

# Role of the ABCG2 drug transporter in the resistance and oral bioavailability of a potent cyclin-dependent kinase/Aurora kinase inhibitor

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## Abstract

Cell cycle kinase inhibitors have advanced into clinical trials in oncology. One such molecule, JNJ-7706621, is a broad-spectrum inhibitor of the cyclin-dependent kinases and Aurora kinases that mediate G<sub>2</sub>-M arrest and inhibits tumor growth in xenograft models. To determine the putative mechanisms of resistance to JNJ-7706621 that might be encountered in the clinic, the human epithelial cervical carcinoma cell line (HeLa) was exposed to incrementally increasing concentrations of JNJ-7706621. The resulting resistant cell population, designated HeLa-6621, was 16-fold resistant to JNJ-7706621, cross-resistant to

mitoxantrone (15-fold) and topotecan (6-fold), and exhibited reduced intracellular drug accumulation of JNJ-7706621. ABCG2 was highly overexpressed at both the mRNA (~163-fold) and protein levels. The functional role of ABCG2 in mediating resistance to JNJ-7706621 was consistent with the following findings: (a) an ABCG2 inhibitor, fumitremorgin C, restored the sensitivity of HeLa-6621 cells to JNJ-7706621 and to mitoxantrone; (b) human embryonic kidney-293 cells transfected with ABCG2 were resistant to both JNJ-7706621 and mitoxantrone; and (c) resistant cells that were removed from the drug for 12 weeks and reverted to susceptibility to JNJ-7706621 showed near-normal ABCG2 RNA levels. ABCG2 is likely to limit the bioavailability of JNJ-7706621 because oral administration of JNJ-7706621 to *Bcrp* (the murine homologue of ABCG2) knockout mice resulted in an increase in the plasma concentration of JNJ-7706621 compared with wild-type mice. These findings indicate that ABCG2 mediates the resistance to JNJ-7706621 and alters the absorption of the compound following administration. [Mol Cancer Ther 2006;5(10):2459–67]

## Introduction

JNJ-7706621, a novel 3,5-diamino-1,2,4-triazole, is a potent cell cycle inhibitor that targets cyclin-dependent kinases (CDK) and Aurora kinases. This compound delays the progression of cancer cells through G<sub>1</sub> phase and arrests the cell cycle in G<sub>2</sub>-M phase. It is cytotoxic to a wide range of tumor cell types, and produces antitumor activity in human tumor xenograft models (1). This compound is one of the first promising antineoplastic agents being developed as a dual CDK/Aurora kinase inhibitor. However, a major obstacle to successful cancer therapy is the acquired resistance of tumor cells to therapeutic drugs. Therefore, this work was undertaken to assess the potential for development of resistance to JNJ-7706621 prior to the clinical use of this compound.

Although the basis for resistance is often complex, a common resistance phenomena known as multidrug resistance (MDR) frequently occurs when cells are repeatedly treated with a single cytotoxic drug. Prominent among possible mechanisms of MDR are the broad specificity drug efflux pumps of the ATP-binding cassette (ABC) transporter family, including P-glycoprotein (P-glycoprotein/ABCB1; ref. 2), multidrug resistance-associated protein (MRP1/ABCC1; ref. 3), and the breast cancer resistance protein (BCRP/MXR/ABCP/ABCG2; refs. 4–7). The overexpression of ABC transporters in cancer cells can result in MDR by limiting the intracellular accumulation of cytotoxic agents through the active extrusion of these agents. ABCG2/BCRP overexpression has been observed in

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several drug-resistant cell lines and tumors, which indicates its importance in the multidrug-resistant phenotype of cancer cells (4, 8–10). The ABCG2 drug efflux pump can transport a variety of chemotherapeutic agents including mitoxantrone, the camptothecins, topotecan, SN-38, doxorubicin, and flavopiridol (4, 5, 7, 9, 11–16).

Certain ABC transporters also mediate extensive protection of the body against the toxic action of anticancer drugs. ABCG2, in particular, is expressed in a number of normal tissues; the canalicular membrane of liver hepatocytes, the apical membrane of the epithelium in the small and large intestine, the ducts and lobule of the breast, the luminal surface of brain capillaries, and human placenta (17). The localization of ABCG2 in the intestine and liver suggests that ABCG2 has the potential to strongly affect the pharmacokinetics of substrate drugs. In fact, ABCG2 expression was shown to influence both the absorption and secretion of topotecan in mice and in humans (18, 19). Thus, ABCG2 function has the potential to mediate resistance to chemotherapy and to alter the pharmacokinetic properties of certain anticancer drugs.

In the present study, a population of human epithelial cervical carcinoma (HeLa) cells was selected for resistance to JNJ-7706621, and the molecular mechanism involved in the resistance was identified. It was determined that JNJ-7706621 is a substrate of the ABCG2 efflux pump. Overexpression of the ABCG2 transporter was observed in the resistant cells, and inhibition of this transporter restored the sensitivity of these cells to JNJ-7706621. Furthermore, mouse models using male *Bcrp1*<sup>-/-</sup> (the murine homologue of ABCG2; ref. 20) mice showed that the transporter could influence the pharmacokinetics of JNJ-7706621 following oral administration.

## Materials and Methods

### Reagents

JNJ-7706621 was synthesized by the Cancer Therapeutics Research Team (Johnson & Johnson Pharmaceutical Research and Development, LLC) as previously described (21). Fumitremorgin C (FTC) was obtained from Axxora, LLC (San Diego, CA). Vinblastine, doxorubicin, topotecan, mitoxantrone, and paclitaxel were purchased from Sigma-Aldrich (Saint Louis, MO). Flavopiridol was purchased from ChemPacific (Baltimore, MD). All compounds were dissolved in DMSO (Sigma-Aldrich).

### Cell Culture

HeLa cells were obtained from the American Type Culture Collection (Manassas, VA). Sensitive HeLa cells and their resistant variants were grown in minimum essential medium (Eagle) supplemented with 10% fetal bovine serum, 2 mmol/L of L-glutamine, 0.1 mmol/L of nonessential amino acids, and 1 mmol/L of sodium pyruvate. Resistant HeLa cells (HeLa-6621) were selected for growth via continuous exposure to stepwise increased JNJ-7706621 concentrations ranging from 750 nmol/L to 3.5  $\mu$ mol/L over a 12-month period. During selection, cells were monitored for cell cycle kinetics by flow cytometry as

described in Cell Cycle Analysis. When cells achieved a normal cell cycle distribution, exposure to JNJ-7706621 was increased by 0.5  $\mu$ mol/L. HeLa-control cells were maintained in an equivalent volume of DMSO vehicle. Revertant cells were established by maintaining the resistant cells in drug-free medium for 12 weeks. At the 12-week time point, sensitivity to JNJ-7706621 was assessed. All cells were maintained at 37°C in 5% CO<sub>2</sub>.

### Cell Cycle Analysis

Cells were stained with propidium iodide for the analysis of nuclear DNA content as previously described (1).

### Drug Accumulation Studies

Asynchronous cells ( $1 \times 10^6$ ) were treated with 1, 5, and 10  $\mu$ mol/L of JNJ-7706621 for 1 hour. Following incubation, the cells were washed with PBS and lysed in 500  $\mu$ L of HNTG buffer (50 mmol/L HEPES, pH 7.5; 1% Triton X-100, 150 mmol/L NaCl, 10% glycerol, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L Na<sub>3</sub>VO<sub>4</sub>, and 5 mmol/L NaF). Cell lysates were clarified by centrifugation and an aliquot was taken to determine protein content by the bicinchoninic acid protein assay (Pierce, Rockford, IL). Lysates (200  $\mu$ L) were frozen at -80°C and the clarified cell lysate was analyzed for JNJ-7706621 content as described in Pharmacokinetics Analysis. Drug concentration was calculated based on equal amounts of protein content. The results were obtained from the average of three independent experiments.

### Cell Proliferation Assays and Determination of Resistance Factor Values

Antiproliferative activity was assessed in a cell proliferation assay that measured <sup>14</sup>C-thymidine incorporation into cellular DNA as previously described (22). All cell types used in the assay were plated at 4,000 cells/well and exposed to compound for either 48 or 96 hours. Resistance factors were calculated by the ratio of IC<sub>50</sub> values of HeLa-6621 cells to IC<sub>50</sub> values of HeLa-control cells, or by the ratio of IC<sub>50</sub> values of wild-type ABCG2-transfected human embryonic kidney 293 (HEK-293) cells to IC<sub>50</sub> values of vector-transfected HEK-293 cells. To examine the effect of ABCG2 inhibition on sensitivity to JNJ-7706621, the cell proliferation assay was done in the presence of 10  $\mu$ mol/L FTC following a 10-minute preincubation period with the compound.

### RNA Isolation and Quantitative Reverse Transcription-PCR

Total cellular RNA was isolated from cell lines using the RNeasy Mini Kit (Qiagen, Valencia, CA). Quantitative real-time reverse transcription (RT)-PCR was used to measure the mRNA expression levels of the selected ABC drug transporters. The LightCycler RNA Master SYBR Green Kit and LightCycler machine (Roche Biochemicals, Indianapolis, IN) were used in these studies. All primer sets were tested prior to use to ensure that only a single product of the correct size was amplified for all ABC transporter primer sets (23). The RT-PCR reaction was done on 150 ng total RNA with 250 nmol/L of specific primers under the following conditions: reverse transcription (20 minutes at 61°C), one cycle of denaturation at 95°C for 30 seconds, and

PCR reaction of 45 cycles with denaturation (15 seconds at 95°C), annealing (30 seconds at 58°C), and elongation (30 seconds at 72°C with a single fluorescence measurement). The PCR reaction was followed by a melting curve program (65–95°C with a heating rate of 0.1°C/s and a continuous fluorescence measurement) and then a cooling program at 40°C. Negative controls consisting of no-template (water) reaction mixtures were run with all reactions. The specificity of the PCR products was determined with the LightCycler software's melting curve analysis feature. PCR products were also run on agarose gels to confirm the formation of a single product at the desired size. Crossing points for each transcript were determined using the second derivative maximum analysis with the arithmetic baseline adjustment. Crossing point values for each transporter were normalized to glyceraldehyde-3-phosphate dehydrogenase housekeeping gene. Data are presented as the fold change of gene expression for the resistant cells compared with the drug-sensitive control cells. All reactions were done in duplicate.

#### Immunoblot Analysis

A nearly confluent 75 cm<sup>2</sup> flask of cells was washed with PBS and then lysed in 0.5 mL of HNTG buffer (50 mmol/L HEPES, pH 7.5; 1% Triton X-100, 150 mmol/L NaCl, 10% glycerol, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L Na<sub>3</sub>VO<sub>4</sub>, and 5 mmol/L NaF). The protein concentrations of the supernatants were determined using the bicinchoninic acid protein assay (Pierce). Samples were separated on a 10% polyacrylamide-SDS gel and transferred to nitrocellulose (Invitrogen, Carlsbad, CA) using a wet transfer system. Lysates were probed with an anti-BCRP monoclonal antibody, clone BXP-21 (Kamiya Biomedical Company, Seattle, WA) at 1 µg/mL and an anti-β-actin monoclonal antibody, clone AC-15 (Sigma-Aldrich) at 1:2,500 dilution. Primary antibodies were detected using horseradish peroxidase-conjugated sheep anti-mouse secondary antibody (Amersham Biosciences, Piscataway, NJ) diluted 1:2,000 and enhanced chemiluminescence plus Western blotting detection system (Amersham Biosciences).

#### *Bcrp1*<sup>-/-</sup> Mice and Animal Dosing

Animals used in the study were male *Bcrp1*<sup>-/-</sup> (*Bcrp1* knockout) and wild-type mice of a comparable genetic background (FVB; ref. 24) between 4 and 5 weeks of age. Animals were purchased from Taconic (Germantown, NY). A total dose of 30 mg/kg JNJ-7706621, prepared as a 3 mg/mL solution in 100% polyethylene glycol 400, was delivered by oral gavage. After the specified time points, animals were sacrificed by terminal bleeding through cardiac puncture under anesthesia with CO<sub>2</sub>. Blood samples were centrifuged for cell removal, and 200 µL of the plasma supernatant was transferred to a 24-well plate, placed on dry ice, and stored in a -70°C freezer prior to analysis.

#### Pharmacokinetics Analysis

Two hundred microliters of acetonitrile-containing internal standard was added to 100 µL of plasma to precipitate proteins. Samples were then centrifuged at 3,000 × g for 5 minutes and the supernatant was removed for

analysis by liquid chromatography tandem mass spectrometry. Calibration standard and quality control samples were prepared by adding the appropriate volumes of stock solution directly into plasma and treated identically to collected plasma samples. These sample preparation steps were automated using a liquid handling workstation (Tomtec Quadra 96; Tomtec, Hamden, CT) in the 96-well format. Liquid chromatography tandem mass spectrometry analysis was done using multiple reaction monitoring for the detection of characteristic ions of compounds being tested and the internal standard. The internal standard used was propranolol for positive ions or chloramphenicol for negative ions.

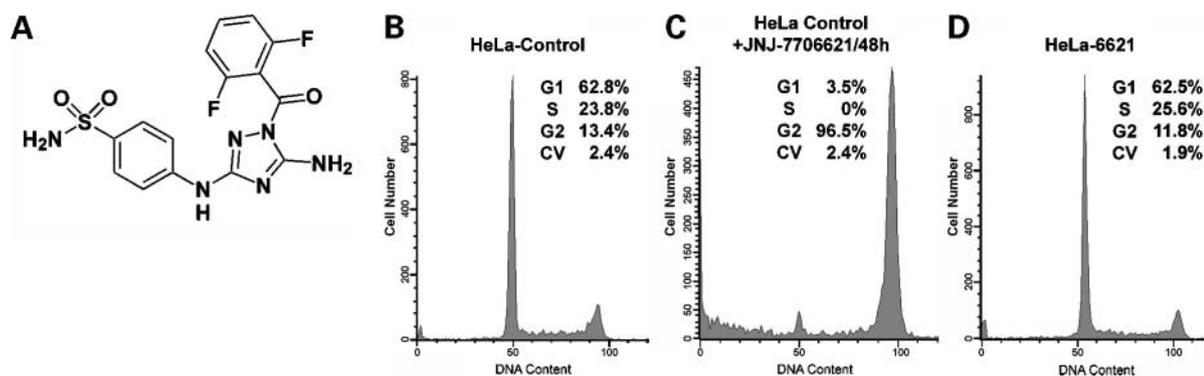
High-pressure liquid chromatography analysis of each sample was done using a gradient elution method with solvent A of 0.1% acetic acid in water and solvent B of 0.1% acetic acid in acetonitrile. The gradient program was carried out from 5% to 100% of solvent B at a flow rate of 1.5 mL/min. A Waters SunFire (Milford, MA) C18 (3.5 µm, 4.6 × 30 mm) reversed-phase column was used for sample analysis. Positive ion turbo spray mass spectrometry was obtained using a Sciex API4000 mass spectrometer. The operating conditions for the mass spectrometer were as follows: source temperature of 550°C, collisionally activated dissociation gas at 10 psi, curtain gas flow at 40 psi, GS1 at 45 psi, GS2 at 45 psi, and an ion spray source of 5,000 V. For the quantitation of propranolol, the internal standard, the dissociation of *m/z* 260.21 to *m/z* 116 was used. For quantitation of JNJ-7706621, the dissociation of *m/z* 395.03 to *m/z* 141.03 was used. Calibration standards and quality control samples were prepared by adding appropriate volumes of stock solution directly to plasma and these samples were treated identically to collected plasma samples.

## Results

### Selection of Resistant HeLa Cells

HeLa cells were selected for resistance to the novel CDK/Aurora kinase inhibitor, JNJ-7706621 (Fig. 1A). Over a period of 12 months, cells were continuously exposed to increasing concentrations of JNJ-7706621. The initial selection concentration, 750 nmol/L, was approximately 3-fold higher than the IC<sub>50</sub> value achieved in this cell line (1). Cells were stabilized and maintained in 3.5 µmol/L of JNJ-7706621. Following selection, the resistant cells (HeLa-6621) were 16-fold resistant to the selecting drug (Table 1).

To further characterize the HeLa-6621-resistant cells, cell cycle analysis was done by examining the DNA content of control and resistant cells that were exposed to JNJ-7706621 (Fig. 1). Treatment of control HeLa cells with 3 µmol/L of JNJ-7706621 resulted in the accumulation of 96.5% of the cells with 4N DNA content, indicative of G<sub>2</sub>-M arrest (Fig. 1C). In contrast, resistant cells that were continually exposed to 3.5 µmol/L of JNJ-7706621 did not arrest or accumulate in G<sub>2</sub>-M (Fig. 1D) and the cell cycle distribution of these cells was similar to that of the untreated control cells (Fig. 1B). Thus, selection resulted in a population of cells that cycle normally in a concentration of drug that induces cell cycle arrest in sensitive cells.



**Figure 1.** Effects of JNJ-7706621 on cell cycle. **A**, the chemical structure of JNJ-7706621 (4-[5-amino-1-(2,6-difluorobenzoyl)-1H-[1,2,4]triazol-3-ylamino]-benzenesulfonamide). **B**, cells were stained with propidium iodide for the analysis of nuclear DNA content by flow cytometry. The number of cells in each phase of the cell cycle was determined for asynchronous HeLa control cells, HeLa-control cells exposed to 3  $\mu\text{mol/L}$  JNJ-7706621 for 48 h (**C**), and HeLa-6621 cells continually exposed to JNJ-7706621 and stabilized in 3.5  $\mu\text{mol/L}$  of JNJ-7706621 (**D**).

### Analysis of Cross-Resistance

The cross-resistance profile of the HeLa-6621 cells was determined in a 48-hour cell proliferation assay (Table 1). Compared with the control cell line, HeLa-6621 cells were highly cross-resistant to mitoxantrone (15-fold) and also exhibited resistance to topotecan (5.6-fold). The resistant cells, however, remained sensitive to vinblastine (1-fold) and only slightly resistant to doxorubicin and paclitaxel (2.5-, and 2.8-fold, respectively).

Cross-resistance properties of the HeLa-6621 cells were also determined for another CDK inhibitor, flavopiridol (25). Only 1.4-fold resistance was observed with flavopiridol. However, appreciable resistance was observed with a CDK inhibitor from the same chemical series as JNJ-7706621 (7-fold), as well as another structurally distinct pyrazolo-pyridine (data not shown). This suggests that a common mechanism of resistance may exist for this structural class of compounds and is not necessarily related to the mechanistic target of these agents.

### Intracellular Accumulation of JNJ-7706621 and Up-Regulation of ABCG2 in HeLa-6621 Cells

The cross-resistance profile of the HeLa-6621 cells to several anticancer drugs suggested the possibility that cell surface drug transporters were actively exporting the

compounds from the resistant cells. To examine this possibility, intracellular drug concentrations were evaluated in HeLa-6621 and HeLa-control cells treated with 1, 5, and 10  $\mu\text{mol/L}$  of JNJ-7706621 for 1 hour. Following treatment, cells were washed, lysed, and intracellular drug accumulation was determined by liquid chromatography tandem mass spectrometry. As shown in Fig. 2, resistant HeLa-6621 cells showed a statistically significant decrease ( $P \leq 0.05$ ) in the intracellular accumulation of JNJ-7706621 at all concentrations tested relative to control cells.

Because the drug transporters of the ABC family have been shown to contribute to multidrug resistance, the potential contribution of ABC transporters in reduced drug accumulation in HeLa-6621 cells was investigated. RNA expression levels for a panel of ABC transporters were examined. Total RNA from both resistant and control HeLa cells was isolated and subjected to quantitative real-time RT-PCR using specific primers for ABC transporters A2, A3, B1, C1, C2, C4, C5, C11, and G2. The expression levels for each of the ABC transporters in the resistant cells were compared with the levels within the drug-sensitive control cells and the fold change is presented in Fig. 3A. All samples were normalized to the expression level of the glyceraldehyde-3-phosphate dehydrogenase housekeeping

**Table 1.** Cross-resistance profile of HeLa control and HeLa-6621 cells

Drug	HeLa control IC <sub>50</sub> (nmol/L) $\pm$ SD*	HeLa 6621 IC <sub>50</sub> (nmol/L) $\pm$ SD*	Resistance factors <sup>†</sup>	P <sup>‡</sup>
JNJ-7706621	306 $\pm$ 6.5	4,812 $\pm$ 1,338	16	0.004
Mitoxantrone	0.4 $\pm$ 0.2	6.1 $\pm$ 3.9	15	0.032
Topotecan	82 $\pm$ 53	456 $\pm$ 56	5.6	0.035
Flavopiridol	208 $\pm$ 51	286 $\pm$ 22	1.4	0.065
Doxorubicin	27 $\pm$ 1.3	68 $\pm$ 18	2.5	0.009
Vinblastine	374 $\pm$ 19	360 $\pm$ 8.6	1	0.096
Paclitaxel	0.8 $\pm$ 0.09	2.2 $\pm$ 0.8	2.8	0.017

\*IC<sub>50</sub>s shown are the means  $\pm$  SD of results from three experiments, each done with  $n = 10$ .

<sup>†</sup>Resistance factors were determined by dividing the mean IC<sub>50</sub> of the HeLa-6621 by the mean IC<sub>50</sub> for the control HeLa cells.

<sup>‡</sup>Differences in the mean IC<sub>50</sub> values were tested with unpaired  $t$  tests at  $P = 0.05$  (two-tailed).

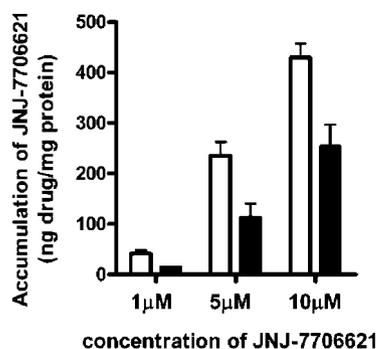
gene. Results showed that the ABCG2 transporter was highly overexpressed (163-fold) in resistant cells. ABC transporters, C2 and B1, also showed an increase in RNA levels but to a lesser extent; 37-fold and 3-fold increase, respectively.

Next, protein expression levels were evaluated for the ABCG2 transporter in resistant and drug-sensitive control cell lines. Figure 3B shows the immunoblot analysis of control and resistant lysates detected by a specific ABCG2 antibody.  $\beta$ -Actin served as a loading control. Analysis revealed increased levels of ABCG2 protein in the resistant cell line. Taken together, these data revealed the overexpression of both ABCG2 transcript and protein in the resistant HeLa-6621 cells and support the cross-resistance data with known ABCG2 substrates mitoxantrone and topotecan.

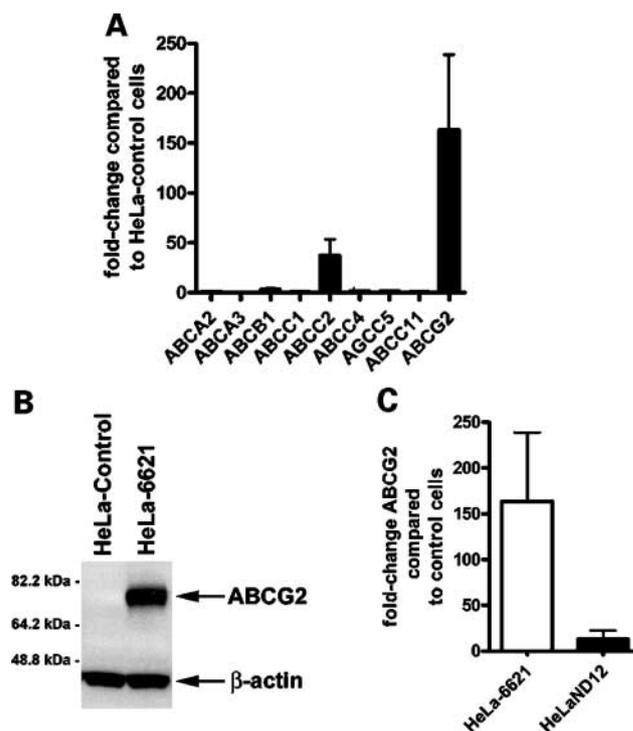
To further characterize the significance of ABCG2 overexpression in the HeLa-6621 cells, ABCG2 expression was evaluated in revertant cells. Revertant cells (HeLaND12) were established by growing the resistant cells in drug-free media. Removal of drug for 12 weeks resulted in cells that were resensitized to the cellular effects of JNJ-7706621. Upon treatment with 4  $\mu$ mol/L of JNJ-7706621 for 48 hours, 79% of revertant HeLaND12 cells arrested in G<sub>2</sub>-M phase, compared with 92% in drug-sensitive control cells, and only 14% in HeLa-6621 cells (data not shown). Total RNA was isolated from the revertant cells and quantitative real-time RT-PCR was done for the panel of ABC transporters as previously described. Data are presented in Fig. 3C. In contrast to the overexpression of ABCG2 transcripts found in resistant cells compared with control cells (163-fold increase), the revertant HeLaND12 cells expressed greatly reduced levels of ABCG2 (13-fold increase).

#### HEK-293 Cells Transfected with Wild-Type ABCG2 Are Resistant to JNJ-7706621

To confirm that JNJ-7706621 is a substrate of the ABCG2 transporter, a 96-hour cell proliferation assay was done on



**Figure 2.** Measurement of intracellular JNJ-7706621 drug accumulation in control and resistant HeLa-6621 cells. Intracellular JNJ-7706621 accumulation was determined for HeLa-control (*open columns*) and HeLa-6621 cells (*filled columns*) following treatment with the indicated concentrations of JNJ-7706621 for 1 h. Accumulation of JNJ-7706621 in nanograms per milligram of protein. *Columns*, mean; *bars*, SD ( $n = 3$ ). At each drug concentration, resistant cells show a statistically significant decrease in intracellular JNJ-7706621 accumulated ( $P \leq 0.05$ ).



**Figure 3.** Examination of the expression levels of ABC transporters in control, drug-selected resistant cell lines, and revertant cells. **A**, quantitative real-time RT-PCR was done using primers specific for ABC transporters A2, A3, B1, C1, C2, C4, C5, C11, and G2. The mRNA expression was calculated relative to the expression of glyceraldehyde-3-phosphate dehydrogenase housekeeping gene. Data are presented as the fold change of gene expression for the HeLa-6621 cells compared with the drug-sensitive control cells. All reactions were done in duplicate. *Columns*, mean; *bars*,  $\pm$ SD. **B**, ABCG2 protein levels in control and resistant cells were determined by immunoblot analysis with a monoclonal antibody directed against ABCG2 (anti-BCRP, clone BXP-21). Detection of  $\beta$ -actin on the blots served as a positive loading control. The positions of migration of the prestained protein standards and their molecular masses are indicated to the side of each panel. **C**, quantitative real-time RT-PCR was done as described in **A** with cells that were maintained in drug-free media for 12 wks (*filled column*). The level of mRNA expression in HeLa-6621 cells is graphed for comparison (*open column*).

HEK-293 cells transfected with either wild-type or with vector control ABCG2. HEK-293 cells with enforced expression of wild-type ABCG2 were previously characterized (26), and the protein expression levels were confirmed via immunoblot at the time of the experiment (data not shown). The results are summarized in Table 2. Cells that overexpressed wild-type ABCG2 were >20-fold resistant to JNJ-7706621 compared with control cells. In agreement with published reports, these cells also exhibited high levels of resistance to the ABCG2 substrate mitoxantrone and conferred low levels of resistance to paclitaxel, a non-ABCG2 substrate.

#### Inhibition of ABCG2 Altered the Sensitivity of HeLa-6621 Cells

The effect of inhibiting the activity of the ABCG2 drug transporter in the resistant cells was assessed by blocking ABCG2 function with a well-characterized, specific ABCG2

inhibitor, FTC (27). HeLa-6621 and HeLa-control cells were preincubated with FTC and cells were treated with JNJ-7706621, mitoxantrone, or paclitaxel in a 48-hour cell proliferation assay. The results are shown in Table 3. Exposure to FTC alone did not alter the sensitivity of control cells to JNJ-7706621 because the IC<sub>50</sub> values in the absence and presence of FTC were similar (268 ± 39 and 197 ± 62 nmol/L, respectively). In contrast, the inhibition of ABCG2 altered the sensitivity of resistant HeLa-6621 cells from 23-fold resistance in the absence of FTC to 4-fold resistance in the presence of FTC. Treatment with FTC also restored the sensitivity of HeLa-6621 cells to the ABCG2 substrate, mitoxantrone. In this case, the observed 13-fold resistance was potentiated to a 3-fold difference in resistance. FTC did not alter drug sensitivity to the non-ABCG2 substrate, paclitaxel. These data show that JNJ-7706621 is a substrate of the ABCG2 transporter and that inhibition of the transporter activity restores sensitivity to resistant cells.

#### Plasma Absorption of JNJ-7706621 Increased in *Bcrp*<sup>-/-</sup> Compared with Wild-Type Mice

Because the localization of ABCG2 in the intestine and liver suggests that ABCG2 has the potential to strongly affect the absorption of substrate drugs, we next examined if oral uptake of JNJ-7706621 would be enhanced in *Bcrp*<sup>-/-</sup> mice which lack functional ABCG2 transporter. JNJ-7706621 was administered orally to *Bcrp*<sup>-/-</sup> and wild-type mice and plasma concentrations were plotted as a function of time (Fig. 4). The time to reach the peak plasma concentration was 0.5 hours for wild-type mice and 1 hour for *Bcrp*<sup>-/-</sup> mice. The peak plasma concentrations (C<sub>max</sub>) were 4,539 and 1,543 ng/mL for *Bcrp*<sup>-/-</sup> and wild-type mice, respectively. Thus, *Bcrp*<sup>-/-</sup> mice had an ~2.9-fold increase in plasma JNJ-7706621 compared with wild-type mice. This indicates that the *Bcrp*<sup>-/-</sup> genotype may alter the oral bioavailability of JNJ-7706621.

## Discussion

The transporter-mediated active efflux of cytotoxic agents is a well-characterized mechanism by which cancer cells develop MDR. ABCG2 (ABCP/BCRP/MXR) has been shown to confer resistance to a number of important anticancer agents such as mitoxantrone, camptothecin, topotecan, SN-38, doxorubicin, and flavopiridol (4, 5, 7, 9, 11–16). The data presented here strongly support the

conclusion that the ABC transport protein family member, ABCG2, is largely responsible for the drug-resistance phenotype of HeLa cells selected for resistance to JNJ-7706621.

The two other major multidrug resistance ABC proteins, MDR1 (P-glycoprotein, ABCB1) and multidrug resistance-associated protein 1 (MRP1, ABCC1) do not seem to significantly contribute to JNJ-7706621 resistance in HeLa cells. Consistent with this assertion, the cross-resistance profile for JNJ-7706621 was previously examined in P-glycoprotein/MDR1 overexpressing human uterine sarcoma/doxorubicin-resistant (MES-SA/Dx5) and sensitive human uterine sarcoma (MES-SA) cell lines. The IC<sub>50</sub> value for JNJ-7706621 was nearly identical in both cell lines, indicating that overexpression of P-glycoprotein does not modulate JNJ-7706621 activity (1). Furthermore, only small changes in P-glycoprotein/MDR1 and MRP1 RNA levels were observed in the HeLa-6621 cells. It is interesting to note, however, that the ABCC2 (MRP2) transporter showed an increase in RNA levels following continuous exposure to JNJ-7706621 (~37-fold), although to a lesser extent than ABCG2. ABCC2 expression has been observed in many solid human tumors and has been shown to confer resistance to brief (4-hour) exposures to very high concentrations of the anticancer agent, methotrexate (28, 29). Although the implication of elevated RNA levels of ABCC2 in HeLa-6621 cells is an interesting topic, the increase in the level of ABCC2 protein needs to be verified by Western blot analysis and warrants further investigation.

In the resistant HeLa-6621 cells, little cross-resistance was observed with the ABCG2 substrates, flavopiridol and doxorubicin (1.4-fold and 2.5-fold resistance, respectively). Similar observations for flavopiridol have been made in HEK-293 cells transfected with ABCG2 (26), despite the fact that flavopiridol-resistant cells overexpress ABCG2 and that cross-resistance to flavopiridol has been found in ABCG2 overexpressing selected cell lines (16). It is conceivable that these compounds bind the ABCG2 proteins expressed on the resistant cells with weak affinity. If this is the case, expression of ABCG2 in the HeLa-6621 cells may not be adequate to confer appreciable resistance to these compounds. Alternatively, genetic variants present in the ABC transporter genes of the resistant HeLa-6621 cells may alter the resistance profile. Variants of ABCG2 have been previously reported to affect protein

**Table 2. Cross-resistance profile of HEK-293 cells transfected with vector control and wild-type ABCG2**

Drug	Vector control IC <sub>50</sub> (nmol/L) ± SD	Wild-type ABCG2 IC <sub>50</sub> (nmol/L) ± SD	Resistance factors*
JNJ-7706621	1,522 ± 322	32,660 ± 854	22
Mitoxantrone	0.7 ± 0.2	14 ± 2	20
Paclitaxel	1.4 ± 0.2	4.2 ± 0.3	3

\*Resistance factors were determined by dividing the mean IC<sub>50</sub> of the wild-type ABCG2-transfected cell line by the mean IC<sub>50</sub> for the empty vector-transfected cell line.

**Table 3. Effect of FTC on sensitivity to JNJ-7706621**

Drug	HeLa control IC <sub>50</sub> (nmol/L) ± SD	HeLa-resistant IC <sub>50</sub> (nmol/L) ± SD	Resistance factors
JNJ-7706621	268 ± 39	6,183 ± 1,187	23
JNJ-7706621 + 10 μmol/L of FTC*	197 ± 62	789 ± 250	4*
Mitoxantron e	1 ± 0.1	13 ± 0.02	13
Mitoxantrone + 10 μM FTC*	1 ± 0.2	3 ± 0.01	3*
Paclitaxel	0.5 ± 0.06	1 ± 0.1	2*
Paclitaxel + 10 μmol/L of FTC	0.5 ± 0.04	0.9 ± 0.1	1.8

\*Significantly different ( $P < 0.05$ ) compared with the level of resistance in the absence of FTC.

function, protein expression, ATPase activity, and localization (30–32). These variants can also alter the MDR phenotype by changing substrate specificity (26, 33–35). Further work is under way to determine the protein sequence of the ABCG2 transporter expressed in the resistant cells.

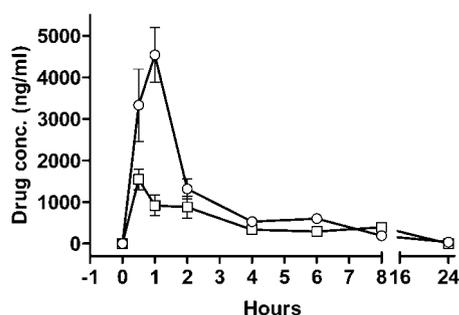
Along with the selection of JNJ-7706621 resistance in HeLa (cervical carcinoma) cells, A375 (malignant melanoma) and HCT116 (colorectal carcinoma) cells were also selected for resistance. RNA levels were evaluated for a panel of ABC transporters in the A375- and HCT116-resistant cells by quantitative real-time RT-PCR. Similar to the results observed with the resistant HeLa-6621 cells, resistant A375 cells showed high levels of ABCG2 transcripts (55-fold compared with control cells; data not shown). Interestingly, analysis of the resistant HCT116 cells revealed no change in the RNA levels of ABCG2 compared with control cells. Instead, these cells showed high RNA levels of the ABCC4/MRP4 transporter (66-fold change; data not shown). The overexpression of multiple ABC transporters in response to exposure to JNJ-7706621 illustrates the complexity involved in understanding mechanisms of resistance and prompts further investigation to better understand the function and regulation of transporters.

The tissue distribution of the mouse *Bcrp1* and human ABCG2 in the small and large intestinal epithelium and in the biliary canalicular membranes, in particular, suggests that *Bcrp1*/ABCG2 may have an important role in limiting intestinal uptake and increasing hepatobiliary excretion of

substrate drugs. Indeed, inhibition of *Bcrp1* in mice markedly increases the oral uptake of topotecan and slows its elimination from the body (18). Furthermore, the increased oral bioavailability of topotecan was observed with the inhibition of ABCG2 and P-glycoprotein in humans (19). In the present study, the results show that *Bcrp1* affects the absorption of JNJ-7706621 into the plasma following oral administration in mice. The  $C_{max}$  or peak plasma concentration was 2.9-fold higher in the *Bcrp* knockout mice compared with the wild-type mice. It is not clear if the majority of CDK-targeted molecules will interact with ABCG2 and face similar issues. The resistant HeLa-6621 cells showed cross-resistance to structural analogues of JNJ-7706621 and other pyrazolo-pyridines, but showed little cross-resistance to flavopiridol and other CDK inhibitors (e.g., purvalanol; data not shown). Thus, the ability of ABC transporters to recognize differences in substrate structure and to have unique, and sometimes overlapping substrate-specificity profiles, makes it necessary to examine each anticancer molecule for transporter interaction.

The therapeutic use of orally administered JNJ-7706621 could be severely limited by poor absorption into the plasma. The extent to which JNJ-7706621 is absorbed, metabolized, and excreted can significantly influence efficacy and toxicity in patients. Beyond this, if ABCG2 is induced by chronic oral delivery of JNJ-7706621, up-regulation of the protein can alter exposure to the drug over time. Therefore, interindividual or intraindividual variation in ABCG2 activity as a consequence of induction, stimulation, or inhibition of ABCG2 by drugs or polymorphisms will certainly play a role in the overall clinical development of JNJ-7706621. Interpatient variation of  $30 \pm 7.7\%$  (range, 21–45%) was calculated for the oral bioavailability of the anticancer compound, topotecan, an ABCG2 substrate (36). Such a high variation could partially account for the fact that the current chemotherapeutic schedules for topotecan are mainly by i.v. injection.

The interaction of JNJ-7706621 with ABCG2 may have further therapeutic significance. Because the levels of ABCG2 expression were found to be different between men and women, particularly in the liver (37), administration of JNJ-7706621 may have to be adjusted according to gender. In addition, recent studies indicate that ABCG2 expression provides an important cell survival advantage under hypoxic conditions. These studies indicate that ABCG2 permits enhanced cell survival in oxygen-poor



**Figure 4.** Absorption of JNJ-7706621 in *Bcrp1*<sup>-/-</sup> and wild-type mice. Plasma concentration versus time curve after oral administration of 100 mg/kg of JNJ-7706621 to wild-type (□) and *Bcrp1*<sup>-/-</sup> mice (ABCG2 knockout; ○;  $n = 3$  mice/group). Points, mean; bars, ±SD.

environments by reducing the accumulation of toxic heme metabolites (38). Therefore, chemotherapeutic treatment of solid tumors that induce hypoxia and up-regulate ABCG2 may be less suitable for combination therapy with JNJ-7706621. On a positive note, because ABCG2 is overexpressed in a subpopulation of uncommitted hematopoietic stem cells (referred to as the side population), ABCG2 may play a role in maintaining these progenitor cells in an undifferentiated state (39). Such cells would be protected (and thereby enriched) from the cytotoxic effects that cancer cells would experience upon treatment with JNJ-7706621, and potentially result in enhanced recovery for patients following chemotherapy.

In summary, our data show that ABCG2 can reduce the intracellular accumulation of the CDK/Aurora kinase inhibitor, JNJ-7706621, resulting in resistance in cultured HeLa cells. Furthermore, mouse models using male *Bcrp1*<sup>-/-</sup> mice (mouse homologue of ABCG2; ref. 20) showed that ABCG2 influenced the absorption of JNJ-7706621 following oral administration. Taken together, it is likely that ABCG2 function will influence both the resistance and pharmacology of JNJ-7706621 in patients. These observations may have profound effects for the development of this compound and illustrate the importance of identifying the transporters that play a role in resistance. The identification of such transporters can result in the development of strategies to prevent resistance and to potentially modulate transporters to increase the bioavailability, and ultimately, the efficacy of anticancer drugs.

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# Molecular Cancer Therapeutics

## Role of the ABCG2 drug transporter in the resistance and oral bioavailability of a potent cyclin-dependent kinase/Aurora kinase inhibitor

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