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ORIGINAL ARTICLE

Differential effects of the immunosuppressive agents cyclosporin A, tacrolimus and sirolimus on drug transport by multidrug resistance proteins

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Abstract
Purpose We sought to determine the effects of the immunosuppressants, cyclosporin A (CsA), tacrolimus and sirolimus, on drug transport by the ATP-binding cassette proteins, P-glycoprotein (Pgp; ABCB1), multidrug resistance protein-1 (MRP-1; ABCC1) and breast cancer resistance protein (BCRP; ABCG2), and the major vault protein lung resistance protein (LRP).
Methods Cellular content of mitoxantrone, a Pgp, MRP-1 and BCRP substrate, was measured by flow cytometry in cells overexpressing these proteins following incubation with and without CsA, tacrolimus or sirolimus. Interaction of BCRP with these compounds was studied by photolabeling and ATPase assays. Nuclear–cytoplasmic distribution of doxorubicin was studied by confocal microscopy in cells overexpressing LRP.
Results CsA increased cellular drug uptake in cells overexpressing Pgp, MRP-1 or BCRP and nuclear drug uptake in cells overexpressing LRP at the clinically achievable concentration of 2.5 μM. Tacrolimus enhanced cellular drug uptake at 1 μM, but not at 0.08 μM, its clinically achievable concentration, and did not enhance nuclear drug uptake. Sirolimus enhanced cellular drug uptake in cells overexpressing Pgp, MRP-1 and BCRP with optimal effects at 2.5 μM, but was effective at its clinically achievable concentration of 0.25 μM if cells were pre-incubated for at least 30 min before drug exposure, and also enhanced nuclear drug uptake at 0.25 μM. BCRP modulation by all three immunosuppressive agents was associated with competitive binding to the drug transport sites.
Conclusions CsA, tacrolimus and sirolimus modulate drug transport by Pgp, MRP-1 and BCRP and CsA and sirolimus modulate drug transport by LRP at concentrations that differ from immunosuppressive concentrations and maximum tolerated concentrations.

Keywords Cyclosporin A · Tacrolimus · Sirolimus · P-glycoprotein · Multidrug resistance protein-1 · Breast cancer resistance protein · Lung resistance protein

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Introduction

Multidrug resistance (MDR) proteins including the membrane-bound ATP-binding cassette (ABC) proteins P-glycoprotein (Pgp; ABCB1), multidrug resistance protein (MRP-1; ABCC1) and breast cancer resistance protein (BCRP; ABCG2), which mediate energy-dependent cellular drug efflux, and the major vault protein lung resistance protein (LRP), which impairs cytoplasmic-nuclear drug transport, are associated with treatment failure in acute myeloid leukemia (AML) [4, 22–24, 26, 38, 44]. Agents that block MDR

protein transport of chemotherapy drugs, termed MDR modulators, are usually MDR protein substrates that act as competitive inhibitors, but, alternatively, they may bind to MDR proteins and cause conformational changes resulting in impaired transport function or may interfere with interactions between substrates and ATP sites [6, 9, 16, 29, 32, 37, 41].

Cyclosporin A (CsA), tacrolimus (FK506) and sirolimus (rapamycin) are immunosuppressive agents used in the setting of solid organ and hematopoietic stem cell transplantation [15, 39]. CsA binds to the immunophillin cyclophilin A [12, 39] and inhibits the calcium-dependent serine/threonine phosphatase calcineurin, abrogating transcription of interleukin-2 (IL-2) and other lymphokines [12, 28]. Tacrolimus is structurally different from CsA (Fig. 1), and binds to a different immunophillin, FK506-binding protein (FKBP12) [28, 39], but the FK506–FKBP12 complex, binds to calcineurin [28, 40], like the CsA–cyclophilin A complex, also resulting in inhibition of IL-2 transcription. Tacrolimus is 10–100 times more potent than CsA as an immunosuppressive agent [40], with effective concentrations of 0.003–0.019 μM (2.8–15.6 ng/mL) [5], compared to 0.083–0.208 μM (100–250 ng/mL) for CsA [18, 21, 36]. The clinically tolerable range of tacrolimus is 0.015–0.083 μM (12–68.5 ng/mL) [40], compared to 0.125–2.58 μM (150–3,100 ng/mL) for CsA [17, 25]. Sirolimus is structurally similar to tacrolimus (Fig. 1), rather than CsA, and also binds to FKBP12, but the sirolimus–FKBP12 complex binds to and inhibits the mammalian target of rapamycin (mTOR), rather than calcineurin, resulting in inhibition of cytokine-mediated lymphocyte signaling, rather than cytokine production. The concentration of sirolimus for immunosuppression is 0.01–1 nM, and its clinically tolerable range is 0.013–0.26 μM [34].

We have previously demonstrated that CsA is a broad-spectrum MDR modulator, effectively impairing drug transport in cells overexpressing Pgp, MRP-1, BCRP and LRP at the clinically achievable concentration of 2.5 μM [35]. Tacrolimus and sirolimus have also been shown to modulate both Pgp [2] and BCRP [16], but these effects have been demonstrated at micromolar concentrations, which are above those that can be achieved clinically with these drugs. Modulation by tacrolimus and sirolimus has not been tested at clinically achievable concentrations, and the effects of tacrolimus and sirolimus on MRP-1 and LRP are unknown. In addition, modulation of BCRP and its mechanism of modulation have been controversial [10, 16, 33, 35].

In the work reported here, we compared the effects of CsA, tacrolimus and sirolimus on substrate drug transport by Pgp, MRP-1, BCRP and LRP as a function of concentration and exposure conditions, and studied the mechanism by which modulation of BCRP occurs.

Materials and methods

Cell lines

Drug-selected cell lines overexpressing Pgp (HL60/VCR), MRP-1 (HL60/ADR and HT1080/DR4), BCRP (8226/MR20) and LRP (8226/MR20 and HT1080/DR4) were obtained and maintained as previously described [35]. Parental HL60 cells, which do not express any of the MDR proteins [30], were studied as a negative control. Parental 8226/S and HT1080 [35] were also studied.

Drugs

CsA and sirolimus were purchased from Sigma Aldrich (St Louis, MO) and tacrolimus from Astellas Pharma US, Inc. (Deerfield, IL). The Pgp-specific modulator PSC-833 (Novartis, East Hanover, NJ), the MRP-1-specific modulator MK-571 (Calbiochem, San Diego, CA), and the BCRP-specific modulator fumitremorgin C (FTC) (from Dr. Susan Bates, National Cancer Institute, Bethesda, MD) were used in comparison studies at their established effective concentrations of 2.5, 5 and 10 μM, respectively [35].

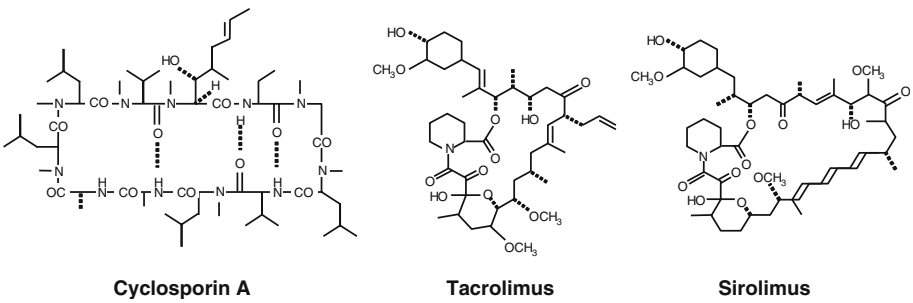

Fig. 1 Chemical structures of CsA, tacrolimus and sirolimus

Mitoxantrone (Sigma Aldrich), a substrate for Pgp, MRP-1 and BCRP [30], was used in cellular uptake experiments, as previously described [35], and doxorubicin (Sigma Aldrich) in studies of intracellular drug distribution, also as previously described [35].

Cellular drug uptake

Approximately 1×10^6 cells were incubated with 3 μM mitoxantrone in the presence and absence of an immunosuppressive agent or an established MDR protein modulator for 30 min at 37°C, washed with cold phosphate-buffered saline (PBS), and kept on ice until analysis. Mitoxantrone content was measured on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) and analyzed with WinList software (Verity Software House, Topsham, ME), as previously described [30, 35]. Mitoxantrone content following uptake in the presence and absence of a modulator was compared using the Kolmogorov–Smirnov (K–S) statistic, expressed as a *D*-value ranging from zero (no difference) to one (no overlap), with values ≥0.2 indicating significant modulation, as previously described [30, 35]. To establish the validity of the K–S statistic, this method was directly compared to calculations of differences in mean fluorescence intensity (MFI). Experiments were performed in triplicate, and *D*-values were reported as mean ± standard error of the mean (SEM).

The magnitude of modulation of drug uptake in cell lines expressing Pgp, MRP-1 or BCRP was compared to the magnitude of modulation in HL60 cells, which

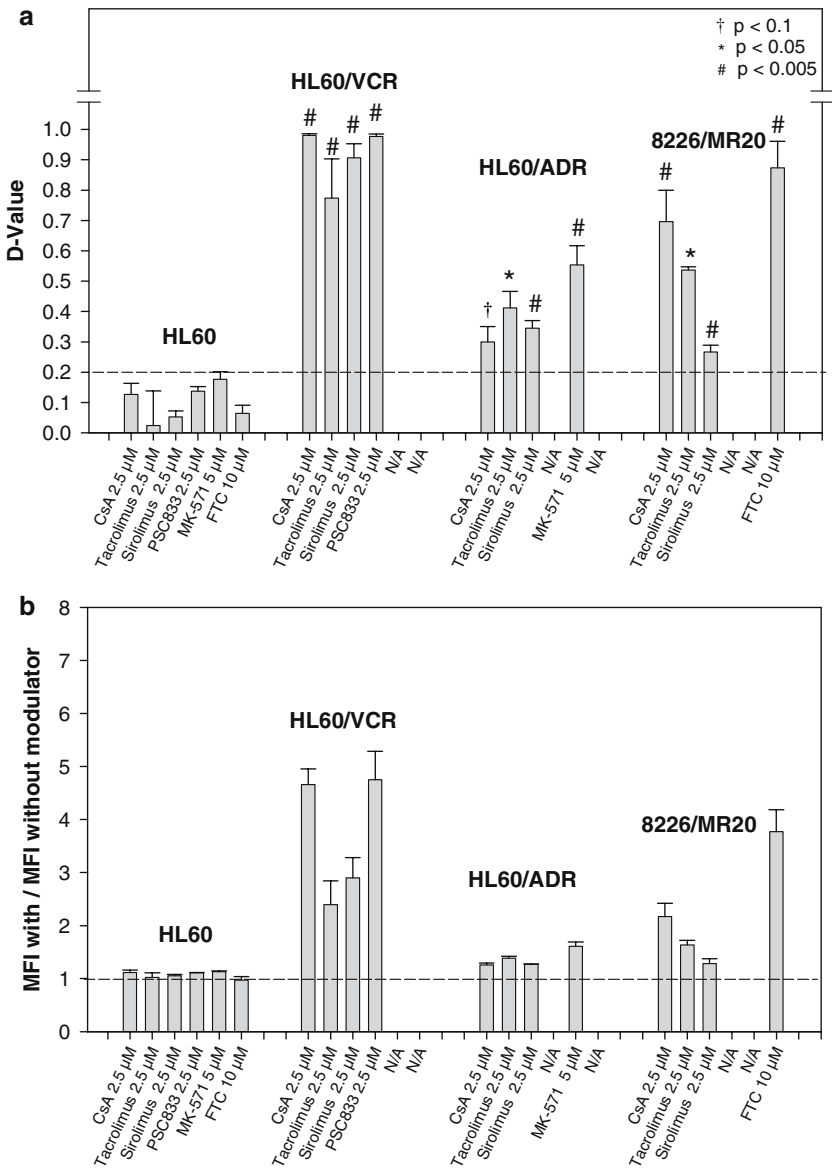

Fig. 2 Modulation of mitoxantrone uptake by 2.5 μM CsA, tacrolimus and sirolimus and by the Pgp-, MRP-1- and BCRP-specific modulators PSC-833, MK-571 and FTC at concentrations of 2.5, 5 and 10 μM, respectively, which have been established to be effective for modulation, in HL60 cells, which express no MDR proteins, and in HL60/VCR, HL60/ADR and 8226/MR20 cells, which express Pgp, MRP-1 and BCRP, respectively. **a** Mitoxantrone uptake in the presence and absence of modulators was compared by the K–S statistic, generating *D*-values. Mean ± SEM of triplicate experiments are shown. The magnitude of modulation of drug uptake in cell lines expressing Pgp, MRP-1 or BCRP was compared to the magnitude of modulation in HL60 cells, which do not express these MDR proteins, using the Student's *t* test. **b** Ratios of MFI following uptake with and without modulator. Mean ± SEM of triplicate experiments are shown

do not express these MDR proteins, using the Student's *t* test.

In experiments aimed at determining the effect of pre-incubation on modulator activity, cells were treated with modulator for periods of 30 min to 6 h, and then incubated with mitoxantrone for 30 min in the presence of the modulator.

Intracellular drug distribution

Intracellular drug distribution was studied by confocal microscopy, as previously described [35]. Briefly, 1×10^6 cells were incubated with 3 μM doxorubicin with and without modulator for 3 h, washed and aliquotted onto slides and studied by confocal microscopy. The excitation light source was set at 488 nm, and emission was captured through a 550 nm long pass filter. For each cell, 10–20 focal planes were evaluated, and images with optimal nuclear–cytoplasmic ratios were stored for analysis. Triplicate experiments were performed.

Pgp, MRP-1 and BCRP expression

MDR protein expression was studied as previously described [45]. Pgp expression was studied on unfixed cells with the MRK-16 monoclonal antibody to an external epitope of Pgp (Kamiya Biomedical Company, Tukwila, WA), and MRP-1 was detected with the MRPm6 antibody to an internal epitope of MRP-1 (Kamiya) in fixed cells. BCRP protein was detected both with BXP-21 antibody to an internal epitope of BCRP (Kamiya) and with phycoerythrin (PE)-conjugated anti-ABCG2 (eBioscience, Seattle, WA), which reacts with an external epitope of the protein, as previously described [45].

Pgp, MRP-1 and BCRP function

Function of the MDR proteins Pgp, MRP-1 and BCRP was measured by modulation of mitoxantrone uptake by the Pgp-, MRP-1- and BCRP-specific modulators PSC-833, MK-571 and FTC. Mitoxantrone uptake in the presence and absence of modulator was compared by the K–S statistic, as described above.

Photocrosslinking of BCRP with [^{125}I]-iodoarylazidoprazosin

BCRP expressed in MCF-7 FLV1000 cells, which is wild type (BCRP^{R482}) [42], was photo-labeled with [^{125}I]-iodoarylazidoprazosin (IAAP) as described previously [42]. Briefly, crude membranes (0.2 mg protein/mL) from MCF-7 FLV1000 cells were incubated with 20 μM CsA, sirolimus, tacrolimus or FTC in dimethyl sulfoxide (DMSO) for 10 min at room temperature in 50 mM Tris–HCl, pH 7.5, and 3–6 nM [^{125}I]-IAAP (2,200 Ci/mmol) (PerkinElmer Life Sciences, Wellesley, MA) were added and the samples were incubated for an additional 5 min under subdued light. The samples were then illuminated with ultraviolet (UV) light

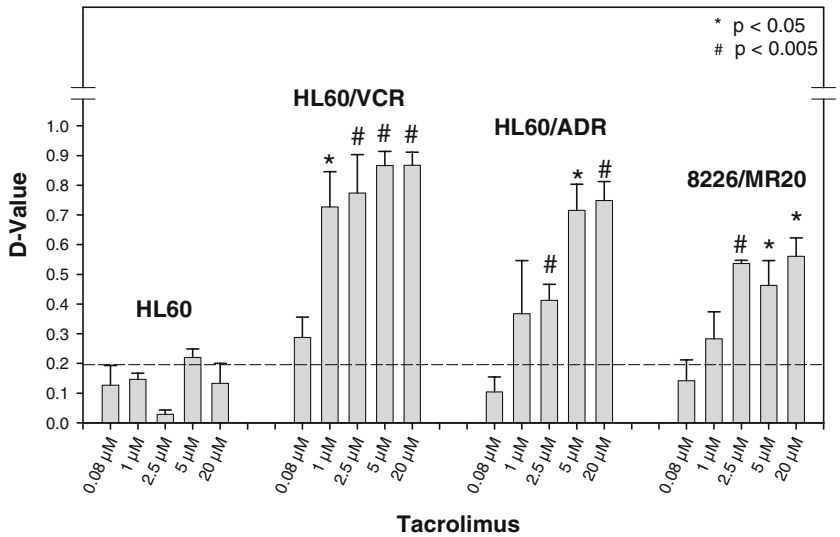

Fig. 3 Concentration-dependent tacrolimus modulation of mitoxantrone uptake in cell lines overexpressing Pgp (HL60/VCR), MRP-1 (HL60/ADR) and BCRP (8226/MR20). HL60 cells, which express no MDR proteins, are shown as a control. Cells were incubated with mitoxantrone for 30 min in the presence and absence of tacrolimus at the concentrations shown. Mitoxantrone uptake in the presence and absence of modulators was compared by the K–S statistic, generating *D*-values. Mean ± SEM of triplicate experiments are shown. The magnitude of modulation of drug uptake in cell lines expressing Pgp, MRP-1 or BCRP was compared to the magnitude of modulation in HL60 cells, which do not express these MDR proteins, using the Student's *t* test

for 10 min, and the labeled BCRP was immunoprecipitated using BXP-21 antibody. The radioactivity incorporated into the BCRP band was quantified using the STORM 860 PhosphorImager system (Molecular Dynamics, Sunnyvale, CA) and ImageQuaNT software (Molecular Dynamics).

ATPase assays

The ATPase activity in crude membranes of High Five insect cells expressing BCRP was measured by the endpoint P_i release assay [1]. Crude membranes (100 μg protein/mL) were incubated with varying concentrations of CsA, sirolimus and tacrolimus at 37°C in the presence and absence of beryllium fluoride (BeFx; 0.2 mM beryllium sulfate and 2.5 mM sodium fluoride) in ATPase assay buffer (50 mM MES–Tris–HCl, pH 6.8, 50 mM KCl, 1 mM ouabain, 5 mM sodium azide, 1 mM EGTA, 2 mM DTT, and 10 mM $MgCl_2$) for 5 min. The reaction was started by the addition of 5 mM ATP and was stopped by the addition of 0.1 mL of 5% SDS solution. The amount of inorganic phosphate released and the BeFx-sensitive ATPase activity of ABCG2 were determined as described previously [1].

Results

We showed previously that CsA modulates drug transport by Pgp, MRP-1, BCRP and LRP at 2.5 μM [35], which is a clinically achievable concentration [25]. Tacrolimus and sirolimus were also tested initially at 2.5 μM, a concentration previously shown to modulate Pgp [2] and BCRP [16]. The effects of CsA, tacrolimus and sirolimus at 2.5 μM on uptake of mitoxantrone in MDR cells overexpressing Pgp (HL60/VCR), MRP-1 (HL60/ADR) and BCRP (8226/MR20) are shown in Fig. 2, in relation to those of the Pgp-, MRP-1- and BCRP-specific modulators PSC-833, MK-571 and FTC at the concentrations that have been established to be effective for modulation. In addition to their known effects on drug transport by Pgp [2] and BCRP [16], both tacrolimus and sirolimus enhanced mitoxantrone uptake in HL60/ADR cells, consistent with modulation of transport by MRP-1, with effects of similar magnitude to those of CsA. Comparison of Fig. 2a (K–S statistic *D*-value) with Fig. 2b (ratio of MFI) validates the K–S statistic as a method for evaluating differences in fluorescence intensity. The K–S statistic method was applied for analysis of subsequent data presented.

Tacrolimus (Fig. 3) and sirolimus (Fig. 4) were then tested at a range of concentrations, including their clinically achievable concentrations of 0.08 [40] and 0.25 μM [34], respectively. The effects of tacrolimus on Pgp, MRP-1 or BCRP, largely plateaued at 5 μM. Tacrolimus did not modulate Pgp, MRP-1 or BCRP at its clinically achievable concentration of 0.08 μM. The effects of sirolimus on Pgp, MRP-1 and BCRP, plateaued at 2.5 μM. Sirolimus did not modulate Pgp, MRP-1 or BCRP at its clinically achievable concentration of 0.25 μM.

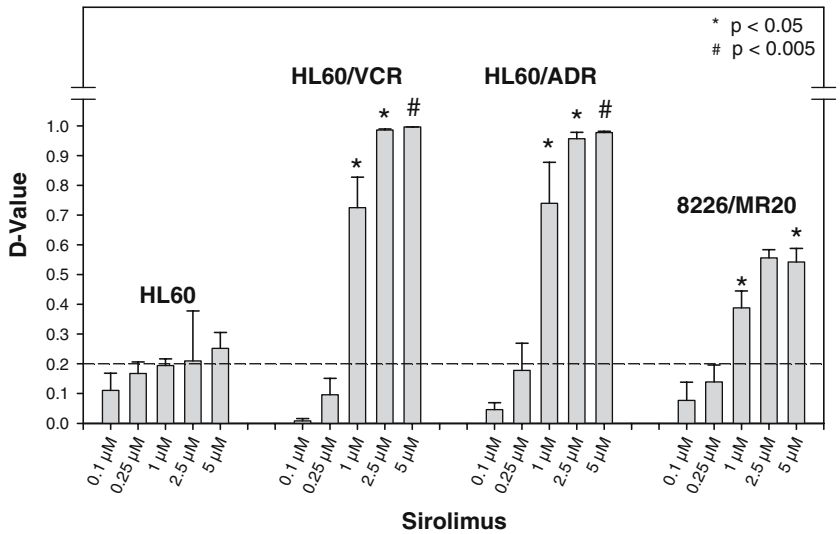

Fig. 4 Concentration-dependent sirolimus modulation of mitoxantrone uptake in cell lines overexpressing Pgp (HL60/VCR), MRP-1 (HL60/ADR) and BCRP (8226/MR20). HL60 cells, which express no MDR proteins, are shown as a control. Cells were incubated with mitoxantrone for 30 min in the presence and absence of sirolimus at the concentrations shown. Mitoxantrone uptake in the presence and absence of modulators was compared by the K–S statistic, generating *D*-values. Mean ± SEM of triplicate experiments are shown. The magnitude of modulation of drug uptake in cell lines expressing Pgp, MRP-1 or BCRP was compared to the magnitude of modulation in HL60 cells, which do not express these MDR proteins, using the Student's *t* test

Since MDR modulation clinical trials commonly use a loading phase with modulator, prior to administration of MDR substrate drug(s) [3, 27], we mimicked this approach in vitro by pre-incubating cells for 30 min to 6 h with CsA, tacrolimus and sirolimus at their clinically achievable concentrations of 2.5, 0.08 and 0.25 μM, respectively, to determine whether pre-incubation allowed modulation at these concentrations (Fig. 5a). Pre-incubation with CsA or tacrolimus did not have a consistent effect on modulation of mitoxantrone uptake in any of the three cell lines, but pre-incubation with 0.25 μM sirolimus for 30 min or more enhanced its subsequent modulation of mitoxantrone uptake in cells expressing Pgp, MRP-1 and BCRP. Sirolimus pre-incubation did not alter cellular expression of Pgp, MRP-1 or BCRP, as detected by the MRK-16, MRPm6 and BXP-21 antibodies, or Pgp, MRP-1 or BCRP function, measured by mitoxantrone uptake. Surface BCRP

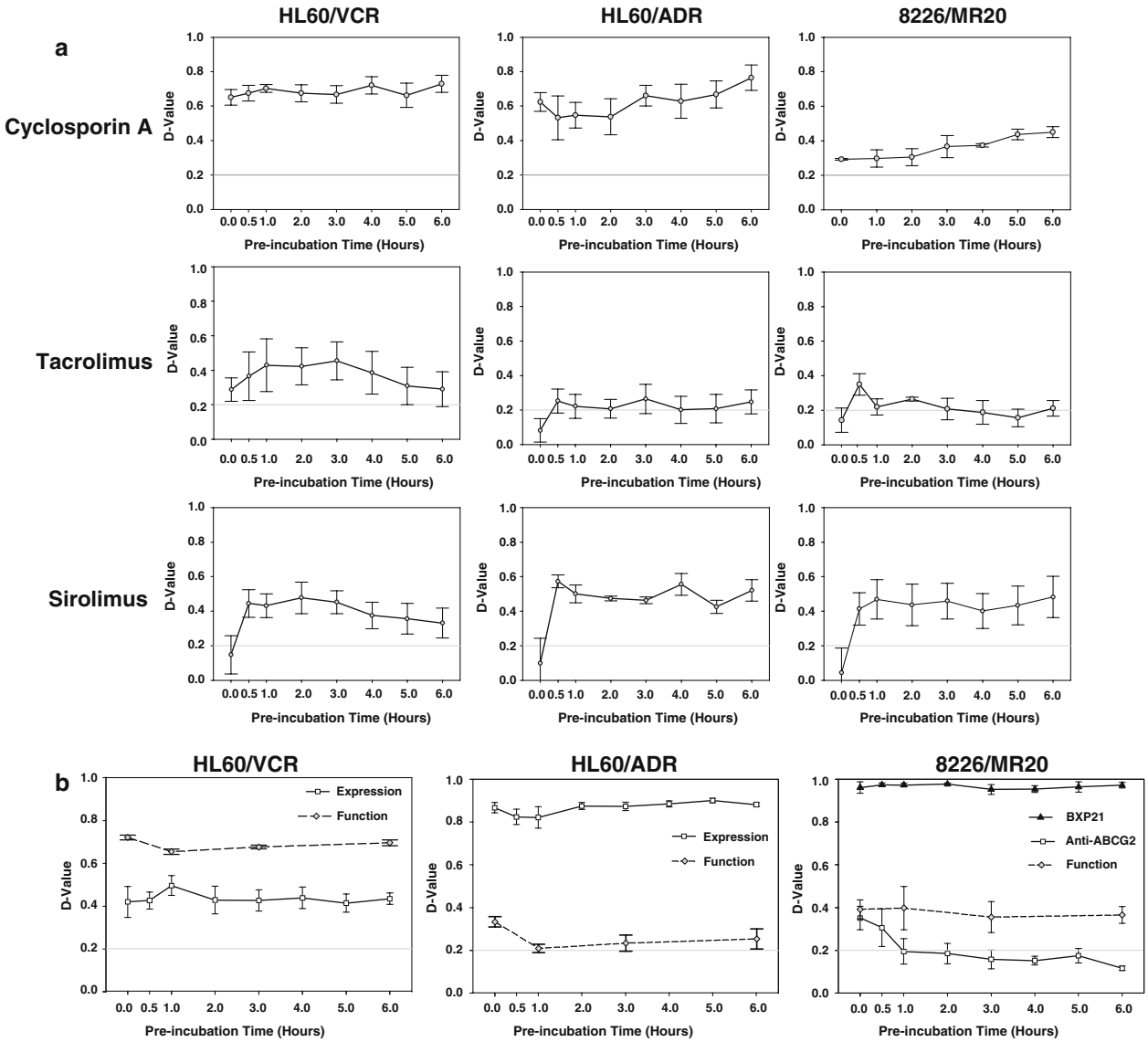

Fig. 5 Effects of sirolimus pre-incubation. **a** Sirolimus pre-incubation enhanced modulation of mitoxantrone uptake. Cells were pre-incubated with 0.25 μM sirolimus for the time periods shown, then incubated with mitoxantrone for 30 min in the presence of 0.25 μM sirolimus. Mitoxantrone uptake modulated by sirolimus following pre-incubation was then compared to mitoxantrone uptake without modulation. In contrast, pre-incubation with CsA and tacrolimus at the clinically achievable concentrations of 2.5 and 0.08 μM, respectively, did not enhance modulation. **b** Sirolimus pre-incubation had no effect on cellular Pgp, MRP-1 or BCRP expression or function, but decreased surface BCRP expression, measured by labeling with the anti-ABCG2 antibody. Function was measured by modulation of mitoxantrone uptake by the Pgp-, MRP-1, and BCRP-specific modulators PSC-833, MK-571 and FTC, respectively. Mitoxantrone content and MDR protein expression were measured by flow cytometry, and both mitoxantrone content following uptake under different conditions and staining with antibodies and with isotype controls were compared by the K–S statistic, generating *D*-values (see materials and methods). Mean ± SEM of triplicate experiments are shown

expression decreased, as measured by staining with the anti-ABCG2 antibody, but this decrease was not accompanied by a decrease in function (Fig. 5b). Efficacy of pre-incubation was also demonstrated in HT1080/DR4 cells, which express MRP-1 and LRP, and HEK-293 482R cells, which are transfected with $BCRP^{R482}$ (data not shown).

We previously demonstrated that CsA at 2.5 μM enhances nuclear uptake of doxorubicin in cells overexpressing LRP [35]. The effects of tacrolimus and sirolimus on nuclear uptake of doxorubicin were also studied in 8226/MR20 and HT1080/DR4 cells, both of which overexpress LRP (Fig. 6). Tacrolimus at 5 μM did not enhance nuclear uptake of doxorubicin in either cell line, while sirolimus at 0.25 μM (Fig. 6) as well as 2.5 μM (not shown) increased doxorubicin nuclear uptake in both 8226/MR20 and HT1080/DR4 cells, but had no effect in parental 8226/S or HT1080 cells (data not shown). The 8226/MR20 cells overexpress BCRP [30], in addition to LRP, but doxorubicin is a poor substrate for BCRP [19] and, additionally, FTC, which modulates BCRP, had no effect on doxorubicin nuclear uptake (data not shown). Similarly, HT1080/DR4 cells also overexpress MRP-1 [43], but MK-571, which modulates MRP-1, had no effect on doxorubicin nuclear uptake (data not shown).

As the mechanism by which the immunosuppressive agents studied here modulate BCRP is unclear in the previous literature [10, 16], we used photoaffinity labeling to determine whether these agents interact at the substrate binding sites of BCRP. [^{125}I]-IAAP, the photoaffinity analog of prazosin, which is also a substrate of this transporter, has been used to study the interactions of different substrates with BCRP [10, 42]. As shown in Fig. 7a, incubation of the crude membranes (20 μg) from MCF-7 FLV1000 cells with 20 μM CsA, tacrolimus, sirolimus or FTC at room temperature for 10 min inhibited photolabeling by 3–6 nM [^{125}I]-IAAP, suggesting that these compounds interact with the substrate-binding sites of the transporter. We therefore examined the effect of these compounds on the ATPase activity of ABCG2. As shown in Fig. 7b, CsA, tacrolimus and sirolimus inhibited ATP hydrolysis by BCRP in a concentration-dependent manner, with IC_{50} values of 1.40, 2.10 and 1.53 μM, respectively.

Discussion

The immunosuppressive agents CsA, tacrolimus and sirolimus, used in the setting of solid organ and hematopoietic stem cell transplantation, have been reported to have activity in reversing MDR mediated by drug transport proteins. We previously demonstrated that CsA is a broad-spectrum MDR modulator, with effects on Pgp, MRP-1, BCRP and LRP [35]. Tacrolimus and sirolimus have also been reported to modulate both Pgp [2] and BCRP [2, 16]. We report here that tacrolimus modulates MRP-1, in addition to Pgp and BCRP, but does not modulate LRP, while sirolimus, like CsA, modulates Pgp, MRP-1, BCRP, and LRP. Moreover, CsA modulates at a clinically achievable concentration, and sirolimus also modulates at a clinically achievable concentration if cells are pre-incubated for 30 min or more prior to exposure to substrate chemotherapy drugs. Of note, peak blood concentrations (T_{max}) are reached within 1 h after oral sirolimus administration [7, 11, 20, 47]. The data suggest that sirolimus will likely have efficacy as an MDR modulator at a clinically achievable concen-

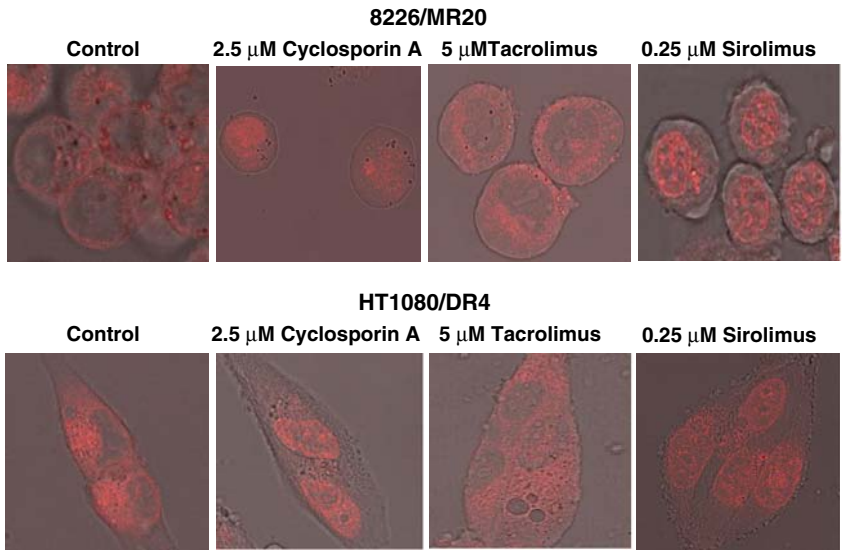

Fig. 6 CsA, tacrolimus and sirolimus modulation of cytoplasmic–nuclear distribution of doxorubicin, studied by confocal microscopy, in 8226/MR20 (*top row*) and HT1080/DR4 (*bottom row*) cells, which overexpress LRP

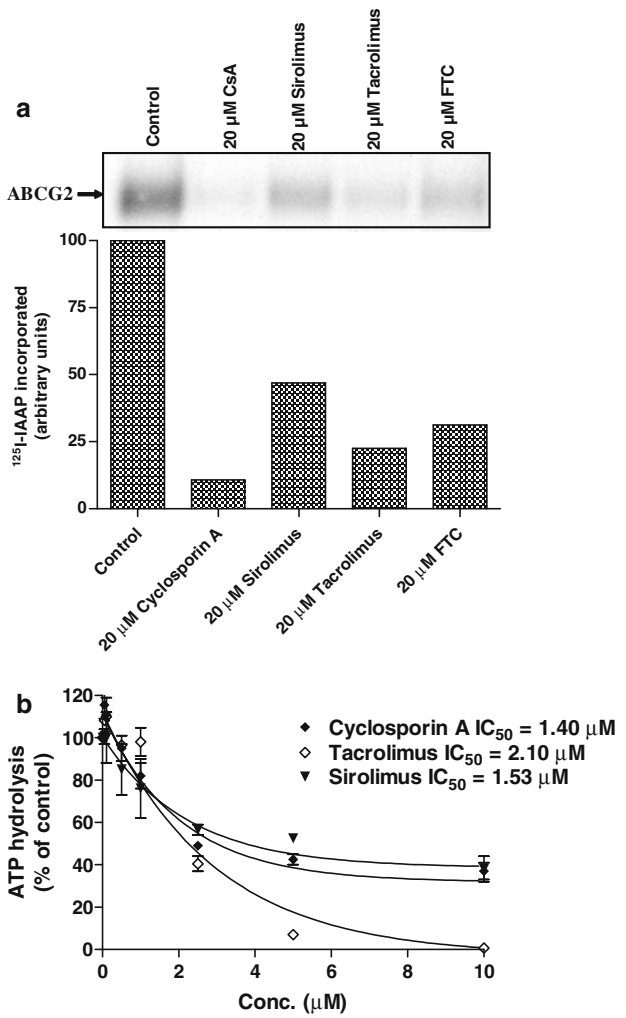

Fig. 7 Mechanism of modulation of BCRP. **a** CsA, sirolimus and tacrolimus inhibit [^{125}I]-IAAP photolabeling of BCRP. Crude membranes (0.2 mg/mL) from MCF-7 FLV1000 cells were incubated with 20 μM of the indicated drugs for 10 min at room temperature in 50 mM Tris–HCl, pH 7.5. 3–6 nM [^{125}I]-IAAP (2,200 Ci/mmol) was then added and incubated was continued for an additional 5 min under subdued light. The samples were then cross-linked with a UV lamp (365 nm) for 10 min at room temperature and were processed after immunoprecipitation with BXP-21 antibody. The autoradiogram from a representative experiment from three independent experiments is shown, with the *arrow* representing the position of BCRP (ABCG2). **b** CsA, sirolimus and tacrolimus inhibit the BeFx-sensitive ATPase activity of BCRP. Crude membranes (100 μg protein/mL) from High Five insect cells expressing BCRP were incubated at 37°C with varying concentrations of CsA, sirolimus and tacrolimus in the presence and absence of BeFx (0.2 mM beryllium sulfate and 2.5 mM sodium fluoride) in ATPase assay buffer for 5 min. The reaction was started by the addition of 5 mM ATP and stopped after 20 min. The graph represents the percent inhibition of the ATPase activity (*Y*-axis) as a function of varying concentrations of different compounds (*X*-axis). The mean value from three independent experiments, which were performed in duplicate is shown, and *error bars* indicate SDs

tration, but that tacrolimus will not modulate at concentrations achieved with immunosuppressive regimens.

The concentrations at which CsA, tacrolimus and sirolimus block drug transport by MDR proteins differ from the concentrations that are effective for immunosuppression. CsA, tacrolimus and sirolimus, mediate their immunosuppressive effects by binding to their specific immunophillins cyclophillin (CsA) and FKBP12 (tacrolimus and sirolimus), and these complexes inhibit either calcineurin (CsA and tacrolimus) or mTOR (sirolimus) in T lymphocytes, impairing T cell cytokine secretion (CsA and tacrolimus) or proliferation (sirolimus). CsA [14], tacrolimus and sirolimus [2] modulate drug transport mediated by Pgp by competitive inhibition, a mechanism that is independent of that by which immunosuppression occurs. Moreover, although these drugs were previously reported not to be transported by BCRP [10, 16], our data suggest transport by BCRP and modulation of BCRP by competitive inhibition. The mechanisms of modulation of MRP-1 and LRP are not yet known.

The literature on CsA modulation of BCRP is inconsistent. While our group demonstrated CsA modulation of BCRP in a previous report [35] as well as in the present report, and other groups [13, 16, 33] also demonstrated the same phenomenon, Ejendal and Hrycyna [10] found that CsA did not modulate BCRP. Our finding that CsA (20 μM) inhibited the binding of [^{125}I]-IAAP to BCRP is also contrary to the report by Ejendal and Hrycyna [10] that CsA (10 μM) does not compete with the binding of this photoaffinity analog to BCRP. The differences observed could be due to the higher concentration of CsA used in our experiments. These authors also showed that CsA does not affect ATP hydrolysis mediated by BCRP. On the other hand, our results here are consistent with those in another previous report [33] demonstrating that CsA inhibits the ATPase activity of BCRP expressed in insect cells. Ejendal and Hrycyna [10] attributed the difference in the activity to the use of the insect cell expression system, as opposed to mammalian cells, which may affect the membrane composition, other cellular factors or the interaction between BCRP and other proteins. In addition, the solvent used for the preparation of stock solution of CsA might affect its potency. CsA dissolved in ethanol appears to be more potent than CsA dissolved in DMSO (SS and SVA, unpublished data). Our IAAP binding and ATP hydrolysis data suggest that CsA, as well as tacrolimus and sirolimus, does interact with the substrate binding site of BCRP, thereby inhibiting its activity. Gupta et al. [16] previously demonstrated that CsA modulates transport by BCRP, but is not transported by BCRP.

The fact that pre-incubation enhances the efficacy of sirolimus modulation is likely explained at least in part by its role as a competitive inhibitor. In cells that express

Pgp, for which sirolimus is a substrate, pre-incubation likely saturates binding sites, while a similar effect is not seen with CsA at 2.5 μM, since it is already highly effective without pre-incubation, nor with tacrolimus at 0.08 μM, which is ineffective with or without pre-incubation. Sirolimus might also saturate MRP-1 binding sites during the pre-incubation period. In addition to its likely effect as a competitive inhibitor of BCRP, sirolimus also decreased surface expression of BCRP, likely via inhibition of mTOR, as has been previously shown to occur with inhibition of Akt [31, 46]. However, the decrease in surface expression of BCRP did not correlate with a decrease in BCRP function, likely because it was only partial, as has also been seen with Akt inhibition [46].

Sirolimus has intrinsic anti-tumor activity by virtue of inhibition of mTOR, which regulates cell growth, transcription and translation [8]. Our data suggest that regimens combining sirolimus with chemotherapy drugs should optimize interactions that exploit not only mTOR inhibition, but also inhibition of transport of MDR protein substrate chemotherapy drugs by sirolimus. The same considerations likely apply to the sirolimus analogs everolimus (RAD001), temsirolimus (CCI-779) and AP23573, which are currently being studied as therapeutic agents in diverse malignancies [8].

Finally, modulation of drug transport by MDR proteins has implications not only for reversal of drug resistance in cancer cells, but also for enhanced absorption of orally administered Pgp and BCRP substrate drugs. As pointed out by Gupta et al. [16], the concentration of CsA, tacrolimus and sirolimus following oral administration should exceed the concentrations required for modulation, and all three drugs therefore likely enhance absorption of Pgp and BCRP substrate drugs by virtue of modulation of Pgp and BCRP expressed in intestinal mucosal cells.

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