confounded by active efflux. The initial influx rate that was due to the saturable active influx transport (eq 3) was determined by subtracting the MXR uptake at 4 °C (linear with concentration, eq 2) from the total uptake measured at 37 °C (eq 1):

$$\text{total influx transport rate} = \frac{T_{max}[C]}{K_m + [C]} + K[C] \quad (1)$$

$$\text{passive influx transport rate} = K[C] \quad (2)$$

$$\text{active influx transport rate} = \frac{T_{max}[C]}{K_m + [C]} \quad (3)$$

where T_{max} is the maximum influx rate due to saturable active transport, K_m is the concentration at half-maximal rate, [C] is the concentration in the bulk media, and K is the proportionality constant representing the influx clearance due to passive diffusion. The uptake rate was normalized based on the total protein content of the cells. Protein was determined by the Pierce BCA Protein Assay Regent.

Effect of MXR Concentration on Directional Flux. Directional flux was determined using previously reported methods.[10] Briefly, confluent cell monolayers (WT and bcrp1 cell line) were washed three times with assay buffer. [^{3}H]-MXR was added into the apical (1.5 mL) or basolateral (2.6 mL) compartments, respectively. In the MXR high concentration groups, all the solutions contained 20 μM unlabeled MXR. At various times up to 180 min (0, 15, 30, 60, 90, 120, 180 min), aliquots of receiver solution (200 μL) were withdrawn and replaced with equal volumes of assay buffer. The [^{3}H]-MXR flux from the donor to the receiver side was estimated from the radioactivity appearing in the receiver compartment. Radioactivity in each sample was determined by liquid scintillation counting.

The apparent directional permeabilities (P_{app}, either in the A-to-B or B-to-A direction) of MXR were calculated from the permeability equation (below) using slopes obtained from the amount transported vs time plots,

$$P_{app} = \frac{dQ/dt}{A \cdot C_0} \quad (4)$$

where A is the membrane surface area, C_0 is initial concentration in donor compartment, and dQ/dt is the slope of the amount transported vs time profile.

Influence of Probenecid and TEA on [^{3}H]-MXR Accumulation. The method used to determine the influence of broad-spectrum inhibitors on cellular accumulation was identical as described above, except with probenecid or TEA added to the incubation buffer to inhibit transport function. The final concentrations of these inhibitors in each well were 0, 20, 200, and 2000 μM.

Statistical Analysis. Experimental groups were compared using the one-way ANOVA with the Tukey test for multiple comparisons. Differences were considered to be statistically significant at $p < 0.05$.

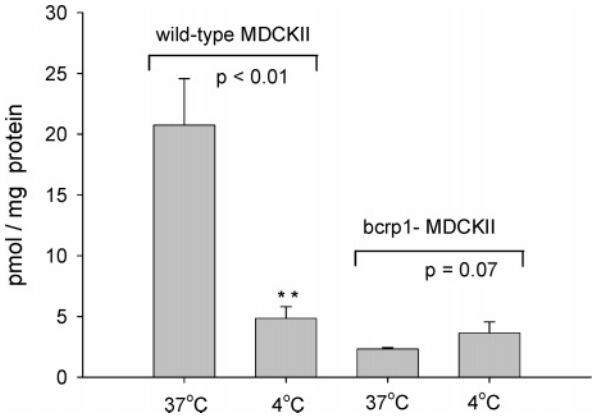

Figure 1. Temperature dependent [^{3}H]-mitoxantrone accumulation. MXR accumulation was significantly reduced in the bcrp1 cells compared to the WT cells at 37 °C. At 4 °C, MXR accumulation in WT cells reduced significantly, while in bcrp1 cells, MXR accumulation increased, but did not reach statistical significance ($p = 0.07$). Results are expressed as mean ± SD ($n = 3$). (**, $p < 0.01$, compared with WT group, 37 °C.)

Results

Temperature-Dependent Cellular Accumulation. There was a significantly reduced ($p < 0.01$) MXR steady-state accumulation at 37 °C in the bcrp1 MDCKII cells when compared to the WT cells (Figure 1). This initial result seemingly verified the function of the efflux transporter bcrp1 in these cells. However, when the incubation temperature was decreased to 4 °C to inhibit active transport processes, the MXR accumulation in WT MDCKII cells was reduced by 77% when compared with 37 °C. Conversely, in the bcrp1 cells, MXR accumulation significantly increased from 11% to 18%. These results indicate that in the WT MDCKII cells there exists an energy-dependent MXR influx transport system that is inhibited at low temperature. However, in the overexpressing bcrp1 cells, the increase in the net intracellular accumulation of MXR at low temperature indicates that the efflux of MXR via the bcrp1 transporter is important in maintaining steady-state intracellular levels of MXR even with the concurrent function of the constitutively expressed influx transporter. These steady-state MXR accumulation results begin to show the complex interplay between influx and efflux transporters, i.e., the magnitude of a net effect on transport will depend upon expression, affinity, incubation time, and available driving force.

Concentration-Dependent Accumulation. (A) Steady-State Accumulation. MXR accumulation at steady state in bcrp1-transfected cells was significantly less when compared to wild-type cells (Figure 1 and Figure 2). However, after a 3 h incubation, as the MXR concentration in the donor media increased, MXR cellular accumulation in both the WT and bcrp1-transfected cells decreased to the same relative degree (Figure 2). Regarding the cellular accumulation of tracer

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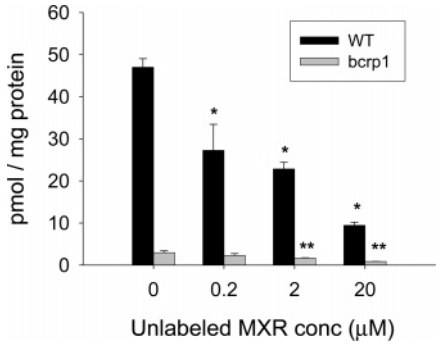

Figure 2. Influence of unlabeled MXR concentration on tracer [^{3}H]-MXR accumulation. [^{3}H]-MXR accumulation in WT and bcrp1 cells decreased significantly with the increasing concentrations of MXR in donor medium. Black columns indicate wild-type MDCKII cells; shaded columns indicate bcrp1-transfected MDCKII cells. There was a similar relative response to increasing concentration in both WT and bcrp1 MDCKII cells. Results are expressed as mean ± SD ($n = 3$). (*, $p < 0.01$, compared with WT control group; **, $p < 0.01$, compared with bcrp1 control group.)

MXR, the bcrp1 cells behaved like the WT cells with an increasing donor concentration of unlabeled MXR. This may be explained by the different affinity of the influx transporter when compared to the efflux transporter bcrp1. In other words, the influx active transport system is more susceptible to saturation within this donor concentration range than is the efflux system (bcrp).

(B) MXR Initial Influx Rate. Model fits of eq 3 to the initial rate data (Figure 3B) showed a saturable influx component with similar K_m values for both the wild-type and bcrp1-transfected cells (Table 1). The apparent K_m values for MXR in wild-type and bcrp1-transfected MDCKII cells were determined to be 4.94 ± 1.74 μM and 8.53 ± 1.31 μM, respectively. The maximum influx rate due to the saturable process was also similar between wild-type (47.6 ± 5.1 pmol/min/mg of protein) and bcrp1-transfected cells (76.4 ± 4.2 pmol/min/mg of protein). The tracer MXR initial uptake rates in wild-type and bcrp1 cells were comparable, which suggests that the expression of the influx transporter in the two cell lines is similar. The intrinsic active influx clearances (V_{max}/K_m for the saturable influx process), indicative of transport functional activity, were very similar between wild-type (9.51 mL/min/mg of protein) and bcrp1-transfected (8.92 mL/min/mg of protein) cells (Table 1). The total initial influx rate and the initial rates due to passive diffusion at 4 °C (linear term in eq 1) are depicted in Figure 3A. The cellular uptake due to passive diffusion was not different between the wild-type and bcrp-transfected MDCKII cells (Table 1).

Directional Flux. The permeability experiments showed no significant difference between WT and bcrp1-transfected cell monolayers in the [^{3}H]-MXR directional flux at tracer concentrations. However, at this low MXR concentration in the donor, the influx permeability, i.e., A-to-B (apical side to basolateral side) flux, was greater than the B-to-A flux in both wild-type and bcrp1-transfected cells (Figure 4). This

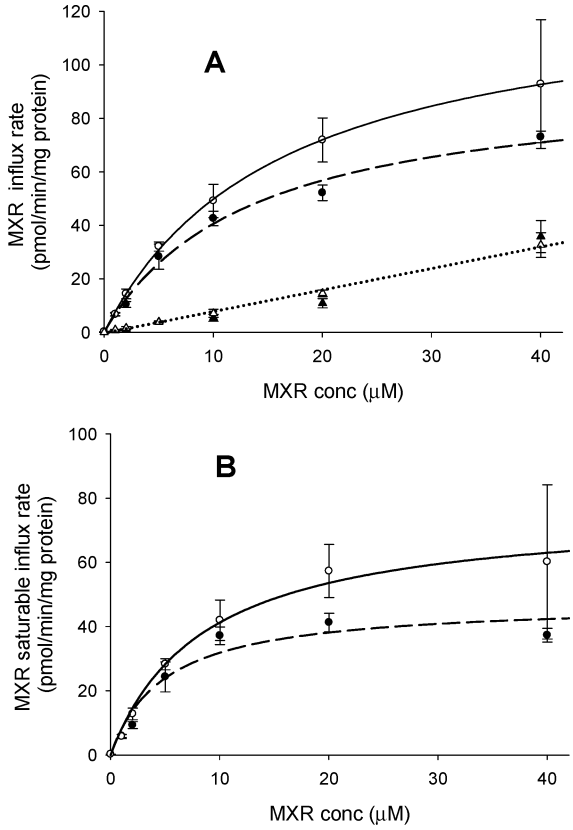

Figure 3. Influence of MXR concentration on initial uptake. (A) The initial total uptake rate (at 37 °C) of [^{3}H]-MXR was measured in the presence of MXR at concentration range from 0 to 40 μM in WT (●) and bcrp1 (○) MDCKII cells. The initial total uptake rate (at 4 °C) of [^{3}H]-MXR in WT (▲) and bcrp1 (△) MDCKII cells. An uptake period of 2 min was chosen to approximate initial rates of transport. [^{3}H]-MXR uptake rate was transformed to total MXR uptake rate according to the specific activity. Solid and dashed lines show model fits to eq 1. The dotted line shows the model fits to eq 2. (B) Uptake specific to an influx transporter was calculated by subtraction from total uptake of the uptake into cells at 4 °C (dotted line in **A**), which is assumed to be the MXR uptake by passive diffusion. The solid and dashed lines are the model fits to eq 3. Shown is the mean ± SD of triplicate determinations ($n = 3$).

Table 1. Model Parameters Determined from Initial Influx Rates

	saturable component			linear component
cell type	V_{max} (pmol/min/mg)	K_m (mM)	Cl_{int} (V_{max}/K_m) (L/min/mg)	K (L/min/mg)
WT MDCKII	47.6 ± 5.1	4.94 ± 1.74	9.51×10^{-6}	8.11×10^{-7}
bcrp1 MDCKII	76.4 ± 4.2	8.53 ± 1.31	8.92×10^{-6}	7.95×10^{-7}

was unexpected given the known location (apical membrane) and orientation (efflux) of the bcrp transporter in the transfected MDCKII cells.

Importantly, increasing the MXR concentration to 20 μM in the donor compartment significantly decreased the A-to-B

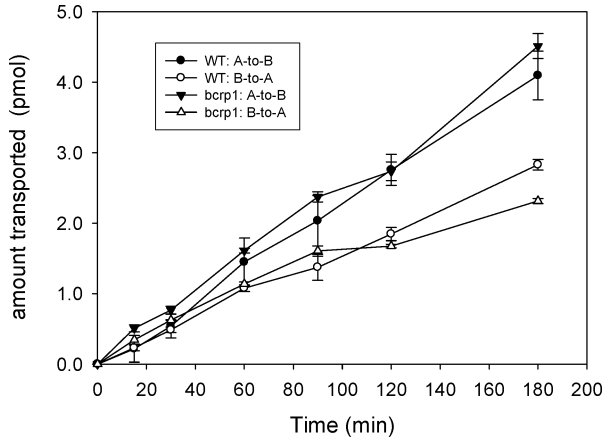

Figure 4. [3H]-Mitoxantrone directional flux across WT and bcrp1 MDCKII cell monolayers. The A-to-B flux of tracer MXR in the WT cells (●) was not different from that in the bcrp1 cells (▼), and the B-to-A flux in the WT cells (○) was not different from that in the bcrp1 cells (△). However, in both the WT and bcrp1 cells, the flux of tracer [3H]-MXR from A to B was greater than the B-to-A flux. Results are expressed as mean ± SD ($n = 3$).

directional flux of [3H]-MXR in both the WT (see Figure 5A) and bcrp1 MDCKII cells (see Figure 5B) ($p < 0.01$). Moreover, increased MXR concentration in the donor compartment significantly increased the B-to-A directional flux of [3H]-MXR ($p < 0.01$) in the wild-type (Figure 5A) and bcrp-transfected cells (Figure 5B). Therefore, in the presence of 20 μM MXR, bcrp1 MDCKII cell monolayers showed the expected significant differences in directional transport given the localization and orientation of bcrp1 in the transfected MDCKII cells. The B-to-A flux was significantly higher than in the A-to-B direction ($p < 0.01$), and it was also higher when compared with the WT group (Figures 5A and 5B).

The apparent permeabilities (P_{app}) in each direction across the monolayer were calculated using eq 4 and are shown in Table 2. In the control group (i.e., MXR at tracer concentrations), the P_{app} A-to-B is higher than P_{app} B-to-A, and there were no significant differences between the WT and bcrp1-transfected groups ($p > 0.05$). However, MXR at 20 μM in the donor compartment revealed a B-to-A flux that was greater than A-to-B. It also resulted in a P_{app} difference between WT and bcrp1 cells that was significant in the B-to-A direction. In summary, there was no significant difference between WT and bcrp1 MDCKII cell monolayer tracer [3H]-MXR permeability at tracer concentrations in the donor, but there was a significant difference when the donor MXR was 20 μM. The efflux ratio (ratio of $P_{app(B-to-A)}$ to $P_{app(A-to-B)}$) was less than unity in the tracer experiments and significantly greater than one when the donor concentration was 20 μM (see Table 2).

Effect of Inhibitors on Accumulation. [3H]-MXR accumulation in WT MDCKII cells decreased significantly in the presence of the OAT inhibitor probenecid (Figure 6A) and OCT inhibitor TEA (Figure 6B). The results depicted in Figure 6 show that probenecid had an enhanced effect on

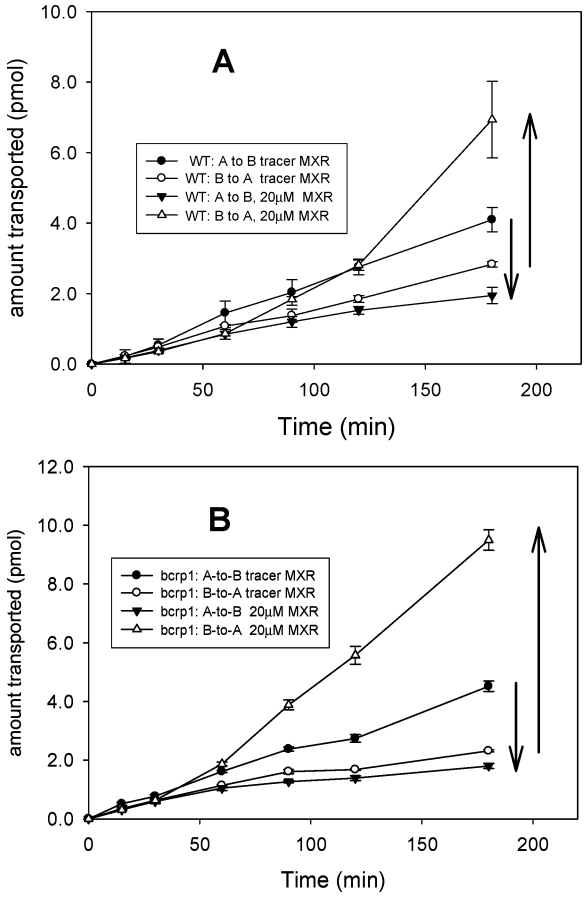

Figure 5. Concentration dependence of tracer [3H]-mitoxantrone flux across WT (A) and bcrp1 (B) MDCKII cell monolayers. The WT and bcrp1 MDCKII cell monolayers were preincubated with 20 μM MXR, and then A-to-B and B-to-A flux of tracer ([3H]-MXR) was measured with 20 μM unlabeled MXR present in both the chambers. 20 μM MXR significantly decreased the directional A-to-B flux of [3H]-MXR, while it increased the B-to-A flux. Results are expressed as mean ± SD ($n = 3$).

MXR accumulation in MDCKII cells compared to TEA. TEA reached its maximum effect at 20 μM, while probenecid showed an increasing dose-response up to 2000 μM. However, in the concentration range of inhibitors tested, the pharmacological inhibition did not decrease MXR accumulation to the same level as substrate saturation via 20 μM MXR did in the WT cells.

Discussion

It is well-known that drug transporters play an important role in drug absorption, distribution, and elimination in vivo.[11] At the cellular level, transporters are also one of the key determinants for drug molecules to transverse cell membranes and eventually determine the active intracellular

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Table 2. Transport of Mitoxantrone (MXR) across MDCKII Cell Monolayers[a]

	MXR tracer donor			MXR 20 μM donor		
P_{app} ($\times 10^{-6}$ cm/s)	A-to-B	B-to-A	efflux ratio	A-to-B	B-to-A	efflux ratio
WT MDCKII	3.37 $\pm$ 0.49	2.02 $\pm$ 0.14	0.60	1.85 $\pm$ 0.12**	3.32 $\pm$ 0.27**	1.78
bcrp1 MDCKII	3.23 $\pm$ 0.15	1.92 $\pm$ 0.09	0.59	1.62 $\pm$ 0.08**	6.50 $\pm$ 0.25**[b]	4.01

[a] Results are expressed as mean $\pm$ SD ($n = 3$). (**) $p < 0.01$ compared with tracer. [b] $p < 0.05$ compared with WT.

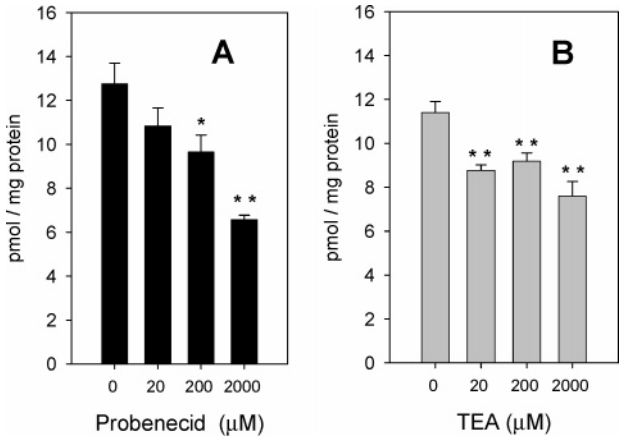

Figure 6. Probenecid and TEA influence on [^{3}H]-mitoxantrone accumulation. In the presence of broad spectrum inhibitors probenecid (A) and TEA (B), [^{3}H]-MXR accumulation in MDCKII cells significantly decreased. Results are expressed as mean $\pm$ SD ($n = 3$). (*, $p < 0.05$, **, $p < 0.01$; compared with control group.)

concentrations.[12,13] Therefore, transporter function has important implications in drug action, i.e., efficacy and toxicity.

In the clinic, MXR use has been limited by the development of resistance.[9] Often this resistance to MXR has been attributed to the overexpression of drug efflux transporters (e.g., P-gp (Abcb1), MRPl (Abcc1), and BCRP (Bcrp1)), an alteration in intracellular drug distribution, reduced topoisomerase II activity, or some combination of these diverse mechanisms.[9,14] It has not been previously reported that the development of resistance to MXR could be related to a change, i.e., decreased expression or function, in active influx transport. The current study is the first report of an MXR influx transporter in mammalian cells, which has been shown to be important to MXR permeability and influences the active intracellular concentrations. If this transporter is widely expressed in tumors and other normal tissues, its expression may have significance in effective use of MXR.

The initial motivation of this study was to verify and characterize the bcrp1 function in the bcrp1-transfected MDCKII cells. The accumulation of [^{3}H]-MXR was used as positive control because it is a classic BCRP substrate.[15] However, we unexpectedly found that the MXR permeability exhibited differing temperature-sensitive changes in cellular accumulation, depending on cell type. When we decreased temperature in an effort to diminish energy-dependent efflux transport activity, MXR accumulation in WT cells decreased, while its accumulation in the bcrp1-transfected cells increased (Figure 1). This suggests an effect on competing transport systems to a varying degree; in the case of the wild-type cells an active influx system was inhibited, and in the case of the bcrp1-transfected cells, the overexpressed efflux system had a greater influence on overall intracellular accumulation.

In a subsequent experiment, the intent was to examine the concentration dependence of the bcrp1 efflux transporter using an increasing concentration of MXR in the donor medium (tracer to 20 μM). If MDCKII cells were serving as simply a background cell for the expression of transfected bcrp1, then an increasing concentration of MXR should result in an increased MXR accumulation in bcrp1 MDCKII cells (saturating efflux), and have no influence on MXR accumulation in the WT MDCKII cells. However, as seen in Figure 2, the MXR accumulation in both WT and bcrp1 cells decreased significantly, and interestingly, to the same relative extent. In this experiment, a 180 min incubation time was chosen because both our preliminary experiments on the time course of accumulation (data not shown) and literature reports indicate that the MXR net accumulation in the cell after 3 h would reach steady state.[16] It was found that tracer MXR accumulation in both wild-type and bcrp1-transfected cells decreased with the increase of MXR concentration, evidence that strongly suggests the existence of influx transporter for MXR in the MDCKII cells. Given that steady-state accumulation represents the net effect of both of influx and efflux transport systems (particularly in the bcrp1-transfected cells), evaluation of an influx process with such a long incubation time may not be adequate. Therefore, initial uptake rates (2 min incubation) were examined to evaluate the saturable influx transporter contribution to MXR permeability and cellular accumulation (Figure 3A). The MXR

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initial uptake rates at tracer concentrations in both the wild-type and bcrp1-transfected cells were similar, suggesting that the activities (functional expression) of the MXR influx transporter in the two cell lines are similar (see Figure 3A and Table 1).

An additional interesting finding in these accumulation experiments is that the effects of increasing the concentration of MXR on the initial influx are similar to its effects on tracer MXR accumulation after 3 h incubation (steady state), when both influx and efflux processes are occurring. This finding may be a result of widely different K_m values for the influx transporter versus the bcrp-mediated efflux transport. However, another more likely possibility is the fact that the relatively high extracellular MXR concentration may saturate an influx system (which presumably is sampling from the bulk), whereas intracellular concentrations, especially the free concentration in the cell, could be significantly below the K_m for bcrp-mediated transport and not show concentration dependence. Lower intracellular concentrations, or significant intracellular binding, could significantly affect the apparent K_m of an efflux transport process. This has been eloquently shown for the transport of paclitaxel in P-glycoprotein overexpressing cells.[17]

Both of these observations, the temperature- and concentration-dependent accumulation, are indicative of a complex process for MXR cellular accumulation and stimulated us to further evaluate the mechanism of MXR cellular permeability using directional flux experiments in MDCKII cells. This would be an additional way to examine and characterize the existence of an active influx transporter, the most plausible explanation for the MXR accumulation results. In the MXR directional flux experiments, it was found that, in wild-type cell monolayers, the tracer [^{3}H]-MXR flux A-to-B was higher than in the opposite B-to-A direction. However, in the presence of 20 μM unlabeled MXR, the A-to-B flux decreased, and the opposing flux (B-to-A) was significantly increased (see Figure 5A). These data strongly suggest the existence of an MXR influx transporter localized on the apical membrane of the MDCKII cells.

One important aspect of these directional flux studies is that they revealed the relationship between MXR competing efflux and influx transporters. In a situation where efflux ratios in bcrp1-transfected MDCKII cells are utilized to screen drugs or compounds in development for bcrp1-substrate status, one could make an erroneous judgment depending on the concentration of the putative substrate in the donor media. In the present case, if only tracer MXR is examined, then the efflux transporter function could not be distinguished for this known prototypical substrate of bcrp1 (see Figures 4 and 5). Comparing P_{app} values (apparent permeability) in Table 2, it can be seen that tracer [^{3}H]-MXR flux from A to B is greater than from B to A in the tracer (control) group, and the directional flux in WT and bcrp1 MDCKII cells was not different. These results indicate that, in some instances, MXR influx transporter function could mask the efflux transporter function. However, under the current experimental conditions, the influx active transport system was more easily saturated than the efflux system. A high concentration of MXR (20 μM) could saturate the influx transport system, revealing the functional efflux transport activity (see Figure 7, in the proposed localization and orientation, an influx transporter would mask efflux transporter function). These interesting directional flux results lead to an appreciation of the influence of competing transport systems (i.e., influx vs efflux), their relative affinities for a common substrate, and the possibility of one transporter concealing the function of the other (Figure 7).

Overall, our results show that not only is MXR net permeability in cells affected by passive diffusion and efflux transport but also a putative influx transporter plays an important role. The cellular accumulation of many drugs is influenced by influx transporters, and that can lead to changes in drug efficacy. For example, lower temperature significantly decreased the accumulation of several HIV protease inhibitors in CEM cells, indicating active influx transport for the protease inhibitors.[18] Also, it is well-recognized that the downregulation of the reduced folate carrier (SLC19A1, an influx transporter that serves as a primary process for cellular accumulation of anti-folates, such as methotrexate), can lead to a clinically important resistance to methotrexate treatment.[19] Moreover, the anti-folate example is particularly germane since the upregulation of the ABCG2 (bcrp1) gene is also involved in the complex development of resistance to methotrexate.[19] A very similar example of this complex interplay between influx and efflux transporters has recently been reported by Thomas et al., who found that the uptake of [^{14}C]-imatinib was temperature dependent. At low temperature, imatinib cellular accumulation decreased, again indicative of an active influx process.[13] Their further investigation suggested that this active influx was mediated by hOCT1, and additional clinical evidence showed the importance of the hOCT1 influx system in imatinib resistance.[21] The authors suggested that the expression of the imatinib influx transporter hOCT1 contributed to its drug resistance as well as a contribution by efflux transporters, such as ABCB1 (P-glycoprotein).[13] Therefore, the balance between the expression of efflux and influx transporters may determine the intracellular levels and hence the response to

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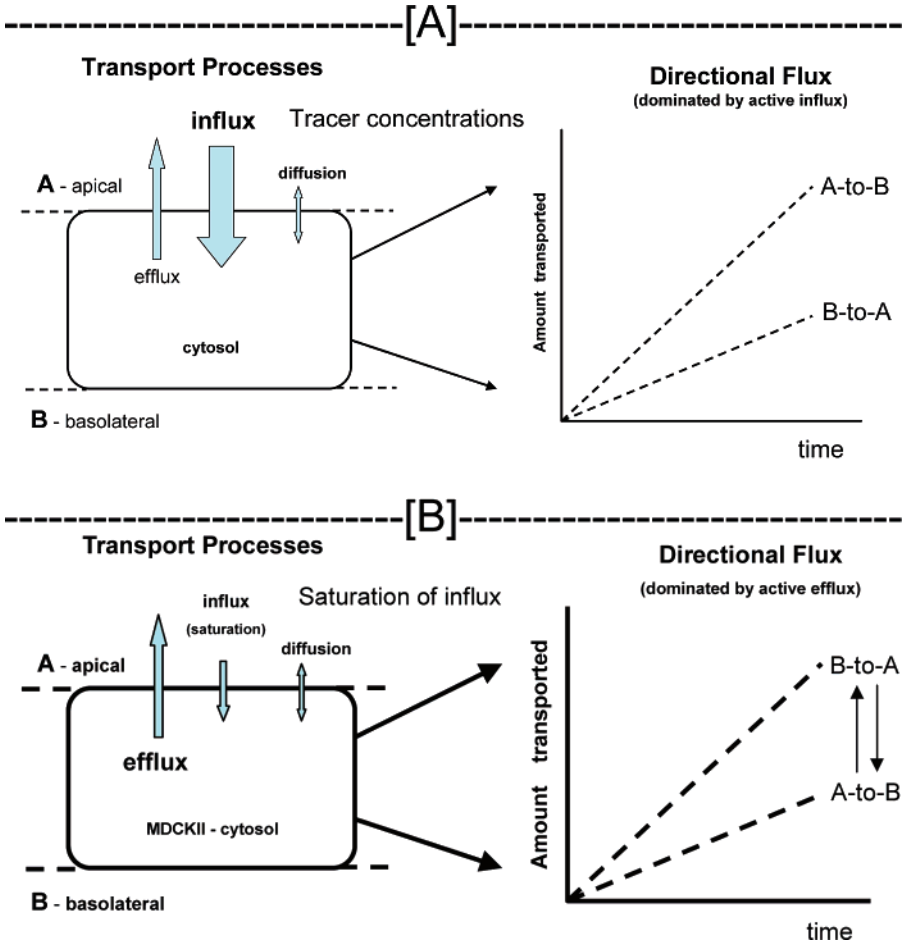

Figure 7. Schematic of proposed transporter roles in mitoxantrone flux in the MDCKII cell apical membrane. (A) At the apical side of the MDCKII cell, MXR permeability is influenced by both efflux and influx transporters, and at tracer concentrations the active influx transport dominates this process resulting in a greater A-to-B than B-to-A transport. (B) However, when a sufficiently high concentration of substrate (e.g., MXR at 20 μM) saturates the influx transporter, it has less effect on efflux transporter function, resulting in a change in direction of the most efficient transport process (B-to-A greater than A-to-B).

imatinib.[20] In a similar manner, the existence of an MXR influx transporter may suggest that either the downregulation or inhibition of active influx transport may be a mechanism of MXR resistance, in addition to efflux transport. If such a system was downregulated in some patients vs others, then this could also be a mechanism of resistance to MXR treatment and lead to significant interpatient variability in drug response.

In drug absorption, distribution, metabolism, and excretion, many of the influx transporters can be classified as organic cation transporters (OCTs), zwitterions/cation transporters (OCTNs), or organic anion transporters (OATs).[22] This putative MXR influx transporter may belong to one of these transporter families, and it is very crucial in maintaining intracellular MXR concentration in MDCKII cells. Although some OATs are expressed in MDCKII cells, it may not be reasonable to hypothesize that this is the MXR influx transporter.[23] This is because MXR is positively charged at physiological pH.[24] Therefore, it seems more likely that the MXR-influx transporter will be one that is responsible for influx transport of cations, such as OCTs.[25] There are few reports about OCT expression in MDCK cells. Shu et al. claimed that there was detectable OCT2 in MDCK cells.[26] However, because both the OAT inhibitor probenecid and

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the OCT inhibitor TEA could decrease MXR uptake (Figure 6), MXR might not be a selective substrate for these transport systems. Another candidate influx transporter subfamily is the OCTNs.[22,27] OCTN2 has an interesting characteristic in that the accumulation of its prototypical substrate, carnitine, could be decreased by both probenecid and TEA in OCTN2-transfected HEK293 cells, exactly like the MXR behavior in the current MDCKII cell model system.[28,29] It is also possible that MXR undergoes active influx by multiple transporters, and additional studies are warranted to examine this possibility. The data in Figure 6 are indicative of the inhibition of active influx; however, the actual magnitude of decreased accumulation may be muted by the effects of either probenecid or TEA on active efflux processes. Additional studies are warranted to examine the identity of this influx transporter, therefore allowing a specific characterization using transfected cell models.

In the current study, we show two important findings relevant to the cellular accumulation of MXR and hence its pharmacological action. First, the uptake of MXR into MDCKII cells is predominantly an active rather than a passive process, as demonstrated by the unlabeled MXR competition and temperature dependency experiments. Second, as shown by the directional flux studies, this influx transporter exists in the apical membrane of MDCKII cells, and has a higher MXR affinity when compared with the bcrp1 efflux transporter. Although the influx transport system has a key role in the maintenance of the intracellular concentration of MXR, it is easily saturated by high dose MXR, and as such the cellular accumulation will be sensitive to extracellular concentrations. This is an important caveat on the use of a common cell culture model for transcellular transport, the MDCKII cell. It has many complicating characteristics with regard to multiple and competing transport systems that may need to be considered when exploring the influence of a single target transport system, such as bcrp1. For example, since MXR influx transporters exist within these cells, the interpretation of cellular accumulation data may be more complex with regard to experimental design, such as accumulation time. It may also be prudent to use a sufficient MXR concentration (such as 20 μM) to saturate the influx transport function when this cell model is used for efflux transporter evaluation, as seen with the directional flux results in the current study (see Figure 7). In a recently published study designed to examine a medium throughput method for determining bcrp activity, Xiao et al. observed significant MXR flux difference in a bcrp-transfected MDCKII cell line at a concentration as high as 10 μM MXR.[30]

The recognition of an MXR influx transporter also has potential clinical implications. If the transporter is widely expressed, the MXR influx and efflux transport can comprise a competing system, which may be critical to MXR antitumor effects and the development of resistance. Effective use of MXR is dependent on the adequate delivery of the drug to its intracellular sites of action and, given the current results, it is probable that both influx and efflux transporters play an important role in achieving this cellular delivery. Further studies are needed to identify and characterize the MXR influx transporter including inhibitor selectivity and substrate structural requirements.

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