Ixabepilone, a Novel Microtubule-Targeting Agent for Breast Cancer, Is a Substrate for P-Glycoprotein (P-gp/MDR1/ABCB1) but not Breast Cancer Resistance Protein (BCRP/ABCG2)

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ABSTRACT
Ixabepilone is the first epothilone to be approved for clinical use. Current data suggest the epothilones have a role in treating taxane-resistant cancers and 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-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) in ixabepilone resistance. A significant decrease in ixabepilone-mediated cytotoxicity was observed in Madin-Darby canine kidney cells transfected with human multidrug resistance 1 (MDR1) comparative with the parental cells (IC50 > 2000 nM versus 90 nM). Overexpression of P-gp also resulted in significantly decreased cell susceptibility to docetaxel, paclitaxel, and vinblastine. Bidirectional transport of ixabepilone across monolayers of porcine kidney-derived cells expressing human MDR1 showed a significantly increased efflux ratio relative to the parental cells. A BCRP-overexpressing cell line was developed by transfecting human embryonic kidney (HEK)-293 cells with BCRP cDNA and confirmed by immunoblotting and bodipy prazosin and mitoxantrone uptake. Neither P-gp nor multidrug resistance protein 2 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 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) 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 anticancer therapies. The epothilones act via stabilizing intracellular microtubules (tubulins), and one of the consequences of this is apoptosis after 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 and Swanton, 2008; Vahdat, 2008). It is noteworthy that current evidence suggests that the epothilones may have a different, more favorable, resistance profile to other tubulin-binding agents. Indeed, ixabepilone has been approved by the Food and Drug Administration for the treatment of advanced breast cancer in patients whose tumors are resistant or refractory to taxanes, anthracyclines, and/or capecitabine (Harrison and Swanton, 2008; Vahdat, 2008; Hortobagyi et al., 2010; Sparano et al., 2010).

One critical cellular process that contributes to drug resistance is the decreased accumulation of drugs within cells caused by drug efflux mechanisms mediated by ATP-binding cassette (ABC) efflux transporters. The ABC transporters, P-glycoprotein (P-gp/MDR1/ABCB1), breast cancer resistance protein 2, multidrug resistance protein 2, MRP2, human embryonic kidney (HEK), Madin-Darby canine kidney (MDCK), LLC-PK1, Lilly laboratory cell for porcine kidney 1, HEK-293 cell line for human embryonic kidney, F13, fumitremorgin C, MDR, multidrug resistance, RR, relative resistance, BP, bodipy prazosin, PBS, phosphate-buffered saline, HPBS, PBS containing 10 mM HEPES, pH 7.4, RT, reverse transcription; PCR, polymerase chain reaction; LC/MS/MS, liquid chromatography/tandem mass spectrometry; ER, efflux ratio; FRT, Flp recombination target; A → B, apical to basal; B → A, basal to apical; GF120918, 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; MDR1, 3-[3-(3-[(1E)-2-(7-chloro-2-quinolinyl)ethenyl]phenyl)][3-(dimethylamino)-3-oxopropyl]thiophenethyl]thio propanoic acid; XTT, sodium 3-[4,5-(3,4-dimethyloxazol-2-yl)-5-(phenylamino)-carboxamido]-2-(2-nitro-5-phenyl)-benzene sulfonyl chloride; FTC, fumitremorgin C; MDR, multidrug resistance; RR, relative resistance; BP, bodipy prazosin; PBS, phosphate-buffered saline; HPBS, PBS containing 10 mM HEPES, pH 7.4; RT, reverse transcription; PCR, polymerase chain reaction; LC/MS/MS, liquid chromatography/tandem mass spectrometry; ER, efflux ratio; FRT, Flp recombination target; A → B, apical to basal; B → A, basal to apical; GF120918, 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; MK-571, 3-[[3-[(1E)-2-(7-chloro-2-quinolinyl)ethenyl]phenyl][3-(dimethylamino)-3-oxopropyl]thiophenethyl]thio propanoic acid; XTT, sodium 3-[4,5-(3,4-dimethyloxazol-2-yl)-5-(phenylamino)-carboxamido]-2-(2-nitro-5-phenyl)-benzene sulfonyl chloride.
tance protein (BCRP/ABCG2), and multidrug resistance protein 2 (MRP2/ABCC2) actively extrude a wide variety of anticancer drugs from tumor cells (Borst and Elferink, 2002; Kruh and Belinsky, 2003; Krishnamurthy and Schuetz, 2006). 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 antimitotubule agents focus on P-gp. These data demonstrate taxanes (i.e., paclitaxel and docetaxel) and many other antimitotubule 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. (2004) 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 cell lines (i.e., MCF-7DOX and MCF-7AX). The presence of BCRP in these cell lines provided an explanation as to the inability of valspodar, 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- to 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 toward vinblastine and paclitaxel (Litman et al., 2002), suggesting that the multidrug-resistant phenotype caused by BCRP expression is overlapping, but distinct, from that caused by P-gp.

In addition to preclinical models, some clinical data are available. Polymorphisms in host genes may also be important for docetaxel metabolism, transport, and action; these include MDR1, BCRP, cytochrome P450 3A4, cytochrome P450 3A5, microtubule-associated protein τ, and microtubule-associated protein 4 (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 with other tubulin-binding agents. Ixabepilone retains antineoplastic activity in cell lines selected for resistance to paclitaxel, including P-gp-overexpressing HCT116/V4M46 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., 2008, 2009). Relative resistance (RR; a ratio of IC_{50} values) in multidrug-resistant versus sensitive lines (i.e., HCT116/V4M46 versus HCT116), was substantially lower for ixabepilone than in paclitaxel (7.77 versus >100), providing initial evidence that it may be a weak substrate for P-gp.

Here, we examined the cytotoxic characteristics of ixabepilone and other antimitotubule agents using established cDNA gene-transfected Madin-Darby canine kidney (MDCK)-MDR1 and HEK-BCRP cells. We also investigated bidirectional transepithelial transport of ixabepilone across LLC-MDR1 cell monolayers. Our aim 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.

Materials and Methods

Materials. Platinum Taq DNA Polymerase, Gene Pool human liver cDNA, subeloning efficiency DH-5α-competent cells, Flp-in human embryonic kidney (HEK) cells, Zeocin, fetal bovine serum, trypsin, Lipofectamine 2000, hygromycin B, Flp-in system, and bodipy prazosin (BP) were purchased from Invitrogen (Carlsbad, CA). Fumitremorgin C (FTC) and the antibodies against P-gp and MRP2 were purchased from Enzo Life Sciences, Inc. (Farmingdale, NY). 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 (GP120918, elacridar) and 3-[[3-(1-((2,7-chloro-2-quinolinyl)ethyl]phenyl)[3-(dimethylamino)-5-oxopropyl]thio)methyl]thiopropanoic acid (MK-571, L-660711) were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). The antibody against BCRP was purchased from Kamiya Biomedical (Thousand Oaks, CA). [3H]mitoxantrone and [3H]digoxin were purchased from American Radiolabeled Chemicals (St. Louis, MO). [4C]Mannitol and [3H]Propranolol were purchased from Sigma-Aldrich (St. Louis, MO). Ixabepilone was supplied by the Process Research and Development Department of Bristol-Myers Squibb (New Brunswick, NJ). Other chemicals were purchased from Sigma-Aldrich unless stated otherwise.

Cell culture reagents including Dulbecco’s modified Eagle medium, RPMI medium, and fetal calf serum were purchased from Invitrogen. MDCK parental cells and those transfected with human MDR1 (MDCK-MDR1) were obtained from The Netherlands Cancer Institute (Amsterdam, The Netherlands). HEK-293 cells containing the Flp recombination target (FRT) recombination site were purchased from Invitrogen. 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 Life Sciences (Lowell, MA).

Generation of a Stable Human BCRP-Transfected Cell Line and Cultivation of HEK-BCRP, MDCK-MDR1, and LLC-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 CTG 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 the pcDNA5-FRT expression system by ligation. The resulting vector construct contains BCRP followed by an inter-
medium was replaced and replaced with complete medium supple-
mented with selecting antibiotic hygromycin B (100 μg/ml). Single
hygromycin-resistant colonies 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 BP accumulation using a CytoFluor Microplate Reader 4000
(Applied Biosystems, Foster City, CA) 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 multi-
drug resistance gene (MDCK-MDR1 cells) were obtained from the
Netherlands Cancer Institute and cultured in Dulbecco’s modified
Eagle’s medium supplemented with 10% fetal bovine serum, 0.1 mM
nonessential amino acids, 2 mM l-glutamine solution, 100 U/ml peni-
cillin, 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, San Jose, CA) were cultured in BD Falcon 24-well plates
with 1-μm culture inserts in medium M199 supplemented with 10%
heat-inactivated fetal calf serum, 2 mM glutamine, 100 U/ml peni-
cillin, 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 to 4
days.

Cytotoxicity Assays. Cytotoxicity of drugs in MDCK-MDR1,
MDCK, HEK-BCRP, and HEK cells was measured using XTT color-
metric assay (Roche Applied Science, Mannheim, Germany) per-
formed in 96-well plates. Five thousand cells were seeded into each
well in 50 μl of culture medium. Fifty microliters of 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 spectrophotometri-
cally 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-me-
diated transport of ixabepilone was also evaluated in LLC-PK1 wild-
type and LLC-MDR1 cell lines. Before conducting transport experi-
ments, the integrity of monolayers was confirmed by measurement of
transepithelial electrical resistance using an electrical resistance
system with chopstick electrodes (World Precision Instruments, Inc.,
Sarasota, FL) and determining permeability of paracellular marker
[14C]mannitol. Moreover, monolayer integrity was further evaluated by
postexperimental lucifer yellow apical to basal side (A → B) flux
determination for each cell monolayer. As a transcellular high per-
meability marker, the permeability of [3H]propranolol was also de-
termined. P-gp expression was functionally tested by determining
bidirectional transport of [14C]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 × 10^5 cells/well with 0.3 ml of medium in
the upper compartment and 35 ml of medium in the lower nonsub-
divided 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 baso-
lateral 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. [3H]digoxin (5 μM)
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 basolat-
eral 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 two micro pumps
(PerkinElmer Life and Analytical Sciences, Waltham, MA), 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
(Milford, MA) symmetry C18 column (2.1 × 50 mm, 5 μm).

The mobile phase used was 0.1% formic acid in water (A) or in acetoni-
trile (B) at 0.3 ml/min with a gradient from 70 to 40% A in 3 min.
Ixabepilone concentration was determined by multiple-reaction
monitoring at a transition of m/z 505.3 to 405.3 in negative electro-
 spray ionization mode.

Apparent permeability (P_app) was calculated using:

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

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 \(t = 0\), \(A\) 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:

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

where \(P_{app \rightarrow A}\) is the \(P_{app}\) value measured in the basal to apical
direction, \(P_{app \rightarrow B}\) 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
dithiothreitol and protease inhibitor cocktail (Roche Applied Science,
Indianapolis, IN). The lysates were centrifuged, and the supernatant
proteins (30 μg) were separated by NuPAGE 4 to 12% Bis-Tris gel
electrophoresis. Gels were transferred to nitrocellulose membranes
overnight at 5 mA. Membranes were blocked for 1.5 h at room
temperature (SuperBlock Blocking buffer in TBS-Tween; Thermo
Fisher Scientific, Waltham, MA) 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 enhanced chemiluminescence detection
agents (Thermo Fisher Scientific, Waltham, MA), 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 Cell Studies. The uptake
experiments using BP and mitoxantrone in HEK-BCRP and HEK
cells were conducted with the cell monolayers grown in 96- and
6-well Biocoat tissue culture plates, respectively. Before initiating
uptake experiments, cell monolayers were washed twice with phos-
phate-buffered saline (PBS) containing 10 mM HEPES, pH 7.4
(HPBBS). 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
PTC (10 μM) or chrysirin (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 a
CytoFluor Microplate Reader 4000. The excitation and emission
wavelengths were 485 and 530 nm, respectively. Nonspecific uptake
for HEK-BCRP cells caused by passive diffusion was determined in
parallel experiments in parental HEK cells. The baseline was deter-
mined by omitting BP and incubating cells with HPBBS only.

To measure intracellular amount of mitoxantrone, the cell mono-

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layers were quickly washed three times with 3 ml of ice-cold HPBS, and the cells were detached by 10-min incubation with 500 μl of 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 obtained in the study were analyzed by a scintillation counter (LS 6500; Beckman Coulter, 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 and 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 IC50 values are shown in Table 2, and representative IC50 curves are shown in Fig. 2. As shown in Fig. 2A, the addition of cyclosporine A, verapamil, and GF120918 restored the sensitiv-

![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 the XTT method. Each point represents the mean value of eight individual assays with S.D.](image)

<table>
<thead>
<tr>
<th></th>
<th>MDCK, IC50</th>
<th>MDCK-MDR1</th>
<th>HEK, IC50</th>
<th>HEK-BCRP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nM</td>
<td>IC50</td>
<td>RR</td>
<td>IC50</td>
</tr>
<tr>
<td>Ixabepilone</td>
<td>90.0</td>
<td>&gt; 2000</td>
<td>NC</td>
<td>3.9</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>323.9</td>
<td>&gt; 2000</td>
<td>NC</td>
<td>2.2</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>74.7</td>
<td>&gt; 2000</td>
<td>NC</td>
<td>1.0</td>
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<tr>
<td>Epothilone B</td>
<td>7.1</td>
<td>12.4</td>
<td>1.7</td>
<td>0.3</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>32.1</td>
<td>&gt; 500</td>
<td>NC</td>
<td>1.6</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>234.6</td>
<td>&gt; 2000</td>
<td>NC</td>
<td>2.7</td>
</tr>
</tbody>
</table>

NC, not calculated.
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>IC_{50} (nM)</th>
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<tr>
<td>No inhibitor (ixabepilone only)</td>
<td>60.4</td>
</tr>
<tr>
<td>GF120918 (1 μM)</td>
<td>15.7</td>
</tr>
<tr>
<td>Cyclosporin A (2.5 μg/ml)</td>
<td>23.4</td>
</tr>
<tr>
<td>Verapamil (10 μM)</td>
<td>24.7</td>
</tr>
<tr>
<td>FTC (5 μM)</td>
<td>52.0</td>
</tr>
<tr>
<td>MK-571 (50 μM)</td>
<td>51.8</td>
</tr>
</tbody>
</table>

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

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 symbols) and from the apical to basolateral compartment (A → B; open symbols) were plotted. Data are expressed as mean ± S.D. (n = 3).

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Characterization of the Stable BCRP Transfection Model. Initial RT-PCR and immunohistochemistry analyses with specific primers and antibodies revealed the expression of a human BCRP 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 human BCRP sequence was confirmed by sequence analysis.

Transfection of HEK cells with the construct revealed 11 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 determine the approximate level of P-gp and MRP2, Western blotting of the cell lysate of HEK-BCRP was conducted. The results are 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 after 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 trans-
fected 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 with 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 analogs developed at Bristol-Myers Squibb also support the conclusion that epothilones are not substrates for BCRP. To determine whether resistance to the drugs is solely caused by 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 anticancer 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 nontaxane entities with superior activity and better resistance profiles could potentially provide greater clinical benefit for patients with cancer.

Here, we used established preclinical models to assess the roles of P-gp and BCRP in microtubule-binding agent resistance mechanisms. First, 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. Second, 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; Meerum Terwogt et al., 1999; Maligré et al., 2001; Bardelmeijer et al., 2002). 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; Kruijtzer 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 sensitiveness of HEK-BCRP cells to paclitaxel and docetaxel was probably the result of BCRP antagonism, because 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 that 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. Taxanes have been shown to be substrates of MRP2 (Huisman et al., 2005; Lagas et al., 2006), suggesting the increased levels of MRP2 in the MDCK-MDR1 cell line could augment the effect of P-gp in reduced cytotoxicity. To confirm

![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 the XTT method. Each point represents the mean value of eight individual assays with S.D.](image-url)
the involvement of P-gp in ixabepilone resistance in P-gp-overexpressing MDCK-MDR1, further experiments using the coinubcation of chemical inhibitors with ixabepilone were conducted. The results confirmed the involvement of P-gp in MDCK-MDR1 resistance to ixabepilone, because P-gp inhibitors cyclosporine A, verapamil, and GF120918 restored the sensitivity of MDCK-MDR1 to ixabepilone, whereas inhibitors of MRP2 and BCRP had no effect. Consistent with the Western blot experiment in which a 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 IC_{50} values 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, LLCP/K-MDR1; ixabepilone exhibited marked active \( B \rightarrow A \) 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 have been 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/Vh46 cells as published by 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 because of 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 for 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 \( \beta \)-III-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 Arg482 site were demonstrated to alter the substrate specificity and sensitivity to multiple substrates including doxorubicin, daunorubicin, methotrexate, and R1023 (Robey et al., 2003). Although the clinical relevance of the Arg482 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.

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Authorship Contributions

Participated in research design: Shen, Lee, and Gan.
Conducted experiments: Shen.
Contributed new reagents/analytic tools: Shen and Lee.
Performed data analysis: Shen and Gan.
Wrote or contributed to the writing of the manuscript: Shen and Gan.

References

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