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ORIGINAL ARTICLE

Sirolimus and everolimus intestinal absorption and interaction with calcineurin inhibitors: a differential effect between cyclosporine and tacrolimus

Fabien Lamoureux[a,b], Nicolas Picard[a,b,c,*], Belkacem Boussera[a,b], François-Ludovic Sauvage[a,b,c] and Pierre Marquet[a,b,c]

[a]INSERM UMR-S850, 2 rue du Dr marcland, 87025 Limoges, France
[b]Faculty of Medicine, University of Limoges, 2 rue du Dr marcland, 87025 Limoges, France
[c]C.H.U. of Limoges, Department of Pharmacology – Toxicology, 2 avenue Martin Luther-King, 87042 Limoges, France

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*Correspondence and reprints:
nicolas.picard@unilim.fr

ABSTRACT

The mTOR inhibitors (ImTORs) sirolimus (SRL) and everolimus (EVR) have been increasingly used in renal transplantation as part of calcineurin inhibitor (CNI) sparing or avoidance regimens. Those drugs have low and variable oral bioavailability that is increased when combined with cyclosporine or tacrolimus (TAC). We investigated the mechanisms involved in ImTORs intestinal absorption in vitro and associated it with their drug–drug interactions with CNIs. The transport of ImTORs across Caco-2 cells was studied in the apical (A) to basolateral (B) and B to A directions, in the absence or presence of cyclosporine, TAC, and GF120918 (P-gp inhibitor). In Caco-2 cells, EVR and SRL displayed a polarized transport with 8.7- and 5.9-fold higher $P_{app,B\rightarrow A}$ than $P_{app,A\rightarrow B}$, respectively. P-gp inhibition by GF120918 resulted in a 70 and 41% decrease in EVR and SRL efflux, respectively. Cyclosporine and TAC led to a comparable and significant decrease in the efflux ratio of ImTORs, suggesting inhibition of a P-gp-mediated efflux transport. Cyclosporine also exhibited a specific increase of $P_{app,B\rightarrow A}$, which may be attributed to the inhibition of other transporters and/or metabolizing enzymes. In conclusion, EVR and SRL are both subject to an apically directed efflux mediated by P-gp. TAC mainly inhibits this efflux mechanism, while the effect of cyclosporine appears to be more complex with mechanisms to be confirmed by further studies.

INTRODUCTION

Sirolimus (SRL), formerly known as rapamycin, and everolimus [EVR; 40-O-(2-hydroxyethyl)-rapamycin] are mTOR inhibitors (ImTORs) commonly used to prevent allograft rejection after solid organ transplantation. ImTORs are being used increasingly in combination with low doses of calcineurin inhibitors (CNIs), cyclosporine A (CsA), or tacrolimus (TAC).

mTOR inhibitor s as well as CNIs present a low average oral bioavailability (approximately 25%) with wide interindividual variations (range: 4–89%) [1,2]. The causes of this remain unclear but are presumably linked with the variable activity of metabolic enzymes [cytochrome P450 (CYP) 3A] and of active efflux transporters such as P-glycoprotein (P-gp) or members of the multidrug resistance-associated proteins (MRPs) family (especially MRP1 and MRP2) in the small intestine [3]. CsA, TAC, SRL, and EVR are indeed extensively metabolized by CYP 3A4 and, to a lesser extent, by CYP3A5 [4–6]. These drugs are also known substrates of P-gp [7,8]. In addition, we recently showed that ImTORS are not transported by uptake transporters of the organic anion-transporting polypeptides (OATPs) family [9].

Although structurally similar, EVR and SRL undergo different metabolism. In contrast to SRL, the presence of

Table I Concentration-dependent effect of cyclosporine (a), tacrolimus (b) and GF120918 (c) on the transport of digoxin (1 μM), a P-gp probe, across Caco-2 cell monolayers.

(a)

CsA (μM)	$P_{app,A\rightarrow B}$ (10^{-6} cm/s)	$P_{app,B\rightarrow A}$ (10^{-6} cm/s)	Efflux ratio $P_{app,B\rightarrow A}/P_{app,A\rightarrow B}$
Control	1.02 ± 0.28	21.25 ± 2.21	20.79
1	0.84 ± 0.14	17.20 ± 1.82	20.43
10	3.10 ± 0.23	3.49 ± 0.10	1.13**
50	2.41 ± 0.60	3.73 ± 0.32	1.55**

(b)

TAC (μM)	$P_{app,A\rightarrow B}$ (10^{-6} cm/s)	$P_{app,B\rightarrow A}$ (10^{-6} cm/s)	Efflux ratio $P_{app,B\rightarrow A}/P_{app,A\rightarrow B}$
Control	0.98 ± 0.25	20.76 ± 2.32	21.18
1	0.57 ± 0.14	17.92 ± 1.52	31.37*
10	3.40 ± 0.72	3.64 ± 0.36	1.07*
50	2.53 ± 0.59	3.31 ± 0.43	1.31*

(c)

GF120918 (μM)	$P_{app,A\rightarrow B}$ (10^{-6} cm/s)	$P_{app,B\rightarrow A}$ (10^{-6} cm/s)	Efflux ratio $P_{app,B\rightarrow A}/P_{app,A\rightarrow B}$
Control	1.02 ± 0.28	21.25 ± 2.21	20.79
2	2.77 ± 0.64	6.22 ± 1.04	2.25*

Data are expressed as mean ± standard deviation and are representative of three independent triplicate experiments.

$P_{app,A\rightarrow B}$ and $P_{app,B\rightarrow A}$, apparent permeability in the apical-to-basal and basal-to-apical directions, respectively, calculated according to the equation described in the 2.8 section.

*$P \leq 0.05$ and **$P \leq 0.01$ (measured efflux ratio vs. control).

a 40-O-2-hydroxyethyl group on EVR prevents its 39-O-demethylation and also decreases two major hydroxylation pathways, which results in an overall decrease in metabolism as shown using human liver microsomes [10]. However, Crowe et al. suggested on the basis of experimental studies in rats and Caco-2 cells that EVR is metabolized by the intestine to a greater extent than SRL, which is balanced in terms of bioavailability by a higher intrinsic permeability [11,12].

Concurrent administration of CsA significantly increases SRL area under curve (area under the plasma drug concentration vs. time curve), C_{max}, and t_{max} [13], while TAC seems to have a lower effect [14,15]. Similar effects of CNIs on the pharmacokinetics of EVR have recently been described [16]. The mechanisms of these drug–drug interactions at the intestinal level are still not fully understood but are likely to be linked to the inhibitory effect of CNIs on ImTORs intestinal and/or hepatic metabolism and transport. CsA and to a lesser extent TAC are inhibitors of CYP3A4 and P-gp [17,18].

The human colon adenocarcinoma cell line Caco-2 is widely used as a model to study drug transport in intestinal epithelium. When fully differentiated, polarized Caco-2 cells exhibit morphological and functional similarities with human intestinal enterocytes, expressing efflux transporters (e.g., P-gp, MRP1, and MRP2) [19] as well as metabolic enzymes at significant levels [20]. A predictive relationship between the permeability of Caco-2 monolayers and human in vivo intestinal absorption has been reported by several authors [21,22].

The present study aimed to compare the transepithelial passage of ImTORs across Caco-2 cells and their interaction with CNIs at this level.

MATERIALS AND METHODS

Materials and chemicals

The human colon adenocarcinoma cell line Caco-2 was obtained from the American Type Culture Collection (Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), glutamine, nonessential amino acids (NEAA), penicillin–streptomycin (10 000 units/mL and 10 mg/mL in 0.9% sodium chloride, respectively), 0.05% trypsin – 0.53 mM EDTA-4Na, Hank's balanced salt solution (HBSS), HEPES solution, and Dulbecco's phosphate-buffered saline (PBS) were purchased from GibcoBrl Life Technology (Cergy-Pontoise, France). Matrigel was purchased from BD biosciences Discovery Labware (Le Pont de Claix, France). EVR and CsA were kindly provided by Novartis Pharma AG (Basel, Switzerland), SRL by Wyeth-Lederlé (Paris, France), and TAC by Astellas Pharma (Levallois-Perret, France). The MDR1 chemical inhibitor GF120918 (9,10-dihydro-5-methoxy-9-oxo-N-[4-[2(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-4-acridinecarboxamide hydrochloride salt) was kindly donated by GlaxoSmithKline (Marly-le-Roi, France). Digoxin and atenolol were obtained from Sigma (Saint Quentin Fallavier, France). Organic solvents and chemicals used for drug analysis were of analytical grade.

Caco-2 cells culture conditions

Caco-2 cells were routinely cultured at a density of 7×10^6 cells on 75-cm^2 plastic culture flasks (BD Biosciences Discovery Labware), containing DMEM, 10% inactivated FCS, 2 mM glutamine, 1% NEAA, 100 units/

mL of penicillin, and 100 μg/mL of streptomycin. All the cells used in this study were passaged 45–60 times.

For the transepithelial transport study, Caco-2 cells were seeded on the apical side of matrigel-treated filter inserts (12 mm diameter and 0.4-μm pore size, Millipore, Molsheim, France) at a density of 6×10^5 cells per filter. The cells were grown until differentiation for at least 21 days and used for experiments between 21 and 28 days postseeding. All transport studies were conducted in transport buffer (HBSS supplemented with 10 mM HEPES; pH 7.4) in both apical-to-basolateral (A to B) and basolateral-to-apical (B to A) directions under iso-pH conditions (pH 7.4 on both sides).

Cell toxicity and viability assessment

The viability of CNI- or ImTOR-treated cells was assessed by the trypan blue dye exclusion test and the MTT assay. Cytotoxicity experiments were conducted by applying 1–100 μM CNI and 1–10 μM ImTOR for 2 h at 37 °C on differentiated Caco-2 cells. Cells were then incubated for 4 h with 5 mg/mL MTT (Sigma) in PBS and lysed in 200 μL SDS 10%/HCl 0.01 N for 4 h. Aliquots of the lysates were transferred in 96-well plates, and absorbance was recorded at 550 nm using a Multiskan EX (Labsystems, Milford, MA, USA) microplate spectrophotometer system.

Trypan blue exclusion was also used to qualitatively assess cell viability. After exposure to increasing concentrations of CNIs (1, 10, 50, or 100 μM) or ImTORs (1, 5, or 10 μM), Caco-2 monolayers were washed with PBS, detached from the support with trypsin – EDTA, incubated with 0.4% trypan blue solution for 1 min, and counted in a hemocytometer using a light microscope.

Exposure to CNIs or ImTORs for 2 h did not decrease mitochondrial activity or viability of Caco-2 cells in comparison with controls, as assessed during these assays.

Assessment of cell monolayer integrity

The integrity of cell monolayers was evaluated by two different methods. The transepithelial electrical resistance (TEER) was measured using a Millicell-ERS equipment (Millipore) as described previously [23]. Only monolayers with TEER > 600 Ω/cm^2 were used for further experiments. Additionally, TEER was measured after each permeability experiment to confirm monolayer integrity. Exposure of the cell monolayers to ImTORs did not induce a significant decrease in TEER before and after transport studies (n = 120, 688 ± 35 Ω cm^2 at day 28 postseeding vs. 600 ± 61 cm^2 just after transport experiments).

The integrity of the cell monolayers was also studied by determining the paracellular permeability (apparent permeability coefficient, P_{app}) of atenolol, as previously recommended [24,25]. The permeability of atenolol through untreated cells was the same for both transport directions ($P_{app,A\rightarrow B}$: 1.44 ± 0.09 × 10^{-6} cm/s, $P_{app,B\rightarrow A}$: 1.38 ± 0.11 × 10^{-6} cm/s), consistent with the literature values [21,26] and unaffected after incubation of the monolayers with the immunosuppressive agents tested in comparison with the control.

Transport studies

Transport of ImTORs accross Caco-2 monolayers

Fresh medium containing EVR or SRL at various concentrations (1, 5, and 10 μM, corresponding to levels expected after oral dosing) was added to either the apical (for absorption studies: A → B direction) or basal (for secretory studies: B → A direction) side. An equal volume of incubation medium without drugs was systematically added to the opposite side of the monolayers. Monolayers were then incubated for 2 h at 37 °C in a humidified 5% CO^2 atmosphere, in three independent and triplicate experiments. Incubation time was set at 2 h according to ImTORs pharmacokinetic data: the t_{max} reported are 1–1.75 h for EVR [16,27] and 1.3 + / 0.5 h for SRL [28], and concomitant administration of CNIs with mTORs tends to delay the t_{max} [13,16,29]. Samples were taken from the apical and basolateral compartments at the end of the 2-h period and kept at −22 °C prior to liquid chromatography - Tandem Mass Spectrometry (LC-MS/MS) analysis.

Infuence of CsA, TAC, and GF120918 on the transport of ImTORs

The transport of ImTORs (EVR and SRL at 1, 5, and 10 μM) was also studied in both the A → B and B → A directions, in the absence or presence of CsA (10 μM), TAC (10 μM), and GF120918 (2 μM; a third-generation P-gp inhibitor with no effect on CYP3A) for 2 h at 37 °C, in triplicates. Samples from the A and B compartments were then taken and kept at −20 °C until analysis.

As a positive inhibition control, the effect of CsA, TAC (1-10-50 μM), and GF120918 (2 μM) on the efflux of the P-gp substrate digoxin (1 μM) [30] across Caco-2 cell monolayers was investigated using similar experimental conditions.

LC-MS/MS analysis

The concentrations of EVR and SRL in compartments A and B were determined using turbulent flow chromatography–tandem mass spectrometry (TFC-MS/MS). The LC system used for all analyses was a high turbulence

liquid chromatography 2300 turbulent-flow chromatography system (Cohesive Technologies, Milton Keynes, UK) equipped with a CTC HTC Pal autosampler (CTC Analytics AG, Zwingen, Switerland) kept at 4 °C, two binary high-pressure Agilent 1100 pumps (Agilent Technologies, Palo Alto, CA, USA), and three-six-port switching valves controlled by the Aria OS software package (Cohesive Technologies, Franklin, MA, USA).

Specific and sensitive detection and quantitation were performed using a triple stage quadrupole mass spectrometer Quantum Discovery tandem mass spectrometry system (Thermo-Fischer Scientific, Les Ulis, France) equipped with an orthogonal electrospray ionization source and controlled by the Xcalibur computer program. The drugs were monitored in the positive ion, selected reaction monitoring mode, following two transitions per compound. Quantitation limits were 10 μg/L for all drugs (10.4, 10.8, 8, 12, and 13 nM/L for EVR, SRL, CsA, TAC, and digoxin, respectively). Excellent calibration curves were obtained using quadratic regression from the limit of quantitation up to 2000 μg/L (2.09, 2.19, 1.66, 2.49, and 2.56 μM/L for EVR, SRL, CsA, TAC, and digoxin, respectively).

Calculation of transport data

The flux (J) was calculated using the following equation:

$$JX = \mathrm{d}Q/\mathrm{d}t$$

where Q (pM) is the amount of drug transported within a given time period dt (s).

X denotes transport direction, either absorptive (A to B, A → B) or secretory (B to A, B → A).

Permeability was estimated by calculating the apparent permeability coefficient ($P_{app} \times 10^{-6}$ cm/s) across Caco-2 monolayers in both the A → B$\left(P_{app,A\to B}\right)$ and B → A$\left(P_{app,B\to A}\right)$ directions, according to the following equation:

$$P_{app} = J/(A * C0)$$

where A is the surface area of the monolayer exposed to the compound (0.6-cm^2) and C0 (ng/ml) the initial concentration of test compound in the donor compartment.

The polarization (Efflux) ratio (ER) was defined as:

$$\text{Efflux ratio (ER)} = P_{app,B\to A}/P_{app,A\to B}$$

where $P_{app,B\to A}$ and $P_{app,A\to B}$ represent the apparent permeability coefficient in the B to A and A to B directions, respectively.

Statistical analysis

Results are presented as mean values ± SD of triplicate A → B or B → A experiments. Comparisons of Papp and ER across experiments were performed using the non-parametric one-tailed Mann–Whitney test with Statview® (SAS Institute, Cary, NC, USA), with a level of significance set at 0.05.

RESULTS

Transepithelial transport of mTOR inhibitors across Caco-2 cell monolayers

As shown in *Figure 1a,b*, respectively, EVR and SRL displayed a polarized transport. At all the concentrations tested (1, 5, and 10 μM), their $P_{app,B\to A}$ was significantly higher than their $P_{app,A\to B}$ ($P = 0.003$ for both EVR 1 μM and SRL 1 μM; $P = 0.05$ for EVR and SRL 5 μM and 10 μM), suggesting an apically directed efflux of ImTORs.

The $P_{app,A\to B}$ of EVR and SRL increased up to $1.59 \pm 0.45 \times 10^{-6}$ cm/s and $2.84 \pm 1.40 \times 10^{-6}$ cm/s at the highest concentration (10 μM), respectively, while their $P_{app,B\to A}$ also varied over the concentration range, with a trend to decrease when ImTOR concentrations increased (*Figure 1a,b*). These variations resulted in a steadily decreasing efflux ratio (ER) over the concentration range examined ($P = 0.012$), from 9.2 to 2.3 and from 7.15 to 2 for EVR and SRL, respectively, suggesting a concentration-dependent and saturable transepithelial transport of ImTORs across Caco-2 monolayers. As a result of these observations, all the subsequent transport studies were performed at a non-saturating level of 1 μM for EVR and SRL.

At this concentration, we observe no difference in transepithelial transport between EVR and SRL, but a trend toward a higher ER for EVR ($P = 0.139$; *Table II*).

GF120918, a known chemical P-gp inhibitor, was used to evaluate the P-gp-mediated efflux of EVR and SRL in Caco-2 cells, while digoxin was employed as a P-gp substrate (positive control for inhibition). In the presence of 2 μM GF120918, the mean ER of digoxin decreased from 20.79 to 2.25 ($P = 0.05$) as the result of both a decreased $P_{app,B\to A}$ and an increased $P_{app,A\to B}$ (*Table Ic*); the $P_{app,B\to A}$ of EVR and SRL (1 μM) was also significantly decreased ($P = 0.012$), but their $P_{app,A\to B}$ was not significantly modified (*Table II*), resulting nevertheless in a 70% decrease in EVR ER ($P = 0.012$) and a 41% decrease in SRL ER ($P = 0.13$) (*Figure 2a,b*).

Effect of Cyclosporine A and Tacrolimus on the transepithelial transport of ImTORs

To confirm the inhibitory effect of CsA and TAC on P-gp, incubations were first performed with the P-gp substrate digoxin. Addition of CsA or TAC resulted in a significant

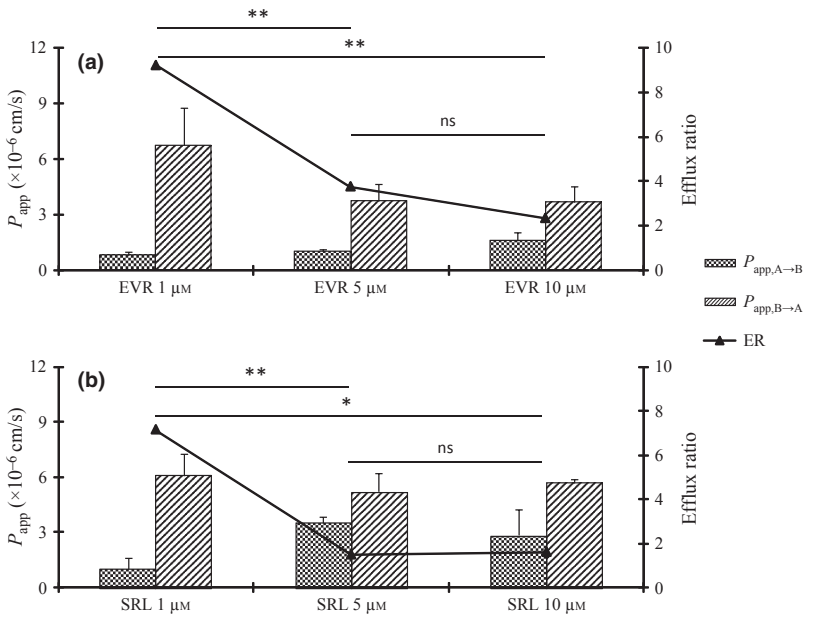

Figure 1 Everolimus (a) and sirolimus (b) $P_{app,A\rightarrow B}$, $P_{app,B\rightarrow A}$ and efflux ratio across Caco-2 cell monolayers over a 1–10 μM concentration range (data shown as mean ± SD; results are representative of three independent triplicate experiments). Efflux ratios were compared using the nonparametric Mann–Whitney test (*$P \leq 0.05$ and **$P \leq 0.01$).

Table II Effects of GF120918 (a P-gp inhibitor) and calcineurin inhibitors on the transepithelial transport of everolimus 1 μM (a) and sirolimus 1 μM (b) across Caco-2 cell monolayers. Data are expressed as mean ± SD and are representative of three independent triplicate experiments.

	$P_{app,A\rightarrow B}$ (10^{-6} cm/s)	$P_{app,B\rightarrow A}$ (10^{-6} cm/s)	Efflux ratio $P_{app,B\rightarrow A}/P_{app,A\rightarrow B}$
(a)			
EVR 1 μM (control)	0.78 ± 0.20	6.76 ± 1.98	9.23 ± 3.20
+GF120918 2 μM	0.99 ± 0.04	2.74 ± 0.36	2.77 ± 0.47**
+Cyclosporine A 10 μM	12.94 ± 1.53	15.66 ± 0.74	1.22 ± 0.09**
+Tacrolimus 10 μM	2.48 ± 0.19	3.11 ± 0.28	1.26 ± 0.07*
(b)			
SRL 1 μM (control)	1.03 ± 0.58	6.11 ± 1.20	7.15 ± 3.05
+GF120918 2 μM	0.84 ± 0.19	3.27 ± 0.56	4.16 ± 1.81
+Cyclosporine A 10 μM	9.89 ± 2.58	10.24 ± 1.71	1.09 ± 0.32**
+Tacrolimus 10 μM	2.76 ± 0.21	2.85 ± 0.14	1.04 ± 0.11*

*$P \leq 0.05$ and **$P \leq 0.01$ (measured efflux ratio vs. control).
$P_{app,A\rightarrow B}$ and $P_{app,B\rightarrow A}$: apparent permeability in the apical-to-basal and basal-to-apical directions, respectively, calculated according to the equation described in the 2.8 section.

and concentration-dependent increase in digoxin $P_{app,A\rightarrow B}$ and decrease in $P_{app,B\rightarrow A}$ (*Table Ia,b*). At the highest CsA or TAC concentrations tested, the mean digoxin ER was 1.55 and 1.31, which is consistent with an almost complete inhibition of active efflux (*Table Ia,b*, respectively), similar to that obtained with the P-gp inhibitor GF120918 (2 μM) (*Table Ic*).

The effects of CsA and TAC on the apically directed efflux of EVR and SRL are presented in *Figure 2* and *Table II*. In the presence of CsA (10 μM), both the $P_{app,B\rightarrow A}$ and the $P_{app,A\rightarrow B}$ of EVR and SRL (1 μM) were significantly and markedly increased ($P = 0.012$ and $P = 0.014$, respectively). TAC also increased significantly their $P_{app,A\rightarrow B}$ ($P = 0.014$) but, in contrast to CsA, decreased their $P_{app,B\rightarrow A}$ ($P = 0.012$) in a significant manner. In both cases, these variations resulted in a significant and important decrease in EVR and SRL mean ERs (*Table II*).

DISCUSSION

Using the enterocyte-like Caco-2 cell line, we found that EVR and SRL exhibit a polarized transport, with permeability in the basal-to-apical direction significantly higher than that observed in the apical-to-basal direction. We also found that equal levels of CsA and TAC interacted with their transepithelial flux by different mechanisms.

Few studies investigated the intestinal transport mechanisms of EVR or SRL [11,12,31–33]. Some of these studies, using either rats or cell models, indicated a major role of CYP3A in limiting EVR [12] or SRL [31,33] oral absorption. On the other hand, the role of P-gp in the intestinal absorbtion of ImTORs was not clearly

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