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Role of P-glycoprotein in cyclosporine cytotoxicity in the cyclosporine–sirolimus interaction

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Cyclosporine nephrotoxicity remains a major side effect in solid organ transplantation, and can be exacerbated by concomitant administration of sirolimus. Cyclosporine and sirolimus are P-glycoprotein (Pgp) substrates. We hypothesized that the Pgp activity level may affect cyclosporine cytotoxicity by interfering with the ability of Pgp to remove cyclosporine from within tubular cells, and that an interaction between cyclosporine and sirolimus on Pgp function may explain the enhancement of cyclosporine nephrotoxicity by sirolimus. Cyclosporine cytotoxicity was evaluated in primary cultures of normal human renal epithelial cells (HRECs) by cell viability and cytotoxicity assays. Verapamil, quinine, PSC833, and PGP-4008 were used as Pgp inhibitors. Rhodamine-123 (R-123), a fluorescent substrate of Pgp, was used to assess Pgp-mediated transport. Cellular cyclosporine concentration was measured by high-performance liquid chromatography coupled to tandem mass spectrometry. Pgp expression and function were confirmed in HRECs and cyclosporine and sirolimus were shown to be Pgp inhibitors in this model. Verapamil-induced inhibition of Pgp led to a significant increase in cellular concentration of cyclosporine ($P<0.05$). Cyclosporine exerted a concentration-dependent cytotoxic effect on HRECs that was significantly increased by inhibition of Pgp activity. Sirolimus exerted an inhibitory effect on R-123 efflux in HRECs and increased cellular cyclosporine concentrations in a dose-dependent manner. These data demonstrate that Pgp plays a critical role in protecting renal epithelial cells from cyclosporine toxicity. The inhibitory effect of sirolimus on Pgp-mediated efflux and the cellular concentration of cyclosporine could explain the exacerbation of cyclosporine nephrotoxicity observed clinically.

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Cyclosporine A, a member of the calcineurin inhibitor class of drugs, remains a mainstay of immunosuppressive regimens to prevent allograft rejection following solid organ transplantation.[1] In renal transplantation, the major limiting factor for use of cyclosporine is nephrotoxicity, which may reduce the overall benefit of cyclosporine therapy with respect to long-term graft survival.[2] In a prospective study of the natural history of chronic allograft nephropathy, cyclosporine appeared to be the chief cause of late ongoing histologic injury, even in grafts with excellent early histologic findings.[3] Two forms of cyclosporine nephrotoxicity have been described. Acute (or 'functional') nephrotoxicity is mediated by an imbalance of vasoconstrictors and vasodilators within the renal vasculature, leading to vasoconstriction and sustained renal ischemia.[2] Chronic (or 'structural') cyclosporine nephropathy is defined as irreversible renal dysfunction associated with morphological injuries such as tubular atrophy, interstitial fibrosis and arteriolar hyalinosis.[4] Cyclosporine may induce these changes by promoting multiple, inter-related pathophysiological processes.[5] A combination of cyclosporine-induced hemodynamic changes and direct toxic effects of cyclosporine on tubular epithelial cells is thought to play a role in the development of interstitial fibrosis via the release of factors such as transforming growth factor-beta, endothelin-1, plasminogen activator inhibitor-type 1, inducing fibroblast proliferation, and matrix synthesis.[6] Cyclosporine may also activate apoptosis genes and increase apoptosis in tubular and interstitial cells, thereby inducing tubular atrophy.[7]

Another mechanism that may contribute to cyclosporine-related nephrotoxicity, but which has not been completely explored, involves the P-glycoprotein (Pgp). The *ABCB1* (previously multidrug-resistance-1) gene product Pgp is a membrane protein, which functions as an ATP-dependent exporter of intracellular xenobiotics. Its importance was first recognized as a consequence of its role in the development of multidrug resistance in cultured tumor cells against various anticancer agents. In the kidney, Pgp is constitutively expressed on the brush border of the proximal tubular cells and on the distal tubule[8] and it has been suggested that Pgp may be instrumental in cyclosporine nephrotoxicity.

Cyclosporine is a substrate of Pgp,[9] and variations in expression and/or function of Pgp could lead to accumulation of cyclosporine, along with other cytotoxic agents, within the tubular cells. In support of this hypothesis, immunohistological studies by Del Moral *et al.*[10] have shown an inverse relationship between cyclosporine deposits in renal tissue and the level of Pgp expression in proximal tubular cells in animal models, suggesting that the normal Pgp response may be defective in patients susceptible to cyclosporine-related nephrotoxicity, leading to retention of excess amounts of cyclosporine in the cells.[11,10] More recently, Koziolek *et al.*[8] showed that low expression of Pgp in renal parenchymal cells was associated with the occurrence of cyclosporine nephrotoxicity. Moreover, a *ABCB1* polymorphism in kidney allograft donors, which is associated with decreased expression of Pgp in renal tissue, has been shown an independent risk factor for the development of cyclosporine-related nephrotoxicity.[12] Together, these findings suggest that factors that modulate Pgp expression may have an impact on cyclosporine-related nephrotoxicity by causing an accumulation of cyclosporine within the renal cells.

The new immunosuppressive agent sirolimus is also a Pgp substrate,[13] and an interaction between cyclosporine and sirolimus is seen clinically. Although perceived as a non-nephrotoxic drug, administration of sirolimus reduces renal function when given concomitantly with cyclosporine.[14] We hypothesized that Pgp may be the site of this interaction. Experimental studies in salt-depleted rats have demonstrated that sirolimus increases intrarenal cyclosporine concentrations to a greater extent than blood concentration of cyclosporine.[15] In addition, even subtherapeutic doses of cyclosporine and sirolimus exert a synergistic effect on the development of chronic nephrotoxicity in rats[16] and recent studies have shown that administration of sirolimus around the time of renal injury can exacerbate the injury and delay repair, an effect that may be due to a potent antiproliferative effect of sirolimus on renal tubular cells.[17]

We therefore postulated that an interaction between cyclosporine and sirolimus could limit Pgp-mediated drug efflux and increase cyclosporine cytotoxicity by limiting the removal of cyclosporine from tubular cells. The present study was undertaken using human renal epithelial cells (HRECs) in primary culture to investigate firstly, whether impaired Pgp function may contribute to nephrotoxicity by interfering with the ability of Pgp to remove cyclosporine from the tubular cell, thereby increasing cyclosporine intracellular toxicity, and secondly, to assess the effect of sirolimus on Pgp-mediated efflux of cyclosporine.

RESULTS

Expression of Pgp in HRECs

Expression of Pgp by the HRECs was confirmed with Western blot analysis of crude membranes using the anti-Pgp monoclonal antibody C219. Results showed varying levels of expression of Pgp on the crude membrane from different kidneys (Figure 1a).

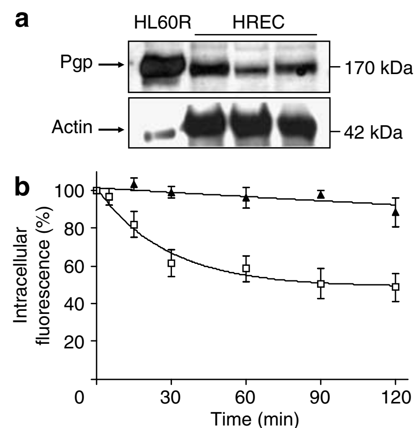

Figure 1 | Expression and function of Pgp in normal HRECs. (**a**) Immunoblot analysis of Pgp in crude membranes from HL60R cells, overexpressing Pgp and used as positive controls (left) and from HRECs derived from three different kidneys using the C219 monoclonal anti-Pgp antibody (10 μg/ml). (**b**) Immunoblotting for actin confirmed equal loading for HREC samples Cellular efflux of R-123. with or without the Pgp inhibitor verapamil. Cells were preloaded for 1 h with 5 μg/ml R-123 and thereafter incubated with R-123-free medium with verapamil 40 μM (triangles) or without verapamil (squares). Intracellular fluorescence was quantified at successive timepoints after the removal of R-123 from the medium. Results are expressed as percentage of the initial intracellular fluorescence, corrected by the total protein content of each sample, before any R-123 efflux. All determinations were performed in triplicate plates. Data shown represent the mean $\pm$ s.e.m. of five independent experiments performed with cells from three different kidneys.

To ensure that the efflux properties of Pgp are not altered by the isolation procedure or culture of HRECs, Pgp activity was evaluated by measuring the effect of Pgp inhibition on cellular efflux of the fluorescent Pgp substrate R123 in the presence or absence of 40 μM verapamil. Figure 1b plots the decrease in intracellular fluorescence in a time-dependent manner. Cellular fluorescence decreased by 40% after an efflux time of 30 min without verapamil, a reduction that was dramatically attenuated in the presence of verapamil. Pgp blockage limited the efflux to $<10\%$ after 120 min.

Effect of cyclosporine on Pgp-mediated efflux

In the absence of cyclosporine, 55% of R123 remained within HRECs after an efflux time of 30 min. Cyclosporine increased cellular retention of R123 in a concentration-dependent manner (Figure 2a). The maximum inhibitory effect was obtained with clinically relevant concentrations of cyclosporine concentrations (>1 μM), above which R123 efflux was almost entirely blocked and R123 intracellular retention was 90%.

In order to analyze the consequences of Pgp inhibition on cyclosporine accumulation, intracellular concentration of cyclosporine was measured after varying durations of cellular efflux, with and without verapamil. Cyclosporine concentration gradually decreased over 60 min (Figure 2b). In the presence of verapamil, this decrease was dramatically

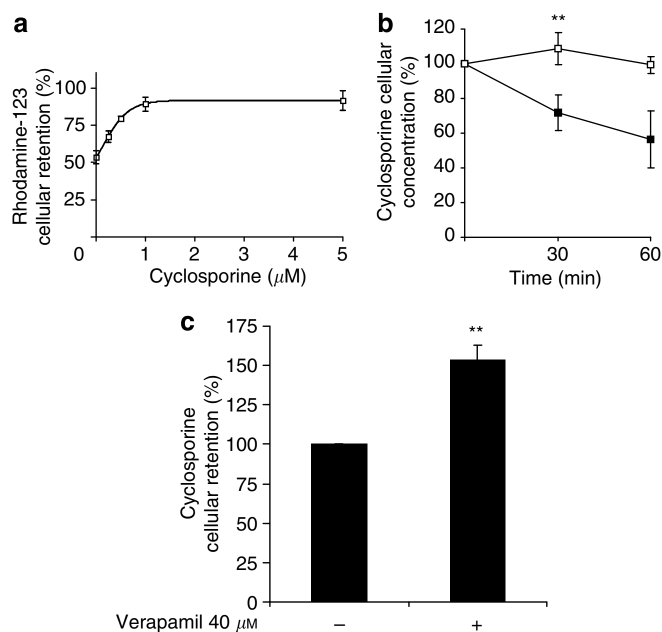

Figure 2 | Cyclosporine transport by Pgp. (**a**) HRECs were preloaded with 5 μg/ml R-123 and incubated with R-123-free medium containing increasing concentrations of cyclosporine (0–5 μM). Intracellular fluorescence was quantified, and corrected by the total protein content of each sample, after an efflux time of 30 min. Results were expressed as percentage of intracellular fluorescence before any efflux. Data shown represent the mean ± s.e.m. of six independent experiments performed with cells from four different kidneys. (**b**) Cells were preloaded for 2 h with 0.5 μM cyclosporine and incubated with cyclosporine-free medium with 40 μM verapamil (white squares) or without verapamil (black squares). Intracellular cyclosporine concentration was measured at successive timepoints after removal of cyclosporine (see Materials and Methods). Data shown represent the mean ± s.e.m. of two independent experiments performed with cells from two different kidneys. (**c**) Cells were preloaded for 2 h with 0.5 μM cyclosporine and incubated with cyclosporine-free medium with or without 40 μM verapamil. Intracellular cyclosporine concentration was measured 30 min after removal of cyclosporine. Data are expressed as a percentage of cyclosporine concentration after 30 min efflux time in the absence of verapamil, and represent the mean ± s.e.m. of four independent experiments performed with cells from three different kidneys. $^{**}P \leqslant 0.01$ (Mann–Whitney test).

curtailed, with a 50% increase in cyclosporine intracellular concentration ($P<0.01$) (Figure 2c).

Effect of Pgp inhibition on cyclosporine cytoxicity

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay showed that cyclosporine reduced viable cell numbers in a dose-dependent manner compared to vehicle-treated cells (Figure 3a). Four unrelated Pgp inhibitors, even though they have no cytotoxic effect on HRECs, significantly decreased the LC_{50} of cyclosporine (i.e. the concentration needed to decrease cell viability by 50%). As shown in Figure 3a, the LC_{50} of cyclosporine decreased from 7.4 ± 0.5 to 3.7 ± 0.5 and 5.9 ± 0.1 μM, when the two highly specific Pgp inhibitors PSC833 and Pgp-4008 were added in the culture medium, respectively ($P<0.01$). Similar results were obtained by measuring lactate dehydrogenase (LDH) release in the medium (Figure 3b).

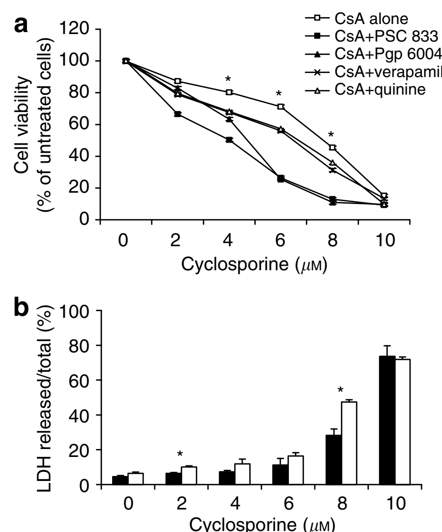

Figure 3 | Impact of Pgp inhibition on cyclosporine-induced cytotoxicity in normal HRECs. (**a**) HRECs, seeded in 96-well plates, were cultured with complete medium containing cyclosporine (CsA, 0–10 μM) in the presence or absence of Pgp inhibitors (40 μM verapamil, 5 μM PSC833, 5 μg/ml Pgp-4008 or 50 μM quinine). After incubation at 37°C for 6 days, the relative number of living cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. The effect of cyclosporine is expressed as percentage cell viability compared to untreated cells. $^{*}P \leqslant 0.05$ (Mann–Whitney test) for all inhibitor-treated cells versus cells treated with cyclosporine alone. (**b**) HRECs, seeded in 96-well plates, were cultured with complete medium containing cyclosporine (0–10 μM) in the presence (black bars) or absence (white bars) of 40 μM verapamil. Cytotoxicity was quantified by LDH release into the culture medium. The effect of cyclosporine is expressed as percentage of LDH released into the culture medium compared to total LDH content. Data represent the mean ± s.e.m. of three independent experiments performed with cells from three different kidneys. $^{*}P \leqslant 0.05$; $^{**}P \leqslant 0.01$ (Mann–Whitney test).

Effect of sirolimus on Pgp-mediated efflux and intracellular cyclosporine retention

In the absence of sirolimus, 55% of R123 was retained within the HRECs after an efflux time of 30 min. The addition of sirolimus resulted in concentration-dependent intracellular retention of R123, with a maximal inhibitory concentration of 1 μM (Figure 4). Sirolimus also induced concentration-dependent intracellular retention of cyclosporine, with a sirolimus dose of 5 μM resulting in 80% increase in cyclosporine concentration within the HRECs after 30 min efflux time (Figure 5).

DISCUSSION

Cyclosporine remains a pivotal immunosuppressive drug in renal transplantation. However, the side effects of cyclosporine include nephrotoxicity, which is one of the major causes of late allograft loss. Although many *in vitro* and *in vivo* studies have explored the pathological changes induced by cyclosporine nephrotoxicity, such as its fibrogenic effects,

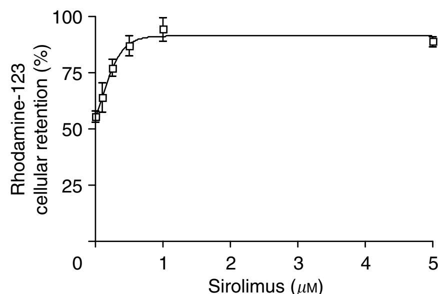

Figure 4 | Effect of sirolimus on Pgp-mediated efflux. HRECs were preloaded with 5 μg/ml R-123 for 1 h and thereafter incubated with R-123-free medium containing increasing concentrations of sirolimus (0–5 μM). Intracellular fluorescence was quantified, and corrected by the total protein content of each sample, after an efflux time of 30 min. Data are expressed as percentage of intracellular fluorescence before any efflux and represent the mean ± s.e.m. of four independent experiments performed with cells from four different kidneys.

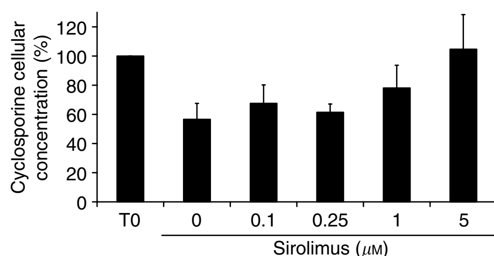

Figure 5 | Effect of sirolimus on intracellular cyclosporine accumulation. HRECs were preloaded with 0.5 μM cyclosporine for 2 h, after which increasing concentrations of sirolimus were added in a cyclosporine-free medium. Intracellular cyclosporine concentration was measured 60 min after removal of cyclosporine from the medium (see Materials and Methods). All determinations were performed in triplicate plates. Data represent the mean ± s.e.m. of four independent experiments performed with cells from three different kidneys.

secretion of vasoactive compounds and the direct tubular cell toxicity caused by apoptosis or necrosis,[5,18] the mechanisms underlying these changes remains undefined. In the current study, we have examined the role of the export protein Pgp on susceptibility to nephrotoxicity in HRECs in order to investigate the location of the cyclosporine–sirolimus interaction within renal cells. Our results demonstrate that Pgp protects human renal tubular cells from the cytotoxic effect of cyclosporine and suggest that Pgp may be the site of the interaction between cyclosporine and sirolimus.

Several studies have previously investigated a causal relationship between renal Pgp expression and function, intrarenal cyclosporine accumulation, and drug-induced nephrotoxicity. Preliminary experiments in human kidney tissue confirmed the presence of Pgp in proximal and distal tubular cells using immunohistochemistry techniques. Moreover, Koziolek *et al.*[8] demonstrated that significantly lower Pgp expression was observed in allograft biopsies from patients with cyclosporine nephrotoxicity compared to patients with biopsy-proven acute tubular necrosis, acute vascular or chronic allograft rejection who did not have cyclosporine-related toxicity.[8] In an animal model, it was shown that intrarenal angiotensin II deposits, peritubular fibrosis, and cyclosporine deposits were inversely related to renal Pgp expression in rats receiving cyclosporine, that is, the lower the expression of Pgp in tubular cells, the greater the histologic signs of severe nephrotoxicity.[10] These data suggest that cyclosporine elimination from tubular cells by Pgp represents a detoxification process.[19] It thus seems clear that low expression of Pgp in renal graft tissue leads to insufficient clearance of cyclosporine from the cells, thereby leading to an accumulation of cyclosporine with consequent nephrotoxic effects. Although these histopathologic data suggest a role for Pgp in detoxification of renal tubular cells, demonstration of a direct link to Pgp was still lacking. To our knowledge, our study is the first to prove that Pgp is a detoxicating factor for cyclosporine in human renal tubular cells, and that blocking Pgp-mediated transport by Pgp inhibitors increased cyclosporine cytotoxicity.

According to this hypothesis, different patterns of Pgp expression may account for variations in susceptibility to cyclosporine nephrotoxicity between individuals. Although variability owing to the cell isolation procedure cannot be excluded, we confirmed the interindividual variability in Pgp expression in HRECs prepared from different subjects by both Western blot (Figure 1) and Northern blot (data not shown). Numerous studies have already demonstrated a high interindividual variability in Pgp expression in the kidney,[20] liver,[21] and intestine.[22] These interindividual variations in Pgp expression may be due to genetic factors;[23] one of these, which may alter Pgp expression, has recently been associated with susceptibility to cyclosporine-related nephrotoxicity.[12]

Other ABC proteins might contribute to the efflux process we studied. ABC-transporters recognized for their ability to modulate drug resistance and toxicity of xenobiotics include multidrug resistance-associated protein (MRP)1, MRP2, and BCRP.[24–26] BCRP is not expressed in the human kidney.[25] In the kidney, MRP1 has been showed to be mainly expressed at the basolateral membranes of distal tubular cells.[27–29] This particular distribution raises questions about its physiological significance in cellular protection from toxic xenobiotics.[27] MRP2 is expressed by the proximal tubular brush border in human kidney and plays an important role in tubular cell detoxication.[29] However, the increased cyclosporine cytotoxicity we observed with the two highly specific Pgp inhibitors Pgp-4008 and PSC833, that inhibit neither MRP1 nor MRP2,[30–32] strongly suggest the pivotal role of Pgp on cyclosporine cellular efflux, and that MRP1 and MRP2 are not involved in this efflux process.

Sirolimus is a potent new immunosuppressive agent that modulates the immune response at different targets to those of cyclosporine. To minimize cyclosporine dose-dependent toxicities, and to augment the therapeutic efficacy of cyclosporine-based immunosuppressive regimens, several clinical studies have evaluated combination therapy with sirolimus. However, a 1-year phase III clinical trial revealed

that sirolimus (2 or 5 mg) with full-dose cyclosporine resulted in higher serum creatinine concentrations and lower creatinine clearance than cyclosporine with azathioprine.[14] Another study gave very similar results,[33] and together the results of these trials led to the European Agency for the Evaluation of Medicinal Products recommendation to withdraw cyclosporine from sirolimus-treated patients at 3 months.

The potentiation of CNI-induced nephrotoxicity by sirolimus has been confirmed in animal models.[16,34,35] Sirolimus does not appear to be nephrotoxic when used alone, but potentiates the nephrotoxic effects of cyclosporine even when cyclosporine is given at low doses. To date, the most frequent explanation for this interaction has been pharmacokinetics, that is, sirolimus increases cyclosporine exposure. This suggestion was supported by the observation that significantly lower cyclosporine doses were required to achieve target concentrations of cyclosporine in sirolimus-treated patients versus those given azathioprine in the pivotal US trial,[14] but in the registration studies blood concentrations of cyclosporine were similar with sirolimus, azathioprine, or placebo.[14,33] A local interaction between sirolimus and cyclosporine within the kidney was firstly suggested by Napoli *et al.*[36] Using a rat model, they demonstrated that a pharmacokinetic interaction between sirolimus and cyclosporine increases the concentrations of both agents in whole blood and, especially, in the renal tissue. More recently, Podder *et al.*[15], reported that combined administration of various doses of cyclosporine and sirolimus in rats increased cyclosporine whole-blood concentrations compared to cyclosporine alone, but that the effect was even greater in kidney tissue. At each dose of cyclosporine, intrarenal cyclosporine concentration increased when animals were given sirolimus concomitantly. This led to speculation that a pharmacokinetic interaction between sirolimus and cyclosporine may alter cyclosporine exposure to a greater extent in the kidney than in the blood, thus explaining the results of the registration trials. In accordance with this hypothesis, in our study, the effects of sirolimus on Pgp-mediated efflux and on cyclosporine intracellular concentration suggest that the increased cyclosporine nephrotoxicity seen clinically in patients receiving sirolimus may be due to inhibition of Pgp-mediated cyclosporine cellular efflux in HRECs by sirolimus. We tried to study the effect of sirolimus on the cytotoxicity induced by cyclosporine and discovered that sirolimus was responsible for an antiproliferative effect by itself on HRECs.[17] This anti-proliferative effect did not allow us to study the impact of sirolimus on the cytotoxicity induced by cyclosporine in this cellular model.

The main limitation of the present study remains the difficulties to extrapolate the results of an *in vitro* study to *in vivo* effects in treated patients. In clinical practice, intrarenal cyclosporine and sirolimus concentrations during conventional immunosuppressive treatment are currently unknown. As cyclosporine and sirolimus are lipophilic compounds, their respective concentrations might be higher in the cellular membrane than in blood. It is then difficult to ensure that *in vitro* concentrations we used have an *in vivo* relevance. In various experiments, HRECs were loaded with a non-toxic cyclosporine concentration of 0.5 μM, already used in other *in vitro* models of cyclosporine toxicity.[37–39] Concerning sirolimus, the concentration ranges were also in accordance with previous studies that have evaluated Pgp inhibition by sirolimus,[13,40] as well as the potentiation of cyclosporine nephrotoxicity by sirolimus *in vivo*.[15]

In conclusion, these results confirm in an *in vitro* model of HRECs, that Pgp plays a critical role in protecting renal epithelial cells from cyclosporine toxicity. Moreover sirolimus inhibits Pgp-mediated efflux and leads to cyclosporine intracellular accumulation in HRECs. These results suggest that Pgp could play a role in the *in situ* interaction between cyclosporine and sirolimus, that would lead to the potentiation of cyclosporine nephrotoxicity by sirolimus seen clinically.

MATERIALS AND METHODS

Reagents and antibodies

Sirolimus was donated by Wyeth Research (Monmouth Junction, NJ, USA). Cyclosporine and PSC833 were donated by Novartis Pharma AG (Basel, Switzerland). All other chemicals were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). Cyclosporine, sirolimus, PGP-4008, and PSC833 were diluted in ethanol and verapamil in water. The cell culture medium and the other cell culture products were obtained from Life Technologies (Cergy Pontoise, France). C219 monoclonal antibody was purchased from DAKO (Trappes, France). Anti-actin antibody was obtained from Sigma (Saint Quentin Fallavier, France).

Cell culture

Normal HRECs were harvested from human nephrectomy specimens removed for renal cell carcinoma, and isolated according to previously published methods, with minor modifications.[14,15,41,42] Fragments of non-malignant renal cortex were minced and digested with collagenase IV (250 IU/ml) for 3 h at 37°C. Cells were centrifuged and the pellets washed three times with phosphate-buffered saline (PBS). Cells were then cultured in Dulbecco's modified Eagle's medium containing 5 μg/ml insulin, 10 μg/ml human apotransferrin, 500 ng/ml hydrocortisone, 10 ng/ml epidermal growth factor, 6.5 ng/ml triiodothyronin, 5 ng/ml sodium selenite, 1% fetal calf serum, 25 IU/ml penicillin, 25 μg/ml streptomycin and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer. Cells were incubated at 37°C in 5% CO_2 and 95% air. The characterization of our cellular model has been published previously,[17] confirming the proximal descent of the vast majority of the cultured tubular epithelial cells. Experiments were not performed with cells beyond the third passage as it has been shown that no phenotypic changes occur up to this passage number.[41]

Isolation of tissue crude membranes fraction and Western blot

Crude membrane fractions were prepared from untreated HRECs. The multidrug-resistant, Pgp-overexpressing, variant HL60R of the acute myeloid leukaemia cell line HL60[43] was used as positive control. Freshly confluent cells (2×10^6 cells) were washed with PBS and lysed with ice-cold hypotonic lysing buffer (Saccharose 0.25 mM,

Tris 10 mM, ethylendiaminetetraacetic acid 1 mM, pH 7.4) containing protease inhibitors (Complete, Roche Diagnostics, Manheim, Germany). After sonication for 20 s, homogenate was centrifugated at 2000 *g* for 10 min. The supernatant was then centrifugated at 30 000 *g* for 30 min and the pellets containing the crude membranes were resuspended in NaH_2PO_4 100 mM, $MgCl_2$ 10 mM, glycerol 20%, pH 7.4 and stored at $-80^{\circ}C$ until immunoblot analysis.

Proteins (40 μg for HRECs or 10 μg for HL60R cells) were separated on sodiumdodecylsulfate-polyacrylamide gel electrophoresis gel. Pgp was detected by Western blot analysis according to standard protocols or manufacturer's instructions using C219 monoclonal antibody (10 μg/ml). A horseradish peroxidase-conjugated anti-mouse IgG was used as secondary antibody and Pgp was revealed with enhanced chemiluminescence reagent (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's instructions.

Determination of Rhodamine-123 efflux

Rhodamine-123 (R-123) is a good fluorescent substrate for Pgp.[44] Cellular efflux of R123 was used to evaluate the activity of Pgp using an adaptation of the method proposed by Chieli *et al.*[45] Confluent cells cultured in 60 mm dishes were loaded for 1 h with 5 μg/ml R123. The medium was then replaced by R123-free medium with or without the Pgp inhibitors under investigation. For determination of Pgp activity, R123 cellular concentration was quantified at 0, 30, 60, and 120 min after removal of R123 from the medium. For evaluation of the inhibitory effect of sirolimus or cyclosporine on Pgp function, the R123-containing medium was replaced by medium containing cyclosporine or sirolimus and quantification of the intracellular concentration of R123 was performed 30 min after its removal. Cells were then washed three times with PBS, lysed with 150 μl ice-cold water and harvested with a rubber policeman. Fluorescence intensity was measured with a spectrofluorimeter (excitation 518 nm, emission 532 nm) and compared to a calibration curve of pure R123. The amount of R123 obtained from cells was corrected on the basis of protein content assessed by the Bicinchroninic acid method (Pierce Chemicals, Rockford, IL, USA).

Cyclosporine cytotoxicity

HRECs were seeded in 96-well plates 103 cells/well. Twenty-four hours later, cells were treated with cyclosporine or vehicle with or without Pgp inhibitors (40 μM verapamil, 5 μM PSC 833, 5 μg/ml Pgp-4008, and 50 μM quinine).[46,30–32] Six days later, the relative number of living cells per well was determined on the basis of mitochondrial integrity by assay with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (Promega, Charbonnieres, France), according to the manufacturer's instructions. Cell death was determined by measuring the amount of LDH released into the medium after treatment with cyclosporine. LDH activity was determined by the CytoTox 96 non-radioactive cytotoxicity assay (Promega, Charbonnieres, France) and quantified by measuring absorbance at 490 nm.

Measurements of intracellular cyclosporine concentration

Freshly confluent cells cultured in 60 mm dishes were loaded with 0.5 μM cyclosporine for 2 h. The medium was then replaced by cyclosporine-free medium containing various concentrations of Pgp inhibitors. Two hours later, cells were washed once with cold PBS and harvested with a rubber policeman using 80 μl ice-cold PBS. Intracellular cyclosporine was then measured by high-performance liquid chromatography coupled with tandem mass spectrometry in the positive ion, selected reaction monitoring mode, following the transitions 1220/1203 and 1220/425 for cyclosporine, 1234/1217 and 1234/1199 for cyclosporine D (internal standard). The cell lysates were diluted 1/5 with blank human serum, then proteins were precipitated with acetone. Quantification was performed against a calibration curve of cyclosporine in human serum. The limit of quantitation was 10 ng/ml and the method was linear from this limit of quantitation up to 2000 ng/ml ($r^2 = 0.9981$). Seven commercial quality controls in whole blood at four levels between 100 and 1725 ng/ml were prepared as cell lysates, by 1/5 dilution in blank serum, and analyzed in the same series as the cell samples. The results showed that the method accuracy was very good, with bias values between -8.6 and $+8.7\%$.

Cyclosporine–sirolimus interaction

The cyclosporine–sirolimus interaction was assessed by measuring cyclosporine cellular retention induced by sirolimus. Briefly, cells loaded with 0.5 μM cyclosporine for 2 h were incubated with increasing sirolimus concentrations (0–5 μM) for 1 h. Cells were then washed and harvested with a rubber policeman and intracellular cyclosporine concentrations were quantified as described above. Results were expressed as percentage of the initial intracellular cyclosporine concentration before any cyclosporine efflux.

Statistical analysis

All data were expressed as the mean $\pm$ s.e.m. of $\geqslant 3$ separate experiments in which triplicates were obtained, unless otherwise specified. Statistical significance was tested by non-parametric tests (Mann–Whitney *U*-test for unpaired comparisons and Wilcoxon's test for paired comparisons). Statistical analyses were performed using Statview 5.0.1® software. *P*-values <0.05 were considered significant.

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