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Therapeutic concentrations of cyclosporine A, but not FK506, increase P-glycoprotein expression in endothelial and renal tubule cells

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Therapeutic concentrations of cyclosporine A, but not FK506, increase P-glycoprotein expression in endothelial and renal tubule cells.

Background. The immunosuppressive drugs cyclosporine A (CsA) and tacrolimus (FK506) are extruded from cells by the multidrug resistance P-glycoprotein (P-gp), an efflux pump for drugs and xenobiotics, which may limit their therapeutic effectiveness and/or incidence of toxic side effects. In the present study, we investigated the effect of therapeutic concentrations of CsA and FK506 on the expression of P-gp in cultured endothelial and proximal tubule cells.

Methods. P-gp expression in human arterial endothelial (HAEC) and rat proximal tubule cells (RPTC) was determined by immunoblotting and immunocytochemistry, and correlated with P-gp-mediated transport by measuring the intracellular accumulation of the fluorescent probe calcein.

Results. Following incubation of HAEC with therapeutic concentrations of 0.1 to 1.6 μM CsA up to seven days, P-gp expression increased in a time- and concentration-dependent manner, maximally to 291 $\pm$ 42% of controls with 0.8 μM CsA for seven days. Similar effects of CsA were observed in RPTC. In contrast, therapeutic concentrations of FK506 (0.01 to 0.2 μM up to 7 days) did not change P-gp expression in either cell type, though at higher, supratherapeutic concentrations of FK506 (0.6 to 1.2 μM) P-gp expression was also increased. Immunocytochemistry revealed increased P-gp expression in the plasma membrane of HAEC and RPTC treated with 0.8 μM CsA, which was reflected by a decrease of P-gp-mediated accumulation of calcein in both cell types.

Conclusions. The data suggest that the induction of P-gp expression in HAEC and RPTC at concentrations of CsA or FK506 above 0.5 μM is part of the protective answer of cells to toxic concentrations of the drugs and could therefore interfere with the therapeutic effectiveness of CsA *in vivo*.

Key words: MDR1, xenobiotics, nephrotoxicity, immunosuppressive drugs, C219, MRK-16, rejection therapy.

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Multidrug resistance P-glycoprotein (P-gp) is a 170 kD transmembrane protein, which is encoded by a small gene family comprising two members in humans (*MDR1* and *MDR2*) and three members in rodents (*mdr1*, *mdr2* and *mdr3*) [1]. Only the P-gp MDR1 in humans and mdr1 and mdr3 in rodents convey drug resistance [2]. MDR1 is abundant in many drug resistant human tumors, but is also present in various epithelial tissues, such as liver, intestine, kidney and endothelia of the blood-brain barrier [3, 4]. In these cells, MDR1 is expressed in the luminal plasma membrane where it acts as ATP-dependent efflux pump for various hydrophobic, potentially toxic endogenous and xenobiotic compounds [5, 6], including chemotherapeutics and immunosuppressive drugs [7].

The cyclic undecapeptide cyclosporine A (CsA) and the cyclic macrolide tacrolimus (FK506) are two potent immunosuppressants used for renal transplant recipients. Whereas both drugs have the same immunosuppressive mode of action, namely binding to immunophilins and inhibition of calcineurin-calmodulin-induced dephosphorylation of the NF-AT factor required for transcription of "early" T-cell activation genes (IL-2, *c-myc*, IL-3, TNFα, IFN-γ) by calcineurin-phosphatase [8], on a molar basis FK506 is 10- to 100-fold more potent than CsA [9–11]. This explains why FK506 is effective at lower concentrations than CsA in transplant recipients [12] and can also be used for anti-rejection therapy [13]. Acute and chronic nephrotoxicity are major adverse effects of both drugs; they occur with a similar frequency [14, 15] and involve tubular and endothelial damage resulting in some degree of renal dysfunction [16–18].

CsA and FK506 are also actively transported by P-gp [7]. The affinity of CsA and FK506 for P-gp transport is

unrelated to the immunosuppressive effect. This transport may decrease their therapeutic effectiveness, but also the incidence of toxic side effects in target cells. Since exposure of cells to supratherapeutic concentrations of CsA increases the expression of P-gp *in vitro* [19] and *in vivo* [20], it seemed pertinent to investigate whether therapeutic concentrations of CsA or FK506 increase the expression level of P-gp. To that end, therapeutic and supratherapeutic concentrations of CsA or FK506 were tested on endothelial and kidney proximal tubule cell lines expressing substantial basal levels of P-gp.

METHODS

Materials

The following reagents were obtained from the listed sources and used at the concentrations indicated in the text. Stock solutions of calcein-AM ester (Molecular Probes, Eugene, OR, USA), cyclosporine A (Sigma, Deisenhofen, Germany or kindly provided by Novartis, Basel, Switzerland) and FK506 (kindly provided by Fujisawa, Munich, Germany) were made by solubilization in dimethyl sulphoxide (DMSO). Verapamil (Aldrich Chemie, Steinheim, Germany) was dissolved in ethanol. Rat tail collagen type I (Sigma) was dissolved in 100 mM acetic acid. C219 monoclonal antibody (mAb; Alexis Deutschland, Grünberg, Germany) and MRK-16 mAb (Biozol, Eching, Germany) were used for the detection of P-gp. The monoclonal antibody to the α-subunit of the Na^+/K^+-ATPase (monoclonal antibody α5-IgG) [21] was a kind gift of Dr. J.-P. Hildebrandt (II. Physiologisches Institut, Universität des Saarlandes, Homburg/Saar, Germany). A monoclonal horseradish peroxidase (HRP)-conjugated rabbit anti-mouse antibody, peroxidase (POD)-conjugated goat anti-rabbit antibody and the chromogenic substrate 3-amino-9-ethylcarbazole (AEC) were from DAKO Diagnostika (Hamburg, Germany). HRP-conjugated sheep-anti-mouse IgG and enhanced chemiluminescence reagents were purchased from Amersham-Buchler (Braunschweig, Germany). Non-fat dry milk and prestained protein standards were from Bio-Rad (Munich, Germany). Polyvinylidene difluoride (PVDF) membranes were from NEN-Dupont (Bad Homburg, Germany). All other substances were from commercial sources and of analytical grade.

Cell culture

Human arterial endothelial cells (HAEC) from the iliac artery of an organ donor were obtained from the Coriell Institute for Medical Research (Camden, NJ, USA). The endothelial cell nature of the cultures was confirmed by immunofluorescence staining for the presence of von Willebrand factor and the homogenous expression of PECAM-1 (CD31) [22]. HAEC were cultured in medium M199, enriched with 20% fetal calf serum (FCS), bovine hypothalamic growth factor (ECGF), and heparin. The cells were plated on gelatin-coated flasks, split 1:2 once a week and used for experiments between passages 8 and 20.

Immortalized cells (WKPT-1292 Cl.8) of the S1 segment of the proximal tubule of normotensive Wistar-Kyoto rats (RPTC) were cultured as described earlier [23]. Briefly, cells were maintained in renal tubular epithelium medium composed of Dulbecco's modified Eagle's Medium (DMEM):F-12 [nutrient mixture F-12 (Ham)] 1:1 and supplemented with 15 mM HEPES, 1.2 mg/ml $NaHCO_3$, 5 μg/ml insulin, 5 μg/ml transferrin, 10 ng/ml epidermal growth factor, 4 μg/ml dexamethasone, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate and 5% fetal calf serum. Cells were plated on rat tail collagen type I (125 μg/ml 100 mM acetic acid)-coated flasks, passaged at 80% confluency and split 1:10 twice a week.

To validate comparisons of different drugs and experiments, only cultures at confluency were employed that allowed the same cell to drug ratio.

Immunocytochemistry of P-gp in single cells (cytospin)

Cells were incubated in the presence or absence of CsA (0.8 μM) or FK506 (0.1 μM) for seven days, trypsinized and resuspended in culture medium plus 20% FCS. After cytospinning onto glass slides, cells were briefly fixed in 4% paraformaldehyde, rinsed in phosphate buffered saline (PBS) and stained according to the following protocol: Endogenous peroxidase activity was quenched for 15 minutes with 0.3% H_2O_2 in methanol (vol/vol) at room temperature (RT). To reduce unspecific binding, slides were incubated for 30 minutes with pooled heat-inactivated human serum diluted 1:5 in PBS. Slides were incubated for 60 minutes with mAb MRK-16 (1:100) or mouse IgG as a negative control. The first secondary antibody, a HRP-conjugated rabbit anti-mouse antibody, was applied for 30 minutes at RT at a dilution of 1:50. To amplify the immunoperoxidase reaction, another secondary antibody, a goat anti-rabbit POD-conjugated antibody, was also applied at a dilution of 1:100 for 30 minutes at RT. After washing, the slides were incubated with the chromogenic substrate 3-amino-9-ethylcarbazole for three minutes at RT and counterstained with hematoxylin. This resulted in a red reaction product in positively labeled cells. Slides were washed once more, mounted in glycerol-gelatine and viewed with a non-inverted microscope using objectives of 10×, 20× and 40×. Images were taken with a Zeiss camera using an Agfachrome RSX50 film.

SDS-PAGE and Western blotting

After washing three times with PBS, HAEC or RPTC were scraped off the culture flasks with a rubber policeman, centrifuged and the cell pellet was resuspended in 80 μl of a buffer containing (in mM) mannitol 280, HEPES 10, EDTA 5, $MgSO_4$ 0.1, Pefabloc® 0.2 and 10 μg/ml leupeptin (pH 7.0 adjusted with Tris). Cells were solubilized by addition of 40 μl of three-times concentrated SDS sample

buffer and sonicated for 30 seconds on ice. Following centrifugation at 12,000 × g for five minutes, the protein content of the supernatant was determined by the method of Lowry et al [24]. Following incubation of samples at 37°C for 30 minutes, 50 μg of membrane protein was loaded onto each lane of the gel. Electrophoresis and blotting procedures were performed essentially as described earlier [25]. Proteins were separated by SDS-PAGE on 7.5% acrylamide Laemmli [26] minigels and transferred onto PVDF membranes overnight. The efficiency of protein transfer was monitored with prestained protein standards. Blots were blocked with 3% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 for six hours and incubated with primary antibodies against P-gp (5 μg/ml C219) or Na^+/K^+-ATPase (0.5 μg/ml Abα5) overnight. Following incubation with HRP-conjugated secondary antibody (1:6,000 dilution) for 60 minutes, blots were developed in enhanced chemiluminescence reagents and signals were visualized on X-ray films. X-ray films were scanned with a single pass flat-bed scanner (Linotype-Hell, Eschborn, Germany) and processed for documentation using a JASC Paint Shop Pro 4.1 software (Jameln, Germany). The intensity of the Western blot signals was further enhanced with a Corel Photo-Paint 5.0 software (Corel Corporation Limited, Rüsselsheim, Germany). Signals from different experiments were scanned, and the intensity (optical density) of the chemiluminescence signals was quantified on a Bioprofil computer assisted imaging and scanning system (Vilber-Lourmat, Marne La Vallée, France).

Calcein-AM assay for P-gp activity

Twelve hours before each experiment, the medium of cells incubated for seven days with DMSO or the immunosuppressive drugs CsA (0.8 μM) or FK506 (0.1 μM) was replaced by medium without drugs or solvent. Cells were then trypsinized, washed and stored in DMEM at 25°C. Calcein accumulation was measured in constantly stirred cell suspensions (5×10^5 cells/ml) in 3 ml of Tyrode solution containing (in mM) NaCl 140, KCl 5, $CaCl_2$ 1, $MgCl_2$ 1, HEPES 10, adjusted to pH 7.4 with NaOH, as described previously [27]. Calcein-AM is a nonfluorescent, highly lipid soluble dye that rapidly penetrates the plasma membrane of cells. Once inside the cell, ester bonds are cleaved by endogenous esterases, transforming calcein-AM into hydrophilic and intensely fluorescent calcein. Cells expressing high levels of P-gp rapidly extrude non-fluorescent calcein-AM from the plasma membrane, thereby reducing accumulation of fluorescent calcein in the cytosol. The amount of P-gp activity is therefore inversely proportional to the accumulation of intracellular calcein fluorescence. Calcein-AM (Molecular Probes, Eugene, OR, USA) was used at 0.25 μM. Fluorescence was measured with a FluoroMax-2 (Jobin Yvon-Spex; Instruments S.A., Edison, NJ, USA) fluorimeter (37°C, λ_{ex} 493 nm; λ_{em} 515 nm; band width 5 nm).

Statistics

All experiments were repeated at least three times with different batches of cell preparations and representative data or means ± SD are shown. Statistical analysis was carried out with the Statgraphics program using the unpaired Student's *t*-test. Results with levels of $P < 0.05$ were considered significant.

RESULTS

Human arterial endothelial cells

In immunohistochemical studies of specimens from renal tissue and human vessels we observed a preferential expression of P-gp in arterial as compared to venous endothelia. We also confirmed the differential expression of P-gp in arterial and venous vascular endothelia in a cell culture system with human endothelial cells from the iliac artery and vein from an organ donor (I.A. Hauser, M. Koziolek, F. Thévenod, manuscript in preparation). Since immunosuppressive drug-induced nephropathy is also associated with degenerative changes in the walls of renal arterioles [16–18], we investigated the effect of the immunosuppressants CsA and FK506 on the expression and function of P-gp in cultured human arterial endothelial cells (HAEC).

The presence of P-gp was ascertained by Western blot analysis with the P-gp specific mAb C219 (5 μg/ml) [28]. C219 mAb is directed against two intracellular domains of P-gp close to its ATP-binding site [29]. Figure 1A shows a representative immunoblot of lysates from HAEC that had been incubated with therapeutically relevant concentrations of CsA (0.1 to 1.6 μM) or FK506 (0.01 to 0.2 μM) for seven days. The first lane of each blot (Fig. 1A) shows constitutive expression of P-gp in HAEC. Concentrations of FK506 chosen were 8 to 10 times lower than those of CsA, as FK506 is at least 10 times more potent in terms of immunosuppression [12]. Figure 1B summarizes a quantitative analysis of immunoblots from five different experiments. P-gp expression was quantified by measuring the optical density of immunoreactive bands with controls set to 100%. The C219 mAb labeled a band of ~170 kD, as expected for full size P-gp. CsA treatment for seven days increased P-gp expression, starting with a concentration of 0.2 μM (Fig. 1 A, B). P-gp expression was increased maximally to 291 ± 42% of controls by 0.8 μM CsA ($P < 0.05$; Fig. 1B). FK506 treatment essentially did not change P-gp expression at the therapeutically relevant concentrations of 0.01 to 0.2 μM tested for seven days (Fig. 1 A, B). However, at higher concentrations FK506 also increased P-gp expression to similar levels as CsA (284 ± 34% at 1.2 μM FK506, $N = 5$; $P < 0.05$; Fig. 1B). In contrast, no changes in the expression of the α-subunit of the Na^+/K^+-ATPase, as determined with the monoclonal antibody

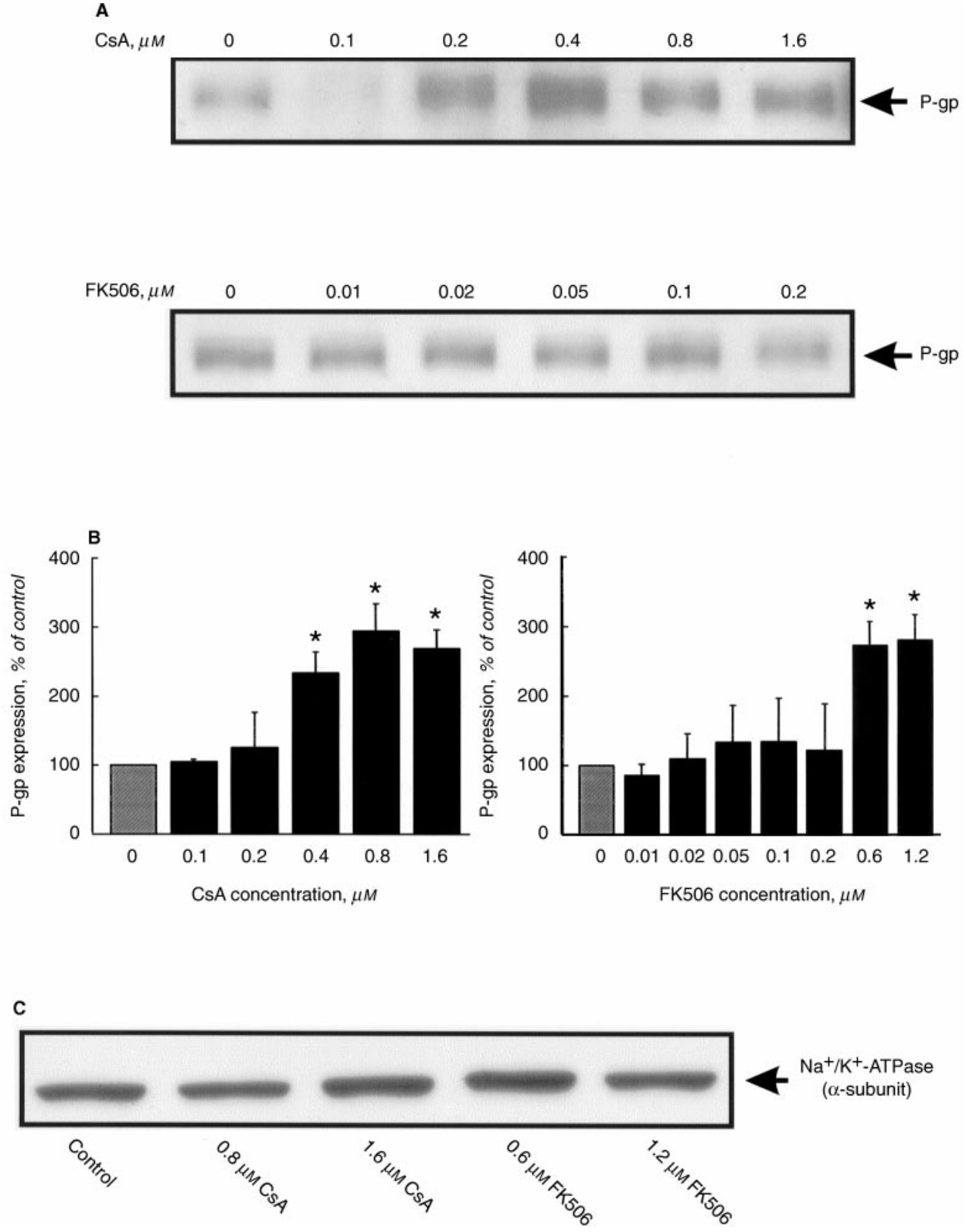

Fig. 1. Expression of P-gp and Na^+/K^+-ATPase in lysates from human arterial endothelial cells (HAEC) treated for seven days with different concentrations of cyclosporine A (CsA) or FK506. (*A*) The immunoblot was probed with mAb C219 (5 μg/ml). HAEC were pretreated for seven days with either 0.1% (vol/vol) DMSO in controls (lane 1) and 0.1 to 1.6 μM CsA (upper immunoblot) or 0.01–0.2 μM FK506 (lower immunoblot). (*B*) Quantification of P-gp expression in HAEC treated with 0.1 to 1.6 μM CsA for seven days (left) or HAEC treated with 0.01 to 1.2 μM FK506 for seven days (right). Immunoreactive bands were analyzed by densitometry, and P-gp expression was expressed as a percentage of the total amount of immunoreactive protein present in HAEC that had been treated with 0.1% (vol/vol) DMSO for seven days. Values represent means ± SD of 5 different experiments. $^*P < 0.05$ using unpaired Student's *t*-test. (*C*) The immunoblot was probed with mAb Abα5 (0.5 μg/ml). HAEC were pretreated for seven days with either 0.1% (vol/vol) DMSO in controls, 0.8 and 1.6 μM CsA, or 0.6 and 1.2 μM FK506. The blot is typical for three different preparations.

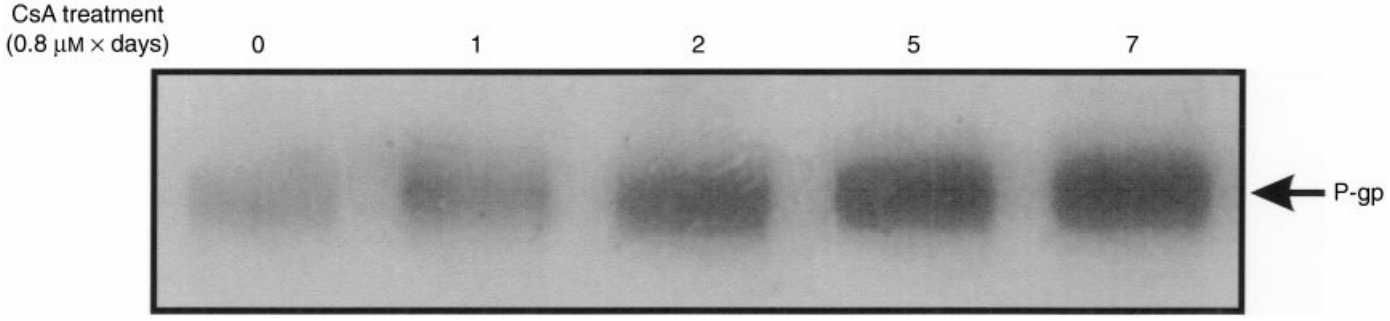

Fig. 2. Time course of the effect of cyclosporine A (CsA) on the expression of P-gp in lysates from human arterial endothelial cells (HAEC). HAEC were left untreated [0.1% (vol/vol) DMSO for 7 days] or exposed to 0.8 μM CsA for one to seven days. The immunoblot was probed with mAb C219 (5 μg/ml). Typical for three different preparations.

Abα5 (0.5 μg/ml), was detected following a seven-day treatment of HAEC with 0.8 to 1.6 μM CsA or 0.6 to 1.2 μM FK506 (Fig. 1C).

Next, we investigated the time-dependence of the effect of CsA on the expression of P-gp in HAEC, which had been treated with 0.8 μM CsA for one to seven days. After 24 hours of incubation with 0.8 μM CsA P-gp expression was slightly, but not significantly, increased compared to control conditions (172 ± 52%, $N = 3$; NS). After two days, P-gp expression was significantly increased (246 ± 32%, $N = 3$; $P < 0.05$) and remained significantly elevated at five days (292 ± 45%, $N = 3$; $P < 0.05$) and seven days of CsA incubation (312 ± 37%, $N = 3$; $P < 0.05$) compared to control conditions (Fig. 2).

Whereas Western blot analyses of HAEC showed that CsA exposure for seven days increased P-gp expression by about two- to threefold in these cells, the functionally relevant localization of P-gp in the plasma membrane of HAEC remained to be determined. The mAb MRK-16 binds to an external domain of P-gp [30] and is therefore suitable for detection of P-gp expressed in the plasma membrane of intact cells. Immunocytochemical detection of P-gp (cytospin) was carried out with the mAb MRK-16 (dilution 1:100) in controls and HAEC that had been treated for seven days with 0.8 μM CsA or 0.1 μM FK506. MRK-16 immunoreactivity on the cell surface of control HAEC turned out to be weak, as shown in Figure 3A. In cells treated with 0.8 μM CsA for seven days, immunostaining on the cell surface was enhanced (Fig. 3B). In contrast, MRK-16 immunostaining in HAEC treated with 0.1 μM FK506 for seven days did not differ from controls (Fig. 3C).

The function of P-gp in the plasma membrane of HAEC was assessed by measuring the time course of intracellular accumulation of fluorescent calcein in the absence or presence of known inhibitors of P-gp. This assay utilizes the fluorogenic dye calcein acetoxymethyl ester (calcein-AM) as a substrate for efflux activity of P-gp [27]. In control HAEC, the addition of 0.25 μM calcein-AM resulted in a high and constant rate of calcein accumulation in the cells following an initial lag time of ~100 seconds (Fig. 4). Addition of the P-gp inhibitor verapamil [31] did not further increase the slope of the fluorescence curve (Fig. 4, control curve). To account for possible differences between control and drug-treated cells, for example, in the activity of intracellular esterases, a dye accumulation ratio was calculated by dividing the rate of net calcein uptake in the presence of the inhibitor by the rate of uptake in the absence of inhibitor [32]. Slopes (rates) were calculated from linear regression. Table 1 shows the accumulation ratios (means ± SD) obtained with the solvent DMSO or with two inhibitors of P-gp, verapamil and CsA [31, 33]. The accumulation ratios of calcein uptake in the presence of DMSO or of the inhibitors of P-gp were not different in control HAEC, suggesting that no or very low levels of functional P-gp were expressed in the plasma membrane of these cells (see also Fig. 3A). In HAEC, which had been pretreated with 0.8 μM CsA for seven days, the accumulation rate of calcein was decreased (Fig. 4, CsA curve). This is expected for cells that overexpress P-gp in their plasma membranes (Fig. 3B) because P-gp extrudes non-fluorescent calcein-AM from the cell, thereby reducing accumulation of fluorescent calcein in the cytosol. Addition of 100 μM verapamil to the cuvette increased the slope of the fluorescence time course (Fig. 4). A total of 100 μM verapamil or 5 μM CsA, which also inhibits P-gp at these supratherapeutic concentrations [33], accelerated the net accumulation of calcein 3.2- to 4-fold (Table 1). In contrast, in HAEC that had been pretreated with 0.1 μM FK506 for seven days, calcein uptake in the absence and presence of inhibitors of P-gp was not different from controls (Fig. 4 and Table 1). This observation is in agreement with the low levels of P-gp detected in FK506-treated HAEC (Figs. 1 and 3C).

Rat proximal tubule cells

Toxic lesions induced by CsA and FK506 have been described in both, proximal and distal tubules [16–18]. From immunohistochemical studies it is known that P-gp is constitutively expressed in the brush border membrane of human [3] and rat proximal tubule cells (RPTC) [34]. In the present study, we also tested the effect of therapeutic concentrations of CsA and FK506 on P-gp expression in a

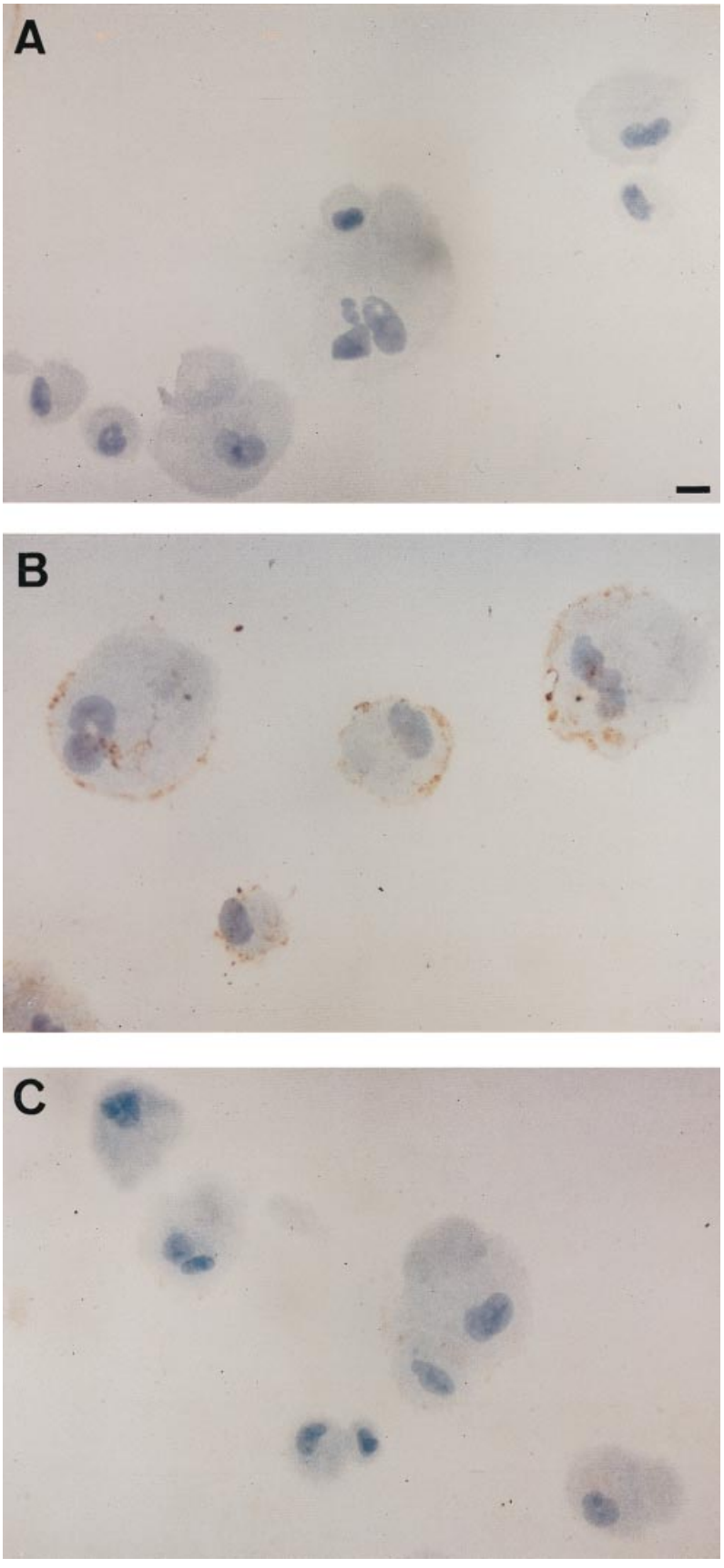

Fig. 3. Immunocytochemical staining of P-gp in the plasma membrane of human arterial endothelial cells (HAEC) incubated without and with cyclosporine A (CsA) or FK506 for seven days. Cytospins of HAEC were fixed in 4% paraformaldehyde, incubated with MRK-16 mAb (1:100) and stained by immunoperoxidase reaction. In (*A*), control HAEC had been incubated with 0.1% (vol/vol) DMSO for seven days. In (*B and C*), HAEC had been exposed to 0.8 μM CsA or 0.1 μM FK506 for seven days, respectively. Typical for four different cytospin preparations. Original magnification $\times$400. Bar = 15 μm.

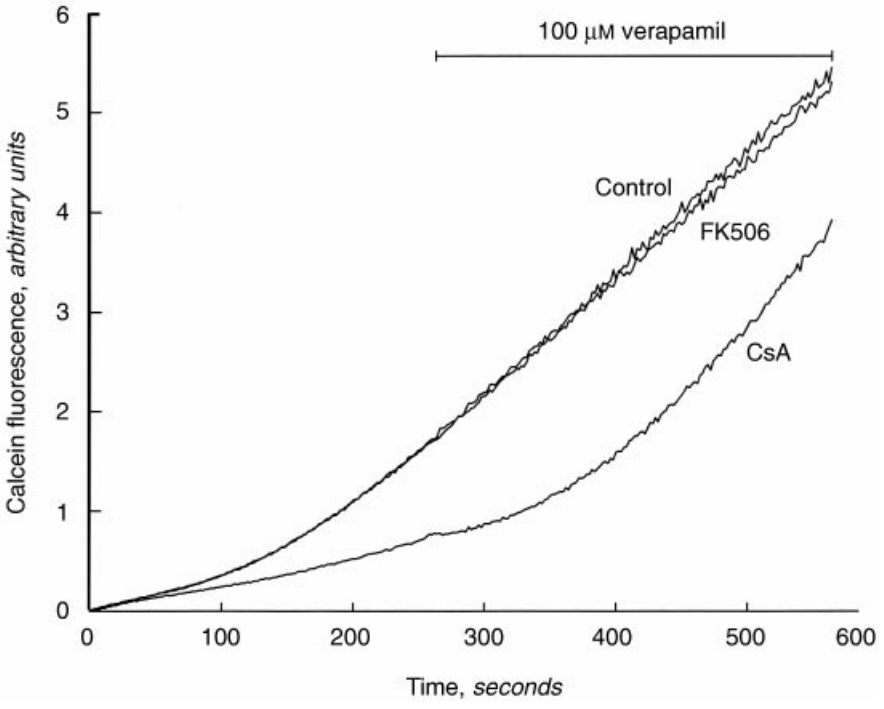

Fig. 4. Fluorimetric time course of calcein accumulation in suspensions of human arterial endothelial cells (HAEC) incubated in the absence and presence of verapamil. Five $\times 10^5$ cells/ml Tyrode solution were incubated in the presence of 0.25 μM calcein-AM at 37°C. The increase in fluorescence was monitored as the dye was hydrolyzed intracellularly. The plot shows calcein fluorescence (arbitrary units) against time. The inhibitor of P-gp verapamil (100 μM) was added where indicated. The different curves represent experiments with cells which had been exposed for seven days to either 0.1% (vol/vol) DMSO (control), 0.8 μM cyclosporine A (CsA), or 0.1 μM FK506 (FK506). Typical for three different experiments.

Table 1. Effect of inhibitors of P-gp on calcein accumulation in human arterial endothelial cells

Pretreatment of cells	Accumulation ratio		
	DMSO	CsA *5 μM*	Verapamil *100 μM*
DMSO	1.63 ± 0.12[a]	1.52 ± 0.13	1.71 ± 0.11
CsA 0.8 μM × 7 days	1.52 ± 0.13	3.31 ± 0.10[b]	3.74 ± 0.36[b]
FK506 0.1 μM × 7 days	1.83 ± 0.09	1.85 ± 0.14	1.99 ± 0.11
N	3	3	3

[a] The accumulation ratios shown (means ± SD) were calculated by dividing the rate of net calcein accumulation after addition of a drug (or solvent DMSO) by the rate observed without drug. Slopes (rates) were calculated from linear regression.

[b] $P < 0.01$ using unpaired Student's *t*-test

cell culture model of renal proximal tubules using immortalized cells derived from the S1 segment of kidney proximal tubule isolated from normotensive Wistar-Kyoto rats [23]. P-gp was constitutively expressed in RPTC, as reflected by an immunoreactive band of about 170 kD (Fig. 5A, first lane). CsA treatment (0.1 to 1.6 μM) for seven days increased P-gp expression in RPTC as a function of the CsA concentration applied (Fig. 5A), similarly to that observed in HAEC (Fig. 1), but the expression of the α-subunit of Na^+/K^+-ATPase was not affected by CsA treatment (Fig. 5B). Application of FK506 (0.1 to 0.2 μM) for the same period of time did not significantly change the expression of P-gp or Na^+/K^+-ATPase in RPTC cells (Fig. 5 A, B). However, higher concentrations of FK506 (0.6 to 1.2 μM) increased the expression of P-gp in RPTC by about threefold (data not shown), in a similar manner to that shown in HAEC (Fig. 1).

The transport function of P-gp was also investigated in RPTC by measuring accumulation of the fluorescent dye calcein. After the addition of 0.25 μM calcein-AM to control RPTC, fluorescent calcein accumulated linearly over time as a consequence of intracellular hydrolysis of calcein-AM by cellular esterases (Fig. 6). The addition of the P-gp inhibitor verapamil (100 μM; Fig. 6) to the cuvette did not significantly affect the rate of calcein fluorescence accumulation in the control- or FK506-treated cells, but increased the rate in cells that had been incubated with 0.8 μM CsA for seven days (Fig. 6, CsA curve). The presence of a verapamil-sensitive component of calcein accumulation indicates a contribution of P-gp to calcein-AM extrusion. This component had been induced by the CsA treatment.

DISCUSSION

The novel aspect of the present study is a comparison of the effect of therapeutic concentrations of the immunosuppressive drugs CsA and FK506 on P-gp expression using cultured cell lines of endothelial and proximal tubular origin. Both cell lines were selected for these studies, because they seemed to represent suitable *in vitro* models of the renal structures in which immunosuppressive drug-induced toxic lesions may occur [16–18], but also of the human tissues that normally express P-gp [3, 4]. P-gp serves as a major pump for cellular extrusion of CsA and FK506 [7] and thus its levels are expected to determine in part cytosolic drug concentrations. Increased P-gp expression

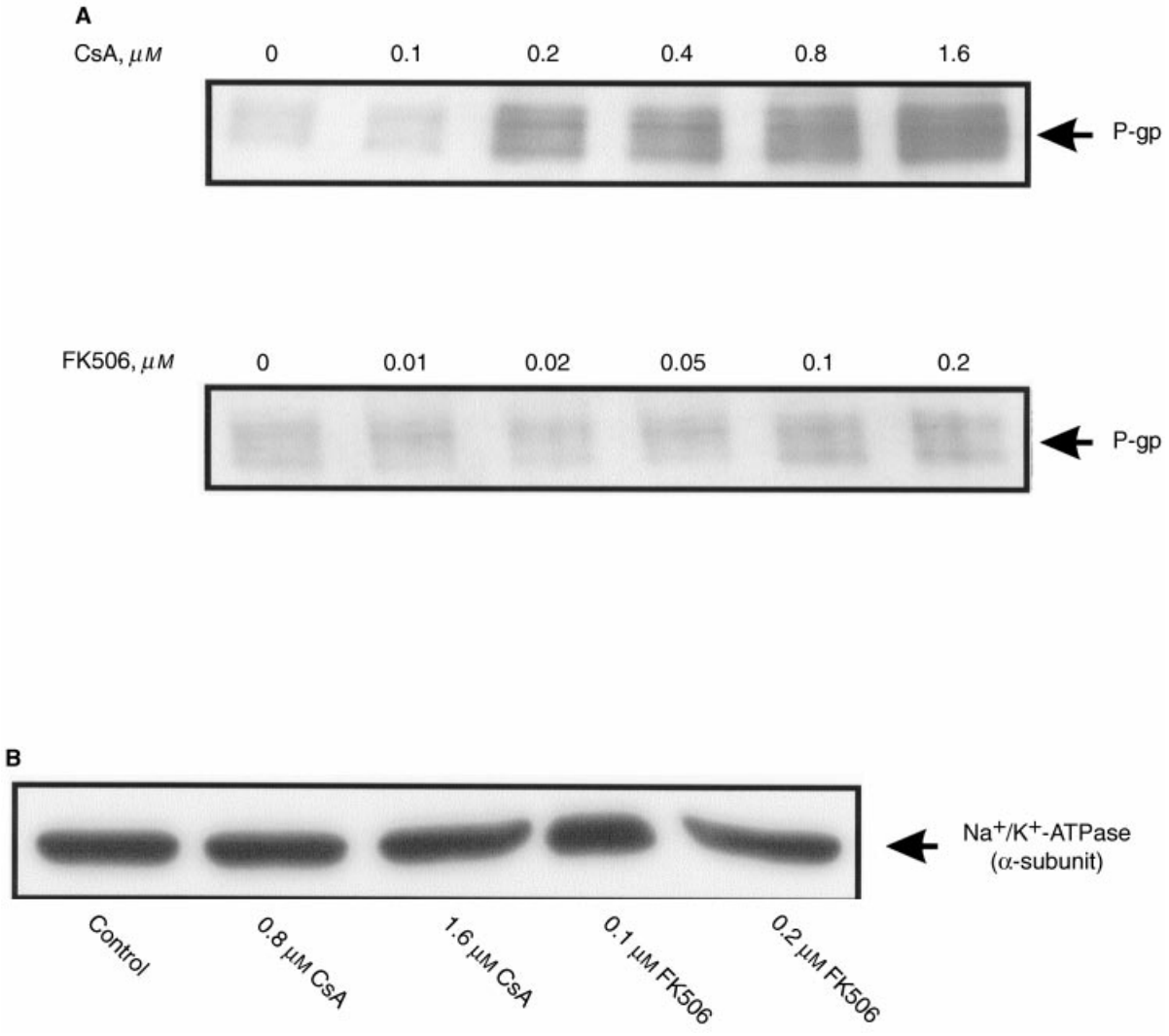

Fig. 5. Expression of P-gp (*A*) and Na^+/K^+-ATPase (*B*) in lysates from rat proximal tubule cells (RPTC) treated for seven days with different concentrations of cyclosporine A (CsA) or FK506. (A) RPTC were exposed for seven days to either 0.1% (vol/vol) DMSO in controls (lane 1) and 0.1 to 1.6 μM CsA (upper immunoblot) or 0.01 to 0.2 μM FK506 (lower immunoblot) dissolved in 0.1% (vol/vol) DMSO. The immunoblot was probed with mAb C219 (5 μg/ml). One out of five similar experiments is shown. (B) The immunoblot was probed with mAb Abα5 (0.5 μg/ml) directed against the α-subunit of Na^+/K^+-ATPase. RPTC were pretreated for seven days with 0.1% (vol/vol) DMSO in controls (lane 1), 0.8 or 1.6 μM CsA, and 0.1 or 0.2 μM FK506. One out of three similar experiments is shown.

should therefore protect cells to some degree from the noxious effects of the immunosuppressive drugs.

To evaluate modulation of P-gp levels by both drugs, immortalized rat proximal tubule cells (RPTC) and human arterial endothelial cells (HAEC) were incubated for prolonged periods (up to 7 days) with FK506 and CsA at therapeutic concentrations. Therapeutic concentrations for CsA and FK506 are well established. FK506 is *in vivo* at least an order of magnitude more potent than CsA in terms of immunosuppression [9–11]. Maximal plasma concentrations of FK506 rarely exceed 0.1 μM [35], whereas for CsA plasma peak values of 1 μM may be reached in renal transplant recipients [36]. The results of this study indicate that therapeutically comparable concentrations of the two drugs differentially affect expression of P-gp, that is, of a major transporter responsible for cellular extrusion of the drugs. Treatment with 0.4 to 0.8 μM CsA for several days increased P-gp expression and transport function two- to threefold in endothelial as well as proximal tubule cells, while treatment with 0.05 to 0.1 μM FK506 had no effect in this respect (see Figs. 1 and 5 for expression, Figs. 4 and 6 and Table 1 for transport). At concentrations exceeding 0.5 μM FK506 P-gp expression was also increased in HAEC and RPTC (Fig. 1B), but concentrations above 0.5 μM are not in the therapeutic range of FK506.

In agreement with our results, Garcia del Moral et al [37] have previously demonstrated overexpression of P-gp in kidney biopsies of renal transplant recipients treated with CsA, and also showed that chronic administration of CsA to MDCK cells (2 μM for >60 days) increased P-gp

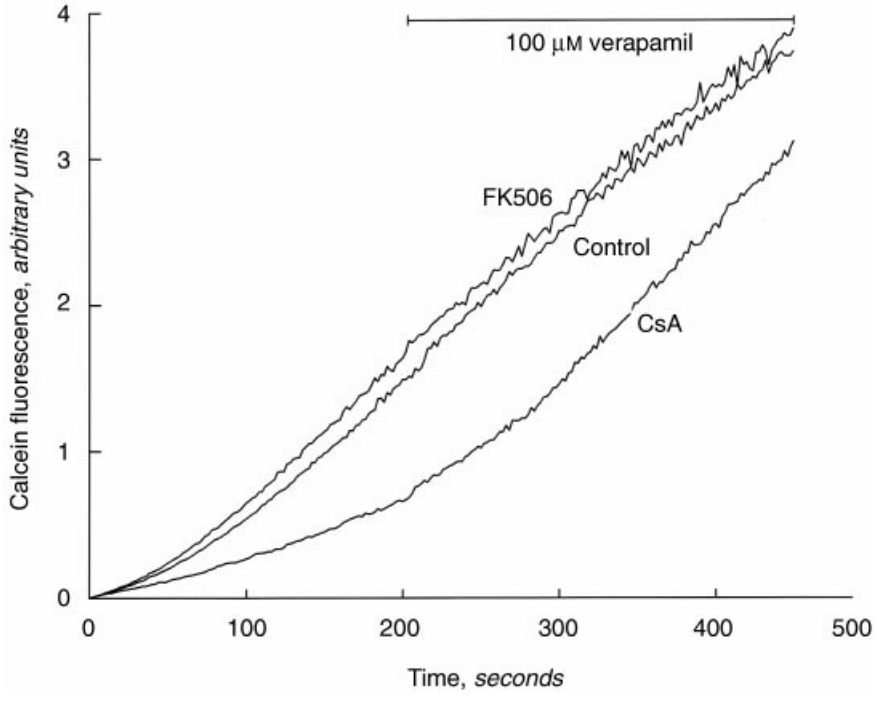

Fig. 6. Fluorimetric time course of calcein accumulation in suspensions of rat proximal tubule cells (RPTC) incubated in the absence and presence of verapamil. Five $\times$ 10^5 cells/ml Tyrode solution were incubated in the presence of 0.25 μM calcein-AM at 37°C. The plot shows calcein fluorescence (arbitrary units) against time. The inhibitor of P-gp verapamil (100 μM) was added where indicated. The different curves represent experiments with cells which had been exposed for seven days to either 0.1% (vol/vol) DMSO (control), 0.8 μM cyclosporine A (CsA), or 0.1 μM FK506 (FK506).

expression *in vitro*. Other studies have also shown an increased expression of P-gp after exposure to supratherapeutic concentrations of CsA, both in cultured cells [19] and in experimental animals [20]. Induction of P-gp by CsA treatment also occurs in typical target cells of the immunosuppressive effect of the drug, that is, in mononuclear cells, which are of critical importance for the development of rejection processes after organ transplantation. Kemnitz et al described an increased incidence of rejection in heart transplant recipients, who showed an induction of P-gp in mononuclear cells of the peripheral blood after transplantation under an immunosuppressive therapy with CsA, azathioprine and dexamethasone [38]. Similar observations have been made by Zanker et al, who correlated the development of acute or chronic rejection with the percentage of P-gp expressing peripheral blood mononuclear cells in kidney transplant recipients [39, 40]. Since FK506 did not induce P-gp expression in HAEC and RPTC at therapeutic concentrations (Figs. 1, 2 and 5), this lack of induction of P-gp may explain in part the efficacy of FK506 as an anti-rejection agent in CsA-resistant refractory allograft rejection [13].

The mechanisms of T-cell immunosuppression by both CsA and FK506 are relatively well understood [8]. In contrast, the molecular basis of nephrotoxicity has not yet been elucidated. The results of this study have implications that need to be taken into account in the interpretation of dose-response curves of both therapeutic and toxic side effects of CsA and FK506. It is surprising that CsA and FK506 display a comparable incidence of nephrotoxicity [14, 15], despite a ~tenfold difference in the drug concentrations used for immunosuppression. From our results it is tempting to speculate that the cytosolic concentration of CsA or FK506 may be of similar magnitude, since CsA in the concentration range of interest increases the level of a transporter that lowers its cytosolic concentration. This finding would suggest that efficacy for therapeutic as well as toxic side effects of CsA will level off if P-gp is induced. However, one should also be aware that peak CsA plasma concentrations of 0.5 to 1 μM may also partially inhibit P-gp mediated drug transport, as indicated by Bennett et al [41]. On the other hand, comparable cytosolic concentrations of CsA and FK506 could have a major influence on the "specific" immunosuppressive effect of both drugs in target cells *in vivo*. The immunosuppressive activity of FK506 occurs at concentrations 10- to 100-fold lower (on a molar basis) than that of CsA [9–11]. Therefore, cytosolic concentrations of FK506 in target cells would fall within the immunosuppressive range, when compared to CsA, because of the higher immunosuppressive efficacy of the drug and the lack of P-gp induction at the interesting concentration range. However, a variable sensitivity of individual patients to therapeutic and toxic side effects of CsA and FK506 may also depend on baseline P-gp protein levels and sensitivity for induction of additional P-gp by CsA.

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APPENDIX

Abbreviations used in this article are: calcein-AM, calcein acetoxymethyl ester; CsA, cyclosporine A; FK506, tacrolimus; HAEC, human arterial endothelial cells; mAb, monoclonal antibody; MDR1, human multidrug resistance P-glycoprotein; mdr1/mdr3, rodent multidrug resistance P-glycoprotein; P-gp, P-glycoprotein; RPTC, rat proximal tubule cells; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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