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BRIEF REPORT

In vitro assessment of the risk of ABCB1-mediated drug–drug interaction between rivaroxaban and tacrolimus in human embryonic kidney 293 recombinant cell lines

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Abstract

Background: In lung transplant patients, direct oral anticoagulants are often taken in combination with immunosuppressive drugs such as tacrolimus. Since tacrolimus is a substrate and inhibitor of the efflux protein ABCB1, also transporting direct oral anticoagulants, a possible drug–drug interaction mediated by competition for this transporter needs to be investigated.

Objectives: To determine the *in vitro* effect of tacrolimus on ABCB1-mediated rivaroxaban transport in order to support clinician practice.

Methods: Recombinant cell line models, based on human embryonic kidney 293 cells, were generated by a stable transfection process to overexpress ABCB1 or not (control cells). The impact of tacrolimus on ABCB1-mediated rivaroxaban transport was assessed by accumulation experiments.

Results: ABCB1 expression decreased the cellular accumulation of rivaroxaban and tacrolimus at their respective clinically relevant concentrations when compared with control cells. This confirms the involvement of ABCB1 in the active transport of tacrolimus and rivaroxaban. However, tacrolimus had no significant influence on rivaroxaban disposition at those clinically relevant concentrations.

Conclusion: Our study does not provide evidence for a possible interaction between tacrolimus and rivaroxaban when used together in practice.

KEYWORDS
ABCB1, anticoagulants, drug interactions, HEK293 cells, lung transplantation, rivaroxaban, tacrolimus

Essentials

- Lung transplant patients are polymedicated and hence at risk of drug–drug interactions.
- ABCB1 expression reduces the cellular accumulation of rivaroxaban and tacrolimus.
- A clinically relevant concentration of tacrolimus has no impact on rivaroxaban disposition.
- This result suggests no clinically significant interaction between tacrolimus and rivaroxaban.

1 | INTRODUCTION

Direct oral anticoagulants (DOACs) such as apixaban, edoxaban, and rivaroxaban are frequently used in clinical practice. Their indications include stroke prevention in nonvalvular atrial fibrillation as well as the treatment and secondary prevention of venous thromboembolism [1,2]. Both are common complications in lung transplant patients, with an incidence of 25% for nonvalvular atrial fibrillation [3] and 8% to 44% for venous thromboembolism [4–6]. Anticoagulant therapy is therefore often required in this population. Lung transplant patients are polymedicated and hence at risk of drug–drug interactions [2]. Immunosuppressive drugs are administered lifelong to transplant patients for the prophylaxis of allograft rejection, among which tacrolimus, a calcineurin inhibitor, shares metabolic and transport pathways with DOACs [7,8]. Notably, tacrolimus is a substrate and inhibitor of ABCB1 (P-glycoprotein) [9], also known to transport DOACs [2,8]. Nevertheless, this potential interaction remains poorly investigated. While the US Food and Drug Administration and a prospective pilot study reported no interaction [10,11], the European Heart Rhythm Association calls for caution in case of concomitant use [12]. Consequently, this lack of sufficient data leads to heterogeneity in guidance and clinical practice [13]. This study therefore aimed to assess the *in vitro* impact of tacrolimus on ABCB1-mediated rivaroxaban transport in order to support clinician practice.

2 | METHODS

2.1 | Materials

Rivaroxaban and (^{13}C6)-rivaroxaban-d5 (internal standard) were supplied by Alsachim; tacrolimus (TAC), rhodamine 123 (Rh123), and LY335979 (zosuquidar [Zo] HCl) were obtained from Sigma-Aldrich; and ascomycin (internal standard) was purchased from Enzo Life Sciences. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, and enzyme-free cell dissociation buffer were purchased from Gibco (Thermo Fisher Scientific); Geneticin (G418) was purchased from Roche.

2.2 | Cell culture

Human embryonic kidney (HEK) 293 cells were grown in DMEM with 4.5 g/L glucose, L-glutamine, and sodium pyruvate supplemented with 10% volume per volume (v/v) of FBS and 1% (v/v) of penicillin/streptomycin solution at a temperature of 37 °C in a 5% CO_2 atmosphere. For subculturing, cells were detached with an enzyme-free cell dissociation buffer.

2.3 | Generation of ABCB1 recombinant cells

The generation of stable recombinant cell lines has been previously described [14,15]. In short, HEK293 cells were transfected by a pcDNA3.1 vector, with complementary DNA encoding either the ABCB1 wild-type (HEK_{ABCB1}) or an empty vector ($HEK_{control}$) using Lipofectamine (Thermo Fisher Scientific). Stable recombinant cells were selected by culture with G418 (1 mg/mL).

2.4 | Characterization of ABCB1 cell lines

HEK293 cells show low endogenous levels of ABCB1 expression, and overexpression of the transporter in transfected cells was previously demonstrated using flow cytometry, immunofluorescence, and western blot [14,15]. The functionality of the overexpressed transporter was further assessed here using the reference substrate Rh123 and the specific ABCB1 inhibitor Zo [14,16]. The day before the experiment, 350,000 cells were plated in 12-well plates in complete medium (DMEM + 10% FBS). Cells were then incubated for 120 minutes at 37 °C in a 5% CO_2 atmosphere in the dark with 5 μM Rh123 alone or combined with 0.2 μM Zo. At the end of the incubation, supernatants

were discarded, and cells were washed twice with phosphate-buffered saline (PBS) at 4 °C and lysed by addition of 200 μL of water per well and overnight agitation. The fluorescence of Rh123 was measured in the supernatant after centrifugation at 14,000 rpm for 10 minutes using a Spectramax M3 fluorimeter (Molecular Devices) with excitation and emission wavelengths set at 485 nm and 530 nm, respectively. Quantitative real-time polymerase chain reaction (PCR) was also performed to quantify the number of *ABCB1* gene copies in the different cell lines. In brief, RNA was extracted from cells (5×10^6 cells) using RNeasy Mini Kit from Qiagen and reverse-transcribed in complementary DNA following the GoScript Reverse Transcription Mix, Oligo(dT) protocol (Promega). Real-time PCR was carried out with SYBR Green (Bio-Rad) using the CFX96 Touch Real-Time PCR detection system (Bio-Rad). *18S* expression was used to normalize *ABCB1* expression. The primer sequences for *18S* and *ABCB1* were (forward) 5′-CGG CTA CCA CAT CCA AGG AA -3′ and (reverse) 5′-ATA CGC TAT TGG AGC TGG AAT TAC C -3′ and (forward) 5′-AGC AAA GGA GGC CAA CAT AC -3′ and (reverse) 5′-GGC TTC ATC CAA AAG CAA AA -3′, respectively.

ABCB1 expression was also characterized by western blot according to a previously published method, with some modifications [14]. The cell pellets were recovered with an adapted RIPA (Radio-Immunoprecipitation Assay) lysis buffer (sodium deoxycholate 1%, tetraethylammonium bromide 100 mM, NaCl 300 mM, IGEPAL (Sigma-Aldrich) 2%, sodium dodecyl sulfate (SDS) 0.2%, n-dodecyl-β-D-maltoside (DDM) 0.4%, pH 7.2-7.5) supplemented with a complete protease inhibitor cocktail (Roche) and incubated for 15 minutes on ice. The cell lysates were then vortexed for 30 seconds and further diluted in LDS Sample Buffer (Invitrogen, Thermo Fisher Scientific) containing 25% of sample reducing agent (Invitrogen, Thermo Fisher Scientific) and water to obtain 15 μg of total protein in 20 μL of sample. Twenty microliters of each sample and 4 μL of protein ladder (PageRuler Prestained, Thermo Fisher Scientific) were loaded on a 4% to 12% Bis-Tris gel (NuPAGE, Thermo Fisher Scientific). Migration and transfer of proteins onto a polyvinylidene difluoride (PVDF) transfer membrane (Thermo Fisher Scientific) were then achieved using a blotting pad (VWR) at 175 V for 60 minutes and 30 V for 60 minutes, respectively. Blots were blocked with Bovine Serum Albumin (BSA) 1% in Tris-Buffered Saline with 0.02 % Tween (TBS-T) pH 8 for at least 60 minutes. After 3 washing steps with TBS-T for 5 minutes, membranes were incubated overnight at 4 °C with a monoclonal primary antibody depending on the targeting protein, namely anti–P-glycoprotein (ab170904, rabbit, 1:10,000 dilution, Abcam) for ABCB1 or monoclonal anti–β-actin antibody (A1978, mouse, 1:2000 dilution, Sigma-Aldrich) diluted in TBS-T with milk powder (150 mg/5 mL of TBS-T). The next day, after 3 steps of 5 minutes of washing in TBS-T, membranes were incubated for 60 minutes with a secondary antibody diluted in TBS-T with milk powder (antirabbit immunoglobulin G HRP; A27036, 1:2500 dilution, Invitrogen) for ABCB1 and antimouse immunoglobulin G (H + L) (A27025, 1:2500 dilution, Invitrogen) for β-actin. Finally, after 2 5-minute washing steps in TBS-T and 1 washing step in Tris-Buffered Saline (TBS), proteins were detected by chemiluminescence using horseradish peroxidase (HRP) substrate (Immobilon Classico, Millipore).

2.5 | Rivaroxaban accumulation

One day before the experiment, 350,000 cells were seeded in poly-L-lysine-coated 24-well plates in 500 μL of complete medium. After 24 hours of culture, the medium was removed and replaced by a fresh complete medium containing rivaroxaban alone or combined with tacrolimus or Zo. Rivaroxaban was used at 100 or 500 ng/mL to cover the range of plasma concentrations measured in treated patients [17], and tacrolimus was used at 80 nM (66 ng/mL) and 1 μM (822 ng/mL), corresponding to peak plasma level [18] and a supratherapeutic concentration shown to inhibit ABCB1 in cell lines overexpressing the transporter, respectively [19]. Zo was used at 0.2 μM as an ABCB1-specific inhibitor (Ki, 59 nM) [16]. After 120 minutes of incubation at 37 °C in a 5% CO_2 atmosphere, the medium was discarded, and cells were washed twice with 1 mL ice-cold PBS and detached with ice-cold PBS containing 0.2% EDTA. Cell pellets were stored at −80 °C until drug extraction and quantification by liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis.

2.6 | Rivaroxaban quantification

Rivaroxaban was extracted and quantified by LC-MS/MS analysis according to a previously described and published method with a calibration curve ranging from 0.03125 ng/mL to 12.5 ng/mL of rivaroxaban [20]. For each experiment, the absolute amount of rivaroxaban recovered from the cell extracts was normalized to the amount of proteins measured with a DC (detergent compatible) Protein Assay from Bio-Rad according to the manufacturer's instructions.

2.7 | Tacrolimus quantification

Tacrolimus was extracted and quantified from cell pellets according to a previously published LC-MS/MS method [21], and concentrations were normalized to the concentration of proteins in the samples.

2.8 | Statistical analysis

All experiments were replicated at least thrice ($N = 3$) in triplicate ($n = 3$). Results are presented as mean with SEM. Statistical analysis was performed using GraphPad Prism version 8.0 (GraphPad Software). Unpaired *t*-test (cell lines) and 2-way analysis of variance (cell lines + conditions) were performed under the null hypothesis that the means of the compared groups were equal. When the differences between the means were significant ($P < .05$), Bonferroni's or Tukey's multiple comparison tests were carried out.

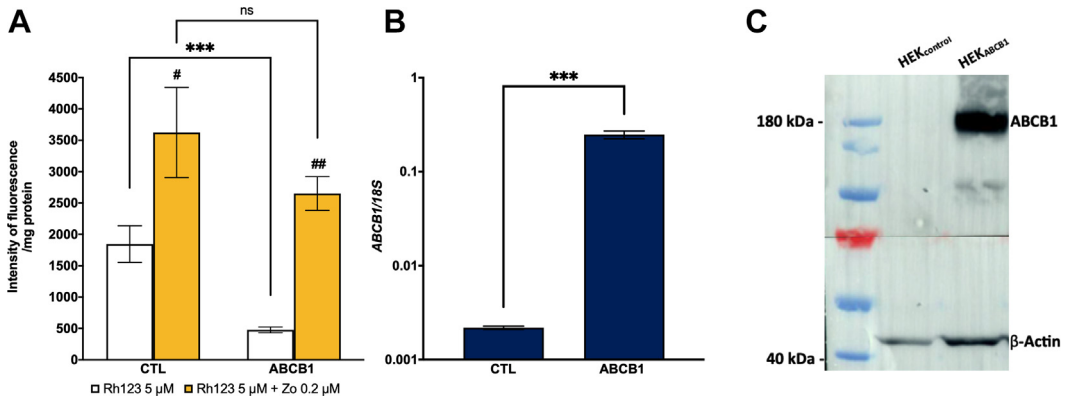

FIGURE 1 (A) Intracellular accumulation of rhodamine 123 (Rh123) is influenced by ABCB1 transporter. Intracellular accumulation of Rh123 (5 µM) with (0.2 µM) or without the ABCB1 inhibitor zosuquidar (Zo; $N = 3$; $n = 3$) in human embryonic kidney $(HEK)_{control}$ (CTL), and HEK_{ABCB1} (ABCB1). Statistical analysis: comparison with CTL, $^{***}P < .001$; comparison between Rh123 alone and Rh123 + Zo in each cell line, $\#P < .05$; $\#\#P < .01$. (B) ABCB1 transporter RNA transcripts. *ABCB1* RNA expression levels in $HEK_{control}$ (CTL) and HEK_{ABCB1} (ABCB1) normalized to the *18S* expression level (housekeeping gene; $N = 1$; $n = 3$). Statistical analysis: ABCB1 compared with CTL, $^{***}P < .001$. (C) ABCB1 expression analysis by western blot. Cells transfected with empty plasmid ($HEK_{control}$) and cells transfected with vector complementary DNA encoding the ABCB1 transporter (HEK_{ABCB1}). ns, not significant.

3 | RESULTS AND DISCUSSION

3.1 | Model validation

The HEK293 transfected cell model, which specifically overexpresses the transporter of interest, is commonly employed to investigate drug–drug interactions [22–24]. To validate the model, the ability of ABCB1 to export Rh123, a known fluorescent ABCB1 substrate [14], was assessed. The results (Figure 1A) demonstrate significantly lower fluorescence levels in cells overexpressing ABCB1 compared with control (CTL) cells, indicating higher Rh123 efflux in ABCB1 recombinant cells. Zo restored cellular Rh123 accumulation in ABCB1 transfected cells, eliminating the difference between both cell lines under these conditions. These results confirm that the difference in accumulation between CTL and ABCB1 transfected cells is due to the efflux activity driven by a functionally active ABCB1 in recombinant cells. Of note, Zo also significantly enhanced Rh123 accumulation in CTL cells. This observation could be attributed to a low but detectable basal expression of *ABCB1* in HEK293 cells (Figure 1B). Protein expression of ABCB1 in HEK293 cells was also evaluated by western blot analysis of total cell extracts (Figure 1C). The results confirmed that HEK_{ABCB1} recombinant cell line overexpressed ABCB1 protein while ABCB1 basal expression was undetectable in $HEK_{control}$ cells.

3.2 | Impact of tacrolimus on rivaroxaban ABCB1-mediated transport

First, we assessed the cellular disposition of rivaroxaban in CTL cells and cells overexpressing ABCB1 (Figure 2). The cellular accumulation of rivaroxaban increased proportionally to its extracellular concentration in the range investigated. In cells overexpressing ABCB1, it was reduced to 30% of its value in CTL cells. Rivaroxaban cellular accumulation was substantially increased by Zo, especially in ABCB1-overexpressing cells (3.5-fold increase in ABCB1 vs 1.7-fold increase in CTL cells). The fact that rivaroxaban concentrations are (i) decreased when ABCB1 is overexpressed and (ii) increased upon coincubation with Zo confirms that rivaroxaban is a substrate for ABCB1 [20,25].

Next, we tested whether tacrolimus could counteract the defeating effect of ABCB1 on rivaroxaban accumulation. At a low, clinically relevant concentration (80 nM [66 ng/mL]), tacrolimus did not modify rivaroxaban accumulation in any cell type. At a higher concentration (1 µM [822 ng/mL]), tacrolimus significantly increased the accumulation of rivaroxaban used at 100 ng/mL in both cell types, but only in cells overexpressing ABCB1 when the extracellular concentration of rivaroxaban was 500 ng/mL. This observation is coherent with a previous work coming to the same conclusion as the ABCB1 substrate mitoxantrone [19]. Furthermore, our data show that Zo is a more potent ABCB1 inhibitor than tacrolimus since it increases rivaroxaban concentrations to higher levels than tacrolimus even when used at lower concentration (0.2 µM vs 1 µM [822 ng/mL]). Lastly, rivaroxaban itself at high concentration (500 ng/mL) seems to inhibit the basal activity of ABCB1, blunting ABCB1 inhibition by tacrolimus in these conditions.

Based on this last observation, we have measured tacrolimus cellular concentration (Figure 3). The accumulation was similar in the presence of low (100 ng/mL) or high (500 ng/mL) rivaroxaban concentrations. This is in line with a clinical study showing that rivaroxaban caused small but nonclinically relevant changes in tacrolimus trough values [26]. However, tacrolimus is a known substrate of ABCB1 [9], and as already demonstrated, the cellular concentration of tacrolimus was significantly decreased ($P < .05$) in the recombinant cell line overexpressing ABCB1 in comparison with CTL cells, and this effect was also concentration-dependent [14].

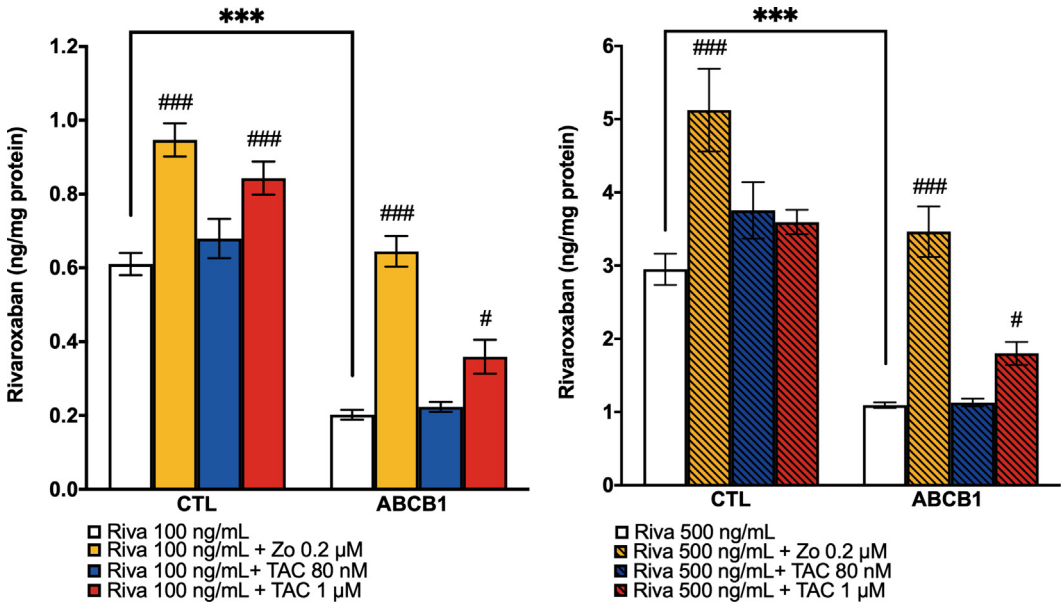

FIGURE 2 Tacrolimus (TAC) does not affect ABCB1-mediated rivaroxaban (Riva) transport at clinically relevant concentrations. Cellular accumulation of Riva after 120 minutes of incubation ($N = 3$; $n = 3$) with an extracellular concentration of 100 ng/mL (left) or 500 ng/mL (right) in human embryonic kidney$_{control}$ (CTL) and human embryonic kidney$_{ABCB1}$ (ABCB1) cells. Riva cellular concentrations are normalized to the total amount of cellular proteins. Statistical analysis: comparison with CTL cells, $^{***}P < .001$; comparison between Riva alone and Riva with inhibitor in each cell line, $\#P < .05$; $\#\#\#P < .001$. Zo, zosuquidar.

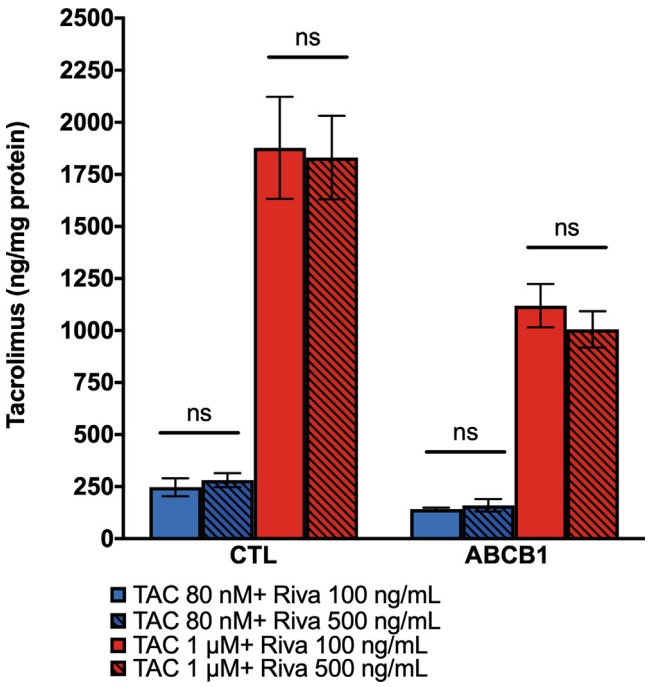

FIGURE 3 ABCB1-mediated tacrolimus (TAC) transport is not impacted by low (100 ng/mL) or high (500 ng/mL) concentrations of rivaroxaban (Riva). Cellular accumulation of tacrolimus after 120 minutes of incubation ($N = 3$; $n = 3$) with an extracellular concentration of 80 nM (blue) or 1 µM (red) in human embryonic kidney$_{control}$ (CTL) and human embryonic kidney$_{ABCB1}$ (ABCB1). Tacrolimus cellular concentrations are normalized to the total amount of cell proteins. Statistical analysis: comparison between tacrolimus + 100 ng/mL Riva and tacrolimus + 500 ng/mL Riva, $P > .05$. ns, not significant.

4 | CONCLUSION

Our *in vitro* study confirms that ABCB1 expression reduces the cellular accumulation of rivaroxaban and tacrolimus at their respective clinically relevant concentrations, confirming that both drugs are ABCB1 substrates. However, tacrolimus does not seem to impact rivaroxaban disposition at clinically relevant concentrations, while the concentration of tacrolimus is not affected by the presence of low or high clinically relevant concentrations of rivaroxaban. In conclusion, our study does not provide evidence against the concurrent use of these 2 molecules in lung transplant patients, which is in line with other studies showing no interaction between tacrolimus and rivaroxaban mediated by ABCB1 transport [11,27]. Pending further confirmation through a well-designed prospective clinical trial including therapeutic drug monitoring of both drugs, our work suggests to reconsider the cautious current recommendations of European scientific associations.

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AUTHOR CONTRIBUTIONS

G.M., A.-L.S., F.V.B., A.S., and L.E. designed the research; V.H. designed and provided the cell models; G.M. conducted the experiments; L.P. and V.H. conducted the high-performance LC/MS-MS analysis; G.M. drafted the manuscript; all authors reviewed and approved the final version of the document.

RELATIONSHIP DISCLOSURE

There are no competing interests to disclose.

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