**MDR1** Synonymous Polymorphisms Alter Transporter Specificity and Protein Stability in a Stable Epithelial Monolayer

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Abstract

The drug efflux function of P-glycoprotein (P-gp) encoded by *MDR1* can be influenced by genetic polymorphisms, including two synonymous changes in the coding region of *MDR1*. Here we report that the conformation of P-gp and its drug efflux activity can be altered by synonymous polymorphisms in stable epithelial monolayers expressing P-gp. Several cell lines with similar *MDR1* DNA copy number were developed and termed LLC-MDR1-WT (expresses wild-type P-gp), LLC-MDR1-3H (expresses common haplotype P-gp), and LLC-MDR1-3HA (a mutant that carries a different valine codon in position 3435). These cell lines express similar levels of recombinant mRNA and protein. P-gp in each case is localized on the apical surface of polarized cells. However, the haplotype and its mutant P-gps fold differently from the wild-type, as determined by UIC2 antibody shift assays and limited proteolysis assays. Surface biotinylation experiments suggest that the non-wild-type P-gps have longer recycling times. Drug transport assays show that wild-type and haplotype P-gp respond differently to P-gp inhibitors that block efflux of rhodamine 123 or mitoxantrone. In addition, cytotoxicity assays show that the LLC-MDR1-3H cells are more resistant to mitoxantrone than the LLC-MDR1-WT cells after being treated with a P-gp inhibitor. Expression of polymorphic P-gp, however, does not affect the host cell’s morphology, growth rate, or monolayer formation. Also, ATPase activity assays indicate that neither basal nor drug-stimulated ATPase activities are affected in the variant P-gps. Taken together, our findings indicate that ‘silent’ polymorphisms significantly change P-gp function, which would be expected to affect interindividual drug disposition and response. Cancer Res; 74(2); 598–608. ©2013 AACR.

Introduction

*MDR1* [P-glycoprotein (P-gp), ABCB1] is one of the major drug transporters found in humans. This gene encodes P-gp, an efflux transporter in the plasma membrane that actively transports a broad range of drugs in an ATP-dependent manner (1). It is found in multiple organs (2), and is expressed in the trophoblast layer of the placenta during pregnancy (3). Mice carrying null *abcb1a* and *abcb1b* genes are viable, but have altered pharmacokinetics of many drugs that are P-gp substrates (4–6). American collies carrying truncated *MDR1* genes have lower tolerance to vincristine and the deworming agent ivermectin, a substrate of P-gp (7, 8). Overexpression of P-gp is a common cause of acquired drug resistance in cultured cancer cells (9–13). In polarized epithelia, P-gp is located on the apical membrane, facilitating transport in a directional manner (14, 15).

P-gp contains 2 important functional domains: the substrate binding site and the ATPase domain. It is well documented that mutations in these domains change P-gp function (reviewed in refs. 16 and 17). In humans, the *MDR1* gene is highly polymorphic, with at least 50 coding single-nucleotide polymorphisms (SNP) in the *MDR1* coding region documented. In particular, 3 SNPs at positions 1236C>T, 2677G>T, and 3435C>T, which form the most common haplotype, have been studied extensively (16, 18–20). Since the first report showing the alteration of P-gp function with these SNPs (18), many studies have been done to define the influence of these SNPs individually, or of the complete haplotype. However, the results of these population-based studies are indefinite, possibly because of variations in terms of experimental settings including inadequate population sizes to assure statistical significance, incomplete sequence of individuals, differences...
in tissue-specific P-gp expression, and other unknown environmental factors (21).

The synonymous SNP 3435C>T, usually part of the haplotype noted above, plays an influential role in P-gp function, including elevated digoxin, cyclosporin A (CsA), and fexofenadine bioavailability (22–24). Our previous study using a vaccinia virus–based transient expression system showed that wild-type P-gp and its haplotype are different in function (25). We also suggested that differences in protein characteristics of 3435C>T, such as those mentioned earlier, might be related to the introduction of a rare codon that alters the translational rhythm and folding of P-gp. However, there are technical limitations in vaccinia virus–based high-level transient expression systems that led us to conduct transport studies and protein stability experiments in polarized cells. To study haplotype P-gp and compare its function with wild-type P-gp under conditions more physiological than those in the transient expression experiments, we developed stable cell lines in which the human MDR1 gene and its variants were translated from recombinant DNA and inserted into genomic DNA in a subclone of LLC-PK1 cells that can form polarized monolayers.

Materials and Methods

Cell culture and materials

The LLC-PK1 cell line was obtained from American Type Culture Collection, and cultured in Medium199 + 3% (v/v) FBS + 1% penicillin/streptomycin. The recombinant cell lines were incubated in the same medium with 500 μg/mL geneticin. KB-3-1, KB-V1, and KB-8-5 cells were cultured in Dulbecco’s Modified Eagle Medium + 10% FBS and 1% penicillin/streptomycin. Cells were cultured at 37°C with 5% CO₂ and relative humidity maintained at 95%.

Cell culture media and geneticin were purchased from Invitrogen. Biotin, paraformaldehyde, verapamil, vinblastine, rodamine 123, calcein-AM, mitoxantrone, trypsin, soybean trypsin inhibitor, MTT, and valinomycin were obtained from Sigma. Bodipy-FL–vinblastine was obtained from Molecular Probes. Restriction enzymes were obtained from New England Biolabs. The antibodies were purchased from the following companies: DAKO (C219, MRK16), Invitrogen (IgG2a-Alexa Fluor 488, CY3-streptavidine), ebioscience (ULC2-PE, 17F9, IgG2a-HRP, Streptavidin-PE), and Jackson Immuno Research (IgG2a-FITC). ECL reagents obtained from GE Healthcare. 125I–iodoarylazidoprazosin (125I-IAAP; 2,200 Ci/mmol) was obtained from PerkinElmer Life Sciences.

Preparation of pcDNA-MDR1 constructs

Details about the preparation of constructs can be found in Supplementary Materials and Methods.

Generation of LLC-MDR1 cell lines

Generation of cell lines is detailed in Supplementary Materials and Methods.

Protein extraction and immunoblot analysis

Total protein extraction from cell culture and protein concentration estimation methods were reported previously (26).

For SDS-PAGE, protein samples (50 μg) were loaded onto a 3% to 8% Tris-acetate gel (Invitrogen). Separated proteins were transferred to a nitrocellulose membrane by iBlot (Invitrogen). The membrane was blocked in PBS + 20% milk and incubated overnight with C219 antibody (1:2,000) followed by incubation with anti-IgG-2a-HRP antibody and ECL+ (GE Healthcare).

Southern blot analysis

Total genomic DNA was extracted using a GenElute Mammalian Genomic DNA Purification Kit (Sigma). Total DNA (50 μg) and pcDNA-MDR1 (1236C-2677G-3435C; as copy number standard) were digested with NdeI for 24 hours and separated in a 0.7% agarose gel. DNA in the gel was denatured and was transferred onto a Hybond-XL membrane (GE Healthcare). The membrane was washed briefly with 2 × saline-sodium citrate (SSC) and soaked with hybridisd in 42°C for 2 hours. A NdeI-digested DNA [32P]-labeled DNA probe was prepared by Ready-To-Go DNA Labeling Kit (GE Healthcare) and was added on the membrane for 16-hour incubation at 42°C. The membrane was washed with 2 × SSC/0.1% SDS at 65°C for 30 minutes, 2 × 0.1 × SSC/0.1% SDS at 65°C for 10 minutes. The image was recorded by a phosphorimager and was analyzed by ImageQuant TL (GE Healthcare).

TaqMan qRT-PCR

Expression levels of human MDR1 genes were measured using the TaqMan method. One microgram of total RNA, isolated by the RNeasy Mini Kit (Qiagen), was used to synthesize cDNA using the high-capacity cDNA Reverse Transcription Kit (Invitrogen). cDNA was mixed with TaqMan Universal PCR Master Mix (Invitrogen), run on an ABI Prism 7900HT Sequence Detection System (Invitrogen) as per the manufacturer’s instructions. Porcine plasma membrane calcium ATPase 4 gene (PMCA4) was used as the reference gene.

Confocal microscopy

Cells were grown on Transwell 3470 membrane inserts (Corning) until confluent. Tight cell monolayers were fixed by treatment with 4% paraformaldehyde for 10 minutes, then incubated with PBS + 1 mmol/L MgCl₂ + 0.1 mmol/L CaCl₂ + 1% bovine serum albumin for 1 hour. To label P-gp, cell monolayers were incubated with MRK16 (1:100) for 1 hour, followed by incubation with anti-IgG2a-Alexa Fluor 488 antibody. To label nuclei, cells were incubated with 4',6-diamidin-2-phenylindole (DAPI; 300 nmol/L). For cell surface labeling, biotin was incubated for 15 minutes before cell monolayers were fixed. Streptavidin-PE (1:100) was added to the fixed cell monolayers for 1 hour. Fluorescence images were acquired with a Zeiss LSM 510 confocal microscope, and the images were analyzed by LSM Image Browser (version 4.2.0; Zeiss).

Cell growth assay

Cells were seeded on 60-mm culture dishes and cultured at 37°C. Starting from day 3 after cell seeding, 3 dishes of cells from each cell line were trypsinized. Cell numbers were recorded daily until day 14. Cell counting was measured by the AutoT4 cellometer (Nexelom Bioscience).
Cell surface immunolabeling by flow cytometry

Cell surface P-gp expression was detected by incubating cells with 1 μg of MRK16, U1C2, and 17Fl antibodies per 200,000 cells for 30 minutes. For experiments using CsA (10 μmol/L), cells were preincubated with drug for 10 minutes. After primary antibody incubation, cells were incubated with anti-mouse IgG2a FITC-conjugated antibody for 40 minutes.

Drug influx/efflux assay

Actively growing cells (2 × 10⁶) were harvested by trypsinization and were incubated in Iscove’s modified Dulbecco’s medium (IMDM) + 5% FBS medium, with rhodamine 123 (0.5 μg/mL), mitoxantrone (5 μmol/L), or bodipy-FL–vinblastine (0.5 μmol/L) in the presence or absence of CsA (10 μmol/L), DCPQ (50 nmol/L), Taridiquar (50 nmol/L), verapamil (5 μmol/L), digoxin (125 μmol/L) at 37°C. Cells were washed and further incubate with IMDM or IMDM with drugs for 40 minutes before FACS analysis.

Limited trypsin digestion assay

Limited protein digestions by trypsin were performed with crude membranes. Three micrograms of the crude membranes were treated with different concentrations of trypsin for 5 minutes at 37°C. The reaction was stopped with 5 × excess of soybean trypsin inhibitor. Remaining P-gp was detected by Western blot analysis using C219 antibody.

Cell surface P-gp biotinylation

For cell surface biotinylation, cells were washed with ice cold PBS, and then incubated with 0.2 mg/mL EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific) in ice-cold PBS for 30 minutes. Excess biotin was washed with glycine/PBS and PBS. For flow cytometry analysis, cells were trypsinized, washed, and incubated with MRK16 + IgG2a-FITC (0.5 μg per 200,000 cells) + CY3-streptavidine (1:50). Labeling of cells with sulfo-NHS-LC-biotin just before trypsinization was considered as time 0. The percentage of biotinylated P-gp remaining at each time point was determined by measuring the percentage of biotinylated P-gp cells remaining compared with the number of biotinylated P-gp cells at time 0. Data calculations were performed by CellQuest software BD Biosciences.

Cytotoxicity assay

The cytotoxicity of drugs on LLC-MDR1 cells was measured by a colorimetric viability assay using MTT as previously described (26). Cytotoxicity (IC₅₀) was defined as the drug concentration that reduced cell viability to 50% of the untreated control.

Crude membrane preparations and ATPase assays

The crude membranes from cells were prepared as described previously (27). ATPase activities were measured as reported previously (28). Briefly, crude membrane protein (10 μg protein per 100 μL) was incubated at 37°C for 30 minutes in 100 μL of reaction buffer (50 mmol/L MOPS-KOH, pH 7.5, 50 mmol/L KCl, 5 mmol/L sodium azide, 1 mmol/L EGTA, 1 mmol/L ouabain, 10 mmol/L MgCl₂) in the presence and absence of 0.3 mmol/L sodium orthovanadate (V₁). The reaction was initiated by the addition of 5 mmol/L ATP for 20 minutes at 37°C. SDS solution (2.5%, v/v) was added to terminate the reaction, and the amount of inorganic phosphate released was quantified with a colorimetric reaction, as described previously (28). The basal P-gp ATPase activity was determined by subtracting the ATPase activity in the P-gp–containing membranes with the ATPase activity in the control membranes in the same experiment. Drug-stimulated P-gp ATPase activity was measured in an ATPase reaction mixture that contained 2.5 μmol/L paclitaxel, 10 μmol/L vinblastine, 10 μmol/L valinomycin, and 30 μmol/L verapamil, and the activity obtained was corrected for the basal ATPase activity.

Photoaffinity labeling with 125I-IAAP

Photoaffinity assays were performed by incubating crude membranes (1 mg/mL) with drugs for 10 minutes at room temperature in 50 mmol/L Tris-HCl, pH 7.5 followed by the addition of 3-6 nmol/L [125I]-IAAP (2,200 Ci/mmol). The samples were cross-linked and incorporation of [125I]-IAAP was determined as described previously (29).

Statistical analysis

Statistical significance of the experimental results was obtained by the 2-sample t test. Results were considered statistically significant at P < 0.05.

Results

Characterization of LLC-PK1 cell lines expressing wild-type, haplotype, and mutant P-gps

To characterize the effect of P-gp variants, stable cell lines expressing human P-gp were developed. The original LLC-PK1 cell line, isolated from porcine kidney proximal tubule epithelial cells (30), expresses a low level of P-gp (31). Taking this into consideration, the LLC-PK1 subclone termed LLC-PK1#7 was selected by clonal selection. LLC-PK1#7 has undetectable endogenous P-gp and is unable to efflux P-gp substrates including rhodamine 123 and calcine-AM (data not shown).

The MDR1 wild-type [pTM1-MDR1(1236C-2677G-3435C)], haplotype [pTM1-MDR1(1236T-2677T-3435T)], and a MDR1 haplotype mutant encoding the isoleucine at 1145 using a rarer haplotype [pTM1-MDR1(1236T-2677T-3435A)] were used for this study. After transfection, LLC-PK1 clones were isolated and grown under the same culture conditions. During establishment of stable cells, we did not select P-gp expression with P-gp substrates such as colchicine or vinblastine to prevent endogenous P-gp induction and/or alteration of other endogenous resistance mechanisms (32, 33). We designated the cell lines in this study as follows: LLC-vector (vector-transfected cells), LLC-MDR1-WT [MDR1(1236C-2677G-3435C)-expressing cells], LLC-MDR1-3H [MDR1(1236T-2677T-3435T)-expressing cells], and LLC-MDR1-3HA [MDR1(1236T-2677T-3435A)-expressing cells]. As shown inFig. IA, all the P-gp–expressing cell clones, numbered 1 to 4, have one copy of MDR1 cDNA. qRT-PCR analysis determined that the transfected DNA expressed a comparable level of MDR1.
transcripts (Fig. 1B, 2–4). Recombinant P-gp expression was identified by Western blotting using C219 antibody. In Fig. 1C, P-gp was not detectable in the LLC-vector cells (lane 1), but was evident in the P-gp–expressing cells (lanes 2–4). P-gp in all P-gp–expressing LLC-PK1 cell lines was found as a single band with an apparent molecular weight of ~160 kDa and no immature bands were detected (Fig. 1C and Supplementary Fig. S1). The P-gp protein from LLC-MDR1-WT cells had lower mobility than the P-gp expressed in High-Five insect cell membranes, indicating that P-gp is likely N-glycosylated in the LLC-PK1 cells (Supplementary Fig. S1). The N-glycosylation pattern of the different P-gps may differ, because we observed that the WT-P-gp (Fig. 1C, lane 2) had slightly higher mobility than the 3H-P-gp (Fig. 1C, lane 3) and the 3HA-P-gp (Fig. 1C, lane 4). P-gp–expressing cell lines expressed high levels of P-gp and all the clones expressed comparable levels. P-gp expression in the KB-V1 cells (Fig. 1C, lane 5) was greater than in KB-8-5 cells (Fig. 1C, lane 6), as expected.

We compared the growth rate of the LLC-PK1 cell lines. The cells were allowed to grow in culture dishes for 2 weeks until confluency. The growth curves indicated that these cell lines had similar growth rates (Fig. 1D).

To determine whether P-gp polarization could be affected by P-gp polymorphisms and synonymous mutations, cell surface immunolabeling was performed on LLC-PK1 cell monolayers. Cells were grown in Transwell chambers until cell monolayers were formed. Light microscopy images showed no significant morphologic differences among the tested cell lines (Fig. 1E, top). Using confocal microscopy, in the P-gp–expressing cells, P-gp signals (green) were detected on
the apical side, but not in the vector-transfected stable cells (LLC-vector). WT-P-gp, 3H-P-gp, and 3HA-P-gp expression on the apical membranes were comparable (Fig. 1E, middle). On the basolateral side, as expected, P-gp was undetectable (Fig. 1E, bottom). These results indicate that in the LLC-PK1 cells, P-gp polymorphisms do not affect protein expression or targeting to the apical cell surface.

**MDR1 mutations alter P-gp conformation in the LLC-PK1 cells**

To determine whether polymorphisms of *MDR1* could change protein conformation, we examined the LLC-MDR1 cell lines with the conformation-sensitive antibody UIC2. As shown in Fig. 2A, the vector cells showed minimal reactivity toward all antibodies tested (black histogram). The wild-type and haplotype P-gp–expressing cells showed high and comparable reactivity with conformation-insensitive MRK16 and 17F9 antibodies. However, these P-gp–expressing cells showed differential reactivity with UIC2 and with UIC2 in the presence of CsA (10 μmol/L).

To further examine the effect of conformation differences, the accessibility to various trypsin concentrations of P-gp was determined. Figure 2B shows Western blots of wild type and polymorphic forms of P-gp with trypsin. Membrane proteins from each P-gp–expressing cell line were prepared and they were digested for 5 minutes with increasing amounts of trypsin, and the remaining P-gp protein was analyzed using C219 antibody. For each protein sample, the concentration of trypsin required for a 50% decrease in P-gp was determined and plotted. We observed differences in trypsin sensitivity with LLC-MDR1-WT (most sensitive) < LLC-MDR1-3H < LLC-MDR1-3HA. The fold change in IC50 for LLC-MDR1-WT and LLC-MDR1-3H was 1.2- and 1.4-fold for LLC-MDR1-3HA. In the presence of 50 μmol/L verapamil, a P-gp inhibitor, there

![Figure 2](image-url)
was no difference in the IC50 values for trypsin digestion. WT-P-gp, 3H-P-gp, and 3HA-P-gp were more resistant to trypsin in the presence of verapamil, as we have previously observed using transiently expressed wild-type and haplotype P-gps (25).

**P-gp synonymous polymorphisms influence protein stability**

To elucidate the impact of conformation changes of P-gp by its polymorphisms in LLC-PK1 cells, we determined the half-life of plasma membrane P-gp in LLC-PK1 cell lines by cell surface biotinylation. Cells were labeled with biotin for 30 minutes and cultured for 24, 48, and 72 hours. Cells were stained with strepavidin-PE and MRK16 antibody. The remaining biotinylated P-gp at each time point was determined by flow cytometry and a plot was drawn to calculate the time required for 50% disappearance of biotinylated P-gp (Fig. 2B). The mean half-lives of plasma membrane P-gp were 45 hours (LLC-MDR1-WT), 47 hours (LLC-MDR1-3H), and 53 hours (LLC-MDR1-3HA), respectively (Fig. 3), which are significantly different from each other ($P < 0.05$), and consistent with the *in vitro* trypsin sensitivity experiments shown in Fig. 3.

**Synonymous mutations affect P-gp function**

The effect of drug transport function of P-gp wild type and its variants in LLC-PK1 cells was tested using rhodamine 123 with various P-gp inhibitors. In Fig. 4, FACS histograms show transport of rhodamine 123 by cells transfected with the LLC-vector, LLC-MDR1-WT, LLC-MDR1-3H, and LLC-MDR1-3HA cells. In the absence of P-gp inhibitors, efflux of rhodamine 123 by all P-gp–expressing LLC-PK1 cell lines was no difference in the IC50 values for trypsin digestion. WT-P-gp, 3H-P-gp, and 3HA-P-gp were more resistant to trypsin in the presence of verapamil, as we have previously observed using transiently expressed wild-type and haplotype P-gps (25).

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was the same. Complete inhibition of rhodamine 123 efflux was observed when a high concentration of verapamil (20 \text{\mu mol/L}) was used (Supplementary Fig. S2). Similar observations were also made for high concentrations of the P-gp inhibitors tariquidar (200 nmol/L) and CsA (20 \text{\mu mol/L}; data not shown).

However, we observed differential inhibition of P-gp function when suboptimal inhibitor concentrations were used. For example, in the presence of 50 nmol/L tariquidar, efflux of rhodamine 123 was least affected in LLC-MDR1-3H cells. Also, in the presence of 10 \text{\mu mol/L} CsA or 5 \text{\mu mol/L} verapamil, the LLC-MDR1-3HA cells showed slightly higher efflux of rhodamine 123. When we tested the efflux of rhodamine 123 in the presence of digoxin (125 \text{\mu mol/L}), the LLC-MDR1-3HA cells showed the poorest inhibition to digoxin compared with the LLC-MDR1-3H cells and the LLC-MDR1-WT cells, consistent with the original clinical observations that the P-gp haplotype affected digoxin levels in patients (18). These results suggest that the variant forms of P-gp have different sensitivities to the inhibitors digoxin, CsA, and tariquidar.

The LLC-vector cells were used as a control. This cell line was not able to efflux rhodamine 123, and P-gp inhibitors did not influence its accumulation of rhodamine 123 (Figs. 4 and 5, black histogram).

**Variant P-gps affect drug efflux and cytotoxicity**

The stable LLC-PK1 cells expressing human P-gps enabled us to conduct short-term assays and long-term assays. We confirmed that WT-P-gp, 3H-P-gp, and 3HA-P-gp expression levels remain unchanged for at least 96 hours after geneticin removal (results not shown). Growth assays revealed that all the LLC-PK1 cell lines tested had comparable growth rates (Fig. 1D), indicating that these cells are suitable for cytotoxicity experiments. We tested 2 anticancer drugs that are P-gp substrates (vinblastine, mitoxantrone) in the presence or absence of P-gp inhibitors (DCPQ, CsA). We compared the efflux function of P-gp in different LLC-PK1 cell lines using drug transport influx/efflux assays (short-term) and growth-inhibition assays (long-term) by the MTT method. In Fig. 5, the P-gp–expressing cells show different mitoxantrone efflux. The LLC-MDR1-3H cells showed significantly higher efflux than the LLC-MDR1-3HA and the LLC-MDR1-WT cells. This difference correlated with increased

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**Figure 5.** P-gp–transfected cell lines show differential sensitivity to cytotoxic drugs. LLC-vector (black), LLC-MDR1-WT (green), LLC-MDR1-3H (red), and LLC-MDR1-3HA (blue) cells were tested with cytotoxicity assays (left) and drug efflux assays (right). For cytotoxicity assays, cells were incubated with increasing concentrations of drugs for 72 hours. For drug accumulation-efflux assays, cells were incubated with mitoxantrone+DCPQ for 30 minutes followed by 45-minute efflux. For bodipy-vinblastine+CsA, cells were incubated for 20 minutes followed by 30-minute efflux. Assays were repeated at least three times. Each point in the cytotoxicity assay charts represents an average cell number from three independent experiments.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Inhibitor</th>
<th>LLC-vector</th>
<th>LLC-MDR1-WT</th>
<th>LLC-MDR1-3H</th>
<th>LLC-MDR1-3HA</th>
<th>LLC-vector</th>
<th>LLC-MDR1-WT</th>
<th>LLC-MDR1-3H</th>
<th>LLC-MDR1-3HA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC50 (μmol/L)</td>
<td>0.01 ± 0.003</td>
<td>1.5 ± 0.3</td>
<td>14.6 ± 1.9</td>
<td>9.2 ± 2.5</td>
<td>4.2 ± 0.4</td>
<td>14.6 ± 1.9</td>
<td>2.5 ± 1.0</td>
<td>9.2 ± 2.5</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>DCPQ</td>
<td>0.065 ± 0.015</td>
<td>0.2 ± 0.04</td>
<td>3.2 ± 0.2</td>
<td>0.3 ± 0.02</td>
<td>0.2 ± 0.03</td>
<td>0.06 ± 0.001</td>
<td>0.06 ± 0.001</td>
<td></td>
</tr>
<tr>
<td>Vinblastine</td>
<td>CsA</td>
<td>0.002 ± 0.0002</td>
<td>0.3 ± 0.02</td>
<td>0.3 ± 0.03</td>
<td>0.05 ± 0.003</td>
<td>0.05 ± 0.003</td>
<td></td>
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</tr>
</tbody>
</table>

The IC50 values were calculated by nonlinear regression analysis in GraphPad Prism using dose-response data from MTT assay. The resistance ratio is the fold of IC50 values relative to the LLC-vector cells.

Effect of substrates on P-gp wild-type and haplotype ATPase activity

The synonymous SNPs 1236C>T and MDR1 3435C>T are in amino acid codons found in the nucleotide binding domains (34). To examine whether P-gp conformation could change drug-stimulated ATPase activity or photoaffinity labeling with a transport substrate, we performed ATPase activity assays and photoaffinity labeling assays with [125I]-IAAP in the presence of tasigna, verapamil, paclitaxel, vinblastine, valinomycin, and verapamil (Fig. 6A). There were no significant differences between the cell lines in basal and drug-stimulated ATPase activities.

Photoaffinity labeling assays with [125I]-IAAP in the presence of tasigna, verapamil, paclitaxel, vinblastine, valinomycin, and tariquidar indicated that all tested compounds except verapamil could effectively inhibit [125I]-IAAP binding to P-gp (Fig. 6B). No significant differences were found between the 2 cell lines. To further examine the effect of IAAP binding, experiments showed that the IC50 value of the LLC-MDR1-WT cell line was 1.9 μmol/L and that of the LLC-MDR1-3H cell line was 2.2 μmol/L (P > 0.05).
mutations are related to key developmental pathways (39). strong purifying selection, and the genes harboring these mutation sites in *Drosophila melanogaster* activity via multiple pathways (35). Nevertheless, mounting evidence strongly suggests these polymorphisms can in influence gene function and cellular activity via multiple pathways (35–38). In fact, Lawrie and colleagues reported that a significant portion of synonymous mutation sites in *Drosophila melanogaster* are subject to strong purifying selection, and the genes harboring these mutations are related to key developmental pathways (39).

In the *MDR1* gene, there are 2 synonymous polymorphisms that frequently occur in certain human populations (34, 40, 41). Many clinical and *in vitro* studies have suggested that the 3435C>T SNP plays an important role in the function of *MDR1*. However, the functional effects of synonymous mutations in *MDR1* have not been studied to any significant degree except after transient, high-level expression (25). Therefore, we decided to use the LLC-PK1 epithelial cell line, which can form a polarized tight monolayer, to evaluate the effect of *MDR1* synonymous polymorphisms, and the effects of codon usage.

Our results show that synonymous mutations do influence protein stability in intact cells in addition to trypsin sensitivity *in vitro* as previously reported (25). The *MDR1* haplotype causes a change in P-gp conformation, as determined using cell surface labeling by UIC2, a P-gp conformation-sensitive antibody (42). We further showed that the 3435C>A synonymous mutation increases binding of UIC2 MAb, and increases resistance to trypsin. The surface protein biotinylation experiments and FACS assays showed the wild-type P-gp had the shortest half-life (45 hours), followed by the haplotype (47 hours) and 3HA mutant (53 hours). These differences are consistent with the protein stability differences demonstrated earlier using the trypsin-sensitivity assay (25). We hypothesize that the difference in protein half-life may be linked to protein recycling. Kim and colleagues (43) have reported that P-gp is recycled through a clathrin-dependent pathway and interacts with the AP-2 adaptor complex. The P-gp variations we are studying might cause a change in protein conformation involving a tight turn structure or a di-leucine motif, which interacts with AP-2 adaptor complexes. Also, our functional assays showed that, in certain cases, the haplotype mutants are more resistant to inhibitors because the frequency of haplotype forms of P-gp varies in different human populations (19, 20). These results suggest an evolutionary force driving *MDR1* to alter its ability to interact with drugs and dietary materials through altered protein conformation resulting from the haplotype studied here.

However, common synonymous mutations of *MDR1* did not influence mRNA expression, total protein expression, or translocation to the apical membrane surface. Also, the presence of P-gp on the cell surface did not affect cell growth or formation of a tight polarized monolayer. Our observation does not support some clinical reports suggesting that the haplotype allele often correlates with lower P-gp expression (18, 44, 45).

Our cell lines with stable P-gp expression allowed us to conduct physiologically relevant experiments, including drug efflux assays and cytotoxicity assays. Drug efflux assays suggested that the transport function of P-gp variants is dependent on the drug of choice. Our results indicated that P-gp polymorphisms do not affect rhodamine 123 transport in the presence of verapamil or CsA. In contrast, the LLC-MDR1-3H cell line is less sensitive to tariquidar than LLC-MDR1-WT cells. The LLC-MDR1-3HA cells are more resistant to inhibition by digoxin than the cells carrying the haplotype and the wild-type forms of P-gp. Our results with mitoxantrone indicate that the
drug transport function of P-gp is significantly influenced by the synonymous mutations in \textit{MDR1}. Mitoxantrone is an anthracycline derivative that is a relatively poor substrate of P-gp. Expression of P-gp is able to reduce mitoxantrone accumulation and therefore increase cytotoxic resistance (46). In this study, we found that P-gp polymorphisms directly affect mitoxantrone efflux and cytotoxicity. The exact cause of these observations is unclear, but we hypothesize that the conformational differences affect the relative efficiency of substrate and inhibitor binding between wild-type and haplotype-expressing cells.

In this study, the \textit{MDR1} 1236C>T and 3435C>T SNPs showed a significant impact on the overall folding of P-gp, and not just a localized effect. These "silent" mutations encode glycine 412 and isoleucine 1145, which are found in the first and second ATP-binding domains and are unchanged in the wild-type and haplotype P-gp. These SNPs do not affect basal and drug-stimulated ATPase activity, suggesting changes in conformation in these polymorphic P-gps do not affect ATP binding or hydrolysis (47). These "silent" polymorphisms do not result in mRNA degradation, nor do the resulting P-gp conformational changes result in obstruction in the protein folding pathway (like CFTR), mis-localization, posttranslational modification defects, protein truncation, and/or misfolding. In fact, P-gp encoded by the \textit{MDR1} common haplotype is expressed properly on the cell surface, targeted to the apical side in polarized monolayers, and functions as a drug transporter. Furthermore, some of our results indicate that the function of haplotype P-gp might be more efficient than the wild-type P-gp. These results in stably transfected cells strongly suggest that human P-gp exists in at least 2 conformational states of equivalent stability. The haplotype form folds differently than wild-type P-gp (48). It seems that the silent polymorphisms encode a protein with alternate (or new) energy minima, similar to the model suggested by Tsai and colleagues (48). However, the results do not demonstrate the mechanism of the effect of synonymous mutations on \textit{MDR1}. More experiments are warranted to examine various hypotheses.

In summary, the role of P-gp synonymous polymorphisms was examined in polarized epithelial cells. We showed that the synonymous mutations do influence protein folding and function in stable cells expressing recombinant P-gp. P-gp conformational alterations subtly changed drug efflux function and interaction with P-gp inhibitors. By comparing data from short-term transport assays and cytotoxicity assays, these subtle changes significantly alter cellular cytotoxicity of drugs such as mitoxantrone that do not bind tightly to P-gp. The P-gp-expressing cell lines will help to identify substrates and inhibitors that are influenced by the common variants of P-gp, allowing better predictions about the pharmacokinetics of drugs that are substrates for P-gp, and improved design of clinical studies that seek to inhibit the function of P-gp to reverse drug resistance.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors' Contributions**

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**Study supervision:** K.L. Fung, S.V. Ambudkar, M.M. Gottesman

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