A Novel Human Multidrug Resistance Gene MDR1 Variant G571A (G191R) Modulates Cancer Drug Resistance and Efflux Transport

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ABSTRACT

The human multidrug resistance gene MDR1 encodes a membrane-bound transporter P-glycoprotein (Pgp) that confers the drug resistance of cancer cells by mediating an ATP-dependent drug efflux transport. We and others have reported a number of functionally significant MDR1 variants, including G1199A and G1199T, that modulate cancer drug resistance and intracellular levels of antivirals. In this report, we describe a novel G571A variant of MDR1 detected in 6.4% of leukemia patients. Because this nucleotide modification gives rise to an amino acid change from Gly to Arg at the 191 amino acid position of Pgp, we have developed and characterized the functional affect of the G571A variant in stable, recombinant cells. Using six chemotherapeutic drugs, doxorubicin HCl, daunorubicin HCl, vinblastine sulfate, vincristine sulfate, taxanes (paclitaxel), and epipodophyllotoxin (etoposide, VP-16), we found that the MDR1571A variant selectively reduced the degree of Pgp-mediated resistance in drug-dependent manner. Although there was a minimal effect on doxorubicin and daunorubicin, the MDR1-dependent resistance on vinblastine, vincristine, paclitaxel, and etoposide was reduced by approximately 5-fold. The increased drug sensitivity in MDR1571A, compared with MDR1wt, paralleled the intracellular drug levels. These data suggest that individuals with this novel MDR1 variant, the 571A genotype, may be more sensitive to the specific anticancer drugs that are Pgp substrates.

The human multidrug resistance gene (MDR1 or ABCB1) encodes the P-glycoprotein (Pgp). As one of the ATP-binding cassette superfamily of transporters, Pgp is expressed broadly in epithelial tissues throughout the body, including intestine, liver, kidney, blood-brain barrier, and placenta (Schinkel et al., 1997). As a transmembrane protein, the active efflux drug transporter, Pgp, recognizes a large number of natural compounds and lipophilic xenobiotics (Lin and Yamazaki, 2003). Substrates of Pgp include anticancer, cardiovascular, anti-HIV, immunosuppressant, and β-adrenergic drugs. Because of the broad substrate recognition, it has been a challenge to predict effects of disrupting Pgp-mediated transport. Nevertheless, Pgp expression has been demonstrated to influence the treatment of cancers, infectious diseases (HIV, tuberculosis) (Camus et al., 2006), inflammatory bowel disease (Annese et al., 2006), and organ transplantation (Pauli-Magnus and Kroetz, 2004). Thus, variation in Pgp functions may lead to significant clinical consequence.

The human MDR1 spans over 100 kb on human chromosome 7q21. The MDR1 genome contain 29 exons that give rise to a 3843-bp sequence of transcripts encoding the 1280-amino acid Pgp protein with a molecular mass of 170 kDa. Pgp has 12 transmembrane domains and two ATP binding sites (Gottesman et al., 1996; Ambudkar et al., 1999). Variations of MDR1 sequences have been well studied, and a long list of single nucleotide polymorphisms (SNPs) has been reported; however, their functional affects are not yet fully understood (Woodahl and Ho, 2004). The high-frequency MDR1 SNPs include C1236T (exon 12), G2677T or A (exon 21), and C3435T (exon 26). Some of these SNPs are genetically linked. Their effects on the protein function have been studied in vivo and in vitro (Kim et al., 2001a). In a recent study, MDR1 SNPs that do not alter amino acid sequence

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ABBREVIATIONS: MDR1 or ABCB1, the human multidrug resistance gene; Pgp, a membrane-bound transporter P-glycoprotein that confers the drug resistance of cancer cells; SNP, single nucleotide polymorphism; MDS, myelodysplasia; AML, acute myelogenous leukemia; HEK, human embryonic kidney; GF120918, Elacridar; [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]; DMEM, Dulbecco’s modified Eagle’s medium; RT, reverse transcriptase; PCR, polymerase chain reaction; R123, rhodamine 123; PBS, phosphate-buffered saline; DPBSG, Dulbecco’s phosphate-buffered saline plus glucose containing medium.
also have been shown to result in functional variations. These sequence variations seemed to slow down Pgp translation, leading to a change in protein conformation and altered substrate specificity (Kimchi-Sarfaty et al., 2007).

Pgp is also expressed in leukocytes, hematopoietic stem cells, and leukemia cells. The high degree of MDR1 expression contributes to multidrug resistance in patients with leukemia. In particular, genetic polymorphisms of MDR1 have been found to affect therapeutic outcome in acute myeloid leukemia patients (Illmer et al., 2002). Our ongoing effort to identify functionally significant MDR1 genetic variations in the coding sequences of MDR1 in leukemia patients with either myelodysplasia (MDS) or acute myelogenous leukemia (AML) has led to the discovery of a number of novel variations that exhibit functional impacts. We found that the MDR1 G1199A variation, which results in a serine-to-asparagine transition at amino acid 400 in a cytoplasmic domain of Pgp, alters efflux and transepithelial transport and the drug sensitivity to chemotherapeutic agents (Woodahl et al., 2004). The MDR1 G1199T, on the other hand, exhibits some degree of reversal on cancer drug resistance compared with the wild-type MDR1.

In this report, we have characterized a new MDR1 variation, G571A, which has given rise to G191R amino acid transition. This MDR1 variation, located in exon 7 and mapped to within the third transmembrane domain of Pgp, exhibits 6.4% frequency in the MDS and AML patient population. Using stable recombinant HEK host cells expressing either MDR1 wild-type or MDR1 571 G→A variant, we have systematically characterized the functional differences between the MDR1wt and MDR1571A cells. Our data indicate that the MDR1571A variant selectively reduced the degree of Pgp-mediated resistance in a drug-dependent manner.

Materials and Methods

Chemicals and Drugs. All chemotherapeutic agents used for this study, doxorubicin HCl, daunorubicin HCl, vinblastine sulfate, vincristine (VCR), paclitaxel, and etoposide, were obtained through the drug service of the University of Washington Medical Center. Each drug was diluted with culture medium to a wide range of concentrations. The multidrug resistance protein inhibitor GF120918 (Elacridar) was kindly provided by GlaxoSmithKline (Cambridge, La Jolla, CA) was used for this purpose. The primer sequences were as follows: forward, 5'-GTATGGTG CGACAAATT AGAA-AGTCTT TCACGTC-3' and reverse, 5'-GACTGAAAGAACATTCTA ATTTGTGCACAAAC-3'.

The generated clones were screened by size exclusion and restriction enzyme digestion. The full length of the cDNA containing mutation clones was verified by DNA sequencing using our DNA sequence center facility (Big-Dye 3.0 Chemistry and an ABI Prism 377 DNA Sequencer; Applied Biosystems).

Development and Characterization of Stable Recombinant HEK Cells Expressing Human MDR1wt or MDR1571A Variant. The pCDNA3.1 vector and MDR1wt and MDR1571A plasmids were transfected into the mammalian HEK cells. In brief, HEK cells were seeded into a 24-well plate. Transfection was conducted when the cells reached approximately 90% confluence. The growth medium of the cells was replaced with serum-free medium (Opti-MEM; Invitrogen) and the cells were allowed to grow in a growth medium containing serum. At 24 h after transfection, cells from one or two wells were trypsinized and plated into a 100-mm Petri dish at a dilution of approximately 1:20 in a DMEM growth medium. At 48 h post-transfection, G-418 at 500 μg/ml was added. The medium containing G-418 was refreshed every 3 to 4 days for 3 to 4 weeks, and cells that survived under G-418 selection pressure were used subsequently for clone selection. Single cell colony was picked into a 12-well plate. Cells grown from the single colony were seeded into new 12-well plates, and rhodamine 123 (R123) uptake and efflux were conducted to select the polyclonal cells with high Pgp activities. The cells with high Pgp activity were further expanded and tested. Cells with high Pgp activity stable for at least three times for R123 uptake were stored and used for functional evaluation.

Characterization of Pgp Expression by mRNA Quantitation. Total RNA from HEK cells transfected and MDR1wt and MDR1571A variant were extracted using the Ultraspec total RNA isolation kit according to the manufacturer’s instruction (Biotech Laboratories, Inc., Houston, TX). To compare the expression level, absolute quantitation of MDR1 mRNA transcripts in transfected cellular samples was performed using the ABI Prism 7900HT Detection System (Applied Biosystems). An MDR1 RNA standard was generated by in vitro transcription using the T7 promoter on the plasmid described previously (Yang et al., 2002b), and concentration
was measured by absorbance at 260 nm and converted to the number of copies of MDR1 RNA by the mol. wt. A dilution series of the MDR1 RNA standard was used to generate a standard curve of the number of copies of MDR1 mRNA versus threshold cycle value. After RNA isolation, 1 μg of total RNA of each sample was subjected to RT. Fifty nanograms of total RNA was analyzed in quadruplicate to obtain a threshold cycle value and an estimation of the number of copies of MDR1 mRNA in recombinant HEK cells. To validate the assay, a housekeeping gene, hGUS, was detected at the same time to eliminate the sample variation.

Characterization of Pgp Expression at the Protein Level. Immunoblot analysis was used to detect protein expression. In brief, approximately 1 × 10⁶ cells were pelleted and washed in PBS and lysed in a lysis buffer containing 1% Nonidet P40 and a protease inhibitor cocktail (Calbiochem). Protein concentration was measured by a bicinchoninic acid microplate assay protocol (Pierce Chemical, Rockford, IL), and all samples were diluted into the same concentration using the lysis buffer and mixed with a 5× loading buffer containing 0.1% SDS and β-mercaptoethanol. Electrophoresis and transfer of membrane protein were done according to instructions for Mini-PROTEAN II Electrophoresis (Bio-Rad, Hercules, CA). Proteins were transferred onto polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA) and blocked with 5% nonfat milk in Tris-buffered saline/Tween 20 buffer (20 mM Tris-HCl, 0.9% NaCl, 0.05% Tween 20, pH 7.6). Immunoblotting was performed with the anti-Pgp monoclonal antibody C219 or F4, followed by a secondary horseradish peroxidase-conjugated goat anti-mouse IgG. ECL plus reagents (GE Healthcare, Chalfont St. Giles, UK) were used as substrate, and blots were exposed to X-ray film for visualization of the protein bands.

Cellular Localization of Bodipy-Labeled Vinblastine, Paclitaxel, and Doxorubicin in MDR1 Recombinant Cells. Control and recombinant MDR1 HEK cells (wild type and 571 variant) were seeded onto eight-cell chamber slides for 2 days at 37°C. Cells were briefly washed with PBS. Doxorubicin HCl (7 μM), bodipy FL-vinblastine (1 μM), and paclitaxel (Tubulin Tracker green reagent, 1 μM) were added to the cell in duplicate in a chamber containing serum-free DMEM and incubated for 30 min. The untreated control wells were filled with the medium only. The cells that were grown in the glass slides were washed three times with ice-cold PBS and dried. They were analyzed using a fluorescence microscope (Zeiss Axiovert 200; Carl Zeiss GmbH, Jena, Germany), and the presented images were recorded under a fixed exposure and identical settings.

Evaluation of Cancer Drug Resistance. The drug resistance of stable recombinant cells expressing MDR1wt and MDR1571A transfected was evaluated by measuring the cell viability after exposing them to a wide range of the selected substrates following our previous description (Woodahl et al., 2004; Yang et al., 2004). In brief, the cells (control, MDR1wt, MDR1571A) were seeded at 1 × 10⁴ density/well in a 96-well plate format in a 100-μl culture medium overnight. Media were replaced with 100 μl of media containing a wide range of dilution of selected chemotherapeutic agents alone or with a Pgp inhibitor, GF120918. Each concentration of the drug was run in quadruplicate at least, or experiments were repeated at least twice. The culture was maintained at 37°C 5% CO2 in a humidified incubator for 72 h. Cell viability was measured with a CellTiter-Glo cell viability assay kit (Promega). Data from each quadruplicate was converted to the percentage of their controls with 0% inhibition at zero concentration of the drug and maximal inhibition at the highest concentration. IC₅₀ values were obtained using SigmaPlot software. The relative resistance ratio was calculated comparing the IC₅₀ value of MDR1wt to the IC₅₀ value of control host cells. These data were replicated at least twice to validate the IC₅₀ values. The variations experiments were less than 10%.

Pgp-Mediated Efflux Transport Studies Using Rhodamin123 as a Substrate. Cells were seeded into 24-well plates precoated with poly-d-lysine at 5 × 10⁶well and incubated for 3 days at 37°C until 95% confluence. Cells were washed briefly with Dulbecco’s phosphate-buff- ered saline plus glucose containing medium (DPBSG), pH 7.2, and loaded with 0.5 ml of DPBSG or inhibitor for 10 min. The DPBSG or inhibitor was then replaced with 1 μM rhodamine 123 or rhodamine 123/inhibitor in DPBSG buffer for 30 min at 4°C. After moving the rhodamine123, 1 ml of DPBSG or DPBSG with inhibitor at 37°C was added into each well, and efflux was started in a water bath at 37°C. At the indicated time point, the cells were washed twice with ice-cold DPBSG. One percent Triton X-100 (0.5 ml) was added into each well. The amount of intracellular rhodamine123 was measured using a flu- orometer (PerkinElmer Victor3V, PerkinElmer Life and Analytical Sciences, Waltham, MA) at λ ex = 485 nm and λ em = 530 nm.

Results

A Novel MDR1 Variant, 571 G→A Variant in Leukemia Patients. As an ongoing project to explore the effects of MDR1 sequence variation in functional impact of Pgp, we collected mononuclear cells from leukemic patients undergoing bone marrow evaluation. We then sequenced the entire coding sequence of the cDNA derived from the RNA samples. These sequences were validated with direct sequence analysis of the genomic DNA for the respective regions. Of 78 samples, we found a novel variant in five subjects exhibiting the heterozygous genotype, G571G/A. The genotypic fre-

Table 1

<table>
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<tr>
<th>Number of Subjects</th>
<th>MDR1 G571 Genotype</th>
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<tbody>
<tr>
<td></td>
<td>GG</td>
</tr>
<tr>
<td>78</td>
<td>73</td>
</tr>
<tr>
<td>Genotype frequency (%)</td>
<td>93.6</td>
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</tbody>
</table>

Table 1. Frequency of human MDR1 571 genotypes in leukemia patients

Total RNA from peripheral blood mononuclear cell was used two times, or bone marrow of patients was extracted and amplified by RT-PCR. The PCR product was purified and sequenced. Data were analyzed and compared with the human MDR1 sequence (GenBank accession no. M14758).

Fig. 1. A representative nucleotide sequence alignment of human MDR1 from wild type and a subject with the 571 A/G genotype. RNA from patients was amplified using RT-PCR. The RT-PCR fragment was sequenced and aligned with the human MDR1 database (GenBank accession no. M14758) using Vector NTI (Invitrogen). One of the subjects exhibiting G/A at 571 was presented. The upper part of the chromatograph comes from a patient exhibiting MDR1 wild type at the 571 position.
quency was recorded at 6.4%, and the allelic frequency for the 571A was estimated to be 3.2% (Table 1). A representative of the sequence chromatograph from patients showing the A/G overlapping is presented in Fig. 1. The G→A transition at position 571 translates into an amino acid change from Gly to Arg at position 191 of the human MDR1 protein Pgp. Based on the predicted Pgp structure, it is mapped to exon 5 and predicted to be located within the third transmembrane domain. Such variation is likely to modulate the Pgp efflux transporter function. Hence, we developed stable, recombinant cells to evaluate functional significance of this novel transporter function. Hence, we developed stable, recombinant HEK cells to evaluate functional significance of this novel transporter function.

**Development and Characterization of an MDR1 Recombinant Cell Expressing G571A.** To evaluate the functional impact of the novel MDR1 variant, 571 G→A (191 Gly→Arg amino acid), we expressed it in a mammalian host, HEK cells. The stable recombinant HEK cells were selected with G-418 before full characterization with respect to the MDR1 sequence and protein expression. The function of the stable recombinant cells expressing G571A was compared with previously established recombinant HEK cells expressing MDR1 wild type (517G at the 191 amino acid position).

By direct sequencing confirmation of the MDR1 DNA isolated from the stable recombinant HEK cells, we found that these cells faithfully retained the G571A variant with no other sequence modifications. Using fluorescence R123 efflux assay, we identified three to five clones that exhibited consistent Pgp functions for at least 2 months. The Pgp protein expression levels were evaluated by immunoblot analysis. As shown in Fig. 2, the selected HEK recombinant cells expressing G571A exhibited comparable levels of Pgp protein and RNA transcript (based on mRNA quantitation) with the cells expressing wild-type MDR1. Furthermore, immunofluorescent staining of the cells using the MDR1 monoclonal antibody F4 exhibited similar profile of Pgp expression for both in MDR1 wild type and G571A, and expression levels are significantly higher than those of the control cells (data not shown). Both MDR1 wild type and G571A exhibited similar molecular mass (~160 kDa) using Western immunoblot analysis (Fig. 2). These cells were used for all subsequent functional analyses.

**Effects of G571A Variant on Pgp Efflux Function and Pgp-Dependent Drug Resistance.** To determine the effects of MDR1 571 G→A transition on Pgp transporter function, we first evaluated the intracellular concentration of HEK recombinant cells expressing either MDR1wt or MDR1G571A. Expression of MDR1 in HEK host cells reduced the intracellular levels of a fluorescence substrate R123 by 37 and 33% in MDR1wt and MDR1G571A variant, respectively (Table 2). However, the differences in intracellular concentration of R123 between the recombinant HEK cells expressing MDR1wt and MDR1G571A were not statistically significant. The reduction in intracellular R123 levels was due to Pgp expression because this function can be reversed with an established Pgp inhibitor, GF120918. In the presence of 1 μM GF120918, both Pgp-expressing cells retained R123 levels similar to that of Pgp-negative control cells (Table 2).

We next determined the effect of 571 G→A transition on cancer drug resistance and we compared cytotoxicity using six commonly used drugs that fall within four classes of chemotherapeutic agents that are known substrates of Pgp. HEK recombinant cells expressing MDR1 variants were exposed to these compounds at varying concentrations for 3 days. Cell survival was recorded and shown in Table 3. We found that the expression of MDR1wt or MDR1G571A conferred varying drug resistance to the HEK cells. The record range was from 10- to 150-fold for the drugs tested. For the two anthracyclines, doxorubicin and daunorubicin, insignificant differences were found between MDR1wt and MDR1G571A-expressing cells. However, effects of 571 G→A transition significantly altered the drug resistance profiles for vinblastine, vincristine, paclitaxel, and etoposide. The G→A transition slightly reduced the MDR1 resistance to vincristine and vinblastine. In contrast, recombinant MDR1G571A-expressing cells exhibited approximately 4-fold reductions in resistance to paclitaxel.

**TABLE 2**

<table>
<thead>
<tr>
<th>Recombinant Cells</th>
<th>Intracellular R123 Concentration</th>
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<tbody>
<tr>
<td></td>
<td>Without Inhibitor</td>
</tr>
<tr>
<td>Control</td>
<td>1.94 ± 0.63</td>
</tr>
<tr>
<td>MDR1wt</td>
<td>3.14 ± 0.68</td>
</tr>
<tr>
<td>MDR1G571A</td>
<td>4.10 ± 0.63</td>
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these cells in the presence or absence of 1 doxorubicin, vincristine, and paclitaxel were incubated with for 3 days. Cell viability was measured by chemiluminescent assay as described. Student’s MDR1 transition at the 571 nucleotide position of twice. Taken together, these data indicate that the These data are reproducible and were replicated at least 3 in 3 days. Cell viability was measured by chemiluminescent assay as described. Student’s t test analysis was used to compare MDR1 wt and MDR1571A variants. Relative resistance (R value) was obtained by dividing the IC50 value with the value of the corresponding control from each drug tested. 

<table>
<thead>
<tr>
<th>Drug</th>
<th>Control: IC50</th>
<th>MDR1wt (191Gly)</th>
<th>MDR1571A (191Arg)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>IC50</td>
<td>R Value</td>
<td>IC50</td>
</tr>
<tr>
<td>nM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>25.1 ± 2.55</td>
<td>250.2 ± 24.5</td>
<td>9.971</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>22.3 ± 1.40</td>
<td>241.1 ± 13.8</td>
<td>10.81</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>3.2 ± 2.5</td>
<td>142.6 ± 13.5</td>
<td>45.56</td>
</tr>
<tr>
<td>Vincristine</td>
<td>4.1 ± 0.80</td>
<td>630.0 ± 72.0</td>
<td>153.66</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>8.5 ± 1.38</td>
<td>382.0 ± 21.6</td>
<td>44.94</td>
</tr>
<tr>
<td>Etoposide</td>
<td>99.6 ± 9.95</td>
<td>2518.3 ± 288.15</td>
<td>25.28</td>
</tr>
</tbody>
</table>

* P < 0.05.  
** P < 0.01.

* Cells were seeded at 1 × 10^6/well in a 96-well plate. Chemotherapeutic drugs were serially diluted in culture medium, put into each well in quadruplicate, and exposed for 3 days. Cell viability was measured by chemiluminescent assay as described. Student’s t test analysis was used to compare MDR1 wt and MDR1571A variants. 

**Effect of a Pgp Inhibitor on Reversing MDR1-Dependent Efflux Transport and Drug Resistance.** To further characterize the time course of Pgp efflux transport variations for the MDR1571A variant and to verify the effect of Pgp, we employed GF120918 as a Pgp inhibitor (Hyafil et al., 1993). GF120918 has been shown to be a potent inhibitor of Pgp but also inhibits breast cancer resistance protein. As the HEK host cell did not express a significant endogenous level of breast cancer resistance protein, GF120918 is used presently as a specific Pgp inhibitor. We proceeded to determine the time course of transport efflux in recombinant HEK cells expressing either MDR1wt or MDR1571A. The recombinant cells expressing MDR1wt exhibited a faster decline to lower intracellular R123 concentrations compared with that of MDR1571A, suggesting that Pgp derived from MDR1571A is less efficient in efflux transport of this substrate (Fig. 3). The time and the MDR1 dependent decline in R123 activity was Pgp dependent because the efflux activity was reversed by the presence of 1 μM GF120918. The GF120918 did not influence the time course of intracellular R123 in control host cells.

We next determined whether the Pgp inhibitor GF120918 could reverse drug resistance in HEK cells expressing MDR1wt or MDR1571A. To do so, varying concentration of doxorubicin, vincristine, and paclitaxel were incubated with these cells in the presence or absence of 1 μM GF120918. Fifty percent effective inhibitory concentrations (IC50 values) were derived from a respective dose-response curve. As shown in Fig. 4, the IC50 values for the three drugs were reduced significantly in the presence of GF120918 irrespective of whether the recombinant cell expressed MDR1wt (Fig. 4A) or MDR1571A (Fig. 4B). Taken together, these data suggest that both R123 efflux transports and higher IC50 values

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![Fig. 3](https://i.imgur.com/3.png)
observed for cancer drugs in the recombinant cells expressing MDR1wt or MDR1571A are Pgp dependent.

**Differential Accumulation of Doxorubicin, Vinblastine, and Paclitaxel in the Recombinant MDR1 Cells.** To evaluate whether the reduction in intracellular drug concentration is a potential mechanism of differences in IC50 values recorded for the wild type and 571A variant of MDR1 recombinant cells, we surveyed the intracellular localization and distribution of three fluorescence anticancer drugs. Doxorubicin (a fluorescent compound), bodipy fluorescent-labeled vinblastine, and paclitaxel (labeled with tubulin tracker green) were used for this purpose. All three compounds produce green fluorescence for evaluation of drug localization and distribution. After exposing the cells to each of the three compounds for 30 min at 37°C, they were recorded with a fluorescence microscope and photographed at fixed exposure parameters. As shown in Fig. 5, doxorubicin, a DNA-interacting drug widely used in chemotherapy, exhibited a high degree of drug localization in the nucleolus but did not seem to produce a differential staining pattern between the two MDR1 variants. Bodipy FL-vinblastine is a fluorescent analog of the vinblastine that inhibits cell proliferation by capping microtubule ends, whereas Paclitaxel bis-acetate provides green fluorescent staining of polymerized tubulin in living cells, specifically, paclitaxel binds to the β-subunit of tubulin. Therefore, both compounds are good candidates for our staining purpose to visualize the drug uptake and distribution in the cells. For vinblastine and paclitaxel, fluorescence intensity of these two drugs in control cells was much higher than the two MDR1 recombinant cells. Parallelized with the differences in IC50 values, the green fluorescence for vinblastine and paclitaxel in 571A variant cells was somewhat brighter than the MDR1 wild-type cells under identical imaging conditions, suggesting a more efficient efflux transport function in cells expressing MDR1wt. Together, these results collected with the three fluorescent anticancer compounds in MDR1 recombinant cells suggest that variations in intracellular drug levels probably lead to reduced drug resistance in MDR1571A, compared with MDR1wt.

**Discussion**

As a continued effort to elucidate the pharmacogenetic impact of MDR1 sequence variations on the drug disposition and pharmacokinetics, we discovered a novel genetic variant G→A transition at 571 nucleotide position of MDR1 in humans. This genetic variation translates into a Gly→Arg transition at the 191 amino acid position of Pgp. This variant was identified as heterozygous in five of 78 leukemia patients, representing a 3.2% allelic frequency (Table 1). Whether the same frequency exists in healthy subjects or patients with other types of diseases remains to be determined. With the aid of well characterized stable recombinant cells expressing MDR1 (191Gly) and the variant (191Arg), we found that both express a similar degree of functional Pgp on cell surfaces. Depending on the cancer drug tested, the variant seemed to be exhibit efflux transport and related drug resistance at equal or lower potency. Although the exact mechanism leading to substrate dependent variations is not clear for the Pgp transition at 191 Gly→Arg, it is possible that the net positive charge may contribute to the altered efflux of Pgp function. Amino acid 191 is mapped to the third transmembrane domain, where the added positive net charge may regulate the substrate affinity and transport. Although this is plausible, Arg in the same position may also alter overall conformation of Pgp leading to 4- to 5-fold IC50 reduction of vinblastine, vincristine, paclitaxel, and etoposide. However, in the absence of Pgp protein crystal, the conformational details at atomic levels remain elusive.

Previous studies using site-directed mutagenesis to probe Pgp functions also found selective alterations of transport function for specific drug substrates (Loo and Clarke,
versus 88.4. The influence of MDR1
paclitaxel, etoposide, vincristine, or vinblastine than those
imply that the patients with
to overcome chemoresistance in other diseases. Our results
and pharmacologic optimization in leukemia treatment and
be used in the future to provide more effective drug selection
ance or elimination. This and other functional results could
including those in gut mucosa, liver, and kidney, in drug clear-
MDR1 is expressed widely in healthy cells and tissues, in-
also be more susceptible to dose-limiting toxicities because
2000; Loo et al., 2003). The observed differential sensitivity
to cytotoxic agents because of
amino acid sequence variations (Martin et al.,
1993a,b, 1994a,b). It is generally believed that multiple bind-
ing domains in the drug-binding pocket exist for Pgp, which
may account for the drug-specific alteration in Pgp efflux
because of amino acid sequence variations (Martin et al.,
2000; Loo et al., 2003). The observed differential sensitivity
cytotoxic agents because of 571 G→A may be important in
modulating the efficacy of chemotherapy. The cancer pa-
ents with MDR1 571A or 571A/G genotype will probably be
more sensitive to vinblastine, vincristine, and paclitaxel and
equally sensitive to doxorubicin or daunorubicin. However,
subjects exhibiting MDR1 571A or 571A/G genotypes will also be more susceptible to dose-limiting toxicities because
MDR1 is expressed widely in healthy cells and tissues, in-
cluding those in gut mucosa, liver, and kidney, in drug clear-
ance or elimination. This and other functional results could
be used in the future to provide more effective drug selection
and pharmacologic optimization in leukemia treatment and
to overcome chemoresistance in other diseases. Our results
imply that the patients with 571A may be more responsive to
paclitaxel, etoposide, vincristine, or vinblastine than those with
the wild-type 571G genotype.

Fig. 5. Intracellular or intracellular accumulation of doxorubicin, vinblastine, or paclitaxel analogs in recombinant MDR1 HEK cells as shown by fluorescence intensity and distribution. Control and MDR1 recombinant HEK cells were incubated with doxorubicin (A, D, and G), bodipy-PL-
viblastine (G, E, and H), or paclitaxel (C, F, and I) for 30 min at 37°C in a chamber slide. Cells were washed with PBS three times and viewed under fluorescence microscopy and photographed at constant exposure for all slides (1000× magnification for doxorubicin and 400× for vinblastine and paclitaxel). Less accumulation of doxorubicin, vinblastine sulfate, or paclitaxel by exhibiting weak fluorescence was observed in both wild-type and 571 variants compared with control cells. The result was consistent with the drug cytotoxicity data and R123 efflux data. A to C, HEK host cells; D to F, MDR1 wt recombinant cells; G to I, MDR1 571A recombinant cells. Intracellular drug concentrations, estimated based on mean ± S.D. fluorescence intensity (arbitrary units for equivalent cell surface area) for MDR1 wt and MDR1 571A recombinant cells are as follows: doxorubicin = 93.1 ± 3.3 versus 88.4 ± 0.8; vinblastine = 44.4 ± 0.5 versus 72.7 ± 0.51 (p < 0.005); and paclitaxel = 37.7 ± 1.8 versus 101.2 ± 2.15 (p < 0.05).
expression or activity of Pgp, with which it has been reported to be associated. In addition, the effect of \textit{MDR1} haplotypes can also be evaluated in this system because multiple SNPs can be expressed at once in the same plasmid. Multiple SNPs found on the same chromosome are assigned to a specific haplotype, and some attempts have been made to determine the role of \textit{MDR1} haplotypes in Pgp variability, but the data are still inconclusive (Kim et al., 2001b; Johne et al., 2002; Kroetz et al., 2003). Our expression system may be useful in sorting out the role of \textit{MDR1} haplotypes. By design, this continuous system focuses on genetic modifications in the coding region of \textit{MDR1} that alter the structure of Pgp. Other strategies specifically designed to evaluate the effects of promoter and intronic SNPs may be needed to address regulation of \textit{MDR1} expression.

In summary, we report here a novel polymorphism of \textit{MDR1} G571A found in the leukemia patient. This \textit{MDR1} polymorphism confers moderate drug resistance to recombinant HEK cells but is less efficient than that of \textit{MDR1} wild type for selected drugs. Further studies to correlate the in vitro data with in vivo finding may help us to understand its clinical significance.

References


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