Ixabepilone, a Novel Microtubule-Targeting Agent for Breast Cancer, is a Substrate for P-glycoprotein (P-gp/MDRI/ABCB1) but not Breast Cancer Resistance Protein (BCRP/ABCG2)

This work was performed by employees of Bristol-Myers Squibb

H Shen, FY Lee, and J Gan

Bristol-Myers Squibb, Princeton, New Jersey
Running title: Relevance of P-gp and BCRP in Drug Resistance to Ixabepilone

Corresponding author: Jinping Gan, Bristol-Myers Squibb, PO Box 4000, Princeton, NJ 08543-4000. Telephone: (609) 252-7885; Facsimile: (609) 252-6802; E-mail: jinping.gan@bms.com

Text: 32 pages
Tables: 4
Figures: 7
References: 39
Abstract: 247 words
Introduction: 785 words
Discussion: 1,295 words

ABBREVIATIONS: P-gp, P-glycoprotein; BCRP, breast cancer resistance protein; MRP2, multidrug-resistance protein 2; ABC, ATP-binding cassette; HEK, human embryonic kidney; MDCK, Madin-Darby canine kidney; LLC-PK1 cells, Lilly laboratory cell for porcine kidney; FTC, fumitremorgin C; MDR, multidrug resistance.

Recommended section: Absorption, Distribution, Metabolism, & Excretion
ABSTRACT

Ixabepilone is the first epothilone to be approved for clinical use. Current data suggests the epothilones have a role in treating taxane-resistant cancers and that ixabepilone is unaffected by at least some of the mechanisms underlying chemoresistance. Here we report a series of cytotoxicity and transport studies to assess the potential role of P-gp and BCRP in ixabepilone resistance. A significant decrease in ixabepilone-mediated cytotoxicity was observed in MDCK-MDR1 cells comparative with the parental cells (IC$_{50}$ > 2000 nM vs. 90 nM). Overexpression of P-gp also resulted in significantly decreased cell susceptibility to docetaxel, paclitaxel and vinblastine. Bidirectional transport of ixabepilone across LLC-MDR1 cell monolayers showed a significantly increased efflux ratio relative to the parental cells. A BCRP overexpressing cell line was developed by transfecting HEK-293 cells with BCRP cDNA, and confirmed by immunoblotting, and bodipy prazosin and mitoxantrone uptake. Neither P-gp nor MRP2 was detected in the cells by corresponding polyclonal antibodies. This HEK-BCRP cell line demonstrated resistance to docetaxel, paclitaxel, vinblastine and mitoxantrone, in comparison with the parental cell line (7.3, 4.3, 2.9, and 11.9 resistance factor, respectively). Transport inhibition by BCRP inhibitor fumitremorgin C and broad efflux inhibitor GF120918 restored drug sensitivity. In contrast, ixabepilone was far less susceptible to BCRP-mediated resistance, resulting in a resistance factor of only 1.2 fold. In summary, these results suggest that P-gp could cause resistance to ixabepilone in tumors and affect the disposition of the drug, but it is unlikely that BCRP mediates any drug resistance to ixabepilone.
INTRODUCTION

The epothilones are a novel class of microtubule-binding agents being investigated as anti-cancer therapies. The epothilones act via stabilizing intracellular microtubules (tubulins), and one of the consequences of this is apoptosis, following G2/M phase cell cycle arrest (Bollag et al., 1995). The epothilones have demonstrated preclinical and clinical activity against a range of chemotherapy resistant and pretreated solid tumor types (Harrison et al., 2008; Vahdat, 2008). Importantly, current evidence suggests that the epothilones may have a different, more favorable, resistance profile to other tubulin-binding agents. Indeed, ixabepilone has been FDA-approved, for the treatment of advanced breast cancer in patients whose tumors are resistant or refractory to taxanes, anthracyclines, and/or capecitabine (Harrison et al., 2008; Vahdat, 2008; Hortobagyi et al., 2010; Sparano et al., 2010).

One critical cellular process which contributes to drug resistance is the decreased accumulation of drugs within cells due to drug efflux mechanisms, mediated by ATP-binding cassette (ABC) efflux transporters. The ABC transporters, P-glycoprotein (P-gp/MDR1/ABCB1), breast cancer resistance protein (BCRP/ABCG2), and multidrug resistance proteins 2 (MRP2/ABCC2) actively extrude a wide variety of anticancer drugs from tumor cells (Borst and Elferink, 2002; Krishnamurthy and Schuetz, 2006; Kruh and Belinsky, 2003). Current evidence suggests that the taxanes are substrates for both P-gp and BCRP.

Several studies on the role of ABC transporters in cancer cell resistance to antimicrotubule agents focus on P-gp. These data demonstrate taxanes (i.e., paclitaxel and docetaxel) as well as many other antimicrotubule agents (e.g., vinblastine) are P-gp substrates. For example, gene transfer experiments using MDR1 cDNAs facilitated the basal-to-apical
transport of paclitaxel (Lehnert et al., 1993; Brouty-Boyé et al., 1995) and docetaxel (Shirakawa, et al., 1999), inducing drug-sensitive cultured cells to become resistant to taxanes. Furthermore, limited oral bioavailability and active intestinal epithelial secretion of paclitaxel and docetaxel have been observed in wild-type animals compared with P-gp knockout mice (Sparreboom et al., 1997; Bardelmeijer et al., 2002). At present, relatively little information on other ABC orthologues (especially BCRP) is available.

Guo et al. investigated mechanisms of cross-resistance of taxane-resistant breast tumor cell lines, and demonstrated that, in addition to P-gp, BCRP protein expression was significantly increased in both doxorubicin- and paclitaxel-resistant cells (i.e., MCF-7DOX and MCF-7AX). The presence of BCRP in these cell lines provided an explanation as to the inability of valespodar, a P-gp inhibitor, to fully re-establish sensitivity to paclitaxel in MCF-7DOX cells, suggesting paclitaxel is a substrate for BCRP (Guo et al., 2004). In another study, BCRP-induced (but not P-gp-induced) cells were 5.9–12.7 fold resistant to tubulin binding agents compared with the sensitive parent cells (Kars et al., 2007). In contrast, a previous preclinical study reported that taxanes are not a substrate for BCRP. Two BCRP overexpressing cell lines, MCF-7 AdVp3000 and S1-M1-80, retained sensitivity towards vinblastine and paclitaxel (Litman et al., 2000), suggesting that the multidrug-resistant phenotype due to BCRP expression is overlapping, but distinct, from that due to P-gp.

In addition to preclinical models, some clinical data is also available. Polymorphisms in host genes may also be important for docetaxel metabolism, transport and action; these include MDR1, BCRP, CYP3A4, CYP3A5, MAPT, and MAP4 (Hahn et al., 2006). A significant association between survival beyond 15 months and the ABCG2 421C>A (Q141K)
polymorphism was observed in a clinical trial ($p = 0.05$) of 64 patients with hormone-refractory prostate cancer randomized to receive docetaxel plus vinorelbine or docetaxel plus estramustine phosphate. Although the effect of the BCRP polymorphism on docetaxel pharmacokinetics is unknown, the increased survival observed in patients with an ABCG2 421C>A polymorphism (4 of 6 [66%] patients survived past 15 months compared with 12 of 44 [27%] patients with wild-type ABCG2 [$p = 0.05$]) suggests that compromised drug efflux resulted in better efficacy.

There is some initial evidence that ixabepilone has a different resistance profile compared to other tubulin-binding agents. Ixabepilone retains antineoplastic activity in cell lines selected for resistance to paclitaxel, including P-gp-overexpressing HCT116/VM46 colon and Pat-7 ovarian cancer cell lines (Lee et al., 2001; 2009). Overcoming taxane resistance with ixabepilone was demonstrated in vivo using preclinical xenograft mouse models (Lee et al., 2009; Lee et al., 2008). Relative resistance (RR; a ratio of IC$_{50}$ values) in multidrug resistant versus sensitive lines (i.e., HCT116/VM46 vs. HCT116), was substantially lower for ixabepilone than that of paclitaxel (7.77 vs. >100) providing initial evidence that it may be a weak substrate for P-gp.

Here we examined the cytotoxic characteristics of ixabepilone and other antimicrotubule agents using established c-DNA gene transfected MDCK-MDR1 and HEK-BCRP cells. We also investigated bidirectional transepithelial transport of ixabepilone across LLC-MDR1 cell monolayers. Our aim here was to establish the potential roles of P-gp and BCRP in the removal of ixabepilone and/or taxanes from cells and the potential for these mechanisms to confer chemoresistance.
Methods

Materials. Platinum® Taq DNA Polymerase, Gene Pool human liver cDNA, subcloning efficiency DH-5α competent cells, Flp-in HEK, Zeocin, fetal bovine serum, trypsin, Lipofectamine 2000, hygromycin B, Flp-in system and bodipy prazosin were purchased from Invitrogen Corporation (Carlsbad, CA). Fumitremorgin C (FTC) and the antibodies against P-gp and MRP2 were purchased from Axxora (San Diego, CA). N-(4-[2-(1,2,3,4-Tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide (GF120918, elacridar) and 3-[[3-[(1E)-2-(7-Chloro-2-quinolinyl)ethenyl]phenyl][3-(dimethylamino)-3-oxopropyl]thio]methyl]thio]propanoic acid (MK-571, L-660711) were purchased from Toronto Research Chemicals (North York, Ontario, Canada). The antibody against BCRP was purchased from Kamiya Biomedical Company (Seattle, WA). [3H]-Mitoxantrone and [3H]-Digoxin was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). [14C]-Mannitol and [3H]-propranolol were purchased from Sigma-Aldrich (St. Louis, MO). Ixabepilone was supplied by the Process Research and Development Department (BMS, New Brunswick, New Jersey). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless stated in the text.

Cell culture reagents including Dulbecco’s modified Eagle medium (DMEM), M199 medium, RPMI medium and fetal calf serum (FCS) were purchased from Invitrogen Corporation (Carlsbad, CA). Madin-Darby canine kidney (MDCK) parental cells and those transfected with human MDR1 (MDCK-MDR1) were obtained from The Netherlands Cancer Institute (Amsterdam, The Netherlands). HEK293 cells containing the Flp Recombination Target (FRT) recombination site were purchased from Invitrogen (Carlsbad, CA). Porcine kidney-derived
LLC-PK1 cells expressing human P-gp (LLC-MDR1) and the control cells containing the vector without human P-gp (LLC-PK1) were obtained from BD Gentest (Woburn, MA). Transwells (24-well, 1-μm pores) were purchased from Corning Costar (Cambridge, MA).

**Generation of a Stable Human BCRP Transfected Cell Line and Cultivation of HEK-BCRP, MDCK-MDR1 and LCC-MDR1 Cells.** The full-length wild-type human BCRP cDNA was amplified from the Gene Pool human liver cDNA by polymerase chain reaction (PCR) using Platinum® Taq DNA Polymerase with appropriate primers [5′CCC AAG CTT ACT CTC CAG ATG TCT TCC AG (sense) and 5′CGC GGA TCC CAA GGG AAC AGA AAA CAA CA (antisense)]. Human BCRP cDNA was then inserted into the HindIII and BamHI restriction sites of pcDNA5-FRT expression system by ligation. The resulting vector construct contains BCRP followed by an internal FRT site, which allows BCRP integrating into the host cell genome via Flp recombinase-mediated DNA recombination at the FRT site. This construct was cotransfected with pOG44, a Flp recombinase expression plasmid, into the Flp-In Human Embryonic Kidney (HEK) cell line (Invitrogen, Carlsbad, CA) using a cationic lipid reagent, Lipofectamine 2000. After 72 h, medium was then removed and replaced with complete medium supplemented with selecting antibiotic hygromycin B (100 μg/mL). Single hygromycin-resistant colonies cells were sorted into 24-well plates containing HEK conditioned medium. After expansion, clones were screened for expression of functional BCRP activity on the basis of reduced bodipy prazosin (BP) accumulation using a CytoFluor Microplate Reader 4000 (PerSeptive Biosystems, Framingham, MA) and mitoxantrone transport. The expression of BCRP in selected clones was verified by RT-PCR and Western blotting analysis.
Madin-Darby canine kidney cells transfected with human multidrug resistance gene (MDCK-MDR1 cells) were obtained from the Netherlands Cancer Institute (Amsterdam, the Netherlands) and cultured in DMEM supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acids, 2 mM L-glutamine solution, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained at 37°C under a humidified atmosphere containing 5% CO₂.

Porcine kidney-derived, BD Gentest™ LLC-PK1 cells expressing human MDR1 cDNA (LLC-MDR1) and the control cell line (LLC-PK1 cells containing the vector without human MDR1 cDNA) (BD Biosciences, Woburn, MA) were cultured in BD Falcon™ 24-well with 1 μm culture inserts in medium M199 supplemented with 10% heat inactivated FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate. For the bidirectional transport experiments, cells were maintained at 37°C, 5% CO₂ and 95% relative humidity for 7 days, with medium changed at 3–4 days.

**Cytotoxicity Assays.** Cytotoxicity of drugs in MDCK-MDR1, MDCK, HEK-BCRP and HEK cells was measured using XTT colorimetric assay (Roche Applied Science, Mannheim, Germany) performed in 96-well plates. Five thousand cells were seeded into each well in 50 μL of culture medium. Fifty microliters culture medium containing various amounts of drugs was added to the wells, and incubations were continued for 3 days. Then 50 μL of yellow XTT labeling mixture was added to each well. After an additional 16-h incubation, the resulting orange formazan was spectrophotometrically quantified at 492 nm using a Micro Plate Reader (Molecular Devices, Sunnyvale, CA). The reference wavelength was used at 690 nm. The results were expressed as mean ± S.D. Percentage of growth was calculated relative to control (untreated cells) after 3 days of culture with control taken as 100%.
**P-gp Bidirectional Transport Assays.** The extent of P-gp-mediated transport of ixabepilone was also evaluated in LLC-PK1 wild-type and LLC-MDR1 cell lines. Prior to conducting transport experiments, integrity of monolayers was confirmed by measurement of transepithelial electrical resistance (TEER) using an electrical resistance system with chopstick electrodes (World Precision Instruments, Sarasota, FL) and determining permeability of paracellular marker \([^{14}\text{C}]\)-mannitol. Moreover, monolayer integrity was further evaluated by post-experimental lucifer yellow apical to basal side (A→B) flux determination for each cell monolayer. As a transcellular high permeability marker, the permeability of \([^3\text{H}]\)-propranolol was also determined. P-gp expression was functionally tested by determining bidirectional transport of \([^{14}\text{C}]\)-digoxin and calculating its efflux ratio (ER) across LLC-MDR1 monolayers.

Cells were cultured in M199 medium as described previously. For transport experiments, cells were cultured in 24-well Transwell plates at a density of 1.5 x 10^5 cells/well with 0.3 ml of medium in the upper compartment and 35 ml of medium in the lower nonsubdivided compartment. After culturing for 7 days, the transport wells were transferred to a 24-well plate. Cells were washed three times with Hanks’ balanced salt solution containing HEPES (10 mM, pH 7.4). Subsequently, ixabepilone in Hanks’ balanced salt solution containing HEPES (500 µl) was added either to the apical or basolateral compartment. Based on the solubility data, a test article concentration of 1 µM was selected to determine time dependence of ixabepilone transport. For permeability assays, ixabepilone was assayed at three concentrations: 3, 6 and 10 µM. 5 µM \(^[^3\text{H}]\)-Digoxin was used as a control P-gp substrate. After incubating for 2 h at 37°C, samples (100 µl) were taken from both the apical and basolateral compartment and transferred to a 96-well microtiter plate for LC/MS/MS quantitation. The LC/MS/MS system consisted of a
PerkinElmer Series 200 HPLC system with 2 Micro pumps (PerkinElmer Instruments, Shelton, CT), a LEAP CTC PAL autosampler (LEAP Technologies, Carrboro, NC), and a SCIEX API 4000 triple quadrupole mass spectrometer (AB Sciex, Concord, ON, Canada). The samples were separated on a Waters symmetry C18 column (2.1 x 50 mm, 5-µm). The mobile phase used was 0.1% formic acid in water (A) or in acetonitrile (B) at 0.3 mL/min with a gradient from 70% A to 40% A in 3 min. Ixabepilone concentration was determined by multiple-reaction monitoring at a transition of m/z 505.3 to 405.2 in negative electrospray ionization mode.

Apparent permeability (P_{app}) was calculated using following equation (1):

**Equation 1.**

\[
P_{app} = \frac{\text{Flux} \cdot V_d}{t \cdot A} = \frac{dQ/dt}{A \cdot C_d}
\]

where Flux is the fraction of the donated amount recovered in the receiver chamber, V\_d is the volume in the donor chamber, C\_d is the concentration in the donor chamber at t = 0, and A is the surface area of insert filter membrane, and dQ/dt is the amount of drug transported within a given time period. To confirm the presence of an efflux transport system, an ER was calculated (Equation 2):

**Equation 2.**

\[
ER = \frac{P_{app}^{B\rightarrow A}}{P_{app}^{A\rightarrow B}}
\]

where \( P_{app}^{B\rightarrow A} \) is the \( P_{app} \) value measured in the basal to apical direction, \( P_{app}^{A\rightarrow B} \) is the \( P_{app} \) value measured in the apical to basal direction. Data are reported as mean ± S.D.

**Western Blotting Analysis.** Cells were lysed on ice in RIPA buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium desoxycholate, 0.1% SDS) supplemented with fresh 1 mM DTT and protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA). The lysates were centrifuged and the supernatants proteins (30 µg) were separated by
NuPAGE 4–12% Bis-Tris gel electrophoresis. Gels were transferred to nitrocellulose membranes overnight at 5 mA. Membranes were blocked for 1.5 hour at RT (SuperBlock Blocking Buffer in TBS-Tween, Pierce) on an orbital shaker and followed by incubation with mouse monoclonal primary antibodies anti-BCRP, P-gp and MRP2. Then, membranes were incubated with anti-mouse peroxidase-conjugated secondary antibodies. Membranes were rinsed, treated with ECL detection agents (Pierce, Rockford, IL), and visualized using a GeneGnome HR chemiluminescence imager (Syngene, Frederick, CA). The ubiquitously expressed β-actin was used as a protein loading control.

**Drug Accumulation in HEK-BCRP Cells Studies.** The uptake experiments using BP and mitoxantrone in HEK-BCRP and HEK cells were conducted with the cell monolayers grown in 96-well and 6-well Biocoat tissue culture plates, respectively. Before initiating uptake experiments, cell monolayers were washed twice with HPBS (PBS containing 10 mM HEPES, pH 7.4). The cell monolayers were then incubated with 100 μl of BP (1 μM) at 37°C for 30 min or 1 mL of mitoxantrone (2 μM) for 60 min. For the inhibition experiments, cell monolayers were incubated with FTC (10 μM) or chrysin (5 μM) containing specific concentrations of test compounds. To measure cytoplasm-associated BP, the uptake was quenched by washing cells three times with ice-cold PBS (pH 7.4). The fluorescence of BP in these cells was read immediately by CytoFluor Microplate Reader 4000 (PerSeptive Biosystems, Framingham, MA). The excitation and emission wavelengths were 485 and 530 nm, respectively. Nonspecific uptake for HEK-BCRP cells due to passive diffusion was determined in parallel experiments in parental HEK cells. The baseline was determined by omitting BP and incubating cells with HPBS only.
To measure intracellular amount of mitoxantrone, the cell monolayers were quickly washed three times with 3 mL of ice-cold HPBS, and the cells were detached by 10 min incubation with 500 μL 0.25% trypsin at 37°C for 10 min, and then lysed in 500 μL of freshly prepared lysis buffer (2% SDS and 0.4 mM NaOH). The cell lysates samples obtained in the study were analyzed by a scintillation counter (LS 6500; Beckman Instruments Inc., Fullerton, CA).
Results

Sensitivity of P-gp Overexpressing Cells to Ixabepilone and other Antimicrotubule Agents. The cytotoxicity of ixabepilone and other antimicrotubule agents in MDCK-MDR1 cells were studied by XTT assays. Human MDR1 conferred significant resistance to ixabepilone as well as paclitaxel, docetaxel, vinblastine, and mitoxantrone (Fig. 1), although RR of the drugs could not be determined because of low cytotoxicity observed in the P-gp overexpressing cells (Table 1). Consistent with earlier literature (Rothermel et al., 2003), Epothilone B showed extremely low susceptibility to P-gp-mediated multidrug resistance. (Fig. 1; Table 1).

To ascertain the contribution of P-gp-mediated resistance to ixabepilone in MDCK-MDR1 cells, the cells were treated with ixabepilone in the presence of P-gp inhibitors cyclosporine A and verapamil, MRP2 inhibitor MK-571, BCRP inhibitor FTC, and multiple efflux transporter inhibitor GF120918. The resulting IC50s were shown in Table 2 and representative IC50 curves were shown in Fig. 2. As shown in Fig. 2A, the addition of cyclosporine A, verapamil, and GF120918 restored the sensitivity of MDCK-MDR1 to ixabepilone; in contrast, MK-571 and FTC did not have an effect on MDCK-MDR1 resistance to ixabepilone (Fig. 2B). Similarly, Table 2 showed that the P-gp inhibitors further increased the chemosensitivity of wild type MDCK cell lines resulting in a left shift of IC50s, while no effect was observed by MRPs and BCRP inhibition.

P-gp-Mediated Ixabepilone Transport Using LLC-MDR1 Cell Monolayers. In order to verify the involvement of P-gp in mediating decreased cytotoxicity to ixabepilone, the permeability of ixabepilone were measured across LLC-MDR1 and LLC-PK1 monolayers.
Integrity of LLC-MDR1 monolayers were confirmed by a low $P_{\text{app} \, A \rightarrow B}$ of mannitol, which averaged 0.7 to $1.3 \times 10^{-6}$ cm/s with acceptable TEER values ($\geq 320 \Omega \cdot \text{cm}^2$). $P_{\text{app} \, A \rightarrow B}$ of propranolol averaged 15 to $25 \times 10^{-6}$ cm/s and P-gp expression was confirmed by an ER of 8.7 to 10.0, with an average of 9.8, for digoxin. Moreover, post-experimental transport of lucifer yellow across LLC-MDR1 monolayers was restricted with only 0.8 to 1.9% detected in receiver chamber.

The transport of 1 µM ixabepilone was substantially greater when introduced from the basolateral (B→A) than from the apical (A→B) surface of LLC-MDR1 monolayers (Fig. 3). In contrast, the B→A and A→B fluxes in non-transfected LLC-PK1 cells (control) did not differ as significantly as LLC-MDR1 cells. Generally a linear transepithelial transport rate was observed over 120 min and, as a result, transport was determined at 120 min for subsequent ixabepilone permeability experiments. Bidirectional permeability of ixabepilone across LLC-MDR1 monolayers is summarized in Table 3. Transport of ixabepilone was studied with three concentrations (3, 6, and 10 µM) in LLC-MDR1 monolayers. Across LLC-MDR1 monolayers, ixabepilone showed polarized transport with higher B→A permeability than the A→B permeability ($27.0$ to $31.7 \times 10^{-6}$ cm/s vs. $0.3$ to $0.5 \times 10^{-6}$ cm/s). The ER ranged from $53.6$ to $96.0$ with the highest efflux observed at 3 µM. In contrast, ixabepilone transport in LLC-PK1 cells was essentially unpolarized with ER close to unity (1.8 to 2.3, Table 3). Mass balance calculations following transport studies showed that good ixabepilone recovery was achieved, ranging from 63% to ≥100%.

**Characterization of the Stable BCRP Transfection Model.** Initial RT-PCR and immunohistochemistry analyses with specific primers and antibodies revealed the expression of
a human breast cancer resistance protein transporter in human liver canalicular membrane (Vander Borght et al., 2006). Therefore, human BCRP primers were designed and optimized PCR conditions were used to obtain the BCRP sequence containing the full coding sequence from liver cDNA. The 2080 bp PCR product was cloned into the pcDNA5-FRT vector, and the identity of the full human BCRP sequence was confirmed by sequence analysis.

Transfection of HEK cells with the construct revealed eleven hygromycin-resistant clones, 2 of which were subjected to RT-PCR analysis (Fig. 4A). The analysis indicated that cells of the two clones generated BCRP transcripts of the expected size of 2.1 kb, which was not detected in nontransfected HEK cells. The results indicated that the exogenously introduced BCRP construct was successfully expressed in the transfected cells. The representative clone of 1C with ample expression of BCRP, named HEK-BCRP, was selected.

To confirm the protein expression level of the cloned BCRP cDNAs and to determine the approximate level of P-gp and MRP2, Western blotting of the cell lysate of HEK-BCRP was conducted and the results shown in Fig. 4B. These studies confirmed overexpression of the BCRP protein in HEK-BCRP, while maintaining very low or undetectable levels of protein expression of P-gp and MRP2 in comparison with hepatocytes, Caco-2, and MDCK-derived cells. Thus, the HEK-BCRP cell line is suitable for the evaluation of BCRP-specific efflux of drugs of interest.

**Further Characterization of HEK-BCRP Cells with BCRP Substrate Uptake.** To explore whether BCRP overexpression could confer functional drug efflux, the accumulation and efflux of BP and mitoxantrone in HEK-BCRP was studied. In the mock HEK cells, BP and mitoxantrone accumulated rapidly following a 10 min incubation with BP (Fig. 5A) and 60 min
incubation with mitoxantrone (Fig. 5B), respectively. Accumulation of both substrates was significantly reduced in HEK-BCRP ($p < 0.005$). The addition of BCRP inhibitors, FTC (10 µM) and chrysin (5 µM), markedly increased BP and mitoxantrone accumulation in the transfected cells to an equivalent level observed in the parent cells. The BCRP inhibitors had no effect on BP and mitoxantrone accumulation in HEK cells. These results suggest that the BCRP protein expressed in HEK-BCRP was functional and was responsible for the efflux of BCRP substrates from these cells.

**Sensitivity of BCRP Overexpressing Cells to Ixabepilone and other Antimicrotubule Agents.** To determine whether antimicrotubule agents are substrates of BCRP, the cytotoxicity of these agents, as well as the well characterized BCRP substrate mitoxantrone, in HEK-BCRP was compared with that in HEK. These results are summarized in Table 1 and Fig. 6. Of particular interest, BCRP-expressing HEK-BCRP cells were 7.3-, 4.3-, 2.9-, and 11.9-fold resistant to paclitaxel, docetaxel, vinblastine, and mitoxantrone, respectively, compared with the control HEK cells, whereas the HEK-BCRP cells did not show resistance against ixabepilone and epothilone B, compared to the parental HEK cells (1.2 and 1.7 relative resistance, respectively). These results suggest that both taxanes, as well as mitoxantrone and vinblastine, are substrates for BCRP whereas epothilones are not. Our unpublished data about other epothilone analogues developed at Bristol-Myers Squibb also support the conclusion that epothilones are not substrates for BCRP. In order to determine whether resistance to the drugs is solely due to BCRP overexpression, the effects of FTC, a specific inhibitor of BCRP, as well as a P-gp and BCRP inhibitor, GF120918 (elacridar), were studied in HEK-BCRP cells. FTC (5 µM) completely sensitized the HEK-BCRP cells to mitoxantrone cytotoxicity and restored most of the
cytotoxic activity to paclitaxel and docetaxel in HEK-BCRP cells compared with parental HEK cells (Fig. 7 and Table 4). Basal expression of P-gp in HEK-BCRP cells, although minimal by Western blotting, could explain the incomplete reversal of resistance by FTC inhibition. In fact, incubation with GF120918 further enhanced the sensitivity to both paclitaxel and docetaxel.
Discussion

Since the isolation of paclitaxel in 1971, the taxanes have evolved into an important class of ant-cancer agents (Wani et al., 1971; Arbuck et al., 1993). Taxanes have provided the proof-of-concept for tubulin binding as an effective therapy in this setting. One of the key issues surrounding the taxanes and other tubulin-binding agents in oncology, however, has been the development of cellular resistance particularly via P-gp and BCRP-mediated mechanisms. The identification of non-taxane entities with superior activity and better resistance profiles could potentially provide greater clinical benefit for patients with cancer.

Here we utilized established pre-clinical models to assess the roles of P-gp and BCRP in microtubule-binding agent resistance mechanisms. Firstly, we confirmed that ixabepilone was a substrate for P-gp, and had a preferential basal-to-apical transepithelial transport across P-gp overexpressing epithelial cells. Secondly, consistent with the other epothilone tested (epothilone B), ixabepilone was not transported by BCRP: in contrast, we confirm that paclitaxel and docetaxel are substrates for BCRP. Given the importance of ABC efflux transporters in resistance to taxanes and other microtubule inhibitors, these results imply that BCRP might play a role in cellular resistance to some microtubule-binding agents but not ixabepilone.

Our results for the taxanes are consistent with previous studies (Mcguire et al., 1996; Rowinsky, 1997). Ineffective delivery of taxanes across cell membranes into cancer cells, leading to insufficient intracellular drug concentrations, is a major challenge in the treatment of cancer. Although paclitaxel and docetaxel can enter cells via passive diffusion with inherent lipophilicity, various ABC efflux transporters, expressed at the cellular membrane, can minimize the effective penetration of these agents by efficiently eliminating them from the cell (McGrogan...
et al., 2008). In particular, paclitaxel and docetaxel are known substrates of the multidrug resistance protein P-gp. In preclinical and clinical studies, MDR1 gene deletion and treatment with P-gp inhibitors resulted in a significant increase in paclitaxel and docetaxel bioavailability (Sparreboom et al., 1997; Bardelmeijer et al., 2002; Meerum Terwogt et al., 1999; Malingré et al., 2001). It is likely that this transporter contributes to limited access of taxanes to cancer cells. Furthermore, P-gp expression can be induced by exposure to one chemotherapeutic agent, leading to cross-resistance to a second chemotherapeutic agent.

By comparing cytotoxic profiles of paclitaxel and docetaxel in the absence or presence of FTC and GF120918 we can speculate that both P-gp and BCRP underlie taxane-resistance. The addition of FTC, a specific inhibitor of BCRP function (Rabindran et al., 2000), significantly (but not fully) restored sensitiveness of HEK-BCRP cells to paclitaxel and docetaxel (Fig. 7 versus HEK cells). In contrast, a significant increase in paclitaxel and docetaxel cytotoxicity was observed in HEK-BCRP cells in the presence of GF120918, a broad transporter inhibitor (versus parent cells) (Maliepaard et al., 2001; Kruijzer et al., 2002). Thus, for HEK-BCRP cells, there was a concordance between the ability to restore paclitaxel and docetaxel accumulation and the degree of cytotoxicity of these two drugs. The effect of FTC on the sensitivity of HEK-BCRP cells to paclitaxel and docetaxel was likely the result of BCRP antagonism, since the addition of FTC had no effect on HEK cell cytotoxicity profiles to paclitaxel and docetaxel (data not shown). However, we cannot rule out a possible synergy between the cytotoxicity of taxanes and GF120918. Although it could not be detected in HEK-BCRP and HEK cells by immunoblotting analysis, MRP2 may contribute to taxane resistance (Huisman et al., 2005; Lagas et al., 2006).
In this study we compared the cytotoxicity of ixabepilone with those of paclitaxel, docetaxel, vinblastine and others in an established P-gp cell model, MDCK-MDR1. Markedly reduced cytotoxicity of the P-gp overexpressing cells to ixabepilone was observed, compared with the parental cell (similarly for paclitaxel, docetaxel, and vinblastine treatment). As shown in Fig. 4B, in addition to the P-gp overexpression, the MDCK-MDR1 cell line had much higher expression of MRP2 and paradoxically less expression of BCRP than the parental cell line. Recently taxanes were shown to be substrates of MRP2 (Lagas et al., 2006; Huisman et al., 2005), suggesting the increased levels of MRP2 in the MDCK-MDR1 cell line could augment the effect of P-gp in the reduced cytotoxicity. In order to confirm the involvement of P-gp in ixabepilone resistance in P-gp overexpressing MDCK-MDR1, further experiments using the co-incubation of chemical inhibitors with ixabepilone was conducted. The results confirmed the involvement of P-gp in MDCK-MDR1 resistance to ixabepilone, as P-gp inhibitors cyclosporine A, verapamil, and GF120918 restored the sensitivity of MDCK-MDR1 to ixabepilone, while inhibitors of MRP2 and BCRP had no effect. Consistent with the Western Blot experiment in which substantial level of P-gp protein was detected in the wild type MDCK cells, the treatment of P-gp inhibitors cyclosporine A and GF120918 further increased the cytotoxicity of ixabepilone, resulting in IC50s similar to those in MDCK-MDR1 with the same inhibitors (Table 2). Furthermore, the role of P-gp in ixabepilone transport is consistent with the transepithelial transport studies with another model cell line, LLCPK-MDR1; ixabepilone exhibited marked active B-A transport, with ERs in P-gp overexpressing cells 30- to 40-fold greater than those in control cells. For comparison, digoxin, a well documented P-gp substrate, demonstrated an ER of 9.8 in P-gp overexpressing cells, and a ratio of 2.0 in the control cells. Western blotting data on
efflux transporters are not available for the BD Gentest LLCPK cell lines, but they were recently shown to be functionally inactive in the transport of MRP2 and BCRP substrates (Kapadnis et al., 2009). Additional evidence to support the role of P-gp in ixabepilone transport was reported in HCT116/VM46 cell lines as published by Lee et al. (Lee et al., 2001). We conclude from these data that ixabepilone is a P-gp substrate. We could not compare and differentiate RR between ixabepilone and taxanes in the current study due to incomplete cytotoxicity profile of the drugs in MDCK-MDR1 cells.

Consistent with epothilone B, ixabepilone showed no difference in cytotoxicity between HEK-BCRP and HEK parent cells, suggesting that the epothilones are not a substrate for BCRP. Given that taxanes are a substrate for BCRP these results provide one of many possible explanations as to the activity of ixabepilone in taxane-resistant tumors. In addition, the antitumor activity of ixabepilone in taxane-resistant tumors may be related to its preferential suppression of cell lines expressing high levels of βIII-tubulin, which has also been associated with taxane resistance (Dumontet et al., 2009). Furthermore, the expression of MRP1, another important drug-resistance related efflux transporter, was observed in ixabepilone-sensitive patient samples (McDaid et al., 2002) and taxane-resistant tumor cell line (Pat-7) (Lee et al, 2001). However, as overexpression of P-gp was also observed, it is difficult to assess the relative importance of MRP1 in conveying taxane resistance to these cells. In clinical trials, ixabepilone demonstrated activity against a range of taxane-resistant tumors, such as metastatic breast cancer, non-small cell lung cancer, and ovarian cancer (Vahdat, 2008). BCRP is likely, therefore, to play a role in intrinsic and acquired drug resistance in these tumors.
It is worth pointing out that functional polymorphic variants of BCRP have been reported in various populations (Robey et al., 2007 and references therein), and point mutations at the Arginine-482 site were demonstrated to alter the substrate specificity and sensitivity to multiple substrates including doxorubicin, danorubicin, methotrexate, and Ro123 (Robey et al., 2003). Although the clinical relevance of the Arginine-482 mutation has not been demonstrated, the effect of BCRP polymorphism on ixabepilone was not tested in this study and needs further investigation.

Taken together, our data suggest that ixabepilone, taxanes and other tubulin-binding agents are substrates of P-gp; however, unlike other tubulin-binding agents, ixabepilone is not a substrate of BCRP. To address the clinical relevance of this difference in susceptibility, prospective studies investigating the antitumor activity of ixabepilone in BCRP-genotyped patients, or patients with tumors that overexpress BCRP, are warranted.

Acknowledgements

Editorial assistance was provided by StemScientific, supported by Bristol-Myers Squibb. The authors thank Drs. David Rodrigues, Punit Marathe, Christine Huang for critically reviewing this manuscript.

Authorship Contributions

Participated in research design: H Shen, FY Lee, and J Gan.
Conducted experiments: H Shen.
Contributed new reagents or analytic tools: H Shen and FY Lee.
Performed data analysis: H Shen and J Gan.
Wrote or contributed to the writing of the manuscript: H Shen and J Gan.
Other: None.
References


Reprint requests: Jinping Gan, F12-03, Rt. 206 & Province Line Rd., Bristol-Myers Squibb, Princeton, NJ 08540. Telephone: (609) 252-7885; Facsimile: (609) 252-6802

This study is supported by Bristol-Myers Squibb and all authors are current employees of Bristol-Myers Squibb.
Legends for Figures

**Fig. 1.** Cytotoxicity of antimicrotubule agents to P-gp overexpressing cells. The cytotoxicity of ixabepilone, paclitaxel, docetaxel, epothilone B, vinblastine, and mitoxantrone was examined in MDCK-MDR1 (■) and MDCK (□) cell lines. Cells were cultured for 3 days at 37°C with increasing amounts of each drug and then assessed by XTT method. Each point represents the mean value of 8 individual assays with SD.

**Fig. 2.** GF120918, cyclosporin A and verapamil (Fig. 2A), but not FTC and MK-571 (Fig. 2B), reverse P-gp-mediated resistance to ixabepilone. The parental MDCK (▲) and MDCK-MDR1 cells (■) were cultured for 3 days at 37°C in the absence or presence of 1 μM GF120918, or 2.5 μg/mL cyclosporin A (CsA), or 10 μM verapamil (Ver), or 5 μM FTC, or 50 μM MK-571 with increasing amounts of ixabepilone and then assessed by XTT method. Each point represents the mean value of 8 individual assays with SD.

**Fig. 3.** Time course for the transepithelial transport of 1 μM ixabepilone across LLC-PK1 or LLC-MDR1 cell monolayers. At t = 0 min, the drug was applied into one compartment (apical or basolateral), and the amount of drug translocated to the opposite compartment at t = 45, 90, and 120 min was measured by LC/MS/MS. Transport from the basolateral to the apical compartment (B→A, solid
labels) and from the apical to basolateral compartment (A→B, open labels) were plotted. Data were expressed as mean ± SD (n = 3).

**Fig. 4.** Characterization of BCRP mRNA expression in BCRP transfected cells (HEK-BCRP) (panel A), as well as BCRP, MRP2 and P-gp protein expression in multiple cell models (panel B). (A): RNA was extracted from parental HEK cells and BCRP transfected HEK cells (HEK-BCRP), and RT-PCR was conducted in parallel. A negative control of genomic DNA was also included in the PCR reactions that showed no BCRP DNA fragment was produced; (B): The electrophoresis was performed by loading 30 μg of crude membrane protein into a NuPAGE 4–12% Bis-Tris gel, and electrophoretically blotted onto nitrocellulose membranes. The membranes were blocked, and probed with the customized antibodies. Detection of the immunoreactions was performed using a Pierce ECL Western Blotting detection system.

**Fig. 5.** Drug accumulation in HEK and BCRP-transfected HEK cells in the absence and presence of FTC (10 μM) and chrysin (5 μM). (A): BP accumulation in the cells. (B): [³H]Mitoxantrone accumulation in the cells. Results are expressed as mean ± SD; n = 8 for BP accumulation study and n = 3 for mitoxantrone accumulation study (***, p < 0.005, compared with HEK-BCRP control group).
Fig. 6. Cytotoxicity of antimicrotubule agents to BCRP overexpressing cells. The cytotoxicity of ixabepilone, paclitaxel, docetaxel, epothilone B, vinblastine, and mitoxantrone was examined in HEK-BCRP (■) and HEK (□) cell lines. Cells were cultured for 3 days at 37°C with increasing amounts of each drug and then assessed by XTT method. Each point represents the mean value of 8 individual assays with SD.

Fig. 7. FTC and GF120918 reverse BCRP-mediated resistance to paclitaxel, docetaxel, and mitoxantrone. The parental HEK (▲) and HEK-BCRP cells (●) were cultured for 3 days at 37°C in the absence or presence of 5 μM FTC (□) or 0.5 μM GF120918 (◊) with increasing amounts of each drug and then assessed by XTT method. Each point represents the mean value of 8 individual assays with SD.
TABLE 1

Resistance to Antimicrotubule Agents Conferred by P-gp and BCRP in Transfected Cells

<table>
<thead>
<tr>
<th>Drug</th>
<th>MDCK IC50 (nM)</th>
<th>MDCK-MDR1 IC50 (nM)</th>
<th>RR&lt;sup&gt;a&lt;/sup&gt;</th>
<th>HEK IC50 (nM)</th>
<th>HEK-BCRP IC50 (nM)</th>
<th>RR&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ixabepilone</td>
<td>90.0</td>
<td>&gt; 2000</td>
<td>NC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.9</td>
<td>4.7</td>
<td>1.2</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>323.9</td>
<td>&gt; 2000</td>
<td>NC</td>
<td>2.2</td>
<td>15.8</td>
<td>7.3</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>74.7</td>
<td>&gt; 2000</td>
<td>NC</td>
<td>1.0</td>
<td>4.3</td>
<td>4.3</td>
</tr>
<tr>
<td>Epothilone B</td>
<td>7.1</td>
<td>12.4</td>
<td>1.7</td>
<td>0.3</td>
<td>0.5</td>
<td>1.7</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>32.1</td>
<td>&gt; 600</td>
<td>NC</td>
<td>1.6</td>
<td>4.8</td>
<td>2.9</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>234.6</td>
<td>&gt; 2000</td>
<td>NC</td>
<td>2.7</td>
<td>31.8</td>
<td>11.9</td>
</tr>
</tbody>
</table>

<sup>a</sup>The relative resistance factor (RR) is calculated as the ratio of IC50 of transporter overexpressing cells over that of parent cells.

<sup>b</sup>NC, not calculated.

Data are reported as mean (n = 7–8).
TABLE 2

Resistance to Ixabepilone Conferred by P-gp in the Absence or Presence of Inhibitors in Transfected MDCK cells

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC50 (nM)</th>
<th>MDCK</th>
<th>MDCK-MDR1</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Inhibitor (Ixabepilone only)</td>
<td>60.4</td>
<td>1443</td>
<td></td>
</tr>
<tr>
<td>GF120918 (1 µM)</td>
<td>15.7</td>
<td>16.0</td>
<td></td>
</tr>
<tr>
<td>Cyclosporin A (2.5 µg/mL)</td>
<td>23.4</td>
<td>26.3</td>
<td></td>
</tr>
<tr>
<td>Verapamil (10 µM)</td>
<td>24.7</td>
<td>80.8</td>
<td></td>
</tr>
<tr>
<td>FTC (5 µM)</td>
<td>52.0</td>
<td>814</td>
<td></td>
</tr>
<tr>
<td>MK-571 (50 µM)</td>
<td>51.8</td>
<td>1678</td>
<td></td>
</tr>
</tbody>
</table>

Data are reported as mean (n = 8).
TABLE 3

Permeability of Ixabepilone and Digoxin across LLC-PK1 and LLC-MDR1 Monolayers

<table>
<thead>
<tr>
<th></th>
<th>LLC-PK1 Cells</th>
<th>LLC-MDR1 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A-to-B permeability (x 10^{-6} cm/s)</td>
<td>B-to-A permeability (x 10^{-6} cm/s)</td>
</tr>
<tr>
<td>Ixabepilone (3 µM)</td>
<td>6.5 ± 0.2</td>
<td>15.0 ± 1.7</td>
</tr>
<tr>
<td>Ixabepilone (6 µM)</td>
<td>5.7 ± 0.1</td>
<td>12.3 ± 0.6</td>
</tr>
<tr>
<td>Ixabepilone (10 µM)</td>
<td>6.0 ± 0.4</td>
<td>11.0 ± 1.0</td>
</tr>
<tr>
<td>Digoxin (5 µM)</td>
<td>3.6 ± 0.1</td>
<td>7.0 ± 0.4</td>
</tr>
</tbody>
</table>

*The efflux ratio (ER) is calculated as the ratio of B→A permeability over A→B permeability.
TABLE 4

Resistance to Paclitaxel, Docetaxel and Mitoxantrone Conferred by BCRP in the Absence or Presence of Inhibitors in Transfected HEK cells

<table>
<thead>
<tr>
<th>Drug</th>
<th>HEK</th>
<th>HEK-BCRP</th>
<th>HEK-BCRP+FTC</th>
<th>HEK-BCRP+GF120918</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paclitaxel</td>
<td>2.2</td>
<td>15.8</td>
<td>4.6</td>
<td>1.1</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>1.0</td>
<td>4.3</td>
<td>1.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>2.7</td>
<td>31.8</td>
<td>5.9</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Data are reported as mean (n = 7–8).
Figure 1

Graphs showing the effect of various concentrations of drugs on cell survival for MDCK-MDR1 and MDCK cell lines.

- Top left: Ixabepilone Concentration vs. Cell Survival
- Top right: Paclitaxel Concentration vs. Cell Survival
- Bottom left: Docetaxel Concentration vs. Cell Survival
- Bottom right: Epothilone B Concentration vs. Cell Survival
- Middle left: Vinblastine Concentration vs. Cell Survival
- Middle right: Mitoxantrone Concentration vs. Cell Survival
Figure 2

A) Cell survival (%) vs. ixabepilone concentration (nM)

B) Cell survival (%) vs. ixabepilone concentration (nM)
Figure 3

Ixabepilone Transport (pmol) vs. Time (min)

- △ - LLC-PK1, A→B
- ▲ - LLC-PK1, B→A
- □ - LLC-MDR1, A→B
- □ - LLC-MDR1, B→A
Figure 4

A) 

<table>
<thead>
<tr>
<th>Sample</th>
<th>DNA ladder</th>
<th>HEK cells (parent cells)</th>
<th>HEK-BCRP, clone 1c</th>
<th>HEK-BCRP, clone 2A</th>
<th>Genomic control</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,036 bp</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,636 bp</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

BCRP (2.1 kb)

B) 

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>MDCK-MDR1</th>
<th>MDCK</th>
<th>Caco-2</th>
<th>HEK</th>
<th>HEK-BCRP</th>
<th>Hepatocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>72 kDa (BCRP)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>190 kDa (MRP2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>160 kDa (P-gp)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>43 kDa (β-actin)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 5

A)  

![Graph A]  

- Control  
- FTC, 10 µM  
- Chrysin, 5 µM  

**Units of Fluorescence**  

- HEK  
- HEK-BCRP  

B)  

![Graph B]  

- Control  
- FTC, 10 µM  

**Intracellular Accumulation of Mitoxantrone (pMols/10^6 cells)**  

- HEK  
- HEK-BCRP  

*** ***
Figure 6

- Ixabepilone Concentration (nM)
- Paclitaxel Concentration (nM)
- Docetaxel Concentration (nM)
- Epothilone B Concentration (nM)
- Vinblastine Concentration (nM)
- Mitoxantrone Concentration (nM)
Figure 7

Graphs showing the effect of different concentrations of Paclitaxel, Docetaxel, and Mitoxantrone on the cell survival of HEK-BCRP, HEK, HEK-BCRP + 5 μM FTC, and HEK-BCRP + 0.5 μM GF120918 cell lines.

- Paclitaxel Concentration (nM)
  - HEK-BCRP
  - HEK
  - HEK-BCRP + 5 μM FTC
  - HEK-BCRP + 0.5 μM GF120918

- Docetaxel Concentration (nM)
  - HEK-BCRP
  - HEK
  - HEK-BCRP + 5 μM FTC
  - HEK-BCRP + 0.5 μM GF120918

- Mitoxantrone Concentration (nM)
  - HEK-BCRP
  - HEK
  - HEK-BCRP + 5 μM FTC
  - HEK-BCRP + 0.5 μM GF120918