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SUBSTRATE-DEPENDENT EFFECTS OF HUMAN *ABCB1* CODING POLYMORPHISMS

Jason M. Gow, Laura M. Hodges, Leslie W. Chinn, and Deanna L. Kroetz

Department of Biopharmaceutical Sciences (JMG, LMH, LWC and DLK), University of California, San Francisco

Abstract

One of the many obstacles to effective drug treatment is the efflux transporter P-glycoprotein (P-gp), which can restrict the plasma and intracellular concentrations of numerous xenobiotics. Variable drug response to P-gp substrates suggests that genetic differences in *ABCB1* may impact P-gp transport. The current study examined how *ABCB1* variants alter the P-gp mediated transport of probe substrates *in vitro*. Nonsynonymous *ABCB1* variants and haplotypes with an allele frequency $\geq 2\%$ were transiently expressed in HEK293T cells and the transport of calcein-AM and bodipy-FL-paclitaxel was measured in the absence or presence of the P-gp inhibitor cyclosporin A. The Ala893Ser, Ala893Thr, and Val1251Ile variants and the Asn21Asp/1236C>T/Ala893Ser/3435C>T haplotype altered intracellular accumulation compared to reference P-gp in a substrate dependent manner. Interestingly, certain variants showed altered sensitivity to cyclosporin A inhibition that was also substrate specific. These functional data demonstrate that non-synonymous polymorphisms in *ABCB1* may selectively alter P-gp transport and drug-drug interactions in a substrate- and inhibitor-dependent manner.

Introduction

P-glycoprotein (P-gp) is an important efflux transporter that can influence the pharmacokinetics and pharmacodynamics of many drugs. Important barrier or excretory tissues express P-gp in an orientation that protects the body from harmful compounds and also limits access of drugs to target tissues (Fromm, 2004). The blood-brain barrier expresses P-gp and brain concentrations of P-gp substrates, such as digoxin and loperamide, are increased in *Abcb1a*^{-/-} mice (Mayer et al., 1996; Schinkel et al., 1996). P-gp activity in the intestines is a major determinant of oral bioavailability for many therapeutic compounds (Fromm, 2000) and modulators of P-gp, such as rifampicin, can change the bioavailability of P-gp substrates (Niemi et al., 2003). P-gp may also be important in limiting intracellular levels of drugs. The multidrug resistance phenotype seen in the treatment of cancer is often caused by an overexpression of P-gp in solid tumors (DeMario and Ratain, 1998; Litman et al., 2001).

Variability in drug response is widely observed for P-gp substrates. One potential mechanism for this variability is interindividual differences in P-gp activity that result in altered pharmacokinetics. The number of P-gp transporters on the cell membrane and the level of P-gp transport function are the two most important factors that control the apparent activity of P-gp. Genetic variation in *ABCB1*, which encodes P-gp, is thought to be one of the factors that influence P-gp expression and function. The statistical analysis of *ABCB1* genetic variation

Corresponding author/reprint requests: Deanna L. Kroetz UCSF Box 2911 1550 4th St. Rm RH584E San Francisco, CA 94158–2911 415–476–1159 (office), 415–514–4361 (fax) deanna.kroetz@ucsf.edu.

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data indicates that there is considerable nucleotide diversity in this gene (Kroetz et al., 2003; Leabman et al., 2003). A polymorphism that affects P-gp activity at the protein level most likely will be an amino acid changing nonsynonymous variant. Amino acid changes may alter key domains necessary for substrate binding, ATP hydrolysis, or protein folding. It is possible that synonymous or promoter region variants can influence the expression level of P-gp, as suggested by studies on the *ABCB1* 3435C>T synonymous variation (Wang et al., 2005), and thereby affect P-gp transport function. The majority of pharmacogenetic studies for P-gp have focused on two polymorphisms, the synonymous 3435C>T variation and the nonsynonymous 2677G>T (Ser893Ala) variation (Schwab et al., 2003; Marzolini et al., 2004; Pauli-Magnus and Kroetz, 2004; Salama et al., 2006; Schaefer et al., 2006). Unfortunately, there is no clear consensus as to how these polymorphisms affect P-gp at the *in vivo* or cellular level. Single nucleotide polymorphism (SNP) identification studies have discovered numerous other nonsynonymous and synonymous variants of *ABCB1* (<http://www.hapmap.org>). The polymorphisms at positions 2677 and 3435 may simply be markers for other functionally relevant sites that have yet to be investigated. Additionally, it is unclear as to the effects certain nonsynonymous P-gp variants have at the cellular level (Kimchi-Sarfaty et al., 2002; Woodahl et al., 2004; Woodahl et al., 2005). The improved design and analysis of P-gp pharmacogenetic research *in vivo* is reliant upon functional studies *in vitro*.

In these studies we investigated how seven nonsynonymous variants and two common haplotypes of *ABCB1* alter P-gp function in an *in vitro* assay that utilizes transient expression of P-gp in HEK293T cells. The fluorescent substrates calcein-AM and BODIPYFL-paclitaxel were selected to test the function of the P-gp variants. The data from these studies suggest that certain P-gp variants have altered function that is substrate specific. In addition, some variants have different sensitivities to the P-gp inhibitor cyclosporin A (CsA) that is also substrate dependent.

Methods

Materials

Fetal bovine serum (FBS) and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA). Calcein-acetoxymethylester (calcein-AM) and 4,4-difluoro-4-bora-3a,4-diaza-s-indacene (BODIPY-FL)-paclitaxel were purchased from Invitrogen and stored diluted in DMSO at -20°C in a desiccated container. Cyclosporin A was purchased from Sigma-Aldrich (St. Louis, MO) and stored in DMSO at 4°C. Murine IgG2a MRK16 antibody was obtained from Kamiya Biomedical Co. (Seattle, WA, USA) and goat anti-mouse IgG allophycocyanin (APC) from Invitrogen. Eagle's minimum essential medium (EMEM) with Earle's BSS and L-glutamine, non-essential amino acids, sodium pyruvate and antibiotics were prepared by the University of California, San Francisco Cell Culture Facility. The pcDNA5/FRT mammalian expression vector was obtained from Invitrogen and the pCIneo mammalian expression vector was purchased from Promega (Madison, WI).

Cell Culture

HEK293T cells were obtained from ATCC and maintained according to their instructions. Briefly, EMEM medium containing 10% FBS, 1% nonessential amino acids, 0.11 µg/mL sodium pyruvate, 100 µg/mL streptomycin and 100 units/mL penicillin was used to propagate the cells in 5% CO₂ at 37°C. Cells were maintained in T75 flasks and passaged every 2–3 days.

ABCB1 Plasmids and Transfection

ABCB1 nonsynonymous polymorphisms were identified previously (Kroetz et al., 2003). A full-length *ABCB1* cDNA in pCIneo was used as a template to create variant plasmids. Selected variants of *ABCB1* were created with the QuikChange site-directed mutagenesis kit

(Stratagene) according to the manufacturer's protocol. Primer sequences are listed in Table 1. Plasmid constructs for 2677T (893Ser) and the 1236T/2677T(893Ser)/3435T haplotype were made previously (Kroetz et al., 2003). The following variants were created from the reference *ABCB1* plasmid in pCIneo: 61A>G (Asn21Asp), 1199G>A (Ser400Thr), 1596T>G (a non-functional nucleotide-binding domain (NBD) mutant), 2005C>T (Arg669Cys), 2677G>A (Ala893Thr), 3421T>A (Ser1141Thr) and 3751G>A (Val1251Ile). The 61G(Asp21)/1236T/2677T(Ser893)/3435T construct was made from the 1236T/2677T(Ser893)/3435T plasmid using the 61A>G primers. All variants and the reference were verified by direct sequencing. Reference *ABCB1* was subcloned into the pcDNA5/FRT vector.

HEK293T cells were seeded in T25 flasks at 1.2×10^6 cells/flask in EMEM supplemented with 10% FBS, 1% nonessential amino acids and 0.11 $\mu\text{g/mL}$ sodium pyruvate, and allowed to grow for 24 hours. The quality of plasmid DNA was confirmed using a 260/280 nm absorbance ratio, and plasmid DNA integrity was verified by gel electrophoresis. Cells were transfected at a confluency of 70–85% with 9.2 μg pcDNA5/FRT, *ABCB1* reference plasmid DNA or variant plasmid DNA with 25 μL Lipofectamine 2000 in a final volume of 5 mL. Fresh medium was replaced ~five hours after the addition of transfection reagents.

Substrate Accumulation Assays

The accumulation assays were repeated a total of three times using 1.5 μM calcein-AM or 100 nM BODIPY-FL-paclitaxel in the absence or presence of 10 μM cyclosporin A. Triplicate samples were used for each P-gp variant in the absence and presence of cyclosporin A. After 24 hours, transfected cells were harvested using 0.05% trypsin and counted in order to achieve a concentration of 5×10^5 cells/sample in PBS. Cells were seeded in 96-well plates at 5×10^5 cells/well in 100 μL PBS and centrifuged at 150g for 3 minutes. The PBS was aspirated and 100 μL substrate with or without 10 μM cyclosporin A in PBS was added to resuspend the cells. Samples were allowed to incubate for 45 minutes in the dark at 37°C with a brief agitation period 25 minutes after substrate addition. All subsequent manipulations were done on ice with cold reagents or at 4°C for the centrifugation steps. Accumulation was stopped by centrifuging the cells at 150g for three minutes, followed by a PBS wash. The cells were resuspended in 75 μL of PBS containing 6.67 $\mu\text{g/mL}$ MRK16 primary antibody and incubated on ice in the dark for 30 minutes. After two wash steps, 60 μL of PBS containing 2.5 $\mu\text{g/mL}$ of secondary antibody with APC was used to resuspend the cells and the mixture was incubated on ice in the dark for 25 minutes. Cells were washed twice, resuspended and transferred into 5 mL tubes in 250–300 μL PBS.

Flow Cytometry

Cell samples were run on a dual-laser FACSCalibur machine (Becton-Dickinson, San Jose, CA) with excitation wavelengths at 488 and 635 nm controlled by CellQuest software (Becton-Dickinson, San Jose, CA). Emission filters at 530 nm (FL1) detected calcein-AM and BODIPY-FL paclitaxel, and >670 nm (FL4) detected APC fluorescence. Spectral overlap controls determined there was negligible interference of substrate fluorescence with the >670 nm filter. Initial studies with control murine and goat IgG antibodies showed there was no crossreactivity. A total of 15,000 events were counted and FlowJo software (Treestar, Ashland, OR) was used to analyze the flow cytometry data. Forward- and side-scatter analysis established the R1 gate for the viable, single-cell population. Substrate (FL1) and APC (FL4) fluorescence for the empty vector samples was determined from cells in the R1 gate. The P-gp positive gate was established in the FL4 channel of R1 and was set so that <0.5% of the empty vector population resided in the gate. Substrate and APC measurements for the P-gp transfected samples were determined from the R1/P-gp positive gate.

Statistical Analyses

Transfection efficiency was calculated in the FL4 channel (APC fluorescence) from the percentage of cells in the R1 gate that also resided in the P-gp positive gate. The P-gp expression level was determined from the median APC fluorescence of the cells in the R1/P-gp positive gate. Median calcein and BODIPY-FL-paclitaxel fluorescence values from the R1/P-gp positive cells were averaged from the three separate experiments for P-gp reference, variants and NBD mutant. The averaged fluorescence for the NBD mutant and each variant was converted to a percent of reference (set as 100) and this represented the substrate accumulation in the absence of cyclosporin A. Inhibitor sensitivity was determined from the median calcein and BODIPY-FL-paclitaxel fluorescence values in the presence of cyclosporin A for P-gp reference, variants and NBD mutant. The percent difference in fluorescence with and without inhibitor for P-gp reference, variants and the NBD mutant was calculated from equation 1.

$$\frac{(\text{Avg (+CsA fluorescence)} - \text{Avg (-CsA fluorescence)})}{\text{Avg (-CsA fluorescence)}} \quad \text{Eqn. 1}$$

Percent difference values for the variants and NBD mutant were normalized to reference. After three separate experiments, a one-way ANOVA followed by Student's *t*-test with Bonferroni's correction was used to calculate the statistical significance of the mean values calculated for substrate accumulation and percent difference of inhibitor.

Results

P-gp Variants

The coding regions and flanking intronic regions of *ABCB1* were previously screened for genetic variants in 247 ethnically diverse subjects from the Coriell Institute (Kroetz et al., 2003). Thirteen nonsynonymous sites were identified that span the entire region of the *ABCB1* gene and those chosen for functional analysis are shown in Table 2. The allele frequencies of these variants in Caucasians and African Americans range from 0.5 to 46.4% and three of the variants are ethnic specific. Amino acid sequence alignments were performed using orthologous amino acid sequences from six other mammalian species. The degree of conservation is often linked to the allele frequency; that is, the reference amino acid for low frequency (<1%) nonsynonymous SNPs tends to be more conserved across the different species. For residue position 21, neither the reference nor variant amino acid for humans is found in the other mammalian species. Grantham values were determined for the nonsynonymous variants to determine how drastic the amino acid change is in terms of chemical properties (Grantham, 1974). All of the amino acid changing variants studied here are located in the loop domains of P-gp (Kroetz et al., 2003). The amino acid altering variants that were studied were chosen based on an allele frequency greater than 2% in any one ethnic group, evolutionary conservation of the reference amino acid and/or a Grantham value >150 (Table 2). There are six polymorphic residues that meet these criteria: Asn21Asp, Ser400Thr, Arg669Cys, Ala893Ser/Thr, Ser1141Thr and Val1251Ile. Residue 893 is interesting because it is triallelic and occurs naturally in haplotypes with the two common synonymous SNPs 1236C>T and 3435C>T as well as Asn21Asp (Kroetz et al., 2003). As a result, two different haplotype constructs were also studied that contain Ser893, 1236T and 3435T with either Asn21 or Asp21.

Transient Expression of P-gp in HEK293T Cells

Fluorescence measurements of intracellular substrate levels in the P-gp reference and variant transfected HEK293T cells were only based upon the subpopulation of cells staining positively for surface P-gp expression with the MRK16 antibody. P-gp staining for empty vector, reference, NBD and variant P-gp expressing cells is shown with a "P-gp positive" gate superimposed to mark the population of cells overexpressing P-gp (Figure 1). Two important expression measurements are determined from the P-gp staining histogram: transfection

efficiency and P-gp expression levels. Transfection efficiency is the percentage of healthy cells located in the P-gp positive gate. Within an experiment and, for the most part, across experiments (N = 3), reference, NBD and variant P-gp samples have consistently similar levels of transfection efficiency (52–60%). The second measurement, P-gp expression level, is the median APC fluorescence of the cells that are P-gp positive. P-gp variant expression differs by less than 15% of reference levels. Although the empty vector has detectable APC staining, the endogenous P-gp levels in the HEK293T cells are ~100-fold less than the P-gp transfected cells. Consistent with the flow cytometry analysis, Western blots showed P-gp transfected HEK293T cells expressed a single, ~170 kDa protein (data not shown).

Calcein-AM Transport by P-gp Variants

Calcein-AM passively and rapidly enters the cell due to its high lipid solubility. High-capacity esterases cleave the acetoxymethylester group that simultaneously enables the calcein metabolite to fluoresce and prevents transport by P-gp (Hollo et al., 1994). Intracellular levels of calcein in the P-gp positive cells for reference and the variants was dramatically less than for the NBD mutant and empty vector (Figure 2a). In general, calcein levels for the P-gp variants were similar to that with the reference and no variant had a complete loss of function. Median calcein fluorescence values for all variant constructs were normalized to the reference sample, which was arbitrarily set at 100. The median calcein fluorescence of all variants ranged from 69–97% (Figure 2c). Variants 893Thr and 1251Ile showed increased function as evident from decreased intracellular calcein levels ($69 \pm 16\%$ and $75 \pm 13\%$ of reference ($p < 0.01$), respectively).

Intracellular calcein levels were also investigated in the presence of 10 μM cyclosporin A (Figures 2b and 2c) and the data were analyzed to show how sensitive each variant is to inhibition. The percent difference in intracellular fluorescence between control and cyclosporin A-treated cells was calculated individually for each variant and then normalized to reference which was arbitrarily set at 100. The NBD mutant has very high levels of calcein accumulation both in the absence and presence of cyclosporin A. As a result, the percent difference for the NBD mutant is very low ($2.1 \pm 0.9\%$) and this corresponds to negligible sensitivity to inhibition. In contrast, the reference and variant P-gp constructs have low calcein levels without cyclosporin A but high calcein levels with cyclosporin A. Most P-gp variants showed similar inhibition of calcein-AM transport compared to reference. Interestingly, the Asn21Asp, Arg669Cys and Ala893Ser variants are more sensitive to cyclosporin A ($136 \pm 28\%$, $139 \pm 43\%$, and $130 \pm 22\%$, respectively; $p < 0.05$) compared to reference (Figure 2c).

BODIPY-FL-Paclitaxel Transport by P-gp Variants

The P-gp positive cells expressing reference or any variant plasmid DNA had significantly less intracellular levels of 100 nM BODIPY-FL-paclitaxel than NBD or empty vector transfected cells (Figure 3a). In addition, the presence of cyclosporin A dramatically increased BODIPY-FL-paclitaxel levels (Figure 3b). Median BODIPY-FL-paclitaxel fluorescence values for all variant samples were normalized to reference, which was arbitrarily set at 100. The Asn21Asp, Ser400Asn, Arg669Cys, Ala893Thr variants and the 1236C>T/Ala893Ser/3435C>T haplotype were within 5% of the reference (Figure 3c). In contrast, the Ala893Ser and Val1251Ile P-gp variants and the Asn21Asp/1236C>T/Ala893Ser/3435C>T haplotype showed intracellular paclitaxel levels that were $114 \pm 13\%$, $118 \pm 12\%$ and $124 \pm 13\%$ of reference, respectively ($p < 0.01$), indicating decreased P-gp function.

P-gp transport of 100 nM BODIPY-FL-paclitaxel was also measured in the presence of 10 μM cyclosporin A. Three variants (Ala893Ser, Ala893Thr and Ser1141Thr) were $30 \pm 5\%$, $30 \pm 8\%$ and $27 \pm 17\%$, respectively, less sensitive to cyclosporin A inhibition than reference while Val1251Ile was $65 \pm 26\%$ less sensitive than reference ($p < 0.01$). The haplotype

containing Asn21Asp/1236C>T/Ala893Ser/3435C>T was $53 \pm 8\%$ less sensitive to cyclosporin A inhibition than reference ($p < 0.01$).

Discussion

The studies described above tested the effect of multiple genetic polymorphisms of *ABCB1* on P-gp function. The main focus is on common P-gp variants since interpatient variability associated with P-gp substrates is not likely caused by rare *ABCB1* variants. Six out of 13 previously described nonsynonymous variants were chosen for study based on an allele frequency $>2\%$: Asn21Asp, Ser400Asn, Ala893Ser, Ala893Thr, Ser1141Thr and Val1251Ile. The Arg669Cys variant has an allele frequency of only 1% in African Americans, but was chosen because the variant amino acid causes a drastic chemical change (Grantham value = 180). Interestingly, five variants with Grantham values <100 showed altered function and/or sensitivity to cyclosporin A inhibition, which suggests this metric may not be useful for predicting if a P-gp variant is functionally significant. Further studies involving the seven low frequency variants are warranted, but the results may not be as relevant at the clinical level. Two haplotypes commonly found in African Americans and Caucasians (Kroetz et al., 2003) were also investigated in this study. These haplotypes contained Ala893Ser, 1236C>T and 3435C>T either with or without Asn21Asp. While the synonymous 1236C>T and 3435C>T changes are not expected to directly affect transport function, they could have indirect effects due to changes in mRNA secondary structure and/or translation (Wang et al., 2005; Kimchi-Sarfaty et al., 2007).

Numerous expression systems have been used to measure the effects of P-gp polymorphisms on transport function. Stable cell lines yield a homogenous population of cells that overexpress P-gp (Kim et al., 2001; Morita et al., 2003; Woodahl et al., 2004; Salama et al., 2006). An obvious drawback is that creating stable cell lines can be very time consuming and in the end many cell lines will have P-gp transport that is similar to the reference transporter. The transient expression system we utilized has consistent transfection efficiency and P-gp expression levels that allow for a reproducible method to quickly screen P-gp variants against multiple substrates. Additionally, the endogenous P-gp expression in the HEK293T cells is negated by the substantial overexpression of P-gp in transfected cells.

P-gp function is characterized by the efflux of intracellular substrates. Direct measure of efflux is generally performed with transcellular transport assays that utilize polarized cells (Rautio et al., 2006). The assay used in the present studies involves incubation of transfected HEK293T cells with substrate and quantitation of intracellular substrate levels. It is assumed that substrate uptake is constant in the transfected cells and that differences in substrate accumulation reflect differences in efflux among the P-gp variants. Calcein-AM is a well-documented substrate with a high affinity for P-gp (Hollo et al., 1994). The Ala893Thr and Val1251Ile variants had lower intracellular levels of calcein, indicating increased efflux of calcein-AM by P-gp (Table 3). To our knowledge, the 1251Ile variant has not been studied previously, but recent data shows 893Thr has increased transport of vincristine (Schaefer et al., 2006). Residues 893 and 1251 are located in the intracellular loops adjacent to the 11th and 12th transmembrane domains (TMD11 and TMD12), respectively, which have been shown to be important for drug-binding and ATP-hydrolysis (Loo and Clarke, 1997; Loo and Clarke, 1999). It is possible that amino acid changes at these sites may alter important functions of TMD11 and TMD12. Previous work consistent with the current findings has shown Asn21Asp, Ser400Asn and Ala893Ser do not change calcein-AM transport (Kimchi-Sarfaty et al., 2002; Kroetz et al., 2003). The transport of digoxin by the Ala893Ser variant was originally reported to be increased (Kim et al., 2001), although a subsequent study found similar digoxin transport by the 893Ser and reference P-gp (Morita et al., 2003). Decreased function for 893Ser in accumulation and transepithelial flux assays has been reported for rhodamine-123, vinblastine and vincristine

(Salama et al., 2006). The Ala893Ser variant has been the focus of many *in vivo* and *in vitro* pharmacogenetic studies but it is still not clear if this variant is a causative SNP or merely a marker. In an LLC-PK1 stable cell line expressing 400Asn P-gp, rhodamine 123 transport was reduced while transport of the HIV protease inhibitors saquinavir and zidovudine was increased (Woodahl et al., 2004; Woodahl et al., 2005). This suggests that the Ser400Asn variation alters P-gp function in a substrate specific manner. Similarly, we found that the functional effects of Ala893Ser, Ala893Thr, Val1251Ile and Asn21Asp/1236C>T/Ala893Ser/3435C>T were substrate dependent. It is important in the design of clinical P-gp pharmacogenetic studies to consider these substrate-dependent effects of P-gp variants.

Paclitaxel is another well-established P-gp substrate (Rautio et al., 2006). The *ABCB1* Ser400Asn variant has been shown to confer higher drug resistance to paclitaxel but the stable cell line overexpressing this P-gp was selected for G418 resistance (Crouthamel et al., 2006). It is unknown what other background changes occur during cell line creation that could affect total cellular resistance. BODIPY-labeled paclitaxel was used in the current studies, and while it is possible that the BODIPY modification may alter interactions with P-gp, multiple studies, including ours, show the compound is transported to a high degree (Kimchi-Sarfaty et al., 2002; Robey et al., 2006).

Limited data exists on the effects of *ABCB1* genetic variation on BODIPY-FL-paclitaxel transport. In one study the Asn21Asp, Phe103Leu, Ser400Asn, Ala893Ser, and Ala998Thr SNPs and three double mutants (Asn21Asp/Ser400Asn, Asn21Asp/Ala893Ser and Ser400Asn/Ala893Ser) were investigated using a vaccinia virus expression system with BODIPY-FL-paclitaxel and no differences in function were noted among the variant and reference P-gps (Kimchi-Sarfaty et al., 2002). Our results are similar for Asn21Asp and Ser400Asn, but we found that Ala893Ser and Asn21Asp/Ala893Ser have decreased transport of BODIPY-FL-paclitaxel (Table 3). The percent change in function compared to reference for these hypofunctional variants is at most 24% and would be considered moderate. It should be noted that Val1251Ile P-gp displays decreased function with BODIPY-FL-paclitaxel but increased function with calcein-AM. The 1251 residue is evolutionarily conserved across five other mammalian species and is only found in the Mexican American population (Kroetz et al., 2003). There is no other data on the function of the Val1251Ile variant and testing additional substrates may elucidate the importance of this amino acid change.

Recently, a *Saccharomyces*-based assay tested nine nonsynonymous P-gp variants for cellular resistance to the antibiotic valinomycin and the anticancer drugs daunorubicin, doxorubicin, and actinomycin D (Jeong et al., 2007). The Arg669Cys variant showed highly increased function for all substrates, while our data showed no difference for calcein-AM and BODIPY-FL-paclitaxel. There was increased function for Ala893Ser and Ser1141Thr depending on the substrate, and in the current study the Ala893Ser variant reduced P-gp transport of BODIPY-FL-paclitaxel. These differences may reflect inherent disparities in the assays used for these measurements. Nonetheless, evidence from the *Saccharomyces*-based study highlights the importance of considering substrate-specific effects of P-gp variants.

Limited research is available on how an inhibitor mechanistically alters the interactions between P-gp and a substrate. Various investigations have shown substrates can bind to different P-gp domains (Dey et al., 1997; Rautio et al., 2006) but it is difficult to predict *a priori* what domains are important for each class of compounds. In addition, predictive 3D modeling of how an amino acid change will affect transport function is still in its infancy. We tested the hypothesis that an amino acid variant can alter sensitivity to an inhibitor and our results are supportive. Furthermore, the variants that function differently in the presence of cyclosporin A do so in a substrate dependent manner. The Asn21Asp, Arg669Cys and Ala893Ser P-gp variants show increased inhibition of calcein-AM transport by cyclosporin A.

In contrast, the Ala893Ser, Ala893Thr, Ser1141Thr variants and the Asn21Asp/1236C>T/Ala893Ser/3435C>T haplotype are less sensitive to cyclosporin A inhibition of BODIPY-FL-paclitaxel transport. The coadministration of paclitaxel and cyclosporin A for the treatment of various cancers is currently under consideration (Malingre et al., 2001; Kuppens et al., 2005), and the current data may improve the design and interpretation of such studies. Based on studies showing that (1) P-gp substrates bind different domains (Loo and Clarke, 2002; Loo et al., 2003; Loo et al., 2006b; Loo et al., 2006a) and (2) cyclosporin A is a competitive inhibitor that alters P-gp conformation while in the drug-binding pocket (Loo and Clarke, 1997; Loo et al., 2003; Ejendal and Hrycyna, 2005; Suzuyama et al., 2007), there may be a synergistic effect with these variants in how they influence drug binding in the presence of cyclosporin A.

In summary, P-gp variants and haplotypes were investigated for possible functional effects on P-gp expression and function. Intracellular accumulation of calcein-AM and/or BODIPY-FL-paclitaxel was altered by Ala893Ser, Ala893Thr, Val1251Ile and Asn21Asp/1236C>T/Ala893Ser/3435C>T. In addition, certain variants and haplotypes showed different sensitivities to cyclosporin A inhibition. Our *in vitro* study generated reproducible data regarding how a genetic variant influences transport function but the consequences at the *in vivo* level are not yet known. Unless a P-gp variant is characterized against multiple substrates it is difficult to predict the extent of its clinical significance. However, *in vitro* data continues to contribute to a better understanding of how P-gp operates at the molecular level, and may influence drug design and discovery.

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Non-standard abbreviations

P-gp, P-glycoprotein; SNP, single nucleotide polymorphism; FBS, fetal bovine serum; calcein-AM, calcein-acetoxymethylester; BODIPY-FL, 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene; APC, allophycocyanine; EMEM, Eagle's minimum essential medium; NBD, nucleotide binding domain; CsA, cyclosporin A; TMD, transmembrane domain.

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Figure 1.

Transient P-gp expression in HEK293T cells. After the accumulation period, HEK293T cells were stained for P-gp expression using a primary MRK16 antibody and a secondary APC antibody. The representative histogram of APC fluorescence for empty vector (gray, dotted), NBD mutant (gray), reference (black) and variant transfected cells (Asn21Asp, yellow; Ser400Asn, cyan; Arg669Cys, purple; Ala893Ser, green; Ala893Thr, orange; Ser1141Thr, pink; Val1251Ile, blue; 1236C>T/Ala893Ser/3435C>T, brown; and Asn21Asp/1236C>T/Ala893Ser/3435C>T, red) shows P-gp expression based on the intensity of APC fluorescence, as indicated on the x-axis. The P-gp positive gate is indicated by the bar and was determined as stated in the Methods section and represents the threshold for cells that overexpress P-gp.

Figure 2.

Certain nonsynonymous P-gp variants alter transport of calcein-AM in the absence or presence of cyclosporin A. Fluorescence of calcein as measured in the FL1 channel was determined for empty vector, P-gp reference, variants and NBD mutant. The data collected in the absence of cyclosporin A for empty vector (gray, dotted), P-gp reference (black), NBD mutant (gray), Ala893Thr (orange) and Val125Ile (blue) are shown in a representative fluorescence histogram (A). The effects of cyclosporin A on calcein-AM accumulation are displayed in a representative histogram for P-gp reference (black, shaded), Asn21Asp (yellow), Arg669Cys (purple) and Ala893Ser (green). Data for P-gp reference in the absence of cyclosporin A (black, not shaded) is shown for contrast (B). Calcein fluorescence data for all variants in the absence of cyclosporin A were normalized to reference, which was set at 100 (C, filled bars). P-gp variant sensitivity to cyclosporin A inhibition of calcein transport was calculated as the percent difference in intracellular fluorescence with inhibitor compared to no inhibitor, and expressed relative to reference (C, striped bars). Significant differences compared to reference in the absence ($p < 0.01$) or presence ($p < 0.05$) of inhibitor are marked with an asterisk. P-gp variants exhibiting decreased calcein transport (f); or increased (I) or decreased sensitivity to inhibition (i) are noted for clarity. Each bar represents the mean $\pm$ SD of three experiments.

Figure 3.

Certain nonsynonymous P-gp variants alter transport of BODIPY-FL-paclitaxel in the absence and/or presence of cyclosporin A. Fluorescence of BODIPY-FL-paclitaxel as measured in the FL1 channel was determined for empty vector, P-gp reference, variants and NBD mutant. The data collected in the absence of cyclosporin A for empty vector (gray, dotted), P-gp reference (black), NBD mutant (gray), Ala893Ser (green), Val1251Ile (blue) and haplotype Asn21Asp/1236C>T/Ala893Ser/3534C>T (red) are shown in a representative fluorescence histogram (A). The effects of cyclosporin A on BODIPY-FL-paclitaxel accumulation are displayed in a representative histogram in panel B for P-gp reference (black, shaded), Ala893Ser (green), Ala893Thr (orange), Ser1141Thr (pink), Val1251Ile (blue) and haplotype Asn21Asp/1236C>T/Ala893Ser/3534C>T (red). Data for P-gp reference in the absence of cyclosporin A (black, not shaded) is shown for contrast. BODIPY-FL-paclitaxel fluorescence data for all variants in the absence of cyclosporin A were normalized to reference, which was set at 100 (C, filled bars). P-gp variant sensitivity to cyclosporin A inhibition of BODIPY-FL-paclitaxel transport was calculated as the percent difference in intracellular fluorescence with inhibitor compared to no inhibitor, and expressed relative to reference (C, striped bars). Significant differences compared to reference are marked with an asterisk ($p < 0.01$). P-gp variants exhibiting decreased BODIPY-FL-paclitaxel transport (f), or decreased sensitivity to inhibition (i) are noted for clarity. Each bar represents the mean $\pm$ SD of three experiments.

Table 1Site-directed mutagenesis primers for *ABCB1* nonsynonymous variants

Base change	Mutagenesis primers ^a	
61A>G	Forward	5'-TTTAACTGAACGATAAAAGTGAAAAAG
	Reverse	5'-CTTTTTCACTTTTATCGTTCAGTTTAAA
1199G>A	Forward	5'-GAAATGTTCACCTCAATTACCCATCTCGAA
	Reverse	5'-TTCGAGATGGGTAATTGAAGTGAACATTTT
1596T>G	Forward	5'-GGGGCCCAGTTGAGGGGTGGGCAGAAAGCAG
	Reverse	5'-CTGCTTCTGCCACCCCTCAACTGGGCCCC
2005C>T	Forward	5'-GAAAAAGATCAACTTGTAGGAGTGTCCTGT
	Reverse	5'-CACGGACACTCCTACAAGTTGATCTTTTTC
2677G>A	Forward	5'-GAAAGAACTAGAAGGTACTGGGAAGATCGC
	Reverse	5'-GCGATCTTCCCAGTACCTTCTAGTTCTTTT
3421T>A	Forward	5'-CAGCCGGGTGGTGACACAGGAAGAGATTG
	Reverse	5'-CAATCTCTTCCTGTGTCAACACCCGGCTG
3751G>A	Forward	5'-GTTTCAGAATGGCAGAAATCAAGGAGCATGG
	Reverse	5'-CCATGCTCCTTGATTCTGCCATTCTGAAAC

^a In each primer the modified base position is underlined

Allele frequencies, evolutionary conservation and Grantham values for selected *ABCB1* nonsynonymous polymorphisms and haplotypes

Variant or haplotype		Allele frequencies (%) ^a		Amino acid conservation ^c		Grantham value ^d
		AA (n=200)	CA (n=200)	Reference	Variant	
Nucleotide change	61A>G	2.5	8	-	-	23
	1199G>A	1	2.5	d, ha, mk	ms, r	46
	2005C>T	1	0	d, ha, mk, r, s	-	180
	2677G>T	10	46.4	ha, ms, r	d, mk, s	99
	2677G>A	0.5	3.6	ha, ms, r	-	58
	3421T>A	11.1	0	d, ha, mk, ms, r, s	-	58
	3751G>A	0 ^b	0	d, ha, mk, ms, r	s	29
	1236C>T/2677G>T/3435C>T	6	34	N/A	N/A	N/A
	61A>G/1236C>T/2677G>T/3435C>T	2.5	8	N/A	N/A	N/A

^aMinor allele frequencies for African American (AA) and Caucasian (CA) populations. The number of chromosomes that were analyzed is indicated. Haplotype frequencies represent the percentage of chromosomes that have the respective predicted haplotype (Kroetz et al., 2003).

^bThe 3751G>A variant has an allele frequency of 5% in Mexican Americans (Kroetz et al., 2003).

^cDetermined from sequence alignment with dog (d), hamster (ha), monkey (mk), mouse (ms), rat (r) and sheep (s).

^dGrantham values were taken from Grantham (Grantham, 1974).

Table 3

Substrate- and inhibitor-dependent effects of P-gp variants on transport function

Variant or haplotype	Calcein-AM		BODIPY-FL-paclitaxel	
	-CsA ^a	+CsA ^b	-CsA	+CsA
Asn21Asp		↑		
Ser400Thr		↑		
Arg669Cys		↑		
Ala893Ser			↓	↓
Ala893Thr	↑			↓
Ser1141Thr				↓
Val1251Ile	↑		↓	↓
1236C>T/Ala893Ser/3435C>T				
Asn21Asp/1236C>T/Ala893Ser/3435C>T			↓	↓

^a Arrows indicate statistically significant changes in P-gp function relative to reference in the absence of cyclosporin A (-CsA).

^b Arrows indicate statistically significant changes in sensitivity to CsA inhibition relative to reference P-gp