**ABCB1 1199G>A Genetic Polymorphism (Rs2229109) Influences the Intracellular Accumulation of Tacrolimus in HEK293 and K562 Recombinant Cell Lines**

Géraldine Dessilly1,9, Laure Elens2,9, Nadtha Panin1, Arnaud Capron3, Anabelle Decottignies4, Jean-Baptiste Demoulin4, Vincent Hauflroid1,3*

1 Louvain Centre for Toxicology and Applied Pharmacology, Institut de Recherche Expérimentale et Clinique, Université Catholique de Louvain, Brussels, Belgium, 2 Louvain Drug Research Institute, Université Catholique de Louvain, Brussels, Belgium, 3 Department of Clinical Chemistry, Cliniques Universitaires Saint-Luc, Université Catholique de Louvain, Brussels, Belgium, 4 De Duve Institute, Université Catholique de Louvain, Brussels, Belgium

**Abstract**

**Objective:** ATP-binding cassette, subfamily B, member 1 (ABCB1) transporter, or P-glycoprotein, is an efflux protein implicated in the absorption and the distribution of various compounds, including tacrolimus and cyclosporine A. In vivo studies suggest an association between the ABCB1 1199G>A single nucleotide polymorphism (SNP) and tacrolimus intracellular accumulation. The aim of the present experimental study was to clarify in vitro the impact of the coding ABCB1 1199G>A SNP on ABCB1 transport activity towards both immunosuppressive drugs.

**Method:** Two recombinant cell lines, i.e. Human Embryonic Kidney (HEK293) and Human Myelogenous Leukemia (K562) cells, overexpressing ABCB1 carrying either the wild-type allele (1199G) or its mutated counterpart (1199A), were generated. The impact of the 1199G>A SNP on ABCB1 activity towards rhodamine (Rh123), doxorubicin, vinblastine, tacrolimus and cyclosporine A was assessed by accumulation, cytotoxicity and/or kinetic experiments.

**Results:** Tacrolimus accumulation was strongly decreased in cells overexpressing the wild-type protein (1199G) compared to control cells, confirming the ability of ABCB1 to transport tacrolimus. By contrast, overexpression of the variant protein (1199A) had nearly no effect on tacrolimus intracellular accumulation whatever the model used and the concentration tested. Unlike tacrolimus, our results also indicate that cyclosporine A, Rh123 and doxorubicin are transported in a similar extent by the wild-type and variant ABCB1 proteins while the variant protein seems to be more efficient for the transport of vinblastine.

**Conclusion:** ABCB1 encoded by the 1199G wild-type allele transports more efficiently tacrolimus in comparison to the 1199A variant protein. This observation indicates that the amino-acid substitution (Ser400Asn) encoded by the 1199A allele drastically decreases the ability of ABCB1 to drive the efflux of tacrolimus in a substrate-specific manner, in agreement with our previously published clinical data. Our study emphasizes the importance of the ABCB1 1199G>A polymorphism for ABCB1 activity and its potential to explain differences in drug response.

**Introduction**

P-glycoprotein (P-gp) is a phosphorylated transmembrane glycoprotein of 170 kDa that mediates the ATP-dependent efflux of numerous endogenous and exogenous substances across biological membranes. P-gp is encoded in humans by the ABCB1 gene which spans 28 exons on chromosome 7. The ABCB1 protein (official nomenclature for P-gp) belongs to the group B of the ATP binding cassette (ABC) superfamily and is composed of 1280 amino acids consisting of two equivalent and symmetric domains of 6 transmembrane segments. Each domain encloses an intracellular nucleotide binding domain (NBD), which binds ATP [1–4]. The expression of ABCB1 is mainly distributed in excretory organs and in the blood brain barrier [1,5]. Consequently, ABCB1 is thought to ensure a protective role with respect to the absorption (enterocytes), the distribution (blood brain barrier, lymphocytes) and the excretion (hepatocytes, kidney) of potential harmful substances by driving their transport from the cytoplasm to the extracellular compartment [1,6–10]. Its substrates spectrum is very broad and includes important exogenous molecules, such as antiretrovirals and immunosuppressive agents (e.g. tacrolimus (Tac)}
and cyclosporine A (CsA) [11]) and also anticancer drugs (e.g. imatinib [12]). Overall, this protein influences the pharmacokinetics of various compounds and its overexpression in cancer has been suggested as a major cause of resistance to treatments [9,10,13–16].

Over the last decade more than 50 single-nucleotide polymorphisms (SNPs) have been identified in \textit{ABCB1} [17–19]. The three most common SNPs in the protein coding region are rs1128503 (1236G>C/T, Gly412Gly), rs2032582 (2677G>T/A, Ala893Ser/Thr), and rs1045642 (3435C>T, Ile1145Ile) [20,21]. These three SNPs are in strong linkage disequilibrium (LD) and have been extensively investigated. However, the results that have been reported so far are inconsistent and the real functional impact of these three SNPs remains controversial [22]. Other less frequent SNPs have been described and could also potentially explain part of the variability observed in the expression and/or function of \textit{ABCB1} [5]. Of particular interest, the \textit{ABCB1} 1199G>A coding SNP located in the exon 11 (rs2229109) is relatively frequent with a reported allelic frequency of about 6% in the Caucasian population. Therefore, according to the Hardy-Weinberg distribution, the expected genotypic frequencies are respectively of 0.4% for the homozygotes mutated AA, 11.2% for the heterozygotes GA and 88.4% for the homozygotes wild-type GG. This SNP is associated with a serine to asparagine substitution at position 412 in the cytoplasmic loop of \textit{ABCB1} which is involved in substrates recognition [2]. A small number of \textit{in vitro} studies have assessed the impact of the \textit{ABCB1} 1199G>A SNP on the \textit{ABCB1} efflux activity towards its substrates. One study reported that cells overexpressing the variant protein (\textit{ABCB1}1199A/mut cells) were characterized by a decreased efflux of Rhodamine 123 (Rh123), a substrate of the \textit{ABCB1} protein [23]. In contrast, an increased efflux activity of the 1199A variant towards several anticancer drugs was reported [24]. It can be thus rationally hypothesized that the impact of this SNP, may differentially affect the affinity and/or the activity of this protein in a substrate-dependent manner.

Tacrolimus (Tac) is an immunosuppressive drug, belonging to the calcineurin inhibitor group, and is widely used in kidney and liver transplantation to prevent graft rejection [25,26]. Tac is characterized by a very narrow therapeutic window and a huge inter-individual variability in its pharmacokinetic and pharmacodynamic behavior [25,27,28]. Despite the use of therapeutic drug monitoring, a relatively high rate of therapeutic failure and toxicity events are still encountered. The identification of genetic markers influencing Tac pharmacokinetics and/or pharmacodynamics might potentially help the clinician to better adjust Tac therapy directly after transplantation. In this respect, we have previously linked the 1199A variant to an increased accumulation of Tac in liver biopsies [29] and in peripheral blood mononuclear cells (PBMCs) which include T-lymphocytes, the cellular target of Tac [30] and suggesting a decrease in \textit{ABCB1} mediated efflux activity towards Tac \textit{in vivo}.

Cyclosporin A (CsA) is another powerful immunosuppressive drug, used in transplantation to prevent graft rejection but to a lesser extent than Tac partly because the concentrations used in clinic are more nephrotoxic. It was demonstrated \textit{in vivo} that the 1199G>A SNP influences the CsA concentration in PBMCs of patients with renal transplantation [31]. Carriers of the 1199A variant presented lower intracellular levels compared to homozygotes wild-type patients, arguing for an opposite functional effect of the \textit{ABCB1} 1199G>A SNP considering CsA-mediated efflux when compared to Tac transport.

The aim of the present experimental study was to clarify and further confirm \textit{in vitro} the impact of the \textit{ABCB1} 1199G>A polymorphism on \textit{ABCB1} expression and transport activity towards both immunosuppressive drugs (Tac and CsA) using two stable transfected cell lines.

### Materials and Methods

#### Materials

Tac (Prograf IV) and CsA (Sandimmune) were purchased from Astellas Pharma (Brussels, Belgium) and Novartis Pharma (Vilvoorde, Belgium), respectively. LY335979 (Zosuquidar 3HCl) was purchased from Bio-connect (Huis ten Bosch, Netherlands). G418 was purchased from Roche Applied Science (Vilvoorde, Belgium).

Rhodamine (Rh123) was purchased from Sigma-Aldrich (St-Louis, United States). Doxorubicin and vinblastine were purchased from Pfizer (Brussels, Belgium) and Teva (Wilmington, Belgium), respectively.

HEK293 is a cell line derived from human embryonic kidney cells grown in tissue culture (ATCC CRL-1573™). K562 is a cell line derived from the pleural effusion of a 53-year old female with chronic myelogenous leukemia in terminal blast crises (ATCC CCL-243™).

#### Cell culture

HEK293 were grown in Dulbecco’s Modified Eagle medium (DMEM) 1g/l glucose and glutamine (Gibco, Invitrogen) supplemented with 10% (v/v) of Fetal Bovin Serum (FBS) (Gibco, Invitrogen) and 1% (v/v) of Antibiotic-Antimycotic solution (A-A) (Gibco, Invitrogen) at a temperature of 37°C in the presence of 5% of CO₂. For immunofluorescence staining and intracellular accumulation assays, HEK293 cells were grown in DMEM Glutamax 4.5g/l glucose (Gibco, Invitrogen) supplemented with 10% (v/v) FBS and 1% (v/v) A-A.

K562 were grown in Iscove’s Modified Eagle Dulbecco’s medium (IMDM) (Gibco, Invitrogen) supplemented with 10% (v/v) of FBS (Gibco, Invitrogen) and 1% (v/v) A-A (Gibco, Invitrogen) at a temperature of 37°C in the presence of 5% of CO₂. For intracellular accumulation assay, K562 cells were grown in IMDM Glutamax (Gibco, Invitrogen), 10% (v/v) FBS and 1% (v/v) A-A.

#### Generation of \textit{ABCB1}1199G/wt and \textit{ABCB1}1199A/mut plasmids

The expression vector pcDNA 3.1 with cDNA encoding \textit{ABCB1} (\textit{ABCB1}1199G/wt) was kindly provided by Dr Rodney Ho (University of Washington). The mutated plasmid designated \textit{ABCB1}1199A/mut was generated by site-directed mutagenesis using the QuickChange II XL Site-directed mutagenesis Kit (Stratagene) with the mismatched primers 5’-CAG AAA TGT TGA CTT GAA TTA GCC ATG TCG-3’ (forward) and 5’-CTT TTC GAG ATG CGT AAT TGA AGT GAA CAT-3’ (reverse). The plasmids were sequenced to confirm the presence of the mutation.

#### Generation of stable recombinant cell lines

HEK293 cells were plated the day before transfection in 6 well plates (2 10⁶ cells/well) and transfected with lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol with 4 μg of plasmid DNA. To select stable transfectants, cells were replated at
a low density 24h after the transfection and G418 was added 24h later at a final concentration of 0.8 mg/ml.

K562 cells (10^7 cells) were transfected by electroporation (ECM630 device from BTX, 200 V, 75 Ω, 1300 μF) with 50 μg of plasmid DNA. To select stable transfectants, cells were replated at a low density 24h after the transfection and G418 was added 48h later at a final concentration of 1 mg/ml.

After one week on culture, cell surface ABCB1 protein expression in transfected cells (HEK293 and K562) was analyzed by flow cytometry [FITC Mouse Anti-Pglycoprotein antibody diluted 1:10, clone 557542, BD Pharmingen] or its matched isotypic control diluted 1:10 [FITC Mouse IgG 2bk, clone 557002, BD Pharmingen] (see below).

Characterization of ABCB1 expression

Flow cytometry. 10^6 cells (HEK293 and K562) were recovered by centrifugation. Cells were washed twice with 2 ml of ice-cold HAF solution [filtrated (0.22 μm) Hank’s buffer with 3% decomplemented FBS and NaN3 (20 mmol/l)]. Cells were resuspended in HAF solution containing the primary FITC Mouse Anti-P-glycoprotein antibody diluted 1:10 (clone1F9 557002, BD Pharmingen) or its matched isotopic control diluted 1:10 (FITC Mouse IgG 2bk, clone27-35 555742, BD Pharmingen) and incubated 45 min on ice in the dark. Cells were further washed with 2 ml of HAF solution, centrifugated and finally fixed in 1:1 HAF/paraformaldehyde (2%) (v/v). Samples were analyzed on a Fluorescence-activated cell sorting (FACS) Calibur (BD Sciences).

HEK1199G/wt or K562 1199G/wt and HEK 1199A/mut or K562 1199A/mut cells were sorted with fluorescence parameters gated on the same level of intensity to ensure similar ABCB1 expression. The protocol was identical (see above) except the using of HAF solution without NaN3.

Quantitative Western blot. HEK293 cells were rinsed twice with cold D-PBS (Invitrogen) and recovered with triton lysis buffer (TLB; TritonX100 0.1%, NaCl 150 mM, Tris-HCl pH 7.5 25 mM supplemented with complete protease inhibitors cocktail (Roche) and incubated for 15 min on ice. The solution was centrifuged for 10 min, 4°C at 14000 rpm and the supernatant was recovered. The cell lysate was then sonicated for 5 min and further diluted in Laemmli sample buffer (Bio Rad, Hemel Hempstead, UK) containing 10% of β-mercaptoethanol. The cell lysate was passed five to six times through 26G needle, and samples were boiled at 60°C for 10 min to denature proteins. In order to facilitate the migration, samples were finally centrifuged 5 min, 14000 rpm at room temperature to remove cellular debris. 30 μl of each samples (40 μg of total proteins) and 15 μl of protein ladder (Multicolor High Range Protein Ladder, Fermentas) were loaded on 7.5% Mini-Protean TGX precasted gel as recommended by the manufacturer (Bio Rad). Proteins were then transferred onto a PVDF membrane using the Novex Semi-dry blotter (VWR) at 80 mA/membrane for 120 min. Blots were blocked with blocking buffer (927-40000,LI-COR Biosciences) for at least 60 min. After several washing steps with TBS-T, membranes were incubated overnight at 4°C with a monoclonal primary antibody anti-P-gp (clone P7965, mouse, 1:200 dilution, Sigma-Aldrich) and with a monoclonal anti-calnexin antibody (ADI-SPA-860, 1:15 000 dilution, Enzo Life Sciences) diluted in TBS-T. After three steps of washing in TBS-T, donkey anti-mouse IgG (926-32212, LI-COR Biosciences) and donkey anti-rabbit IgG (926-3223 LI-COR Biosciences) IR dye antibodies were applied to the membranes at a 1:15000 and diluted in TBS-T for 60 min. The transferred proteins were detected using Odyssey IR imaging system (LI-COR Biosciences).

**Immunofluorescence.** One day before the experiment, HEK293 cells were plated at a density of 10^5 cells/well in complete medium. The next day, cells were rinsed with PBS/0.1% BSA, fixed with 4% of paraformaldehyde during 15 min and rinsed with PBS/0.1% BSA. Cell membranes were subsequently permeabilized with 0.1% Triton X100 during 5 min. After a washing step with PBS/0.1% BSA, cells were incubated with the primary monoclonal antibody Ab4E3 (ab103333, Abcam, 5 μg/ml, diluted with PBS/0.1% BSA) or with its isotopic control (Mouse Ig2a kappa Monoclonal, ab10335, Abcam, 25 μg/ml) for 90 min in the dark. After two washing steps with PBS and PBS/0.1% BSA, respectively, cells were incubated for 60 min with goat anti-mouse IgG coupled to FITC (ab6785, Abcam, 1 µg/ml) and with DAPI (Hoechst 33258 pentahydrate (bis-benzimide), H3569, Invitrogen). Finally, the cells were fixed once again with 4% of paraformaldehyde during 5 min. Fluorescence was analyzed in fluorescent mounting medium (Dako) with a digital microscope Evis fluorescence (AMG).

**Rh123 functional test**

One day before the experiment, 10^5 cells (HEK293 or K562) were plated in poly-L-lysine-coated black 96-well plates in complete medium. ABCB1 related transport activity was investigated by loading the cells for 60 min at 37°C with 5 μM of Rh123 in the dark. When indicated, ABCB1 inhibition was performed by pre-incubating the cells for 15 min in the presence of LY335979 (0.1 and 0.2 μM). After incubation with Rh123, the supernatants were discarded. The cells were washed twice with Dulbecco’s PBS at 4°C and cell lysis was performed with Triton X100 1% in DMEM/or IMDM Glutamax at room temperature for 30 min for HEK293 or K562, respectively. Finally, the intracellular fluorescence of Rh123 was analyzed by a fluorimeter Spectramax Gemini Xs (Molecular Devices); excitation wavelength was set at 485 nm and emission at 530 nm.

**Thymidine incorporation assay**

K562 cells were plated at 10^5 cells/well in 96-well plates in complete medium. Cells were incubated for 24h at 37°C with varying concentrations of doxorubicin (from 10 to 2430 nM) or vinblastine (from 1.11 to 270 μM). One 1 μg/ml of [3H] thymidine was then added to each well and further incubated for 24h. The radioactivity was measured with a TopCount NXT liquid scintillation counter (PerkinElmer).

Graphics are represented by the relative proliferation (%); CPM samples/CPM control (without drugs exposure) *100.

**Tac and CsA accumulation**

Tac or CsA accumulation experiments were performed according to a previously described method [32,33], with minor modifications. One day before the experiment, 3.5 10^5 cells (HEK293 or K562) were seeded in 24-wells plates in 500 μl of complete medium. Tac or CsA were added at six different concentrations (from 0.0015 to 0.5 μM and 0.015 to 5 μM, respectively) and cells were incubated for 120 min at 37°C, 5% of CO2. After incubation with Tac or CsA, the supernatant was discarded by spashing the plate and the cells were washed two times in cold PBS and detached with ice-cold PBS supplemented with 0.2% EDTA. After centrifugation at 4°C, the supernatant was discarded and cells pellets were conserved at −80°C until LC-MS/MS analysis.
Tac intracellular kinetics

One day before the experiment, 3.5 $10^5$ cells (HEK293 or K562) were seeded in 24-wells plates in 500 μl of complete medium. Tac was added at a final concentration of 0.05 μM and cells were incubated for 120 min at 37°C, 5% of CO2. After incubation with Tac, the supernatant was discarded by splashing the plate and the cells were allowed to efflux in serum-free medium for 15 sec, 30 sec, 1 min, 5 min, 10 min, 30 min and 1 h. At each time point (including the point measured in preliminary experiments, corresponding to 120 min of accumulation), cells were washed two times in cold PBS and detached with ice-cold PBS supplemented with 0.2% EDTA. After centrifugation at 4°C, the supernatant was discarded and cells pellets were conserved at −80°C until LC-MS/MS analysis.

CsA quantification

We used the chromatography system and the analytic column described above for Tac quantification. CsA was extracted by precipitation with ZnSO4 0.1 M in aqueous solution and with a solution of acetonitrile containing the internal standard, consisting of ascomycin at a concentration of 100 ng/ml. Blank cell pellets were processed in parallel for the calibration curve (from 1.25 nM to 1.25 M). Then, the supernatant was recovered and transferred to a high-performance liquid chromatography vial and 10 μl was injected into the LC-MS/MS. The absolute amount of drug present in cell extracts was normalized to the amount of protein present as assessed using the BCA kit (Thermoscientific).

Impact of ABCB1 1199G>A polymorphism on the intracellular accumulation of Rh123

We further assessed the ability of the 1199A variant to export Rh123, a well characterized fluorescent substrate of ABCB1. As expected, after 90 min of incubation in the presence of 5 μM Rh123, fluorescence levels were lower in both HEK1199G/wt and HEK1199A/mut cell lines compared to control cell lines (Fig 4A, p<0.001), indicating higher Rh123 efflux in ABCB1 recombinant cells. As a control, inhibition by LY335979 (0.1 and 0.2 μM), which is a potent specific inhibitor of ABCB1-mediated efflux, yielded uniform Rh123 intracellular fluorescence values in the two transfected cell lines (Fig 4A, p<0.001), suggesting that the observed differences in fluorescence intensity can be ascribed to a higher ABCB1 efflux activity in HEK1199G/wt and HEK1199A/mut. These experiments also confirmed that the ABCB1 recombinant protein expressed in HEK293 cells was functionally active, at least towards Rh123. Similarly, when performing Rh123 accumulation assay in K562 cells, we observed a lower fluorescence level in both K5621199G/wt and K5621199A/mut cell lines compared to control cell lines (Fig 4B, p<0.001). These differences were abolished when ABCB1-mediated efflux was inhibited by LY335979 (Fig 4B, p<0.001), again suggesting that recombinant proteins were functionally active and correctly inhibited by LY335979.
Impact of ABCB1 1199G>A polymorphism on the cytostatic effects of doxorubicin and vinblastine

Since ABCB1 has been reported to transport doxorubicin and vinblastine [35–37], we assessed the influence of ABCB1 overexpression and of the variant 1199A on K562 cells proliferation in the presence of these cytostatic drugs, to further validate our models. We observed that recombinant models (K562 1199G/wt and K562 1199A/mut) are resistant to both anticancer drugs, doxorubicin and vinblastine (Fig 5A, \( p < 0.05 \), Fig 5B, \( p < 0.01 \) statistics not presented in the figure for clarity). Furthermore, K562 1199G/wt and K562 1199A/mut cells exhibited similar resistance to doxorubicin (Fig 5A, \( p > 0.05 \)), while K562 1199A/mut cells were more resistant to vinblastine (Fig 5B, \( p < 0.05 \)) compared to K562 1199G/wt. This result can be interpreted as an increase of efflux activity of the 1199A variant towards vinblastine and is in agreement with previously published results [13,23].

Impact of ABCB1 1199G>A polymorphism on the intracellular accumulation of Tac and CsA

As it has been previously shown that Tac accumulation in PBMCs and in hepatocytes was correlated with the ABCB1 1199G>A polymorphism in vivo [29,30], we sought to determine
whether this polymorphism could affect the accumulation of Tac in HEK293 and K562 transfected cell lines. HEK293 transfected cell lines were loaded for 120 min with six different concentrations of Tac (from 0.0015 to 0.5 μM) that largely cover the entire range encountered in clinical practice (the whole-blood through level C_min being 0.005 μM and the peak concentration C_max 0.016 μM). K562 cells were only incubated in presence of 0.05 μM of Tac, because this concentration roughly corresponds to the double C_max value and is thus assumed to be of clinical relevance.

As depicted in Fig 6A and 6B, Tac intracellular concentrations were strongly decreased in cells overexpressing the 1199G wild-type allele when compared to control cells (i.e. transfected with empty plasmid) (Fig 6A, p<0.01; Fig 6B, p<0.05) or to cells overexpressing the 1199A variant allele (Fig 6A, p<0.01; Fig 6B, p<0.001), and this effect was observed whatever the concentration engaged in the experiment (Fig 6A) or the model used. Overexpression of the 1199A variant allele had no effect on Tac accumulation when compared to control cell lines (p>0.05), suggesting that substitution of serine 400 for asparagine strongly reduces ABCB1-mediated Tac efflux in HEK293 and K562 cell lines.

As it was also suggested that CsA concentrations in PBMCs were correlated with ABCB1 1199G>A polymorphism in vivo [31], we similarly tested the impact of the ABCB1 1199G>A SNP on CsA accumulation. Six different concentrations were tested (from 0.015 to 5 μM) using HEK293 cell line in order to cover the range of concentrations encountered in patients (the whole-blood through level C_min being 0.03 μM and the peak concentration C_max 0.21 μM). For K562, we selected a clinically relevant concentration of 0.5 μM (which corresponds to the double C_max value). After 120 min of drug accumulation at 0.5 μM, CsA intracellular concentrations were decreased in 1199G/WT cells and in 1199A/mut cells when compared to control cells in HEK293 or K562 cell lines (Fig 6C, p<0.01; Fig 6D, p<0.001) but no difference (p>0.05) was observed between the 1199G/WT and 1199A/mut cells. Similar results were observed when incubating HEK293 cells with other tested CsA concentrations (Fig. 6C).

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**Figure 3. ABCB1 expression analysis by fluorescence microscopy.** (A) Untransfected (CTL) HEK293, (B) transfected with empty plasmid (CTL pcDNA3.1) HEK pcDNA3.1, (C) HEK1199G and (D) HEK1199A cells were stained with anti-ABCB1 antibodies (green fluorescence) as described in materials and methods. DAPI was used to stain nuclei (blue).

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**Figure 4. Intracellular accumulation of Rhodamine123 is not influenced by ABCB1 1199A variant.** Intracellular accumulation of Rhodamine123 (5 μM) in presence (0.1 and 0.2 μM) or absence of ABCB1 inhibitor (LY335979) (N = 6) in (A) CTL HEK293, CTL HEKpcDNA3.1, HEK1199G and HEK1199A and (B) CTL K562, CTL K562pcDNA3.1, K5621199G and K5621199A. * compares without inhibitor p<0.05 **p<0.01 ***p<0.001, # compared to CTL pcDNA3.1 p<0.05 ##p<0.01 ###p<0.001.

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For intracellular kinetics involving Tac, we finally loaded cells using a Tac concentration of 0.05 μM (see above). Both transfected cell lines were thus loaded with Tac (0.05 μM) for 120 min and then placed in a Tac-free medium for up to 60 min to allow for efflux.

As depicted in Fig 7, Tac intracellular concentrations were strongly decreased, at all timepoints in cells overexpressing the 1199G wild-type allele when compared to control cells (transfected with empty vector) (Fig 7A, p<0.01) or to cells overexpressing the 1199A variant allele (Fig 7A, p<0.01; Fig 7B, p<0.01 statistics not presented in the figure for clarity). Overexpression of the 1199A variant allele had no effect on Tac accumulation when compared to control cell lines (p>0.05), suggesting that the ABCB11199A variant abolished ABCB1-mediated Tac efflux in both HEK293 and K562 cell lines.

Discussion

To assess the functional relevance of the ABCB1 1199G>A polymorphism, we have generated stable HEK293 and K562 cell lines overexpressing either the 1199G wild-type or the 1199A variant allele. Importantly, the basal expression of endogenous ABCB1 was negligible in these cells, which present the 1199GG genotype (genotyping results not shown). Using flow cytometry and fluorescence microscopy, we also showed that the transfected ABCB1 protein was properly expressed at the plasma membrane. Basically, our western blot results indicated that the total cellular expression of ABCB1 is slightly higher in HEK293 expressing the wild-type protein when compared to its variant counterpart. However, even if existent, the difference observed in total cell extract expression does not change the interpretation of our accumulation results (see below) as only the protein that is addressed to the membrane is supposed to be active in terms of drug efflux. In fluorescence flow cytometry assays performed on these cell lines, it appeared that the levels of ABCB1 surface expression was the same when we compared HEK293 cells expressing the wild-type with cells expressing the variant protein and this was supported by our immunofluorescence microscopy results. Consequently, our results highlight that the wild-type and the variant cell line were comparable when considering the surface protein pool implicated in the efflux of ABCB1 substrates and that our model is suitable for the analysis of the intrinsic impact of the ABCB1 1199G>A SNP. To test the functionality of the expressed protein in our cell lines and further validate our models, we first analyzed the rate of accumulation of Rh123. We observed that control cells had a higher Rh123 rate of accumulation compared to both transfected cell lines (ABCB11199G/wt and ABCB11199A/mut) regardless of the model used, i.e HEK293 or K562. Altogether, our expression as well as functionality test results confirmed that ABCB1 protein is expressed similarly on the surface of recombinant cell lines and is functionally active. We also showed that Rh123 accumulation in control cells was not influenced by the presence of the LY335979 inhibitor, consistent with the very low basal expression of ABCB1 in our models. By contrast, and as expected, Rh123 accumulation in transfected cell lines was sensitive to the LY335979 inhibitor.

Moreover, we made cytotoxicity experiments using doxorubicin and vinblastine, to test whether the 1199A variant influenced the sensitivity of our recombinant models towards these two antican- cer drugs. We observed that controls cells had a higher sensitivity to doxorubicin and vinblastine, thereby confirming that recombinant models transport both drugs outside of the cell more efficiently than cells not overexpressing ABCB1. We showed also that K5621199G/wt and K5621199A/mut cells exhibited similar resistance to doxorubicin, while K5621199A/mut cells were more resistant to vinblastine compared to K5621199G/wt. This result suggests an increase of efflux activity of the 1199A variant towards vinblastine but not towards doxorubicin. These observations are in agreement with a previous study published by Woodahl et al. [13] (see below in discussion) further validating our model.

The key finding of the present study highlights that the ABCB1 1199G>A polymorphism has a marked influence on the accumulation of Tac in transfected cultured cell lines. First, we observed that Tac intracellular concentrations were much reduced by overexpressing ABCB1 whatever the drug concentration tested or the model used, confirming that Tac is indeed a good ABCB1 substrate [11]. Furthermore, Tac intracellular concentrations in cells overexpressing the variant ABCB11199A protein were significantly higher than in cells overexpressing wild-type ABCB11199G. These results provide an important molecular
clarification of our previous \textit{in vivo} results \cite{29,30}. Indeed, the 1199A variant was previously linked to a decreased \textit{ABCB1} activity towards Tac \textit{in vivo}, as reflected by the observation that carriage of the 1199A variant allele is associated with an increased Tac accumulation in PBMCs or hepatocytes isolated from renal or hepatic transplant patients, respectively \cite{29,30}. The present study suggests a more severe decrease of \textit{ABCB1} 1199A activity towards Tac than observed \textit{in vivo}. However, one should keep in mind that

\begin{figure}
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\includegraphics[width=\textwidth]{figure6}
\caption{The \textit{ABCB1} 1199A variant differentially affects Tac and CsA efflux. Intracellular accumulation of tacrolimus after 120 min of incubation (\(N=3\)) (A) at six different concentrations (0.0015–0.5 \(\mu\)M) in CTL HEK293, CTL HEKpcDNA3.1, HEK1199G and HEK1199A and (B) at 0.05 \(\mu\)M in CTL K562, CTL K562 pcDNA3.1, K562\textsubscript{1199G} and K562\textsubscript{1199A}. Intracellular accumulation of CsA after 120 min of incubation (\(N=3\)) (C) at six different concentrations (0.015–5 \(\mu\)M) in CTL HEK, CTL HEKpcDNA3.1, HEK\textsubscript{1199G} and HEK\textsubscript{1199A} and (D) at 0.5 \(\mu\)M in CTL K562, CTL K562pcDNA3.1, K562\textsubscript{1199G} and K562\textsubscript{1199A}. The intracellular accumulation of Tac or CsA in each cell line was normalized by reporting the absolute amounts of Tac or CsA (in ng) on the total amount of proteins in cell extracts, expressed in mg. * compared to 1199G/WT *\(p<0.05\) **\(p<0.01\) ***\(p<0.001\). doi:10.1371/journal.pone.0091555.g006}
\end{figure}
in vivo, some possible compensatory mechanisms, probably involving other ABC efflux proteins, might counterweight this loss of activity and, consequently, limit the impact of the ABCB1 1199A SNP considering in vivo Tac pharmacokinetics. In sharp contrast to Tac data, we observed a similar efflux of CsA in cells expressing the 1199A protein when compared to cells expressing the 1199G protein. Such discrepancy between Tac and CsA has been also observed in vivo where lower CsA concentrations were reported in PBMCs of renal transplant patients carrying the 1199A variant allele [31]. These results suggest thus an opposite effect of the ABCB1 1199G A SNP.

The differential activity of ABCB1 1199G>A SNP towards Rh123, doxorubicin, vinblastine, Tac and CsA could be explained by the fact that the ABCB1 Ser400Asn substitution is located in a cytoplasmic loop involved in substrate recognition. This variant could thus affect differentially the affinity of ABCB1 towards its specific substrates. This hypothesis is thus corroborated when looking at the differential results observed with the substrates investigated in our study. Indeed, our results suggest a decreased activity of the variant protein towards Tac while it seem to indicate no or reverse effect for doxorubicin, CsA, Rh123 and vinblastine. These in vitro observations reinforce our initial hypothesis that the effect of the 1199A variant depends on the investigated substrates.

Table 1. Influence of ABCB1 1199G>A polymorphism on drug transport and/or efficacy.

| Compound                  | 1199A activity* (in vitro assay) | Clinical observations in 1199A carriers | References |
|---------------------------|----------------------------------|----------------------------------------|------------|
| Rhodamine                 | = or ↓ (fluorescence)            | n.a.                                   | 23 & herein |
| Doxorubicin               | = (cytotoxicity)                 | n.a.                                   | 13 & herein |
| Vinblastine               | ↑ (cytotoxicity)                 | n.a.                                   | 13 & herein |
| Vincristine               | ↑ (cytotoxicity)                 | n.a.                                   | 13         |
| Paclitaxel                | ↑ (cytotoxicity)                 | n.a.                                   | 13         |
| Etoposide (VP-16)         | ↑ (cytotoxicity)                 | n.a.                                   | 13         |
| HIV protease inhibitors   | ↑ (accumulation)                 | n.a.                                   | 24         |
| Cyclosporin A             | = or ↑ (accumulation)            | Lower concentration in PBMCs           | 31 & herein |
| Tacrolimus                | ↓ (accumulation)                 | Higher concentration in PBMCs and hepatocytes | & herein |

*Compared to 1199G wild-type; n.a.: not available.
See text for details.
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It is interesting to stress that no difference in Rh123 export was observed between ABCB1_1199G/wt and ABCB1_1199A/mut transfected cell lines. Indeed, these observations seem a priori in contradiction with the study by Woodahl et al suggesting that cells overexpressing the mutated protein (1199A) exhibited a higher accumulation rate of Rh123 compared to cells overexpressing wild-type protein (1199G) [23]. Some differences in study design could explain those discrepancies. First, a different cell model was used: the porcine kidney epithelial LLC-PK1 cell line which consists of differentiated and polarized cells. Secondly, the experimental approach was different as Woodahl et al analyzed the transmembrane directional transport mediated by ABCB1 while we analyzed the intracellular accumulation. This could potentially lead to differences in sensitivity to detect changes in ABCB1 activity towards Rh123.

Interestingly, the biological impact of the ABCB1 1199G>A polymorphism seems not limited to tacrolimus and vinblastine. This SNP was also reported to influence the pharmacokinetic and/or pharmacodynamic parameters of antiretrovirals and other anticancer agents. Woodahl et al. demonstrated in vitro that, for all investigated antitumor protease inhibitors (Amprenavir, Indinavir, Lopinavir, Ritonavir and Saquinavir), the transepithelial permeability ratio was significantly greater in ABCB1_1199A/mut than in ABCB1_1199G/wt LLC-PK1 cells, reflecting a higher efflux of the drugs across the epithelial barrier. These observations suggest that the ABCB1 1199G>A SNP increases the ability of ABCB1 to transport antitumor PIs and that it might decrease their oral bioavailability and their penetration into cells and tissues [24]. However, the clinical relevance of this observation has still to be confirmed by clinical studies.

Another in vitro study published by Woodahl et al. evaluated the potential alterations in ABCB1-mediated transport of anticancer agents in relation to the ABCB1 1199G>A SNP [13]. The authors demonstrated that the recombinant (LLC-PK1) cells overexpressing either the variant or the wild-type protein displayed comparable doxorubicin resistance, suggesting no change in ABCB1 activity towards this drug. However, the ABCB1 1199A variant cells displayed greater resistance to vinblastine (as in our study), vincristine, paclitaxel, and etoposide (VP-16) in comparison to 1199G expressing cells, illustrating a potential increase in ABCB1 activity related to the variant 1199A allele towards those drugs. Taken together, these studies indicate that the effect of the ABCB1 1199G>A SNP seems to be substrate-specific (Table 1). Indeed, while wild-type ABCB1_1199G appears to be more efficient towards Tac, the ABCB1_1199A variant would be more active towards CsA, PIs (Amprenavir, Indinavir, Lopinavir, Ritonavir and Saquinavir) and four anticancer drugs (Vinblastine, Vincristine, Paclitaxel and Etoposide). As mentioned above, this hypothesis is supported by the fact that this coding SNP leads to the hypothesis of a substrate-dependent effect of the ABCB1 1199G>A SNP on the ABCB1 ability to transport its substrates. However, it appears clear that, for anti-cancer drugs in particular, these in vitro observations have to be validated from a pharmacokinetic point of view and obviously warrant further studies.

It is also important to mention the potential relevance of other ABCB1 SNPs. Indeed, the three most common SNPs in the protein coding region are rs1125053 (1256C>T, Gly412Gly), rs2032582 (2677G>T/A, Ala939Ser/Thr), and rs1045642 (3435C>T, Ile1145Ile) [18-19]. The three 1256C>T, 2677G>T and 3435C>T ABCB1 SNPs that are in high LD are particularly relevant to study from a clinical and therapeutic point of view. These SNPs are not only present at a high allelic frequency in the Caucasian population (about 50%) but have been also reported to have functional consequences on the activity of ABCB1 [20]. For instance, the ABCB1 3435C>T SNP has been associated with reduced ABCB1 expression and/or activity in the liver, the intestine, the kidney and the lymphocytes [9,21]. Moreover, several in vitro studies using recombinant expression models were performed and seem to confirm an impact of these three SNPs on ABCB1 transport activity and suggested an influence of these SNPs on pharmacokinetics of drugs [38,39]. However, until now, the in vivo impact of these three SNPs remain controversial and must be further investigated [22].

In summary, and in line with previous clinical observations, our in vitro results demonstrate that ABCB1 1199G>A SNP strongly decreases the ability of ABCB1 to transport Tac across cellular membranes, in sharp contrast to CsA. Our results also confirm the importance to investigate the true clinical significance of this SNP for Tac therapy and to assess its potential impact on the clinical outcome (i.e. graft rejection and toxicity) in prospective clinical trials. Finally, our study emphasizes the importance of systematically including the ABCB1 1199G>A SNP in every pharmacogenetic investigation involving ABCB1 substrates, especially those showing intracellular activity and/or toxicity.

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Author Contributions

Conceived and designed the experiments: GD LE JBD VH. Performed the experiments: GD LE NP AC. Analyzed the data: GD LE AD JBD VH. Contributed reagents/materials/analysis tools: JBD VH. Wrote the paper: GD LE JBD VH.

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