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Functional Analysis of the Human Variants of BCRP: I206L, N590Y and D620N

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BCRP, breast cancer resistance protein; ABC, ATP-binding cassette; SNP, single nucleotide polymorphism; P-gp, P-glycoprotein; HEK cells, human embryonic kidney cells; mAb, monoclonal antibody; MX, mitoxantrone; FTC, fumitremorgin C; PhA, pheophorbide a; NBD, nucleotide binding domain; MRP1, Multidrug Resistance Protein 1; MRP2, Multidrug Resistance Protein 2; TM, transmembrane.

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Abstract

Previous studies have shown that the V12M and Q141K variants of BCRP can affect expression and function of the transporter. In this study, the effects of the I206L, N590Y and D620N variants on protein expression, plasma membrane localization and transport activity of BCRP were investigated. Wild-type BCRP and the three variants were stably expressed in HEK cells. Confocal microscopy analysis showed that the three variants were predominantly routed to the plasma membrane of HEK cells. The expression level of I206L in the plasma membrane was approximately 45% of that of wild-type protein, while the N590Y and D620N levels were increased approximately 3.6-fold and 2.4-fold, respectively, as determined by immunoblotting. All the three variants transported mitoxantrone, pheophorbide a and BODIPY-prazosin. After normalization for differences in BCRP expression, I206L, N590Y and D620N exhibited approximately 2-fold, 0.3-fold, and 0.5-fold wild-type efflux activities, respectively. The variants also conferred resistance to mitoxantrone and topotecan. Mitoxantrone and topotecan resistance by I206L and N590Y was approximately 2-fold and 0.3-fold of the wild-type BCRP resistance levels, respectively. While D620N conferred a similar topotecan resistance as the wild-type protein, its level of mitoxantrone resistance was decreased by 50%. After normalization to BCRP expression levels, ATPase activities of I206L were not significantly different from those of wild-type protein, whereas N590Y and D620N exhibited approximately 30% and 50% of wild-type ATPase activities, respectively. These results suggest that I206L has the lowest protein expression and the highest activity, while N590Y and D620N display higher expression and lower activity, relative to wild-type BCRP.

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Human BCRP (ABCG2) is a 72 kDa efflux drug transporter belonging to the subfamily G of the ABC transporter superfamily (Allikmets et al., 1998; Doyle et al., 1998; Miyake et al., 1999). Previous studies have shown that BCRP confers high levels of resistance to anticancer drugs including mitoxantrone, topotecan and methotrexate by enhanced drug efflux (Litman et al., 2000; Bates et al., 2001; Doyle and Ross, 2003). In addition to chemotherapeutic agents, BCRP is an active transporter of organic anions with diverse chemical structures such as estrone-3-sulfate and 17β-estradiol 17-(β-D-glucuronide) (Imai et al., 2003; Suzuki et al., 2003). BCRP also plays an important role in disposition of drugs including topotecan, irinotecan and methotrexate, as demonstrated in both animal and clinical studies (Jonker et al., 2000; Jonker et al., 2002; Kruijtzer et al., 2002; van Herwaarden et al., 2003; Breedveld et al., 2004; Stewart et al., 2004). This is consistent with the high level expression of BCRP in organs of absorption (small intestine), elimination (liver and small intestine) and distribution (placenta and the blood-brain barrier) of drugs (Maliepaard et al., 2001). Furthermore, BCRP may protect tissues or fetus from toxicities of chemotherapeutics and/or potentially harmful endogenous substances. For example, it has been demonstrated that BCRP protects hematopoietic stem cells under hypoxic conditions by preventing the accumulation of heme that causes mitochondrial death (Krishnamurthy et al., 2004). Therefore, naturally occurring mutations which alter BCRP expression and/or function could affect the pharmacokinetic properties of substrate drugs and influence the tissue protective role of this transporter.

In recent years, a variety of SNP variants of BCRP have been identified in DNA samples of ethnically diverse origins (Honjo et al., 2002; Imai et al., 2002; Zamber et al., 2003; Kondo et al., 2004; Mizuarai et al., 2004). Two variants V12M and Q141K

occurred at particularly high allele frequencies in Chinese and Japanese populations (30 – 60%) and at relatively lower allele frequencies in Caucasians and African-Americans (5 – 26%) (Zamber et al., 2003). Other variants such as I206L, N590Y and D620N are generally much less frequent with allele frequencies of approximately 1% or less (Honjo et al., 2002; Zamber et al., 2003). *In vitro* functional studies have already demonstrated alterations in expression and function of several BCRP SNPs. For instance, Q141K seems to be associated with reduced protein expression compared with wild-type BCRP in mammalian expression systems (Imai et al., 2002; Kondo et al., 2004).

Functional analysis of the I206L, N590Y and D620N variants of BCRP has not been reported so far. In the present study, we stably expressed wild-type BCRP and the variants I206L, N590Y and D620N in HEK cells and analyzed the effects of the variants on BCRP protein expression, plasma membrane localization, transport and ATPase activities as well as drug resistance characteristics.

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Materials and Methods

Materials. BODIPY FL-prazosin was purchased from Molecular Probes (Eugene, OR). Mitoxantrone hydrochloride and daunorubicin were from Sigma (St. Louis, MO). Pheophorbide a was from Frontier Scientific (Logan, UT). Rhodamine 123 was from ICN Biomedicals (Aurora, OH). Topotecan was from Calbiochem (La Jolla, CA). Fumitremorgin C was a generous gift from Dr. Susan E. Bates (NCI, Bethesda, MD). HPLC grade DMSO was from Fisher Scientific (Pittsburgh, PA) and used as the solvent for making stock solutions of all the drugs and FTC. Eagle's minimal essential medium (MEM) and heat-inactivated horse serum were purchased from ATCC (Manassas, VA). Dulbecco's modified Eagle's phenol-free low-glucose medium (DMEM), phosphate-buffered saline (PBS), trypsin-EDTA solution and G418 were purchased from Invitrogen (Carlsbad, CA).

Construction of BCRP Expression Vectors. The pcDNA-482R plasmid containing full length of wild-type BCRP cDNA (Robey et al., 2003) was kindly provided by Dr. Susan E. Bates. The following strategy was used to construct expression vectors for BCRP variants. First, the DNA fragment containing wild-type BCRP cDNA was obtained by digestion of pcDNA-482R with *Not*1 *and BamH*1 and subcloned into the pBluescript SK(+) plasmid (Stratagene, La Jolla, CA) which was digested with the same restriction enzymes. The variants I206L, N590Y, D620N were then generated using the QuickChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) and the pBluescript SK(+) plasmid carrying wild-type BCRP cDNA as a template, according to the manufacturer's instructions. For each variant, two complementary primers were used,

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each containing the specific mutation (substituted nucleotides are high-lighted and underlined) as follows: the primer pairs for I206L were 5'-CTTATCACTGATCCTTCC**CTC**TTGTTCTTGGATGAG-3' and 5'CTCATCCAAGAACAA**GAG**GGAAGGATCAGTGATAAG-3'; the primer pairs for N590Y were 5'-GAATTTTTGGGACAA**TAC**TTCTGCCCAGGACTC-3' and 5'-GAGTCCTGGGCAGAA**GTA**TTGTCCCAAAAATTC-3'; and the primer pairs for D620N were 5'-GTAAAGCAGGGCATC**AAT**CTCTCACCCTGGGGC-3' and 5'-GCCCCAGGGTGAGAG**ATT**GATGCCCTGCTTTAC-3'. The mutations were confirmed by sequencing. To ensure that no other mutations were introduced during mutagenesis, the full length of BCRP cDNA was sequenced completely. The DNA fragments containing the mutations were then digested from the pBluescript SK(+) plasmids with *Not*1 *and BamH*1 and subcloned into the pcDNA3.1/myc-His(-) (version A) expression vector (Invitrogen). The mutations were again verified after insertion into the pcDNA vectors by DNA sequencing. The resulting plasmids contain a *Not*1 site, a Kozak sequence, and an ATG start codon at the 5' end and a stop codon and a *BamH*1 site at the 3' end of the BCRP gene.

Cell Culture and Stable Transfection of BCRP cDNAs into HEK Cells. HEK293 cells were purchased from ATCC and maintained in MEM with 10% (v/v) heat-inactivated horse serum at 37^{o}C in a 5% CO_2 incubator. For stable transfection, approximately 4 × 10^5 cells were seeded in each well of 12-well plates, and 24 h later, cells were transfected with 2 μg DNA in 1 ml of serum free medium at 1:3 ratio of plasmid DNA to Lipofectamine 2000 (Invitrogen). After 12 h, cells were incubated for an additional 48 h

in fresh medium containing 10% heat-inactivated horse serum before adding 1 mg/ml G418. Cell culture was continued in 1 mg/ml G418 for 3 days. Then, cells were pooled and subcultured in limited dilutions in 1 mg/ml G418 with frequent removal of dead cells by changing medium until resistant colonies appeared. After approximately 3 weeks, cell colonies were removed individually using trypsinized sterile cloning discs (Corning, NY) and subcultured in the presence of 500 μg/ml G418. Levels of BCRP variants in G418-resistant cell populations were then determined by immunoblotting. All the cell lines were then grown and maintained in MEM supplemented with 10% (v/v) heat-inactivated horse-serum in the presence of 500 μg/ml G418 at 37^{o}C in a 5% CO_2 incubator. Cells were grown to 80 – 90% confluence and treated with trypsin-EDTA prior to harvesting for subculturing, immunoblotting, confocal microscopy analysis or flow cytometry assays. Only cells within six passages were used in these experiments.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting. Whole cell lysates from HEK cell lines were prepared as previously described (Gupta et al., 2004). Plasma membrane preparations were prepared as described elsewhere in this manuscript. Protein concentrations were determined by the Bio-Rad Dc protein assay kit (Bio-Rad, Hercules, CA) using bovine serum albumin as standard. The whole cell lysates and plasma membrane preparations were then subjected to immunoblotting using BXP-21, a BCRP-specific mAb (Kamiya Biomedical, Seattle, WA) as previously described (Gupta et al., 2004). Relative levels of BCRP protein expression were determined by densitometric analysis of the immunoblots using the NIH Scion Image software (Scion Corp., Frederick, MD).

Confocal Microscopy. Stably transfected HEK cells expressing wild-type BCRP and the variants and the vector control cells were seeded at approximately 5×10^5 cells/well in a four chamber glass slides (BD Falcon, Bedford, MA). Cells were grown to confluence and washed twice with PBS at room temperature. Cells were then fixed with 4% paraformaldehyde in PBS for 30 min, washed twice with PBS and then incubated in permeabilization buffer (0.2% Triton X-100 in PBS) at room temperature for 10 min. Cells were then blocked for 90 min in blocking solution (0.1% Triton X-100/2% horse serum) and incubated with BXP-21 (1:100 dilution in blocking solution) for 1h at room temperature. After the cells were washed with blocking buffer two times, Alexa Fluor 488 conjugated goat anti-mouse IgG (H + L) $(Fab')_2$ fragment (Molecular Probes, Eugene, OR) was added (1:500 dilution in blocking solution) and incubated in the dark for 1h. Cells were then washed twice with PBS and mounted in Fluoromount G (Southern Biotechnology Associates, Birmingham, AL) and observed at 488 nm excitation and 519 nm emission wavelengths using a Leica TCS SP1 MP multiphoton confocal microscope (Leica Microsystems, Exton, PA).

Detection of Surface Expression of BCRP Variants Using Flow Cytometry. The flow cytometry assay detecting cell surface expression of BCRP was essentially the same as previously described (Robey et al., 2003; Polgar et al., 2004). Approximately 5×10^5 cells were harvested by trypsinization and washed once with PBS. The cells were resuspended in 0.75 ml of PBS with 2% BSA and incubated with 20 μl of the phycoerythrin-conjugated anti-BCRP surface mAb 5D3 (eBioscience, San Diego, CA) or

the phycoerythrin-conjugated mouse IgG2b as a negative control for 30 min at room temperature. Cells were then washed once with PBS and placed on ice in the dark until analyzed. Within one hour, cells were analyzed on a BD FACSCAN flow cytometer equipped with a 488 nm argon laser and 585 nm bandpass filter. Ten thousand (10^4) events were collected. Cell debris was eliminated by gating on forward versus side scatter. The differences in phycoerythrin fluorescence between cells incubated with the 5D3 and the control antibodies were used to express the relative cell surface expression levels of wild-type BCRP and its variants.

Flow Cytometric Efflux Assay. The efflux assays were essentially the same as described previously (Wang et al., 2000; Robey et al., 2001; Gupta et al., 2004) with minor modifications. HEK cells expressing wild-type BCRP and the variants or vector control cells were trypsinized, washed once in PBS and suspended in incubation buffer (DMEM supplemented with 10% heat-inactivated horse serum and 5 mM HEPES buffer) at cell concentration of approximately 10^6 cells per reaction in 1-ml volume. In the accumulation phase, cells were incubated with the following fluorescent compounds, 500 nM BODIPY-prazosin, 10 μM MX, 2 μM PhA or 0.5 μg/ml rhodamine 123 in the presence or absence of 10 μM FTC for 30 min at 37ºC. Cells were then immediately transferred on ice, washed once with ice-cold PBS, and resuspended in 1 ml of incubation buffer with or without 10 μM FTC but containing no fluorescent compounds and then incubation was continued for 1 h at 37ºC to allow maximum efflux of fluorescent compounds (efflux phase). Cells were then washed once with ice-cold PBS and resuspended in 500 μl of ice-cold PBS and kept on ice in the dark. Intracellular fluorescence was measured within 1 h

with a 488 nm argon laser and a 650 nm longpass filter for MX and PhA, and a 488 nm argon laser and 530 nm bandpass filter for BODIPY-prazosin and rhodamine 123 in a BD FACSCAN flow cytometer. Ten thousand (10^4) events were collected for all the samples. Cell debris was eliminated by gating on forward versus side scatter. Cells in medium containing fluorescent compounds alone or medium containing fluorescent compounds and FTC were used to generate Efflux and FTC/Efflux histograms, respectively. Cells in medium containing vehicle (0.2% (v/v) DMSO) yielded the control histogram, a measure of cellular autofluorescence. No effects of the vehicle on efflux activity of BCRP were observed at this concentration. The difference (ΔF) in median fluorescence between the FTC/Efflux histogram and the Efflux histogram was used as a measure of FTC-inhibitable drug efflux activity of BCRP. The efflux activities were normalized to the BCRP protein levels to take into consideration differences in BCRP expression. Statistical significance of difference of efflux activities between wild-type BCRP and its variants was calculated by student's t-test. A difference with p-value of < 0.05 was considered significant.

Cytotoxicity Assay. Drug resistance profiles of stably transfected cell lines were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) microtiter plate assay as described previously (Wilson, 2000). HEK cells stably expressing wild-type or variant BCRP or the vector control cells were seeded at a density of 1000 - 2000 cells/well in 96-well plates in 200 μl of MEM supplemented with 10% heat-inactivated horse serum and 500 μg/ml G418. After 24 h incubation at 37^{o}C in a 5% CO_2 incubator, the medium was replaced with fresh medium containing 5% heat-

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inactivated horse serum and without G418. Drugs were then added to the medium at various concentrations, and cells were further incubated with drugs for 72 h. After 72 h, the medium with drugs was replaced with 200 μl of fresh medium containing 10 mM HEPES (pH 7.4). Fifty μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (Sigma) at a concentration of 2.5 mg/ml in PBS was then immediately added in each well. The plates were incubated for 4 h at 37 ºC in the dark. The medium was then removed. The resulting metabolite of MTT, formazon, was solubilized by adding 200 μl of DMSO, followed by addition of 25 μl of the Sorensen's glycine buffer (0.1 M glycine, 0.1 M NaCl equilibrated to pH 10.5 with 0.1 M NaOH). The optical density of the plates was read at 540 nm. Untreated cells were used as control (100% cell survival). IC_{50} values representing cytotoxicities of drugs were calculated by fitting the following model to the data (Fig. 5) using nonlinear regression (WinNonLin, version 3.2 (Scientific Consulting, Mountain View, CA)):

$$I = I_{max} - [I_{max} - I_o] \times [C^{\gamma} / (C^{\gamma} + IC_{50}{}^{\gamma})]$$

where I is the cell survival as percentage of the optical density of the control cells, I_{max} is the maximal cell survival, I_o is the lowest residual cell survival at the high drug concentrations, *C* is the drug concentration, γ is the slope factor, and IC_{50} is the drug concentration leading to 50% cell survival. Six determinations were carried out within each experiment and at least three independent experiments were performed. Relative resistance factors were calculated as ratios of the IC_{50} values of cells expressing wild-type BCRP and its variants to the IC_{50} values of cells transfected with the empty vector

alone. The relative resistance levels were normalized to the BCRP protein levels to take into account differences in BCRP expression. Statistical significance of difference of resistance levels between wild-type BCRP and its variants was calculated by Student's *t* test. A difference with p-value of < 0.05 was considered significant.

Plasma Membrane Preparation. Plasma membrane preparations were prepared essentially the same as described previously (Loe et al., 1996). Aliquots of the plasma membrane preparations were stored at -80ºC until use and protein concentration was determined using the Bio-Rad Dc protein assay kit. Protein concentrations of the plasma membrane preparations were typically 1 - 4 mg/ml. Relative levels of BCRP protein in the plasma membrane preparations were determined by immunoblotting using BXP-21.

Vanadate-sensitive ATPase Assay. The vanadate-sensitive ATPase acivity of BCRP was determined by measuring the release of inorganic phosphate from ATP, using a calorimetric assay as previously described (Chifflet et al., 1988; Mao et al., 2004). The plasma membranes (5 μg of protein per reaction) prepared from the cells expressing wild-type BCRP and its variants or the vector control cells were added in 100 μl of buffer containing 50 mM HEPES, pH 7.0, 5 mM $MgCl_2$, 5 mM MgATP, 10 mM NaN_3, 2 mM EGTA and 2 mM ouabain in the presence or absence of 1 mM vanadate. The mixture was incubated at 37ºC for 30 min. NaN_3, EGTA and ouabain were included in the assay mixtures to eliminate contributions from nonspecific mitochondrial ATPases, Ca^{2+}-ATPases and Na^+/K^+-ATPases. To determine the effects of substrates or inhibitors on ATPase activity of BCRP, MX, prazosin and FTC were also included in the assay

mixtures at 10 μM concentrations. Reactions were stopped and the amount of inorganic phosphate released was determined immedietely by measuring the optical density at 650 nm as described (Chifflet et al., 1988). The differences between the ATPase activities measured in the absence and in the presence of vanadate were used to express the vanadate-sensitive ATPase activities of BCRP and presented relative to the amount of total protein in the plasma membrane preparations. Finally, the ATPase activities were normalized to the BCRP protein levels to take into account differences in BCRP expression. The ATPase activities of the variants were compared with the respective ATPase activities of wild-type BCRP by Student's *t* test. A difference with p value of < 0.05 was considered significant.

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Results

Stable Expression of Wild-type BCRP and the I206L, N590Y and D620N variants in HEK Cells. To examine the effects of I206L, N590Y and D620N on protein expression, plasma membrane localization and function of BCRP, we generated the three variants by site-directed mutagenesis. The pcDNA expression vectors containing cDNAs of wild-type BCRP (482R) and the three variants were used to transfect HEK cells and stable cell lines were generated. Approximately 20 clonal cell lines expressing wild-type BCRP and the variants were established by limited serial dilution and subjected for screening BCRP expression by immunoblotting of whole cell lysates. The expression levels of BCRP in individual clones varied. Fig. 1A shows a typical immunoblot of whole cell lysates prepared from various cell lines (482R-13, 482R-21, I206L-13, I206L-20, N590Y-1, N590Y-13, D620N-9 and D620N-10) which express the highest levels of BCRP. The cell lines, 482R-21, I206L-13, N590Y-1 and D620N-9 were used in all subsequent experiments. Since it is the BCRP protein that is present in the plasma membrane that determines the apparent drug efflux activity of cells, we determined the relative BCRP expression levels in the plasma membrane preparations isolated from cells expressing wild-type BCRP and the variants. A typical immunoblot of the plasma membrane preparations showed that I206L, N590Y and D620N were expressed at levels of approximately 0.45-fold, 3.6-fold and 2.4-fold those of the wild-type protein (482R), respectively (Fig. 1B). Similar results were obtained from the analysis of the immunoblots of whole cell lysates using β-actin as an internal standard (Fig. 1A). Since β-actin is a soluble protein, it was separated from BCRP in the relatively pure plasma membrane preparations. Therefore, we could not detect β-actin in the plasma membrane

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samples. Endogenous BCRP in the vector control HEK cells (pcDNA-7) could not be detected under the conditions used (Fig. 1A).

The I206L, N590Y and D620N Variants Were Predominantly Routed To the Plasma Membrane. To explore whether the variants might influence plasma membrane localization of BCRP, the 482R-21, I206L-13, N590Y-1 and D620N-9 cells were examined by immunofluorescent confocal microscopy. Cells transfected with cDNAs of wild-type BCRP (482R-21) and the three variants (I206L-13, N590Y-1 and D620N-9) showed strong plasma membrane staining (Fig. 2), suggesting that, similar to wild-type BCRP, all the three variants were predominantly routed to the plasma membrane. There appears to be some intracellular staining for the variants, particularly for N590Y. BCRP was not detectable in cells transfected with the empty pcDNA expression vector (Fig. 2).

To further confirm cell surface expression of the variants, the 482R, I206L, N590Y and D620N cells were incubated with the phycoerythrin-labeled anti-BCRP surface mAb 5D3 and the IgG negative control. The phycoerythrin fluorescence associated with the cells was then measured by flow cytometry. All these cell lines demonstrated strong expression of BCRP on the cell surface (Fig. 3). As expected, cell surface expression of BCRP was not detectable in the vector control cells (Fig. 3). The differences in phycoerythrin fluorescence between cells incubated with the 5D3 and the IgG antibodies were used to measure the expression levels of surface expression of wild-type BCRP and the variants. The relative levels of I206L, N590Y and D620N on the cell surface were calculated from three independent experiments to be approximately 0.79-fold, 3.1-fold and 1.3-fold those of wild-type BCRP, respectively. It should be noted that

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a small difference in BCRP expression may not be distinguished by this flow cytometry assay. For example, Robey et al. noticed a 2-fold higher level of 482R than the 482G or the 482T mutant in HEK cells by immunoblotting; however, this 2-fold difference in BCRP expression could not be detected by flow cytometry (Robey et al., 2003). This may explain why the differences in expression levels between wild-type BCRP (482R) and the variants measured by immunoblotting (Fig. 1B) were generally greater than those determined by flow cytometry. For this reason, only the relative expression levels shown in Fig. 1B were used in all subsequent calculations.

Efflux Activities of the BCRP Variants. To determine whether the variants exhibit altered drug efflux activity, flow cytometric efflux assays were performed. Fig. 4 shows that intracellular fluorescence of MX, PhA and BODIPY-prazosin in cells expressing wild-type BCRP and the three variants were increased by addition of 10 μM FTC, while FTC did not affect intracellular fluorescence of these compounds in the vector control cells. These results suggest that, like wild-type BCRP, the three variants were capable of transporting MX, PhA and BODIPY-prazosin, which are known BCRP substrates (Robey et al., 2001; Robey et al., 2004). In both the vector control and BCRP-expressing cells, addition of 10 μM FTC decreased rather than increased intracellular accumulation of rhodamine 123 (Fig. 4), indicating that rhodamine 123 is not transported by wild-type BCRP and the three variants. To further quantitatively analyze efflux activity, the changes of intracellular fluorescence (ΔF) upon addition of FTC were used to express the FTC-inhibitable efflux activities of BCRP and its variants, as FTC is a BCRP-specific inhibitor. The ΔF values were calculated by subtracting the median fluorescence of the

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Efflux histograms from the median fluorescence of the FTC/Efflux histograms. The results are summarized in Table 1. The cells expressing I206L exhibited apparent efflux activities comparable to those of the cells expressing wild-type BCRP for all the three fluorescence compounds tested, whereas the cells expressing N590Y and D620N showed higher efflux activities. Since wild-type BCRP and the variants were expressed at different levels in the plasma membranes of the cells used in efflux assays (Fig. 1B), to compare specific transport activities of 482R and the variants, the apparent efflux activities were normalized to the BCRP protein levels. After normalization, the efflux activities of I206L were approximately 2 – 3-fold those of wild-type BCRP for the three fluorescent substrates, and the efflux activities were reduced by approximately 60 - 70% and 40 – 50% for N590Y and D620N, respectively (Table 1).

Drug Resistance Profiles of Cells Expressing the BCRP Variants. To further confirm whether the variants affected the drug resistance properties of BCRP, we examined the drug resistance profiles of HEK cells expressing wild-type BCRP and the variants to MX, topotecan, rhodamine 123 and daunorubicin. Wild-type BCRP and the three variants conferred resistance to MX and topotecan and did not confer resistance to rhodamine 123 and daunorubicin (Fig. 5). Consistent with these results, previous studies have demonstrated that rhodamine 123 and daunorubicin are not substrates of wild-type BCRP (Robey et al., 2003). IC_{50} values and the relative resistance factors for MX and topotecan were calculated and are shown in Table 2. The I206L cells showed apparent resistance levels to MX and topotecan comparable to the cells expressing wild-type BCRP; however, after normalizing to the BCRP protein levels, I206L demonstrated an

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approximately 2-fold increase in resistance to MX and topotecan compared with wild-type BCRP. In contrast, after normalization to the BCRP levels, N590Y conferred resistance to MX and topotecan at levels only approximately 30% of those of wild-type BCRP. MX resistance conferred by D620Y was decreased by approximately 50%, while its level of topotecan resistance remained essentially unchanged.

Vanadate-sensitive ATPase Activities of the BCRP Variants. We also measured vanadate-sensitive ATPase activities of wild-type BCRP and its variants in the plasma membranes. The basal ATPase activity of wild-type BCRP was not significantly affected by addition of 10 μM MX, and was slightly stimulated by 10 μM prazosin and almost completely inhibited by 10 μM FTC (Fig. 6). A similar pattern of the effects of MX, prazosin and FTC on the basal ATPase activities of I206L, N590Y and D620N was observed. After normalizing to the BCRP protein levels, the basal ATPase activity of I206L and its ATPase activities in the presence of MX and prazosin were approximately 3 times higher than the respective activities of wild-type BCRP; however, the differences between the ATPase activities of I206L and those of wild-type protein were not statistically significant. In contrast, N590Y and D620N exhibited basal ATPase activities and the ATPase activities in the presence of MX and prazosin that were approximately 30% and 50% of the wild-type activities, respectively. Moreover, the basal ATPase activities of N590Y and D620N and their prazosin-stimulated ATPase activities were significantly different from the respective activities of wild-type BCRP (Fig. 6). FTC at 10 μM, fully diminished the basal ATPase activities of the variants, which is consistent with previous observations that FTC inhibits BCRP ATPase activity (Robey et al., 2001).

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Discussion

Functional characterization has already been reported for several BCRP variants. Imai et al. demonstrated that Q141K was expressed at a lower level in transfected murine fibroblast PA317 cells and conferred lower levels of drug resistance compared with wild-type BCRP, whereas V12M exhibited an expression level and drug resistance profiles very similar to those of wild-type protein (Imai et al., 2002). Another study (Mizuarai et al., 2004) showed that V12M and Q141K were expressed in the polarized LLC-PK1 porcine kidney cells at levels comparable to that of wild-type protein; however, both V12M and Q141K conferred significantly lower drug resistance than wild-type protein accompanied with increased drug accumulation and decreased drug efflux. Further analysis of the mechanism of transport dysfunction revealed that the apical membrane localization of V12M was disrupted while the expression and apical membrane localization of Q141K were not affected; however, the ATPase activity of Q141K was decreased. A recent study illustrated that V12M was expressed in HEK cells at levels similar to that of wild-type BCRP, whereas the Q141K level was significantly decreased compared with wild-type protein; however, the transport activity normalized by the BCRP protein expression was almost the same for V12M, Q141K and wild-type BCRP (Kondo et al., 2004). The reason for such apparent discrepancies is currently unknown but might be possibly caused by the inconsistency of experimental conditions used in these studies such as different mammalian expression systems and/or varying transfection efficiencies. However, the lower Q141K expression appears to be consistent with *in vivo* data. When 99 Japanese placental samples were analyzed, the subjects who were homozygous for Q141K were found to express significantly lower amounts of BCRP

protein (Kobayashi et al., 2005). The lower protein expression and/or transport dysfunction of Q141K could affect drug disposition. The recent clinical study has shown that Q141K is associated with significant changes in pharmacokinetics of diflomotecan (Sparreboom et al., 2004). In 5 patients heterozygous for Q141K, the plasma levels of diflomotecan after intravenous administration were 299% of those in 15 patients expressing wild-type BCRP. Moreover, the plasma levels of diflomotecan were not significantly affected by 11 known variants in ABCB1/P-gp, ABCC2/MRP2, CYP3A4 and CYP3A5 genes. Although more extensive and larger clinical studies are needed, this is the first direct evidence linking an ABCG2 variant to altered drug exposure. Since diflomotecan is a weak BCRP substrate, the possibility that the changes in diflomotecan pharmacokinetics are caused by other transporters and/or drug metabolizing enzymes could not be excluded.

Several other BCRP variants occurring at much lower allele frequencies (0.5 - 1%) such as A149P, R163K, Q166E, P269S and S441N have also been characterized (Kondo et al., 2004). Except for S441N, all of these BCRP variants showed similar protein expression, membrane localization and transport function as wild-type protein (Kondo et al., 2004). S441N exhibited impaired membrane localization and lower protein expression, indicating that this variant may also affect disposition of BCRP substrates.

In the present study, we examined the I206L, N590Y and D620N variants. I206L, N590Y and D620N were stably expressed in HEK cells. The immunoblots of the plasma membranes revealed a markedly lower protein level for I206L compared with wild-type BCRP, whereas the levels of N590Y and D620N were increased (Fig. 1B). Although the expression levels were changed, the three variants were all predominantly routed to the

plasma membrane (Fig. 2) and their cell surface expression was demonstrated by flow cytometry using the 5D3 anti-BCRP surface mAb (Fig. 3). The data indicate that these natural mutations do not significantly influence protein trafficking of BCRP to the plasma membrane. The mechanism of lower expression for I206L is not known but might be related to the location of position 206 in the functionally important Walker B motif of the NBD in BCRP. Several mutations in the Walker B motif of NBD1 in MRP1 have been shown to strongly prevent biosynthetic maturation of the protein (Cui et al., 2001). Likewise, the I206L variant may also affect maturation and hence decreases protein expression of BCRP. The apparent transport activities of the cells expressing I206L were not significantly changed (Fig. 4 and Table 1); however, after normalization to the BCRP protein level, I206L exhibited efflux activities and drug resistance capabilities approximately 2 – 3 times higher than those of wild-type protein (Tables 1 and 2). One possible explanation for the increased transport activities of I206L is that the affinity of substrates to I206L and/or transport (turn-over) efficiency of the variant are augmented. Although not statistically significant, the ATPase activities of I206L were also found to be increased approximately 3-fold compared with wild-type protein (Fig. 6). These results suggest that, similar to the mutants in the Walker B motif in MRP1, the I206L variant can reduce protein expression and influence BCRP activity.

The N590Y and D620N variants are predicted to be in the extracellular loop connecting the fifth and sixth TM segments of BCRP. Functional analysis showed that, after normalization to the BCRP protein level, N590Y exhibited less than 50% of wild-type efflux and drug resistance activities (Tables 1 and 2). The efflux activities of D620N for MX, PhA and BODIPY-prazosin were also reduced but to a lesser extent as compared

with N590Y (Table 1). Whereas D620N conferred resistance to MX at a level approximately 50% of that of wild-type BCRP, its resistance to topotecan was essentially unchanged (Table 2). These data indicate that the naturally occurring N590Y and D620N mutations in the extracellular loop can alter function and substrate selectivity of BCRP without significantly affecting cell surface expression, and that a mutation such as D620N in BCRP could affect recognition of one substrate but not the other, which is likely due to the existence of different ligand binding sites on BCRP that has been implicated in previous studies (Nakanishi et al., 2003; Gupta et al., 2004). Consistent with the changes in efflux activities, ATPase activities of both N590Y and D620N were also decreased to approximately 30 – 50% of the wild-type activities (Fig. 6).

Mutations in the extracellular loops of P-gp have been reported to alter function of the transporter. For instance, the W208G mutation located in the second extracellular loop of P-gp was shown to drastically reduce affinity of valinomysin, FK506 and α-factor for P-gp (Kwan and Gros, 1998). In addition, mAb UIC2, which recognizes a conformational epitope in the first extracellular loop of P-gp, inhibits P-gp-mediated resistance to a large number of drugs (Mechetner and Roninson, 1992). These data indicate that transport activity and substrate specificity of P-gp could be modified by conformational changes induced by mutations in the extracellular loops or by binding of mAb to an extracellular moiety. Our data, demonstrating that the efflux activities and drug resistance capabilities of N590Y and D620N, except for the topotecan resistance, are decreased, indicate that amino acid changes in the extracellular loop of BCRP can selectively affect function of the transporter. The mechanism of such effects is unknown. One possible explanation is that amino acids such as those at positions 590 and 620 in the

extracellular loops might be involved in interactions between two BCRP molecules and thus affect dimerization and/or conformational changes which may be essential for substrate recognition and transport activity of BCRP (Doyle and Ross, 2003). While N590 is not predicted to be a glycosylation site, N620 is part of the sequence NLSP which falls within the pattern of putative N-glycosylation site "NXS/TX". However, the immunoblots of both whole cell lysates and the plasma membranes did not illustrate any reduction or increase in molecular weight of N590Y and D620N compared with wild-type protein (Fig. 1). These results rule out the possibility that N590Y and D620N would be involved in N-glycosylation. Interestingly, ATPase activities of N590Y and D620Y were coincidentally impaired as the transport activities, even though the two mutations are not within the NBD. The ATPase activities of the G406L/G410L mutant, which is predicted in the first TM segment of BCRP, were found to be completely abolished (Polgar et al., 2004). These data suggest that, whereas ATPase activities of BCRP could be affected by mutations in the NBD (e.g., Q141K and I206L), mutations in other regions including the TM segments (e.g., G406L/G410L) and extracellular loops (e.g., N590Y and D620N) can also influence ATP hydrolysis.

The I206L variant was identified so far only in Hispanic livers with a 20% allele frequency from a small number of samples (Zamber et al., 2003). The N590Y variant was present in Caucasians with approximately 0.6 – 1.5% allele frequencies (Zamber et al., 2003; Mizuarai et al., 2004). The D620N variant was detected in 1.1% of all DNA samples examined with unknown genetic origin (Honjo et al., 2002). The *in vitro* analysis in this study revealed that, after normalization to the BCRP expression levels, the specific activities of I206L, N590Y and D620N were significantly altered as compared with wild-

type BCRP; however, the overall efflux activities and drug resistance profiles of the cells expressing these variants remained essentially unchanged. Therefore, expression of these BCRP variants may not be detrimental to patients bearing such polymorphisms.

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Footnotes:

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Figure Legends

Figure 1: Expression levels of wild-type BCRP and its variants I206L, N590Y and D620N in stably transfected HEK cells. (**A**) Immunoblot of whole cell lysates (30 μg of protein each lane) prepared from selected cell lines, as indicated, transfected with BCRP cDNA constructs and the pcDNA empty vector. (**B**) Immunoblot of the plasma membrane preparations (2.5 μg of protein each lane) isolated from cell lines expressing the highest levels of wild-type BCRP and the variants. Relative expression levels of the variants as compared with wild-type BCRP were determined by densitometric analysis as described in "Materials and Methods" and are shown under each lane. Shown are representative immunoblots. Similar results were obtained in two or more independent experiments.

Figure 2: Confocal microscopy of HEK cells stably expressing wild-type BCRP and its variants. BCRP proteins were detected using mAb BXP-21 as described in "Materials and Methods" and are indicated in green. No green fluorescence was detected in the pcDNA vector control cells. Selected areas of HEK cells expressing wild-type BCRP (482R) and the variants I206L, N590Y and D620N are shown. Images have been enhanced for maximal contrast between the black background and green fluorescence and were not intended for quantitative determination of BCRP expression.

Figure 3: Cell surface expression of wild-type BCRP (482R) and its variants in HEK cells. Cell surface expression of wild-type BCRP and the three variants were all detectable using the 5D3 antibody as described in "Materials and Methods". The solid

and dotted lines represent the phycoerythrin fluorescence associated cells incubated with the IgG negative control and the 5D3 antibodies, respectively. Histograms shown are from a representative experiment out of three independent experiments performed.

Figure 4: Efflux of fluorescent substrates by wild-type BCRP and its variants. The efflux assays for wild-type BCRP (482R) and the variants were performed with MX (10 μM), PhA (2 μM) and BODIPY-prazosin (500 nM), and rhodamine 123 (0.5 μg/ml) in the presence or absence of FTC (10 μM) as described in "Materials and Methods". The dotted and solid lines represent intracellular fluorescence in the presence and absence of FTC, respectively. Results shown are from a representative experiment out of three or more independent experiments performed.

Figure 5: Drug resistance profiles of HEK cells expressing wild-type BCRP and its variants. The cells expressing wild-type BCRP (▲), the variants I206L (△), N590Y (□), D620N (♢) and the vector control cells (●) were exposed to MX (A), topotecan (B), daunorubicin (C) and rhodamine 123 (D) at various concentrations indicated. The cell toxicity studies were performed as described in "Materials and Methods". The results shown are mean ± S.D. of six determinations in a representative experiment. Similar results were obtained in at least three independent experiments.

Figure 6: The vanadate-sensitive ATPase activities of wild-type BCRP and its variants. The vanadate-sensitive ATPase activities of the plasma membrane preparations isolated from HEK cells expressing wild-type BCRP (482R) and its variants (I206L, N590Y and

D620N) were measured in the absence of exogenous compounds (basal, solid bars) or in the presence of MX (10 μM, grey bars), prazosin (10 μM, vertical bars) and FTC (10 μM, dotted bars), as described in "Materials and Methods". The results shown have been normalized to BCRP expression. The relative BCRP expression levels shown in Fig. 1B were used for these calculations. The basal ATPase activity of wild-type BCRP was set as 100%. Data points represent means ± S.D. of three independent experiments. * The basal and prazosin-stimulated ATPase activities of N590Y or D620N are significantly different ($p < 0.05$) from the respective ATPase activities of wild-type protein as calculated by Student's *t* test.

Table 1: **FTC-inhibitable Efflux Activities of HEK Cells Expressing Wild-type BCRP and its Variants.**

	MX		PhA		BODIPY-prazosin		Rhodamine 123
	ΔF	Ratio	ΔF	Ratio	ΔF	Ratio	ΔF
pcDNA	0		11.4 ± 7.1		0		0
482R-21	42.5 ± 8.4	1.0	121.9 ± 27.5	1.0	127.0 ± 51.5	1.0	0
I206L-13	52.8 ± 3.0	2.76	131.7 ± 17.3	2.40	127.2 ± 80.2	2.23	0
N590Y-1	64.7 ± 4.7*	0.42	149.3 ± 22.2	0.34	147.5 ± 97.1	0.32	0
D620N-9	67.1 ± 7.1*	0.63	168.2 ± 29.8*	0.55	149.1 ± 68.7	0.47	0

The FTC-inhibitable efflux activities of fluorescent compounds are represented by the differences (ΔF) in the median fluorescence between the FTC/Efflux histograms and the Efflux histograms. The ΔF values obtained from the experiments with rhodamine 123 were negative and therefore assigned to zero. The values shown are means ± S.D. of at least three independent experiments. The relative efflux activities of the variants as compared with wild-type BCRP are shown as "Ratios" after normalization for differences in BCRP protein expression. The relative BCRP expression levels shown in Fig. 1B were used for these calculations. * indicates that the unnormalized ΔF values for the 482R cells are significantly different ($p < 0.05$) from the N590Y or D620N cells as calculated by Student's *t* test.

Table 2: Drug Resistance Profiles of HEK Cells Expressing Wild-type BCRP and its Variants.

	MX			Topotecan			Daunorubicin		Rhodamine 123	
	IC_{50} (nM)	RR	Ratio	IC_{50} (nM)	RR	Ratio	IC_{50} (nM)	RR	IC_{50} (nM)	RR
pcDNA	32.5 ± 3.1			8.5 ± 1.0			34.2 ± 4.1		5797.9 ± 1209.4	
482R-21	226.3 ± 27.4	7.0	1.0	122.8 ± 23.3	14.4	1.0	40.0 ± 1.7	1.2	10281.0 ± 1445.4	1.7
I206L-13	209.7 ± 19.5	6.5	2.06	124.9 ± 17.1	14.7	2.25	25.6 ± 2.8	0.7	8829.4 ± 1454.8	1.5
N590Y-1	287.9 ± 24.0*	8.9	0.35	130.5 ± 17.8	15.4	0.30	19.9 ± 2.3	0.6	7867.9 ± 3691.1	1.3
D620N-9	271.9 ± 33.3*	8.4	0.48	278.3 ± 18.9*	32.7	0.91	31.6 ± 1.8	0.9	15469.3 ± 1762.5	2.6

Relative resistance factors (RR) were obtained by dividing the IC_{50} values of cells expressing wild-type BCRP (482R) or its variants by the IC_{50} values of the vector control cells. The "Ratios" represent the relative levels of resistance of the variants as compared with wild-type BCRP after normalization for differences in BCRP protein expression. The relative BCRP expression levels shown in Fig. 1B were used for these calculations. Since wild-type BCRP and the variants did not confer resistance to daunorubicin and rhodamine 123, the "Ratios" for daunorubicin and rhodamine 123 were not calculated. The IC_{50} values shown are means ± S.D. of at least three independent experiments. * indicates that the unnormalized IC_{50} values of the 482R cells are significantly different ($p < 0.05$) from the N590Y or D620N cells as calculated by Student's *t* test.

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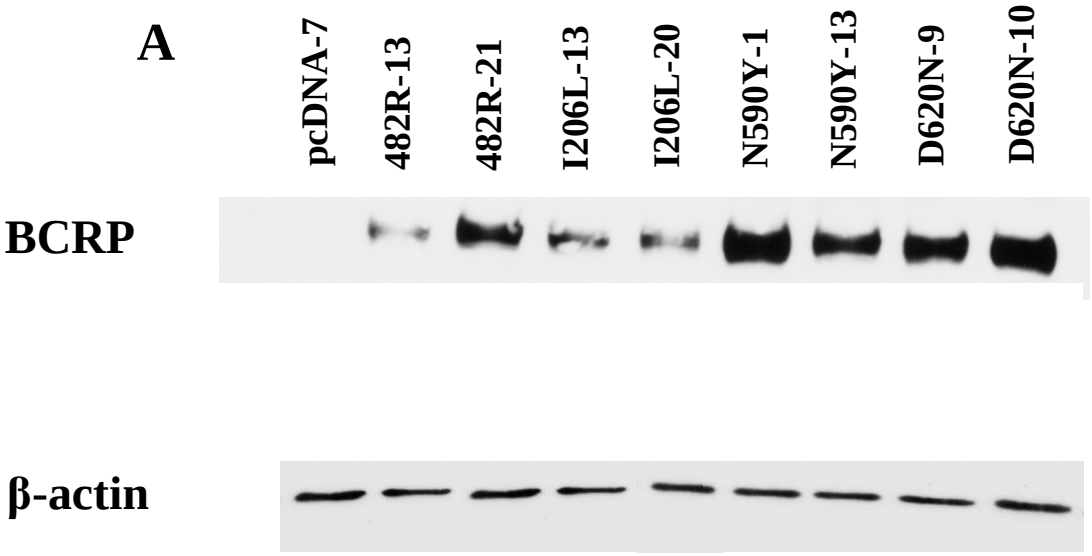
Figure 1



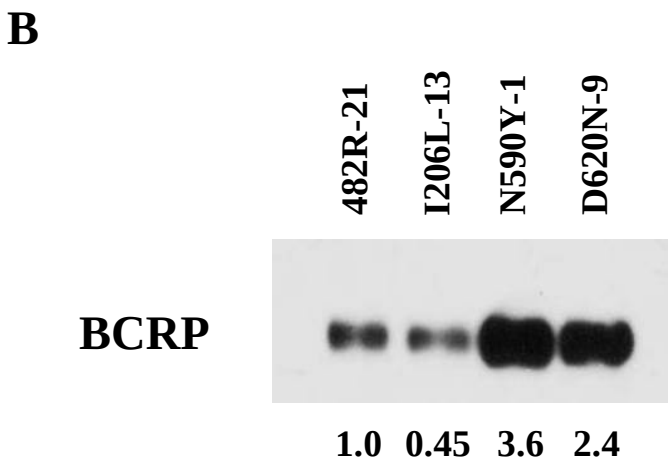


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Figure 2

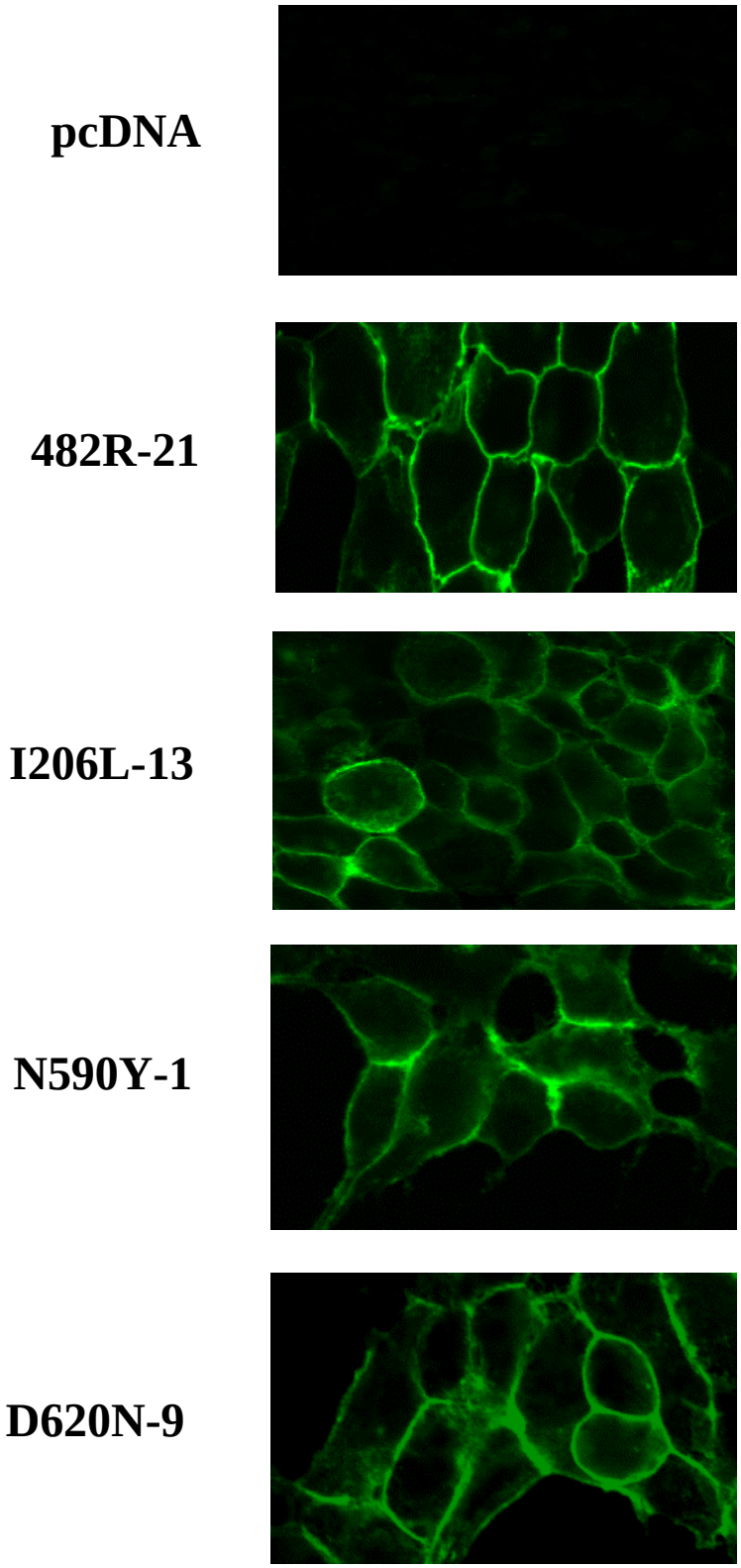

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Figure 3

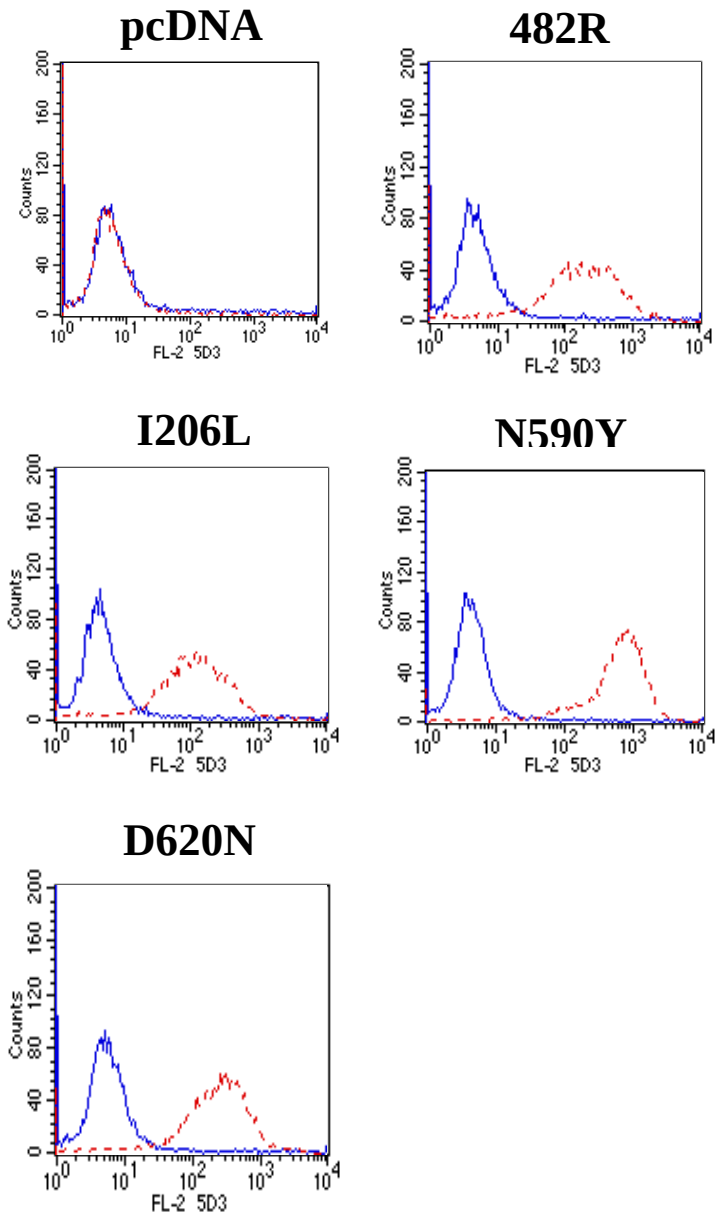

Figure 4

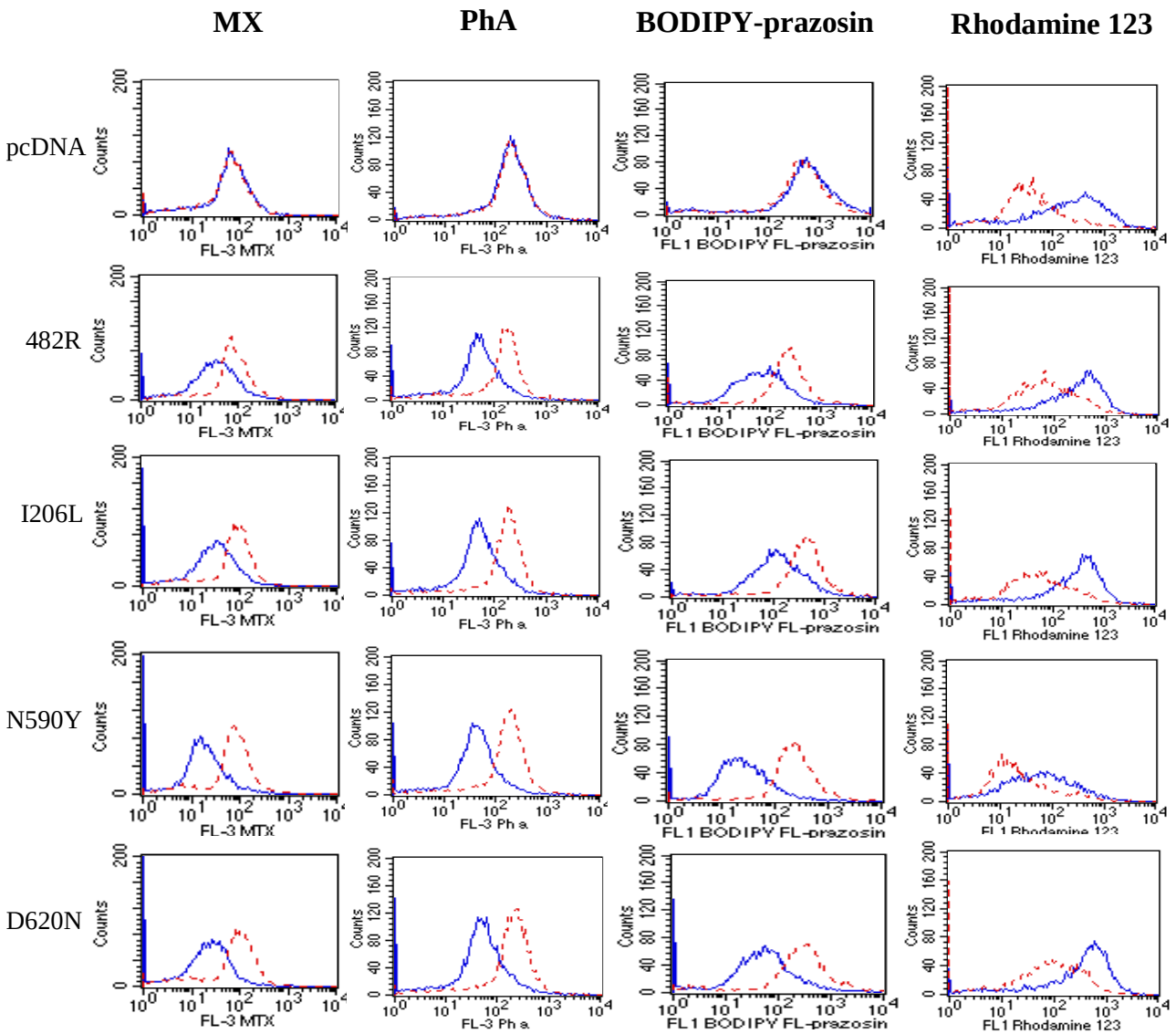

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Figure 5

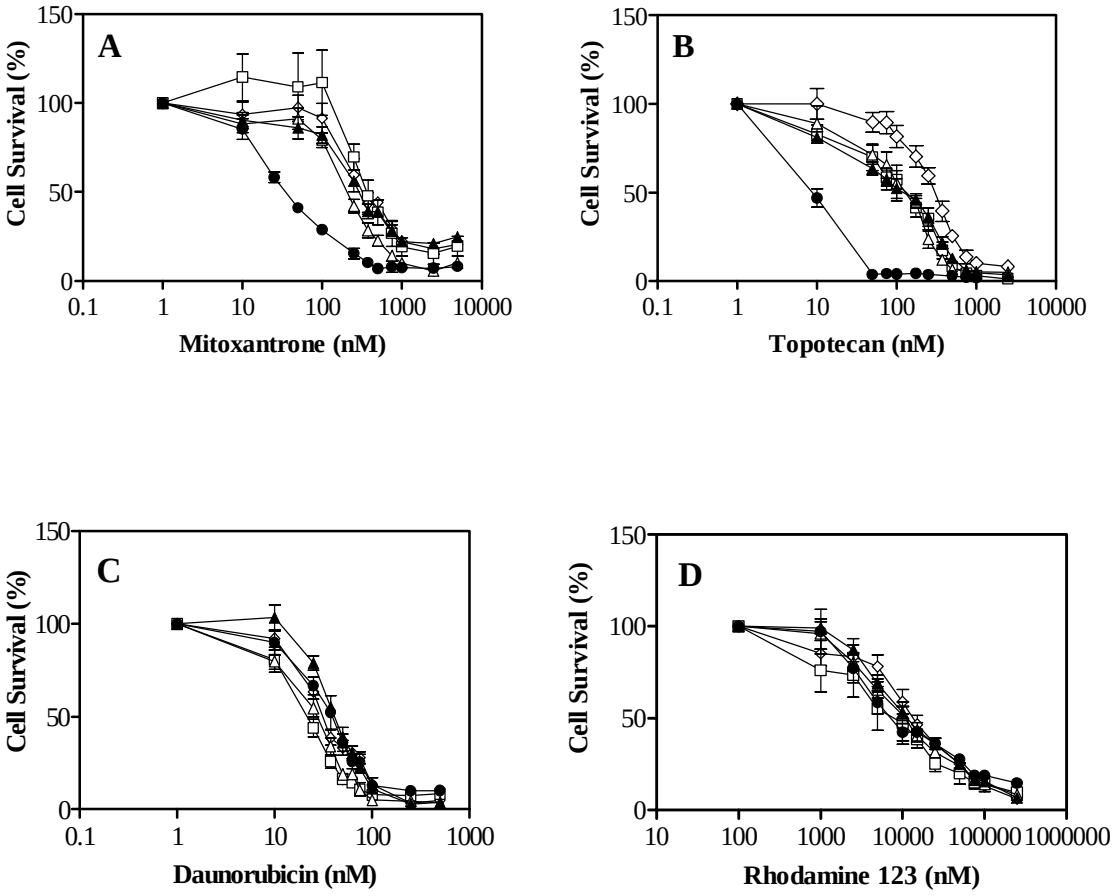

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Figure 6