Research Article

A Novel *MDR1* GT1292-3TG (Cys431Leu) Genetic Variation and Its Effect on P-glycoprotein Biologic Functions

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Abstract. P-glycoprotein (P-gp) is a membrane-bound transporter protein that is encoded by the human multidrug resistance gene *MDR1* (*ABCB1*). P-gp recognizes a wide range of xenobiotics, is pivotal in mediating cancer drug resistance, and plays an important role in limiting drug penetration across the blood–brain barrier. *MDR1* genetic variation can lead to changes in P-gp function and may have implications on drug pharmacokinetics. We have identified a novel *MDR1*_{GT1292-3TG} (Cys431Leu) genetic variation through systematic profiling of subjects with leukemia. The cellular and transport function of this variation was investigated with recombinant human embryonic kidney cells expressing *MDR1*. Compared with the wild type, *MDR1*_{GT1292-3TG} recombinant cells exhibited a lower drug resistance phenotype for a panel of chemotherapeutic agents. When compared with wild type, *MDR1*_{GT1292-3TG} recombinant cells exposed exhibited a 75% decrease in IC₅₀ for doxorubicin (162.6±17.4 to 37.9±2.6 nM) and a 50% decrease in IC₅₀ for paclitaxel (155.7±27.5 to 87.7±9.2 nM), vinblastine (128.0±15.9 to 65.9±5.1 nM), and vincristine (593.7±61.8 to 307.3±17.0 nM). The effects of the Cys431Leu variation, due to *MDR1*_{GT1292-3TG} nucleotide transition, on P-gp-dependent intracellular substrate accumulation appeared to be substrate dependent where doxorubicin, vinblastine, and paclitaxel exhibit an increased accumulation ($p<0.05$), while verapamil and Hoechst33342 exhibit a decreased intracellular concentration compared with wild type ($p<0.05$). Collectively, these data suggest *MDR1*_{GT1292-3TG} variation of P-gp may reduce drug resistance and that subjects with this genotype undergoing chemotherapy with drugs that are transported by P-gp could potentially be more responsive to therapy than those with *MDR1* wild-type genotype.

KEY WORDS: ABC transporter; drug resistance; genetic variation; *MDR1*; P-glycoprotein; polymorphism; transporter.

INTRODUCTION

P-glycoprotein (P-gp), an efflux transporter and a member of the ATP binding cassette (ABC) transporter super-family, resides in the plasma membrane and expresses in both tumor cells and normal cells. A number of cancer cells over express P-gp and can increase transcription of *MDR1* by drug and other compounds (1). The substrates of P-gp-mediated efflux transport include a wide range of natural compounds and lipophilic xenobiotics (2,3). The transport function of P-gp is driven by ATP hydrolysis; however, the stoichiometry of ATP to substrate ratio per transport cycle is yet to be confirmed.

P-gp is expressed on epithelial cells and exerts its largest effect on the pharmacokinetics of drugs in the intestinal mucosa, liver canalicular membrane, kidney proximal tubules,

blood–brain barrier, and placenta (4,5). P-gp is believed to function as a protective barrier against many chemotherapeutic agents and toxic xenobiotics by reducing intracellular and transcellular substrate accumulation. This can be seen through decreased intestinal absorption, enhanced biliary excretion and renal tubular secretion, and limited drug distribution to the fetus and brain. In the case of P-gp expressed in the blood–brain barrier, significant reduction or abolishment of the P-gp function has been demonstrated in mice to significantly increase anti-HIV and cancer drug availability to the brain (6,7). Thus, sequence variations in *MDR1* that potentiate significant change in transport function could affect the outcome of chemotherapy and the disposition of a broad range of xenobiotics.

We have previously reported the functional significance of the G1199A, G1199T, and G571A genetic variations using recombinant *MDR1* cells expressing respective P-gp variants (8–10). *MDR1*_{G1199A} and *MDR1*_{G1199T} variations result in an amino acid substitution at amino acid Ser400 in the first ATP binding domain cytoplasmic loop. Ser400 resides next to the highly conserved Tyr401 and is part of the A-loop responsible for π – π stacking with the adenine base of ATP (11,12). When compared with wild-type *MDR1*, *MDR1*_{G1199A}, resulting in a serine to asparagine transition,

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displayed greater resistance to vinblastine, vincristine, and paclitaxel with no significant difference with doxorubicin, while *MDR1*_{G1199T}, a serine to isoleucine transition, decreased resistance to all of these drugs. *MDR1*_{G571A} (Gly191Arg) is located in the third transmembrane (TM) domain of P-gp and decreased resistance to vinblastine, vincristine, and paclitaxel with no significant difference with doxorubicin.

The aim of the present study is to investigate the functional impact of a novel *MDR1* variant on P-gp transport and drug resistance function. To do so, we have developed and characterized a set of stable recombinant P-gp-expressing cells. These cells were used to investigate the role of the GT1292-3TG novel nucleotide variation on P-gp functions.

MATERIALS AND METHODS

Chemicals and Drugs

Doxorubicin, paclitaxel (Sigma-Aldrich, St Louis, MO), vinblastine (Bedford Laboratories, Bedford, OH, USA), vincristine (Faulding, Paramus, NJ, USA), and topotecan (GlaxoSmithKline, Research Triangle Park, NC, USA) were diluted with culture medium for the cytotoxicity. Multidrug resistance protein inhibitor GF120918 [*N*-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl) ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide] was kindly provided by GlaxoSmithKline. Rhodamine 123 (R123) was purchased from Sigma-Aldrich Co. Bodipy FL-vinblastine, Bodipy FL-verapamil, Paclitaxel-Oregon Green® 488, tissue culture media DMEM, trypsin-EDTA, and antibiotic solutions were supplied by Invitrogen Corp. (Carlsbad, CA, USA). Fetal bovine serum was obtained from Hyclone (Logan, UT, USA). Other reagents were of analytical grades or higher.

Patient *MDR1* Sequencing

Blood or bone marrow samples were used to extract RNA, which was reverse transcribed to obtain *MDR1* cDNA as previously described (13). The *MDR1* nucleotide sequence for each subject (with no identifiable clinical information under an approved human subject protocol by the University of Washington) was determined by directly sequencing the 3.8-kb cDNA in both sense and anti-sense direction with an automated DNA sequencer based on Big-Dye 3.0 chemistry (Applied Biosystems, Foster City, CA, USA). The sequence data were assembled and analyzed with the Vector NT program. Patient demographics were previously described (8).

Cell Culture

Human embryonic kidney cells, HEK293 (HEK) with or without P-gp expression, were grown at 37°C in complete media consisting of DMEM medium (Invitrogen) supplemented with 10% (v/v) fetal calf serum, 1% (v/v) antibiotic-antimycotic, and 1% non-essential amino acid, and under 5% CO₂.

Generation of *MDR1*_{wt} and *MDR1*_{GT1292-3TG} Plasmids

The method was similar to our previously reported method (8). Briefly, *MDR1* cDNA was cloned into a linearized pcDNA-TA vector (Invitrogen) containing cytomegalovirus (CMV) and

T7 promoters capable of transcription, also described previously (13). The mammalian expression plasmid containing the *GT1292-3TG* mutation was generated by site-directed mutagenesis (Stratagene, La Jolla, CA, USA). Details of this expression plasmid design to express P-gp in mammalian cells have been published (9). Clones were screened by restriction enzyme mapping, and the sequence was verified. The variant expression plasmid was designated as *MDR1*_{GT1292-3TG}.

Isolation of Stable Recombinant Cells Expressing *MDR1*_{wt} or *MDR1*_{GT1292-3TG}

Ten million HEK cells were transfected with 10 µg of either *MDR1*_{wt} and *MDR1*_{GT1292-3TG} plasmid by electroporation performed at 250 V and 975 µF in a Gene Pulser® II (Bio-Rad Laboratories, Hercules, CA, USA). Subsequently, cells were kept at 4°C for 10 min before returning to the original cell culture conditions. These cells were exposed to increasing concentrations of G418 (Calbiochem, San Diego, CA, USA), a neomycin derivative, up to 800 µg/ml to select for cells that express neomycin resistance under the direction of the same plasmid vector. The G418 resistance cells were verified to have stable, high levels of P-gp expression and further reduced to two to four clones for final selection. The stable clone that consistently expressed P-gp or variants was picked and used for functional studies and validation.

Western Immunoblot Analysis

Western immunoblot analysis was used to characterize molecular weight and verify P-gp protein expression in the recombinant cells. Control and recombinant P-gp-expressed cells were pelleted and washed in PBS, followed by cell lysis in 1% NP-40 in water. The cell lysate was centrifuged at 400× gravity to pellet the nucleus and unbroken cells. Protein concentration was measured by a microplate assay protocol (DC Protein Assay, Bio-Rad Laboratories). The cell lysate was then placed in Lamelli sample buffer containing SDS and β-mercaptoethanol. A 4–20% gradient SDS-PAGE gel was loaded with 2.5 µg protein per well and ran at 130 V for 60 min. The transfer to a PVDF membrane was run for 90 min at 90 V according to instructions for Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad Laboratories). Non-specific binding sites on the membrane were blocked with 5% evaporated milk in TTBS buffer (0.1% Tween 20; 20 mM Tris-HCl, 0.9% NaCl, pH 7.6). Subsequently, the membrane was incubated with the F4 (Sigma) anti-P-gp monoclonal antibody, followed by a secondary horse reddish peroxidase (HRP)-conjugated goat anti-mouse IgG₁ antibody. ECL-Plus reagent was used as a substrate, and blots were exposed to X-ray film to detect anti-P-gp reactive protein bands.

Rhodamine123 Efflux and Inhibition Assay

Recombinant cells expressing control and *MDR1* variants were seeded into 12-well tissue culture plates at 1 × 10⁶ cells per well and incubated at 37°C overnight. Medium was removed, and cells were preincubated with 0.5 mL of serum-free medium or medium containing 1 µM GF120918 for 5 min before replacing with 0.5 mL medium containing 1 µM R123 or 1 µM R123 plus 1 µM GF120918 and incubated for 30 min at 37°C.

Medium was removed, and the plates were washed twice with warm PBS. One half milliliter of medium or medium containing 1 μ M GF120918 was added, and efflux was performed at 37°C for 30 min. After extensive washing of the cells, cells were lysed with 0.5% deoxycholate (DOC). The intracellular R123 fluorescence (λ_{ex} =485 nm; λ_{em} =535 nm) was measured on a Victor III multiplate reader (PerkinElmer, Waltham, MA, USA).

Drug Sensitivity Assessment

The method was the same as described previously (8). Briefly, cells were seeded out in 96-well plates and grown overnight at 37°C. Varying concentrations of doxorubicin, paclitaxel, vinblastine, vincristine, and topotecan were added to the cells in quadruplicate. Cells were incubated with the indicated drugs for 72 h at 37°C. The viability was measured using CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (MTS) (Promega, Madison, WI, USA). The resultant effective concentration necessary for 50% cell growth inhibition (IC₅₀) for each drug was estimated using a sigmoid E_{max} model in SigmaPlot. The statistics between each group were analyzed with a one-way ANOVA followed by a Tukey post test.

Drug Uptake

Cells were plated at a density of 5.5×10^5 cells/well in 500 μ L in a 24-well plate (Corning) and grown overnight. The growth media were removed and replaced with 1 μ M R123, 5 μ M doxorubicin, 1 μ M Paclitaxel-Oregon Green® 488, 1 μ M Bodipy FL-vinblastine, 0.3 μ M Bodipy FL-verapamil, or 0.4 μ M Hoechst33342 in DMEM for 30 min at 37°C in the presence of 5% CO₂. Cells were washed three times with PBS and lysed in 50 μ L 0.5% DOC; 100 μ L PBS was added to each well, and the fluorescence was read on a Victor III plate reader. Results were normalized for protein content and expressed as percentage of drug uptake in HEK control cells. The statistics between each group were analyzed with a one-way ANOVA followed by a Tukey post test.

Crude Membrane Preparation

Cells were grown in T175 flasks in DMEM+10%FBS to 85% confluence. The cells were washed once with PBS. Cells were then washed with ice-cold hypotonic lysis buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 1 mM MgCl₂, 1 $\times$ protease inhibitor cocktail). The cells were then scraped off, incubated in lysis buffer for 10 min, broken by agitation, and centrifuged at 325 $\times$ g for 5 min at 4°C to pellet the nucleus and unbroken

cells. The supernatant containing the cell membrane was centrifuged at 20,000 $\times$ gravity for 60 min at 4°C. The membrane pellet was resuspended in resuspension buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 50 mM sucrose, protease inhibitor) and pelleted again at 20,000 $\times$ gravity for 60 min at 4°C. The resulting crude membrane pellet was resuspended in 0.2 mL resuspension buffer per T175. The Dc Protein Assay (Bio-Rad) was used to measure protein concentration.

ATPase Activity

In a 96-well plate, crude membrane (20 μ g protein) was incubated in 90 μ L assay buffer (50 mM Tris pH 7.4, 50 mM KCl, 5 mM sodium azide, 2 mM EGTA, 2 mM ouabain, 2 mM DTT, 10 mM MgCl₂, and 0.1% tween 20) with or without drug in the presence and absence of 300 μ M sodium orthovanadate for 5 min at 37°C. The ATPase reaction was started by adding 10 μ L of 50 mM MgATP for a 5 mM final concentration, and incubated for 45 min at 37°C. The reaction was stopped by addition of colorimetric phosphate detection solution (6% (w/v) SDS, 3% (w/v) ascorbic acid, 0.5 M HCl, and 0.5% (w/v) ammonium molybdate). After incubation for 10 min, unreacted molybdate was complexed with a solution containing 2% (w/v) Na₃ citrate, 2% (w/v) Na m-arsenite, and 2% (v/v) acetic acid. Absorbance was measured at 720 nm.

RESULTS

Identification and Validation of the GT1292-3TG Variation in Leukemia Patients

As part of an effort to identify *MDR1* genetic variations in leukemia (8), we sequenced the entire coding sequences of *MDR1* from myeloblast and bone marrow cells collected from leukemia patients. We found a novel GT→TG transition at the 1292-3 nucleotide positions that predicted to produce a Cys→Leu modification of P-gp at amino acid 431. The novel variant 1292-3TG is found to have an estimated frequency of 1.35% and an RNA copy number of $1,126 \pm 137$ copies per nanogram of RNA. The patient carrying the GT1292-3TG variation did not have any other coding region variation within the *MDR1* gene. The RNA copy number is significantly higher ($p < 0.01$) and roughly 2.5-fold higher than the wild-type samples (Table I).

Development and Characterization of Recombinant Cells Expressing *MDR1* and Variants

Following construction of the expression plasmid carrying *MDR1* and its variants, the plasmids were transfected into the

Table I. Allelic Frequency of *MDR1* 1292-3 Variants in Leukemia Subjects

Sequence/variation	Amino acid variation	Estimated allelic frequency in leukemia patients	Copies per ng RNA
Wild type	Cys 431	0.9865	459 $\pm$ 293
GT 1292-3 TG	Cys 431 Leu	0.0135	1126 $\pm$ 137*

The RNA from 74 leukemia patients was collected, and the *MDR1* RNA transcripts were reverse transcribed into complementary DNA. These *MDR1* DNA products were sequenced and analyzed to assign the allele for each individual subject. These data were validated by direct sequencing of DNA from the same subject, and were presented as overall allelic frequencies

* $p < 0.01$ compared with wild type

MDR1 negative mammalian host HEK cells. Cells carrying the *MDR1*_{GT1292-3TG} gene with a high degree of expression similar to that of the previously developed wild-type expressing cells were expanded. Western blot was used to characterize the molecular weight of the protein and the degree of expression in *MDR1*_{wt} and *MDR1*_{GT1292-3TG} stable recombinant HEK cells. The apparent molecular weight of P-gp expressed in representative recombinant cells expressing *MDR1*_{wt} and *MDR1*_{GT1292-3TG} was recorded at approximately 150 kDa, and the level of P-gp expression was equivalent (Fig. 1). Western immunoblot analysis was also performed on the crude membrane fraction to ensure that the expressed P-gp protein was located on cell membrane.

For initial assessment that the expressed P-gp was functional, efflux of R123 was measured after an initial loading of R123 and subsequent incubation in fresh growth media to allow the efflux of R123. These recombinant cells exhibited reduced intracellular accumulation of the P-gp probe substrate R123 and were reversible with addition of a P-gp inhibitor GF120918 (Fig. 2). To evaluate the stability and reproducibility of the recombinant *MDR1* cell lines with respect to the functional stability of P-gp expression, we employed doxorubicin as a P-gp substrate and measured the reduction of intracellular levels due to P-gp expression. This assay was performed on cells prior to freezing in liquid N₂ for storage and on cells recovered after 4 months in storage. We found that these *MDR1* recombinant cells recovered from liquid N₂ exhibit similar characteristics in their ability to reduce intracellular concentration of doxorubicin, a P-gp substrate, with no statistical difference. These cells were stable for more than 2 months of passage in culture (Table II).

Evaluation of the Functional Impact of the GT1292-3TG Variant on *MDR1*-Dependent Drug Resistance

A panel of chemotherapeutic agents that included vinblastine, vincristine, doxorubicin, paclitaxel, and topotecan was used to generate dose–response profiles in the recombinant HEK cells. From these profiles, an IC₅₀ value was calculated. As presented in Table III, *MDR1*_{GT1292-3TG}-expressing cells exhibited increased sensitivity to vinblastine, vincristine, doxorubicin, and paclitaxel compared with

Fig. 1. Comparison of P-gp protein expression in recombinant *MDR1* and host HEK cells. Total cell lysates from recombinant cell expressing *MDR1*_{WT} (lane 2), *MDR1*_{GT1292-3TG} (lane 3), or *MDR1* negative HEK host (lane 1) were separated by gel electrophoresis, then blotted onto a PVDF membrane. The membrane was probed with the F4 anti-P-gp monoclonal antibody, developed with a ECL reagent, and exposed to X-ray film to detect anti-P-gp reactive protein bands at ~150 kDa

Fig. 2. Effects of P-gp inhibitor GF120918 on rhodamine123 efflux in preloaded P-gp recombinant cells. Recombinant cells expressing control and *MDR1* variants were seeded into 12-well tissue culture plates in quadruplicate and grown overnight at 37°C. Medium was removed and preincubated with serum-free medium or medium containing 1 μM GF120918 for 5 min before replacing the medium with 1 μM R123 or 1 μM R123 plus 1 μM GF120918 and incubated for 30 min at 37°C. Medium was removed, and the plates were washed with warm PBS. Serum-free medium or medium containing 1 μM GF120918 was added and incubated at 37°C for 30 min. After extensive washing of the cells, cells were lysed with 0.5% DOC. The intracellular R123 fluorescence (λ_{ex} =485 nm; λ_{em} =535 nm) was measured on a Victor III multiplate reader. White bars rhodamine123, black bars rhodamine123+GF120918

*MDR1*_{wt}, shown by a decrease in drug concentration needed to inhibit growth by 50%.

Expression of *MDR1*_{wt} was able to significantly increase the drug resistance of the HEK host cells doxorubicin 12-fold, vinblastine 20-fold, paclitaxel, 65-fold, and vincristine 88-fold (all $p < 0.001$). While still significantly higher than the HEK host cells, the IC₅₀ values for recombinant cells expressing *MDR1*_{GT1292-3TG} were approximately half of that in cells expressing *MDR1*_{wt} for vinblastine ($p < 0.001$), vincristine ($p < 0.001$), and paclitaxel ($p < 0.005$). Additionally, the IC₅₀ value was decreased by approximately 75% for doxorubicin ($p < 0.001$). This *MDR1*-mediated effect was specific in that the control drug substrate topotecan, a predominantly BCRP substrate, and poor P-gp substrate, exhibited a similar degree of sensitivity between the two *MDR1*-expressing cells.

Intracellular Substrate Accumulation in *MDR1*_{wt} and *MDR1*_{GT1292-3TG} Recombinant Cells

To determine whether *MDR1*_{GT1292-3TG} efflux transport activity is modulated when compared with wild-type P-gp, we evaluated the steady-state levels of intracellular uptake of R123, doxorubicin, Paclitaxel-Oregon Green® 488, Bodipy FL-vinblastine, Bodipy FL-verapamil, and Hoechst33342 (Fig. 3). *MDR1*_{wt}-expressing cells showed a significantly greater reduction in drug uptake compared with *MDR1*_{GT1292-3TG} for R123 ($p < 0.01$), doxorubicin ($p < 0.001$), paclitaxel ($p < 0.05$), and vinblastine ($p < 0.05$). Conversely, there was a significantly lower intracellular accumulation of verapamil ($p < 0.001$) and Hoechst33342 ($p < 0.05$) for *MDR1*_{GT1292-3TG} compared with *MDR1*_{wt} (Fig. 3). Collectively, the intracellular uptake of P-gp substrate profile suggests that the *MDR1*_{GT1292-3TG} variation may increase overall intracellular R123 and cancer drug levels while it reduces the other P-gp substrates Hoechst33342 and verapamil.

Table II. Stability and Reproducibility of P-gp Function in Parent and *MDR1*_{WT} and *MDR1*_{GT1292-3TG} Recombinant Cells

	Doxorubicin (percent of HEK host cells)		
	HEK host cells	<i>MDR1</i> _{wt} (431 Cys)	<i>MDR1</i> _{GT1292-3TG} (431Leu)
Pre-banked cells ^a	100±2.3	61.8±2.8	78.3±3.9
37 days after thawing ^b	100±7.5	62.9±1.7	79.6±1.7
61 days after thawing ^b	100±4.4	61.7±4.0	80.1±3.7

The recombinant cells expressing *MDR1*_{wt} or *MDR1*_{GT1292-3TG} were exposed to 5 μ M doxorubicin for 30 min at 37°C and the doxorubicin concentration were determined. These data were normalized for protein content and expressed as percentage of drug uptake in the HEK host cells. Data expressed were a mean±SD for quadruplicate

^a Cells were banked in 10% DMSO in DMEM+10% FBS growth medium

^b Cells were thawed and passaged following 4 months of storage in liquid N₂

ATPase Activity

ATPase activity was measured to determine if the change from cysteine to leucine at amino acid 431 in the nucleotide binding domain (NBD) would alter the ability of P-gp to hydrolyze ATP to ADP. We used crude membrane preparations in our assay to determine the vanadate sensitive basal activity. Our results showed that the wild-type and GT1292-3TG variant exhibited comparable vanadate sensitive basal activities of 2.93 ± 0.22 and 2.93 ± 0.34 nmol/mg protein⁻¹ min⁻¹, with HEK (-) control cells exhibiting an activity of 1.80 ± 0.21 nmol/mg protein⁻¹ min⁻¹.

DISCUSSION

A number of clinically used chemotherapeutic agents are substrates of P-gp, and expression of P-gp in cancer cells and tumors is a primary cause of drug resistance. Interindividual variation in P-gp activity, due to variation in the *MDR1* sequence, may influence distribution and delivery of anticancer agents to target cells and tissues. Based on our *MDR1* sequence variation analysis, we discovered the novel *MDR1*_{GT1292-3TG} nucleotide variation that translates to an amino acid substitution from cysteine to leucine at position 431. We have shown that the recombinant cell system expressing *MDR1*_{wt}, *MDR1*_{G1199A}, *MDR1*_{G1199T}, or *MDR1*_{G571A} is a highly reliable and consistent method suitable for evaluating functional changes in P-gp due to sequence variation (8–10,14).

In this study, we used *MDR1*_{GT1292-3TG} recombinant HEK cells that express a P-gp variant, with a Cys to Leu substitution at amino acid 431, to evaluate functional changes. Cys431 is

located within the conserved Walker A sequence in the NBD. All ABC transporters, including P-gp, have an ATP binding domain, which includes the A-loop, Walker A, Signature, and Walker B conserved sequences (15). The Walker A region spans the eight amino acid sequence “GxxGxGK(S or T)”, where x is any residue (16). Although Cys431 of the Walker A sequence is not highly conserved among ABC transporters, it is conserved in mammalian P-gp (17).

The Walker A sequence is proposed to form a loop that non-covalently binds to the β and γ phosphates of di- and trinucleotides, and is essential for ATP hydrolysis. It was proposed that Cys431 and Cys1074 (the corresponding cysteine in the second NBD) were responsible for inhibitory disulfide cross-linking between NBDs in the absence of reducing agents, and addition of ATP was able to protect from this modification (18,19). However, recent data based on structural models predict a 35-Å distance between the two cysteine residues, in the absence of nucleotide, that make disulfide bonding improbable (15,20).

The Cys-less P-gp mutant created by Loo and Clarke replaced the seven endogenous cysteines with alanine and retained its ability to confer resistance to multiple drugs compared with wild type (21). In addition, the Cys431Ala mutation did not impact ATPase activity. When the cysteine was re-inserted at amino acid 431 as the only cysteine within the Cys-less (Cys431Ala) P-gp molecule, covalent modification of the thiol side chain with *N*-ethylmaleimide inhibited activity of P-gp, suggesting that both NBDs must be active for function (22). In our studies, we also found that both Cys431 and Leu431 (which does not have the ability to form a disulfide bond) exhibit comparable ATPase activity, consistent with the

Table III. Effects of GT1292-3TG Variation on the Cytotoxicity of Chemotherapeutic Agents in *MDR1* Recombinant Cells

Drug	IC ₅₀ (nM)		
	HEK host cells	<i>MDR1</i> _{wt} (431 Cys)	<i>MDR1</i> _{GT1292-3TG} (431Leu)
Vinblastine	6.32±0.795	128.0±15.9*	65.91±5.1*,**
Vincristine	6.69±1.21	593.7±61.8*	307.3±17.0*,**
Doxorubicin	12.75±0.99	162.6±17.4*	37.93±2.6*,**
Paclitaxel	2.35±0.875	155.7±27.5*	87.69±9.2*,**
Topotecan	13.35±3.71	42.07±8.5*	37.36±3.6*,**

The recombinant cells expressing *MDR1* wild type or variants, along with *MDR1*-negative HEK host cells, were seeded in a 96-well plate. They were exposed to varying concentrations of vincristine, vinblastine, doxorubicin, paclitaxel, or topotecan for 72 h, and cell viability was determined. The dose–response curve for each drug was fitted to estimate IC₅₀ values, as described in “Materials and Methods”. The mean and variations from four replicates were expressed as mean and standard deviation

p*<0.001 compared with HEK host cells; *p*<0.001 compared with *MDR1*_{wt} recombinant cells

Fig. 3. Comparison of intracellular substrate concentration in cells expressing *MDR1*_{WT} and *MDR1*_{GT1292-3TG}. Cells were seeded out into 96-well plates and grown overnight. The growth media was then removed and replaced with 1 μ M rhodamine123 (*R123*), 5 μ M doxorubicin (*Dox*), 1 μ M Paclitaxel-Oregon Green® 488 (*Pac*), 1 μ M Bodipy FL-Vinblastine (*VB*), 0.3 μ M Bodipy FL-Verapamil (*Ver*), or 0.4 μ M Hoechst33342 (*H*) in DMEM for 30 min at 37°C. Cells were washed three times with cold PBS and lysed in 0.5% DOC. The respective drug concentrations were determined based on fluorescent intensity measurement and expressed as a percentage of control host HEK cells without *MDR1*. Results were normalized for protein content. Data expressed are mean and standard deviation of quadruplicate samples. *Black columns* HEK (-) control, *hatched columns* *MDR1*_{WT}, *dotted columns* *MDR1*_{GT1292-3TG}. * $p < 0.05$; ** $p < 0.01$

results of the Cys-less mutant study results. Although a direct comparison of Cys431Ala and Cys431Leu with WT P-gp in HEK recombinant cells remained, such a study is beyond the scope of this report. Regardless, mutating amino acids G427, G430, K433, and S434 within the Walker A sequence do modulate ATPase activity (15), to our knowledge, no study shows that mutations to Cys431 result in a drastic change in ATPase activity.

Due to the conservation of Cys431 within mammalian P-gp, and lack of any previously reported genetic variation at Cys431 within the general population, we hypothesized that the GT1292-3TG genetic variation would result in altered function of P-gp. Our cytotoxicity studies showed that Cys431Leu substitution resulted in a significant increase in sensitivity to chemotherapeutic drugs. The largest decrease in drug resistance was found with doxorubicin. Doxorubicin resistance was lowered by greater than 75%, while resistance was lowered by slightly less than half for vinblastine, vincristine, and paclitaxel (Table III).

The significance of estimating functional impacts of genetic variation using stable recombinant cells expressing P-gp and variants should not be underestimated. We first reported the impact of *MDR1*_{G1199A} genetic variation with recombinant HEK cells to document the increased efflux of chemotherapeutic drugs, resulting in decreased intracellular concentrations of drug, and higher drug resistance in cytotoxicity studies (8). This finding was followed up by Green *et al.* when they analyzed the *MDR1*_{G1199A} variation in ovarian cancer patients treated with paclitaxel and carboplatin to evaluate their survival predictive value *in vivo* (23). Two of the 51 patients they sequenced were heterozygous for the G1199A allele. These two patients' mean progression free survival was only 2 months as compared with 19 months for the wild-type patients. This was

consistent with our prediction that the G1199A variation could potentially decrease the effectiveness of chemotherapy.

However, *MDR1* variations, including GT1292-3TG, may also influence other prognostic factors in addition to the disposition of the chemotherapeutic drugs. These factors may be important in the progression of disease. Without subsequent clinical data for a patient with GT1292-3TG, a definitive conclusion cannot be made as to the impact of the variation to clinical response. This is especially true as we found a greater than 2-fold increase in RNA in the GT1292-3TG patient sample. The increased quantity of transcription of the *MDR1* gene in this patient, who is heterozygous for the GT1292-3TG and wild-type *MDR1*, may offset the decreased transport. These and other possibilities remain to be evaluated.

The results of the drug uptake studies in HEK cells correlated well with the resistance data in the cytotoxicity study. The *MDR1*_{GT1292-3TG}-expressing cells had a higher intracellular accumulation of R123, doxorubicin, paclitaxel, and vinblastine than the wild type, with doxorubicin showing the largest difference (Fig. 3). Although the intracellular concentration of vinblastine in our uptake study was significantly higher in the GT1292-3TG than the wild-type-expressing cells ($p < 0.05$), their values were very close. This is in comparison with the cytotoxicity data where there is a much larger (~50%) decrease in IC₅₀ from the *MDR1*_{wt} to the *MDR1*_{GT1292-3TG}-expressing cells. The uptake assay was performed over a 30-min period, whereas cytotoxicity assay was evaluated over a 4-day period. Therefore, small differences in uptake after a 30-min incubation of vinblastine may not fully predict the magnitude of the cumulative effect of prolonged drug exposure. The decrease in intracellular doxorubicin and paclitaxel in the *MDR1*-expressing cells were better predictors of resistance. For doxorubicin, the intracellular concentration

dropped to 62% of HEK control cells in the MDR1 wild type, with the GT1292-3TG cells only reducing the intracellular by half that amount to 80%. For paclitaxel, the intracellular concentration dropped to 56% of HEK control cells in the MDR1 wild type, with the GT1292-3TG cells reducing the intracellular concentration to 71% of HEK control.

Conversely, for verapamil and Hoechst33342, the intracellular concentration in the *MDR1*_{GT1292-3TG}-expressing cells was less than the wild type. The substrate-specific differences in intracellular accumulation do not appear to link with ATPase activity. Our studies of the ATPase activity showed no difference between the wild-type P-gp and Cys431Leu variant.

The substrate-specific discrepancy in uptake may be explained by the change in TM domain conformation during the transport cycle (24) together with the presence of multiple drug binding sites within the P-gp protein (25–28). Different conformations of the TM domains of P-gp have been detected by binding ATP, binding AMP-PNP (a non-hydrolyzable ATP analogue), during ATP hydrolysis, and through mutation of the catalytic carboxylates within the NBD (24). Using cysteine-scanning mutagenesis, Loo and Clark showed that the packing of the TM segments was changed when different substrates were bound, revealing different residues to the drug binding pocket (28). It is suggested that P-gp undergoes further conformational changes of the TM domains resulting from ATP hydrolysis, finally resulting in drug transport (29).

There are a number of reported structural models that describe drug binding with varying resolutions and confidence. Garrigues *et al.* have stated that there are two pharmacophores located very close together that partially overlap (25). Their results strongly suggest verapamil, cyclosporin A, and actinomycin D bind to P-glycoprotein at one site in the binding domain of P-gp; the binding of vinblastine and tentoxin defined another pharmacophore.

Another proposed model for drug binding within the P-gp protein is the “R” and “H” site model (26,27). The R site binds preferentially R123, daunorubicin, doxorubicin, and other anthracyclines, while the H site binds Hoechst33342, quercetin, and colchicines. Additionally, these two sites have approximately equal affinity for vinblastine, etoposide, and dactinomycin (actinomycin D). Doxorubicin and verapamil were shown to be non-competitive inhibitors (30) and thus proposed to bind to differing regions of the P-gp drug binding pocket. Shaprio and Ling (26) proposed that based on their data, and that of Spoelstra *et al.* (30), verapamil binds preferentially to the H site. In addition, Loo *et al.* found that the binding sites for verapamil and Hoechst33342 do not overlap with rhodamine in the P-gp drug binding pocket (31). Our data for the uptake studies support these proposals as we have similar substrate groupings as the R and H site model. With the GT1292-3TG variation, when compared with wild type, R123 and doxorubicin have increased uptake, Hoechst and verapamil have decreased uptake, and vinblastine has a minor difference in uptake. Clearly, the impact of GT to TG transition at 1292-3 appeared to reduce efflux transport of substrates within the proposed R site and increase those within the proposed H site. While our results are consistent with the R and H sites model, the impact of the GT1292-3TG variation on the Substrate Induced Fit model, as proposed by Loo *et al.* (28), is not known.

In our *MDR1*_{GT1292-3TG} variant, there are no amino acid changes within the TM segments; the *MDR1*_{GT1292-3TG}

variation results in a Cys431Leu substitution in the ATP binding domain. Yet, our data suggest that the differences between drugs that had increased transport and decreased transport correlate well with current models of the drug binding pocket. We hypothesize that the Cys431Leu change may affect the communication between the NBD and the TM domains, resulting in altered conformation of the TM segments during ATP binding and/or ATP hydrolysis. This may result in altered drug transport in a substrate-specific manner, affecting each drug binding site in a different way. We believe that this is not due to the loss of the Cys residue, but due to the transition to a larger, hydrophobic Leu residue. These and other possibilities remain to be investigated. Regardless, genetic transition from GT to TG at 1292-3 of *MDR1* coding sequence will likely have a significant impact on chemotherapeutic agents that are substrates of P-gp, and the extent and direction of impact appeared to be substrate dependent and may relate to whether the compound binds to different sites within the P-gp drug binding pocket.

It must also be noted that this Cys431Leu variation is the result of two mutations occurring simultaneously at nucleotides 1292 and 1293. Single variation at only 1292 or 1293 has not been reported, nor seen in our patient population. If these mutations were to occur independently of one another, they would result in a Cys431Phe transition (G1292T) or Cys431Trp transition (T1293G). The reported 1292-3 variation found in leukemia subjects are yet to be defined in the healthy general population using a larger sample size. Regardless, if our hypothesis is correct in that the change in P-gp-mediated resistance and uptake is due to the increase in size and hydrophobicity of the resulting amino acid 431 compared with Cys, we believe that the Cys431Phe and Cys431Trp may have similar results to the Cys431Leu transition, and remains to be investigated.

CONCLUSION

We investigated a novel human *MDR1*_{GT1292-3TG} (Cys431Leu) variation. Using P-gp recombinant cells expressing *MDR1*_{WT} and *MDR1*_{GT1292-3TG}, functional impacts of this variant on cytotoxicity and drug uptake were analyzed. Sensitivity to our chemotherapeutic drugs was increased in the *MDR1*_{GT1292-3TG}-expressing cells, shown by a decrease in IC₅₀ value, when compared with wild-type-expressing cells. Drug uptake studies showed that the effect of the Cys431Leu variation results in a substrate-specific effect where doxorubicin, vinblastine, and paclitaxel had an increased uptake, while verapamil and Hoechst33342 had a decreased uptake compared with wild type. These differences may be due to small steric changes in the NBD that do not have an effect on ATPase activity, but translate to changes in the drug binding pocket, and warrants further research. These data suggest that patients with *MDR1*_{GT1292-3TG} may be more responsive than their wild-type counterparts, when given chemotherapeutic agents containing doxorubicin, paclitaxel, vinblastine, or vincristine.

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