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ORIGINAL ARTICLE

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Cyclosporin A, tacrolimus and sirolimus are potent inhibitors of the human breast cancer resistance protein (ABCG2) and reverse resistance to mitoxantrone and topotecan

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Abstract *Purpose:* Several studies have demonstrated significant interactions between immunosuppressants (e.g., cyclosporin A) and chemotherapeutic drugs that are BCRP substrates (e.g., irinotecan), resulting in increased bioavailability and reduced clearance of these agents. One possible mechanism underlying this observation is that the immunosuppressants modulate the pharmacokinetics of these drugs by inhibiting BCRP. Therefore, the aim of this study was to determine whether the immunosuppressants cyclosporin A, tacrolimus and sirolimus are inhibitors and/or substrates of BCRP. *Methods:* First, the effect of the immunosuppressants on BCRP efflux activity in BCRP-expressing HEK cells was measured by flow cytometry. *Results:* Cyclosporin A, tacrolimus and sirolimus significantly inhibited BCRP-mediated efflux of pheophorbide A, mitoxantrone and BODIPY-prazosin. The EC_{50} values of cyclosporin A, tacrolimus and sirolimus for inhibition of BCRP-mediated pheophorbide A efflux were 4.3 ± 1.9 μM, 3.6 ± 1.8 μM and 1.9 ± 0.4 μM, respectively. Cyclosporin A, tacrolimus and sirolimus also effectively reversed resistance of HEK cells to topotecan and mitoxantrone conferred by BCRP. When direct efflux of cyclosporin A, tacrolimus and sirolimus was measured, these compounds were found not to be transported by BCRP. Consistent with this finding, BCRP did not confer resistance to the immunosuppressants in HEK cells. *Conclusion:* These results indicate that cyclosporin A, tacrolimus and sirolimus are effective inhibitors but not substrates of BCRP. These findings could explain the altered pharmacokinetics of BCRP substrate drugs when co-administered with the immunosuppressants and suggest that pharmacokinetic modulation by the immunosuppressants may improve the therapeutic outcome of these drugs.

Keywords BCRP · Cyclosporin A · Tacrolimus · Sirolimus · Inhibitor

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Introduction

The breast cancer resistance protein (BCRP, gene symbol *ABCG2*) is an ATP-binding cassette (ABC) transporter [1, 10, 28] which confers high levels of resistance to a wide variety of anticancer drugs including mitoxantrone and the camptothecins (e.g., topotecan and irinotecan) in several drug-resistant cell lines [11, 23]. Recent studies have demonstrated BCRP expression in various types of solid and hematological tumors [2, 11, 46]. For example, a strong correlation of BCRP expression, but not P-glycoprotein (P-gp) or Multidrug Resistance Protein 1 (MRP1) expression, in tumor samples from 72 untreated stage IIIB or IV non-small cell lung cancer patients with the patients' response rate to chemotherapy or survival has been demonstrated [46]. Therefore, BCRP may play a significant role in drug resistance in certain types of cancers.

BCRP is expressed in various normal tissues in the body. In particular, BCRP is highly expressed in the

apical membrane of placental syncytiotrophoblasts, in the apical membrane of the epithelium in the small intestine, and in the liver canalicular membrane [11, 25]. Consistent with this pattern of tissue localization, BCRP has been shown to play a significant role in absorption, distribution and elimination of BCRP substrates [3, 6, 19–21, 43]. Therefore, BCRP inhibitors could increase the systemic exposure of drugs that are BCRP substrates. Indeed, inhibition of BCRP by GF120918 increases the oral bioavailability of topotecan from 40 to 97% in patients with cancer [21].

Cyclosporin A (CsA) affects the pharmacokinetics of irinotecan in clinical studies. A pharmacokinetic study of intravenous irinotecan in 35 patients with fluorouracil-refractory metastatic colon cancer showed that the mean irinotecan clearance was decreased 2.3-fold and the plasma $AUC_{0-24\ h}$ was increased 2.2-fold by co-administration with 5 mg/kg oral CsA twice a day [4]. Similar significant increases in plasma $AUC_{0-24\ h}$ were seen for SN-38 and SN-38G [4]. In a separate clinical study in a larger patient population ($n = 92$) with refractory solid tumors or lymphoma [18], infusion of CsA at a dose of 5–10 mg/kg was found to increase the plasma AUC of SN-38 by 23–630% and decrease irinotecan clearance by 39–64% [18].

The pharmacological mechanism underlying the above clinical observations is currently unknown. Metabolism is not the dominant elimination pathway for irinotecan. A clinical study by Slatter et al. [39] showed that, following intravenous infusion of irinotecan in cancer patients, a majority of the dose (55%) was excreted unchanged in urine and feces. Fecal excretion accounted for approximately 64% of the drug eliminated while urine accounted for 32%. Cytochrome P450-dependent metabolism was a minor pathway in irinotecan disposition. They also found that biliary elimination of the drug was the main source of fecal excretion with 19% of the drug excreted unchanged out of the 30% total drug excreted in the bile. These data suggest that impaired biliary elimination by CsA is likely to be the main mechanism for increased AUC and reduced clearance of irinotecan observed in the clinical studies, rather than inhibition of cytochrome P450 enzymes. Inhibition of efflux transporters expressed in the liver canalicular membrane would inhibit biliary clearance of drugs. Therefore, it is highly possible that CsA modulates pharmacokinetics of intravenous irinotecan and SN-38 by inhibiting efflux transporters which are abundant in the liver and involved in the biliary excretion of these compounds. CsA is an effective inhibitor of P-gp [12, 31]. Although P-gp can transport camptothecins including topotecan, irinotecan and SN-38, these substances are poor P-gp substrates [22–24, 40]. Two independent studies suggest that MRP2 is not involved in irinotecan transport owing to its low affinity for the drug [5, 24]. In contrast, topotecan, irinotecan and SN-38 are high affinity substrates of BCRP [23, 26, 29, 32]. CsA has been shown to be an inhibitor of BCRP [31]. Therefore, in clinical studies, CsA possibly increases the AUC and decreases clearance of intravenous irinotecan and SN-38, in part, by inhibiting BCRP. In this study, we confirmed that CsA is a potent inhibitor but not a substrate of BCRP. In addition, we found that two other macrolide immunosuppressants tacrolimus and sirolimus are also effective BCRP inhibitors. These findings may help understand the mechanism of drug–drug interactions between chemotherapeutic agents and immunosuppressants, and may inform the design of the optimal immunosuppressants dosing strategies to overcome drug resistance in vivo.

Materials and methods

Materials

CsA and mitoxantrone (MX) were purchased from Sigma (St. Louis, MO). [^{3}H]-CsA was from Amersham Biosciences. Tacrolimus and sirolimus were from LC labs (Woburn, MA). Pheophorbide a (PhA) was obtained from Frontier Scientific (Logan, UT). BODIPY-prazosin was from Molecular Probes (Eugene, OR). Topotecan was a gift from GlaxoSmithKline (Research Triangle Park, NC). Fumitremorgin C (FTC) was a generous gift from Dr. Susan Bates (NIH). HPLC grade DMSO (solvent for PhA, MX, BODIPY-prazosin, topotecan, and FTC) and HPLC grade methanol (solvent for CsA, tacrolimus, and sirolimus) were purchased from Fisher Scientific (Pittsburgh, PA). Eagle's Minimum Essential Medium (MEM) and penicillin–streptomycin–glutamine solution were from ATCC (Manassas, VA). Dulbecco's modified Eagle's phenol-free low-glucose medium (DMEM), phosphate buffered saline (PBS), trypsin–EDTA solution, and fetal bovine serum (FBS) were from Invitrogen (Carlsbad, CA). The MTT reagent 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide and the collagen solution used for coating 96-well plates were purchased from Sigma.

Cell culture

HEK293 cells stably transfected with pcDNA empty vector and cDNA coding for wild-type BCRP (482R) were obtained from Dr. Susan Bates [34]. All the cell lines were grown and maintained as described [15]. Cells were grown to 80–90% confluence and treated with trypsin–EDTA prior to harvesting for subculturing or functional assays. Only cells within six passages were used in functional assays.

Flow cytometric efflux assays

The flow cytometric efflux assays were essentially the same as previously described [15, 33, 45]. We have previously reported that, relative to the expression of BCRP, the HEK cells express little endogenous P-gp,

MRP1, or MRP2 [15], therefore, we performed these studies only in HEK cells. The HEK/482R or the HEK/vector control cells were suspended in incubation buffer (DMEM with 5 mM HEPES buffer) at cell concentration of approximately 10^6 cells per reaction in 1 ml of volume. In the accumulation phase, cells were co-incubated with 1 μM PhA or 10 μM MX or 500 nM BODIPY-prazosin at various concentrations of immunosuppressants or 10 μM FTC at 37°C for 30 min. Cells were then washed once with ice-cold PBS and resuspended in 1 ml of incubation buffer, containing only the immunosuppressants or FTC at their respective concentrations, and incubated for 1 h at 37°C to allow efflux of substrate. Cells were then washed once and resuspended in 1 ml of ice-cold PBS. Intracellular fluorescence was measured within 1 h with a 488 nm argon laser and a 650 nm longpass filter for PhA and MX, and a 488 nm argon laser and 530 nm bandpass filter for BODIPY-prazosin in a BD FACSCAN flow cytometer. Ten thousand (10^4) events were collected for all the samples. Methanol and DMSO concentration was limited to 0.5% (v/v) and 0.2% (v/v), respectively, at which no effect on BCRP efflux activity was observed. Cells in medium alone yielded the blank histogram. Cells in medium containing substrate (PhA, MX or BODIPY-prazosin) alone or in medium containing the substrate and FTC or the immunosuppressant generated the control and FTC or immunosuppressant histograms, respectively. To express relative inhibition of BCRP efflux activity by the inhibitors (Fig. 1), the median intracellular fluorescence of HEK/482R or HEK/vector cells in the presence of immunosuppressants or FTC was normalized to the median intracellular fluorescence of the respective cell lines in the absence of immunosuppressants or FTC which was set as 100%. We noted that the fluorescence of PhA, MX or BODIPY-prazosin in the incubation buffer did not quench over at least 3 h, and the addition of the immunosuppressants did not influence the fluorescence of PhA, MX or BODIPY-prazosin.

To determine the inhibitory effectiveness of the immunosuppressants on BCRP efflux activity, the difference (ΔF) between the median PhA fluorescence of the immunosuppressant histogram and the median PhA fluorescence of the control histogram was calculated and plotted over a range of the immunosuppressant concentrations used (0–25 μM) (Fig. 2). EC_{50} values, which are the concentrations of the immunosuppressants leading to half-maximal inhibition of BCRP efflux activity, were then calculated by nonlinear regression using WinNonLin software as previously described [15]. PhA was used in these experiments as it is a specific and high affinity BCRP substrate [35].

Cytotoxicity assays

Cytotoxicity assays were essentially the same as described [44]. The concentrations of various compounds used in the assays were 1–10,000 nM for topotecan, 10–500,000 nM for MX, and 2 μM or 5 μM for CsA, tacrolimus, and sirolimus. Cell survival was determined by adding the MTT reagent and measuring the optical density at 560 nm using a microplate reader. Cells treated with solvent containing no drug were set as 100% cell survival. IC_{50} values were calculated by fitting the following model to the data using nonlinear regression with the WinNonLin software:

$$S = S_{\max} - [S_{\max} - S_o]\ [C^\gamma/(C^\gamma + IC_{50}^\gamma)],$$

where S is the cell survival as percentage of the optical density of the control cells, $S_{\max}$ is the maximal cell survival, S_o is the lowest residual cell survival at the high drug concentrations, C is the drug concentration, γ is the Hill coefficient, and IC_{50} is the drug concentration in the presence or absence of the immunosuppressants leading to 50% cell survival. Six determinations were carried out within each experiment, and at least three independent experiments were performed. The concentrations of DMSO and methanol used as solvents to dissolve drugs were kept at less than 0.1% (v/v). No cytotoxicity of the solvents was observed at this concentration.

Efflux of [^{3}H]-CsA, tacrolimus and sirolimus

Direct efflux assays using [^{3}H]-CsA, tacrolimus and sirolimus were performed to examine if the immunosuppressants are BCRP substrates. Briefly, the HEK/482R and HEK/vector cells (0.5×10^6 cells/ml) were incubated at 37°C with 0.2 μM [^{3}H]-CsA, 1 μM tacrolimus or 1 μM sirolimus in 0.5 ml of incubation buffer (DMEM with 5 mM HEPES buffer) in the presence or absence of 10 μM FTC for 30 min (accumulation phase). The cells were then transferred on ice and washed twice with ice-cold PBS. Incubation was continued in 0.5 ml of incubation buffer in the presence or absence of 10 μM FTC for another 1 h at 37°C (efflux phase). The efflux reactions were stopped by adding 0.9 ml of ice-cold PBS. The cells were then washed once with PBS. For the assay with [^{3}H]-CsA, the cell pellet was dissolved in 1 ml of 1% SDS, and 900 μl of the cell lysate was subjected to scintillation counting. The intracellular concentrations of [^{3}H]-CsA were calculated based on radioactivity associated with the cell pellets. For the assays with tacrolimus or sirolimus, the cell pellet was first suspended in 1 ml of 70% methanol. Cells in 70% methanol were subjected to water-bath sonication for 10 min at room temperature. BSA and ascomycin were added to 900 μl of the cell lysate. The samples were then mixed with 5 ml of ethyl acetate to extract drug in organic phase and centrifuged at 1,200*g* for 10 min. The supernatant was collected and the solvent in the supernatant was evaporated under a stream of N_2 at 37°C. The remaining pellet was dissolved in 90% methanol and the amount of tacrolimus and sirolimus in the pellet was determined by liquid chromatography-mass spectrometry (LC/MS-MS) as briefly described below. The intracellular

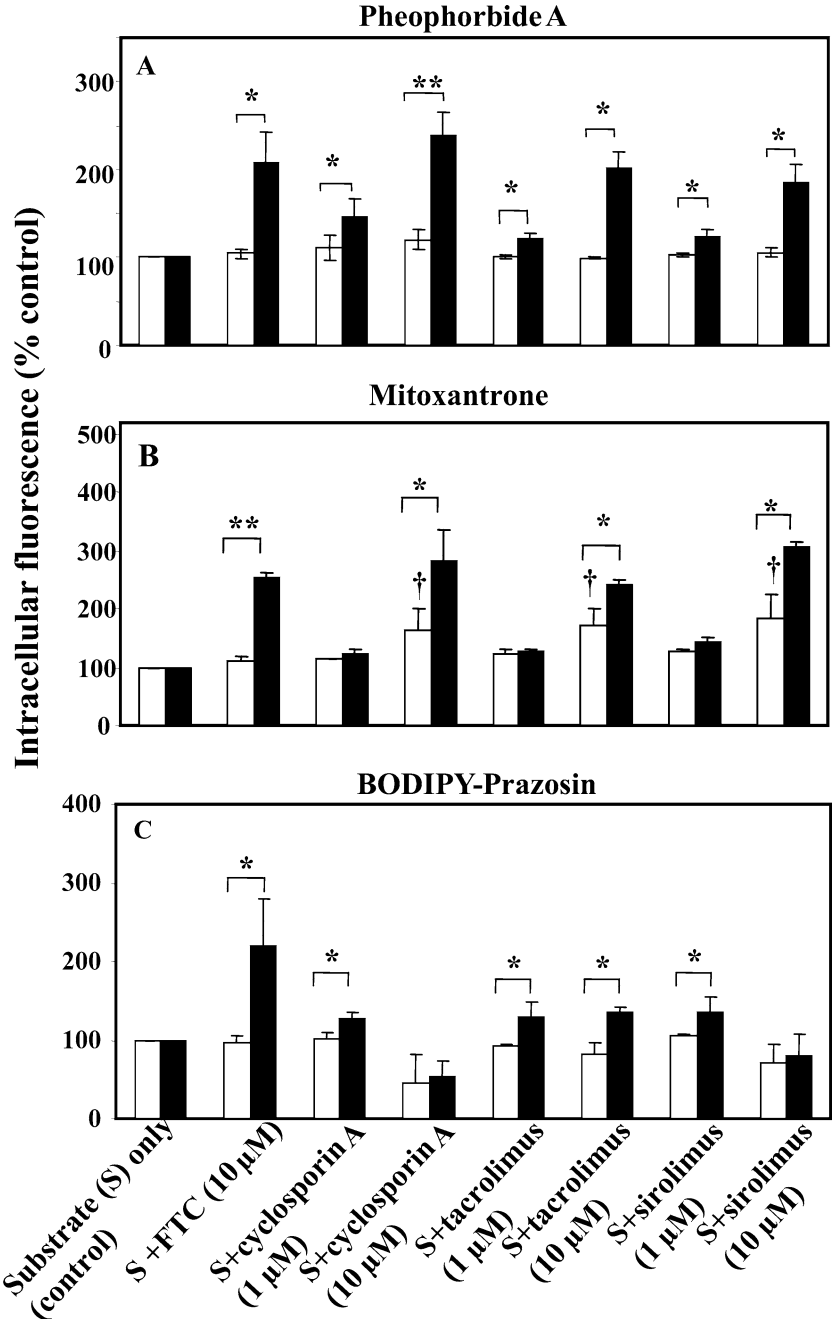

Fig. 1 Increased retention of fluorescent substrates of BCRP by the immunosuppressants in HEK cells. Intracellular fluorescence of 1 μM PhA (**a**), 10 μM MX (**b**) and 500 nM BODIPY-prazosin (**c**) in the presence of 1 μM or 10 μM cyclosporin A, tacrolimus and sirolimus was measured as described in "Materials and methods". FTC at 10 μM, a known BCRP inhibitor, was used as a positive control. The *open bars* represent the intracellular fluorescence in HEK/vector control cells. The *solid bars* represent the intracellular fluorescence in HEK/482R cells. The intracellular fluorescence in the absence of the immunosuppressants was normalized to 100%. Relative levels of intracellular fluorescence in the presence of the immunosuppressants as compared with the controls are shown. The data shown are mean ± S.D. from 3 to 4 independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ (significant difference between the two cell lines with the same treatment and between the HEK/482R cells in the absence and presence of inhibitors); † $P < 0.05$ (significant difference between the HEK/vector control cells in the absence and presence of inhibitors)

concentrations of [^{3}H]-CsA, tacrolimus and sirolimus were finally normalized to the protein concentrations that were measured using the remaining cell lysate by the Bio-Rad Dc protein assay kit and expressed as pmoles of [^{3}H]-CsA or tacrolimus or sirolimus per microgram protein.

LC/MS-MS analysis of tacrolimus and sirolimus

Tacrolimus was separated using a Shimadzu LC-10AD solvent delivery system equipped with a Shimadzu SIL-10ADVP auto-injector and an ODS-HG-5, 150× 2.0 mm, 5-μm column (Shimadzu, Columbia, MD). The mobile phases were A: 0.05% trifluoroacetic acid (TFA) and 40 mM NH_4Ac, and B: acetonitrile. The gradient used was 40% B for 2 min and 95% B for 4.5 min, with a rate flow set at 0.25 ml/min. The elute was then analyzed by mass spectrometry on a Micromass Quattro II tandem quadrupole mass spectrometer (Micromass, Manchester, UK) with a positive electrospray (ESP+) mode. The ion transitions were 821.5 → 768.5 for tacrolimus and 809.5 → 756.5 for the internal standard ascomycin. Tacrolimus was quantified by an external calibration curve. Similar LC/MS-MS method was used to analyze sirolimus except that the mobile phases were A: 0.05% TFA and 0.25 mM NaAc, and B: acetonitrile. Accordingly, sodium adducts were detected with ion

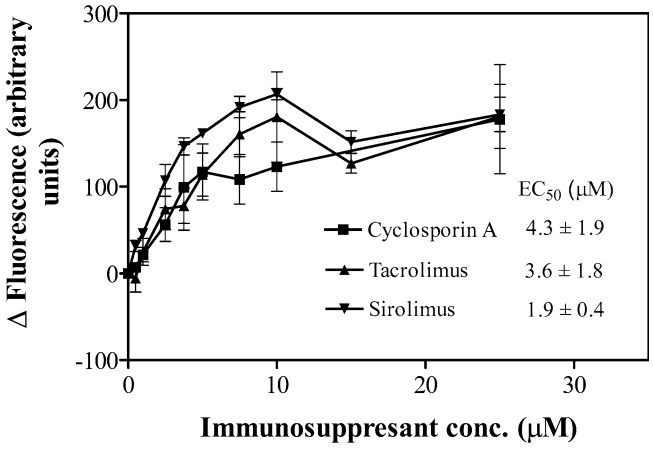

Fig. 2 Effects of the immunosuppressants (0–25 μM) on PhA efflux in HEK/482R cells. The graph represents shift in intracellular PhA fluorescence (ΔF) versus various concentrations of the immunosuppressants cyclosporin A (*solid squares*), tacrolimus (*solid triangles*) and sirolimus (*solid inverted triangles*). The data points shown are mean ± S.D. from 3 to 5 independent experiment

transitions of 937.5 → 937.5 for tacrolimus and 814.5 → 604.3 for ascomycin.

Statistical analysis

Data were analyzed for statistical significance using Student's *t* test. Differences with *P* values of < 0.05 were considered statistically significant.

Results

Inhibition of BCRP-mediated efflux of PhA, MX and BODIPY-prazosin by CsA, tacrolimus and sirolimus

Since PhA, MX and BODIPY-prazosin are fluorescent compounds and known substrates of BCRP [23, 35], they were used to investigate the effects of the immunosuppressants on BCRP efflux activity. FTC significantly increased intracellular fluorescence of PhA, MX and BODIPY-prazosin in HEK/482R cells but not in HEK/vector control cells (Fig. 1), confirming that it is a BCRP inhibitor. CsA, tacrolimus and sirolimus at 1 μM and 10 μM significantly increased intracellular PhA fluorescence by approximately 20–50% and 100–150%, respectively, in HEK/482R cells; however, these compounds did not have significant effects on intracellular PhA fluorescence in HEK/vector control cells (Fig. 1a). These results clearly indicate that CsA, tacrolimus and sirolimus effectively inhibit BCRP-mediated PhA efflux. CsA, tacrolimus and sirolimus at 1 μM had no significant effects on intracellular MX fluorescence in HEK/482R cells but at 10 μM increased intracellular MX fluorescence in both HEK/482R and HEK/vector cells; however, the increase of intracellular MX fluorescence in HEK/482R cells (approximately 150–200%) was significantly greater than the respective increase in HEK/vector cells (approximately 50–100%; Fig. 1b). These results suggest that CsA, tacrolimus and sirolimus also inhibit BCRP-mediated MX efflux. The increase of intracellular MX fluorescence in HEK/vector cells is likely due to inhibition of endogenous efflux transporters for MX by the immunosuppressants. When the effect of CsA, tacrolimus and sirolimus on BCRP-mediated BODIPY-prazosin efflux was measured, we found that CsA, tacrolimus and sirolimus at 1 μM significantly increased intracellular BODIPY-prazosin fluorescence by approximately 30% in HEK/482R cells but had little, if any, effects on intracellular BODIPY-prazosin fluorescence in HEK/vector cells (Fig. 1c). Tacrolimus at 10 μM increased intracellular BODIPY-prazosin fluorescence by approximately 30% in HEK/482R cells and did not affect intracellular BODIPY-prazosin fluorescence in HEK/vector cells. However, although not statistically significant relative to the controls, CsA and sirolimus at 10 μM decreased rather than increased intracellular BODIPY-prazosin fluorescence in both HEK/482R and HEK/vector cells (Fig. 1c).

Concentration-dependent Inhibition of BCRP-mediated PhA efflux by CsA, tacrolimus and sirolimus

To further evaluate inhibitory effectiveness of the immunosuppressants, concentration-dependent inhibition of PhA efflux by CsA, tacrolimus and sirolimus was measured. PhA was used in this experiment since it showed higher specificity to BCRP than the other two substrates. Figure 2 illustrates that intracellular PhA fluorescence in HEK/482R cells is increased by the addition of CsA, tacrolimus and sirolimus in a concentration-dependent manner. The EC_{50} values for inhibition of BCRP-mediated PhA efflux were 4.3 ± 1.9 μM (5.2 ± 2.3 μg/ml), 3.6 ± 1.8 μM (2.9 ± 1.4 μg/ml) and 1.9 ± 0.4 μM (1.8 ± 0.4 μg/ml) for CsA, tacrolimus and sirolimus, respectively. Thus, CsA, tacrolimus and sirolimus are effective BCRP inhibitors with EC_{50} values at low μM concentrations. Sirolimus seems to be the most potent BCRP inhibitor, followed by tacrolimus and CsA.

Reversal of BCRP-mediated topotecan and MX resistance by CsA, tacrolimus and sirolimus

First, we determined cytotoxicity of the immunosuppressants themselves. We found that the survival rates of HEK/482R and HEK/vector cells treated with CsA and tacrolimus at concentrations up to 5 μM for 72 h were greater than 90% (data not shown). Sirolimus showed some toxicity to HEK cells with approximately 85% cell survival at 5 μM. The toxicity profiles of CsA, tacrolimus or sirolimus were almost identical for HEK/482R and HEK/vector cells (data not shown), suggesting that

BCRP does not confer resistance to the three immunosuppressants.

We then examined the reversal capability of CsA, tacrolimus and sirolimus to BCRP-mediated topotecan and MX resistance at 2 and 5 μM at which the three immunosuppressants showed no apparent toxicity (CsA and tacrolimus) or low toxicity (sirolimus) to HEK cells. When topotecan toxicity in the absence of the immunosuppressants was measured, the IC_{50} values of topotecan for HEK/482R and HEK/vector cells were 182.1 ± 30.2 nM and 10.9 ± 0.4 nM, respectively (Table 1). Thus, BCRP conferred 16.7-fold relative resistance (RR) for topotecan in HEK/482R cells. Upon addition of 2 and 5 μM CsA, the IC_{50} values of topotecan for HEK/482R cells were decreased to 66.7 ± 11.2 nM and 47.3 ± 13.4 nM, respectively. Addition of CsA did not significantly affect topotecan toxicity profiles for HEK/vector cells (Table 1). Thus, the RR values of topotecan were reduced from 16.7 to 5.6 and 3.6, respectively, by the addition of 2 and 5 μM CsA. Therefore, CsA can effectively reverse resistance of HEK cells to topotecan conferred by BCRP. The reversal capability of tacrolimus and sirolimus to topotecan resistance was even greater than that of CsA. Upon addition of 2 μM tacrolimus and sirolimus, IC_{50} values of topotecan for HEK/482R cells were decreased to 45.7 ± 14.6 nM and 39.8 ± 25.1 nM, respectively (Table 1). Addition of 5 μM tacrolimus and sirolimus further decreased the IC_{50} values of topotecan for HEK/482R cells to 22.5 ± 17.1 nM and 17.8 ± 14.4 nM, respectively. However, tacrolimus and sirolimus at both 2 μM and 5 μM had no significant effects on the toxicity of topotecan to HEK/vector cells with IC_{50} values almost unchanged around 10 nM. Hence, tacrolimus and sirolimus effectively reversed BCRP-mediated topotecan resistance in HEK cells. Tacrolimus and sirolimus at 5 μM nearly reversed topotecan resistance completely. CsA, tacrolimus and sirolimus showed similar reversal effects on BCRP-mediated MX resistance (Table 1). The RR value for MX was decreased from 13 to below 4.3 by the addition of the immunosuppressants. While resistance of HEK cells to MX conferred by BCRP was reversed by the immunosuppressants, the IC_{50} values of MX for HEK/vector cells were also significantly decreased, possibly due to inhibition of endogenous efflux transporters for MX in HEK cells. These findings are consistent with the observations of the increased intracellular MX fluorescence in HEK/vector cells upon the addition of the immunosuppressants (Fig. 1b). Taken together, CsA, tacrolimus and sirolimus effectively reversed BCRP-mediated resistance to topotecan and MX in HEK cells, confirming that they are effective BCRP inhibitors.

Efflux of [^{3}H]-CsA, tacrolimus and sirolimus

To examine if the immunosuppressants are BCRP substrates, we performed direct efflux studies with these compounds. Figure 3 shows that the intracellular levels of [^{3}H]-CsA, tacrolimus or sirolimus in HEK/482R and HEK/vector cells were not significantly different. Moreover, addition of FTC did not affect the efflux of [^{3}H]-CsA, tacrolimus or sirolimus in either cell line. These results suggest that [^{3}H]-CsA, tacrolimus and sirolimus were not transported by BCRP.

Discussion

The present study shows that CsA, tacrolimus and sirolimus are effective inhibitors of BCRP with EC_{50} values at low micromolar concentrations (Figs. 1, 2). Furthermore, CsA, tacrolimus or sirolimus at 2 and 5 μM effectively restored the sensitivity of BCRP-overexpressing HEK cells to topotecan and MX toxicity (Table 1). Interestingly, the immunosuppressants themselves are not BCRP substrates (Fig. 3). The molecular mechanism by which CsA, tacrolimus and sirolimus inhibit BCRP remains to be elucidated. CsA has been shown to inhibit ATPase activity of wild-type BCRP and its R482G mutant [27, 30], therefore, it is presumed

Table 1 Effect of CsA, tacrolimus and sirolimus on mitoxantrone and topotecan toxicity in HEK/482R and HEK/vector control cells

	DMSO	CsA		Tacrolimus		Sirolimus	
		2 μM	5 μM	2 μM	5 μM	2 μM	5 μM
IC_{50} (nM) of topotecan							
HEK/482R	182.1 ± 30.2	66.7 ± 11.2*	47.3 ± 13.4*	45.7 ± 14.6*	22.5 ± 17.1**	39.8 ± 25.1*	17.8 ± 14.4**
HEK/vector	10.9 ± 0.4	11.9 ± 0.9	13.2 ± 4.4	9.1 ± 2.7	12.9 ± 4.0	12.0 ± 4.0	10.4 ± 1.7
RR	16.7	5.6	3.6	5.0	1.7	2.3	1.7
IC_{50} (nM) of mitoxantrone							
HEK/482R	2574.9 ± 2134.6	63.6 ± 9.7*	36.1 ± 17.8*	64.8 ± 35.7*	17.1 ± 13.8*	51.4 ± 37.3*	22.6 ± 12.8*
HEK/vector	198.8 ± 105.7	14.9 ± 13.2*	21.9 ± 18.1*	24.9 ± 30.4*	16.9 ± 10.5*	20.5 ± 17.9*	24.2 ± 38.2*
RR	13.0	4.3	1.7	2.6	1.0	2.5	0.9

Cytotoxicity studies were performed and IC_{50} values (mean ± S.D., $n = 3$–4 independent experiments) were determined as described in "Materials and methods". Relative resistance (RR) values were calculated by dividing the IC_{50} values of HEK/482R cells by the IC_{50} values of HEK/vector control cells

*$P < 0.05$ and **$P < 0.01$ indicate that the IC_{50} values of the DMSO controls are significantly different from the treatment groups as calculated by Student's t test

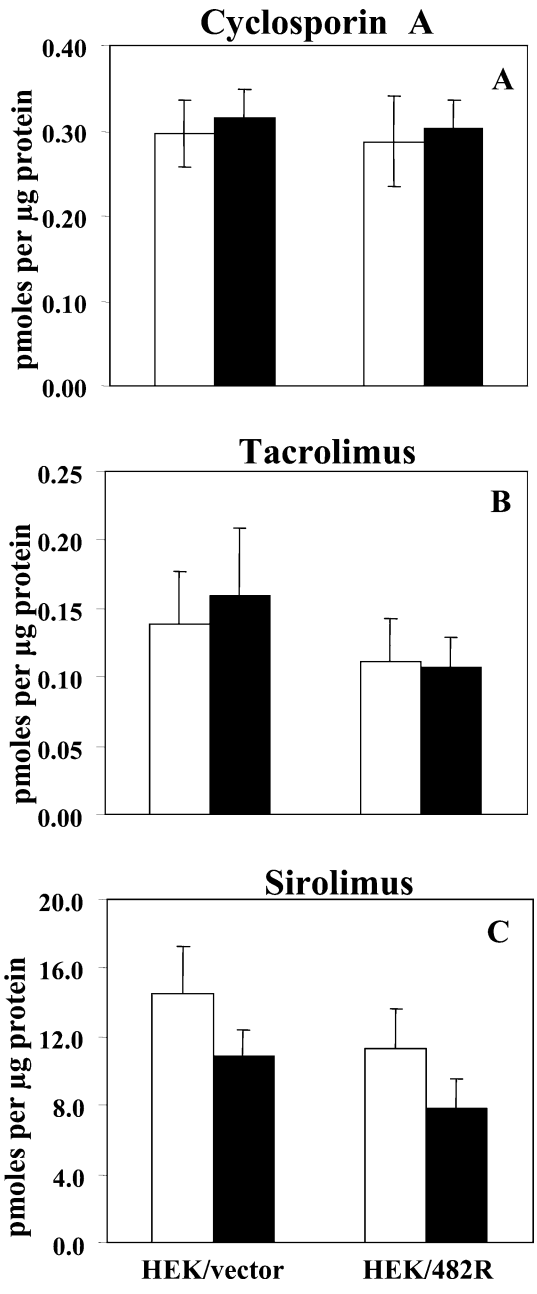

Fig. 3 Efflux of the immunosuppressants in HEK/vector control and HEK/482R cells. Efflux assays were carried out with 0.2 µM [^{3}H]-cyclosporin A (**a**) or 1 µM tacrolimus (**b**) or 1 µM sirolimus (**c**) as described in "Materials and methods". The *open and solid bars* represent intracellular levels of the immunosuppressants in the absence and presence of 10 µM FTC, respectively. The data are mean ± S.D. from five determinations in a typical experiment. Similar results were obtained from 3 to 5 independent experiments

that CsA possibly inhibits BCRP through modulating its ATPase activity.

Garcia-Escarp et al. [13] showed that CsA at 5 µM significantly increased MX retention in drug-resistant cells overexpressing BCRP. Likewise, Qadir et al. [31] also recently demonstrated that CsA at 2.5 µM effectively inhibited MX efflux in various drug-resistant cells overexpressing P-gp, MRP1 and BCRP, indicating that CsA is a broad spectrum inhibitor for ABC transporters [31]. Even before BCRP was cloned, Doyle et al. [9] demonstrated that CsA at 3 µM significantly enhanced daunorubicin accumulation in MCF-7/AdrVp cells, but not in the parental MCF-7 cells. Subsequently, these authors showed that the MCF-7/AdrVp subline, which does not express P-gp, expresses high levels of a BCRP mutant (R482T) which can transport daunorubicin [10]. These results fully agree with the data obtained in this study with respect to inhibition of BCRP by CsA. However, these findings that CsA is a BCRP inhibitor are not consistent with the recent data published by Ejendal and Hrycyna [12]. These authors used flow cytometric analysis to measure efflux of rhodamine 123 and MX in transiently transfected HeLa cells and found that CsA at 5 µM inhibited P-gp but not BCRP function. The reason for this apparent discrepancy is currently unknown but is possibly caused by the experimental conditions used in these studies. First, the expression levels of BCRP in the transiently transfected HeLa cells seem to be quite low as demonstrated by immunoblotting [12]. Therefore, inhibition of relatively low BCRP efflux activity by CsA could possibly have gone unnoticed. Second, rhodamine 123 is not a substrate of wild-type BCRP [34]. MX is also not a highly specific BCRP substrate. As shown in the present study, we found endogenous efflux transporters for MX in HEK cells that can be inhibited by CsA (Fig. 1b, Table 1). Hence, rhodamine 123 and MX do not seem to be ideal substrates for probing transport activity of wild-type BCRP. For this reason, in addition to MX, we used PhA, a highly specific BCRP substrate. Indeed, CsA had no significant effects on PhA efflux in HEK/vector control cells (Fig. 1a).

This study is the first to demonstrate that tacrolimus and sirolimus are potent BCRP inhibitors with EC_{50} values at low µM concentrations. The estimated intestinal luminal concentrations of CsA, tacrolimus and sirolimus are approximately 60 µg/ml, 5 µg/ml and 2 µg/ml, respectively, under currently recommended clinical doses (100 mg daily for CsA, 7.5 mg daily for tacrolimus, and 2 mg daily for sirolimus), assuming rapid complete dissolution of these drugs in the intestine and 1.65 l intestinal fluid volume [7]. Based on the EC_{50} values obtained in this study, and the predicted intestinal concentrations of these drugs, inhibition of intestinal BCRP by the orally administered immunosuppressants, particularly CsA, is expected. In contrast, the target clinical whole-blood trough concentrations in transplantation recipients range from 50 to 400 ng/ml for CsA and 5–15 ng/ml for tacrolimus and sirolimus [41]. Moreover, the unbound fraction of these drugs in blood that is available for rapid equilibration with hepatocytes is less than 10% for CsA, 10–25% for tacrolimus and 60% for sirolimus [42]. Based on these data, a systemic interaction between these immunosuppressants and BCRP substrates (e.g., irinotecan) appears unlikely. Nevertheless, such interactions have been reported as

described below. This lack of an in vitro–in vivo correlation is not novel [8].

CsA significantly affects the pharmacokinetics of irinotecan. Two independent pharmacokinetic studies in patients with different types of cancer demonstrated that co-administration of either oral or intravenous CsA with irinotecan infusion strongly reduced biliary clearance of irinotecan and SN-38 and increased bioavailability of these compounds [4, 18]. Because diarrhea, a major toxicity of irinotecan therapy, is likely caused by direct intestinal damage by SN-38 excreted into the bile and then into the gut, it has been proposed that any drug that can inhibit biliary excretion of SN-38 would reduce the occurrence of diarrhea and improve the therapeutic index of irinotecan [16]. Thus, data from these clinical studies suggest that CsA could be used as a pharmacokinetic modulator of irinotecan, leading to an improved therapeutic index (i.e. increased efficacy and decreased toxicity).

As stated above, quantitative in vitro–in vivo discrepancies for drug–drug interactions involving metabolic and transport processes are not uncommon. Several general explanations have been offered, including failure to accurately determine the inhibition potency in vitro, active hepatic uptake of drug and time-dependent, non-reversible changes to the function of the drug disposition protein. In the case of BCRP inhibition by CsA, it is possible that the EC_{50} values that we report may underestimate the true potency because of uncontrolled non-specific extracellular binding to the walls of the incubation vessel. Although there is no published evidence that CsA is actively transported into the liver, the drug does exhibit a high liver/blood partition ratio and uncharacterized active influx into hepatocytes is possible [36]. Therefore, since irinotecan and SN-38 are high affinity BCRP substrates, and as discussed in the introduction that biliary excretion is the main route of elimination for irinotecan, we suggest that increased AUC and reduced biliary clearance of irinotecan and SN-38 by oral or intravenous CsA observed in clinical studies are mediated, at least in part, by inhibiting hepatic BCRP.

Significant interactions have also been documented between CsA and other drugs. For example, in a clinical study with oral rosuvastatin in heart transplant patients receiving antirejection regimen including orally administered CsA, the plasma $AUC_{0-24\ h}$ and C_{max} of rosuvastatin were increased 7.1-fold and 10.6-fold, respectively, as compared with historic controls not taking CsA [38]. Rosuvastatin has been shown to undergo limited metabolism as approximately 90% of the dose (92% parent compound and 8% metabolites) was eliminated in feces after oral administration while only 10% was excreted in urine in the form of metabolites [37]. Most recently, several statins including rosuvastatin have been shown to be BCRP substrates [17]. Thus, the altered pharmacokinetics of rosuvastatin by CsA is likely mediated, at least in part, by inhibiting BCRP in the liver and small intestine. The recent study using the Bcrp1-deficient mouse model indeed suggests that BCRP is involved in the biliary excretion of pitavastatin, a statin analog of rosuvastatin [17].

CsA, tacrolimus and sirolimus are highly effective immunosuppressants used extensively in post-transplantation regimens. Because the treatment of cancer in transplantation patients is now more common, interactions between the immunosuppressants and anticancer drugs such as irinotecan will occur. For example, hepatocellular carcinoma is one of the indications for liver transplantation. Gornet et al. [14] reported that concurrent treatment of a patient with hepatocellular carcinoma after liver transplantation with irinotecan and tacrolimus resulted in increased systemic exposure of SN-38. The mechanism by which tacrolimus increases SN-38 exposure is currently unknown but may be explained, in part, by inhibition of efflux transporters like BCRP as supported by the data from this study. Collectively, our finding that CsA, tacrolimus and sirolimus are effective BCRP inhibitors is clinically significant with respect to drug–drug interactions with the immunosuppressants in cancer and transplantation patients.

In summary, we have demonstrated that CsA, tacrolimus and sirolimus are effective inhibitors of BCRP with EC_{50} values at low μM ranges. This finding provides new insights into the understanding of the molecular mechanisms underlying the observed clinical effects of the immunosuppressants as reversal agents to multidrug resistance in cancer patients and the pharmacokinetic drug interactions with the immunosuppressants.

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