Effects of Dihydropyridines and Pyridines on Multidrug Resistance Mediated by Breast Cancer Resistant Protein: In Vitro and In Vivo Studies

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dihydropyridine-3,5-dicarboxylate; MDR, multidrug resistance; MRP1, multidrug
resistant-associated protein 1.
ABSTRACT

Breast cancer resistance protein (BCRP, ABCG2) is a recently identified member of the ABC family of cell surface transport proteins. This study was conducted to investigate the effect of a series of newly synthesized 1,4-dihydropyridines and pyridines, designed as potent P-glycoprotein inhibitors, on BCRP-mediated drug efflux both in vitro and in vivo. The effects of 25 synthesized dihydropyridines and corresponding pyridines along with 4 commercially available dihydropyridines (niguldipine, nicardipine, nifedipine and nitrendipine) on the intracellular accumulation of the BCRP substrate mitoxantrone were evaluated in BCRP-expressing human breast cancer MCF7/MX100 and human non-small lung cancer H460/MX20 cells. At a 2.5 µM concentration, 24 out of 25 newly synthesized dihydropyridines and pyridines produced a significant increase of mitoxantrone accumulation in both cell lines. The most potent compound was able to enhance mitoxantrone accumulation approximately 4.5-fold, greater than that obtained with 10 µM of fumitremorgin C, which is a specific BCRP inhibitor. The results from the two cell lines showed good correlation ($r^2 = 0.71$, p<0.01). Niguldipine, nicardipine and nitrendipine also demonstrated potent BCRP inhibition, while nifedipine had no effect. The effects of the dihydropyridine and pyridine compounds on mitoxantrone cytotoxicity paralleled their effects on mitoxantrone accumulation. Co-administration of a selected dihydropyridine compound, I_m (DHP-014) with topotecan, a good BCRP substrate and a moderate to poor P-glycoprotein substrate, resulted in significant increases in the systemic exposure and peak concentration of topotecan in Sprague-Dawley rats when oral topotecan 2 mg/kg was combined with 20 mg/kg DHP-014. The observed increase of
topotecan exposure provides proof-of-concept for in vivo inhibition of BCRP by these agents.

INTRODUCTION

Breast cancer resistant protein (BCRP/ABCG2), was first identified in the MCF-7/AdrVp cell line, which is a multidrug-resistant human breast cancer subline that displays an ATP-dependent reduction in the intracellular accumulation of anthracycline anticancer drugs in the absence of overexpression of known multidrug resistance (MDR) transporters such as P-glycoprotein or the multidrug resistance-associated protein 1 (MRP1) (Doyle et al., 1998). It is a 655 amino acid ‘half-transporter’ and belongs to the G subclass of the ATP-binding cassette (ABC) superfamily (Doyle and Ross, 2003). Cells that overexpress BCRP demonstrate resistance to mitoxantrone and topoisomerase I inhibitors (Maliepaard et al., 1999; Miyake et al., 1999; Kawabata et al., 2001). Increased BCRP mRNA expression in patients’ mononuclear bone marrow cells has been recently reported in relapsed or refractory hematological malignancies and associated with poor response to chemotherapy (Steinbach et al., 2002; van den Heuvel-Eibrink et al., 2002; van der Kolk et al., 2002). It is therefore important to identify clinically applicable chemotherapy modulators in treatment of malignancies such as acute leukemia, where multiple MDR transporters are expressed (Ross, 2000).

1,4- Dihydropyridine calcium channel blockers have been long viewed as P-glycoprotein modulators. The clinical application of calcium channel blockers as P-glycoprotein inhibitors has been limited due to their potent vasodilator effect. The structure of dihydropyridines has been modified in our laboratory to generate a series of analogues that are potent P-glycoprotein inhibitors and demonstrate negligible calcium
channel binding (Zhou et al., 2005). Because of the substantial overlap of substrates and inhibitors for BCRP and P-glycoprotein (Borst et al., 2000; Litman et al., 2000), it is reasonable to speculate that dihydropyridines may also possess BCRP modulation effects. In the present study, these synthesized dihydropyridines and pyridines, as well as four commercially available dihydropyridines (niguldipine, nicardipine, nifedipine and nitrendipine) were evaluated for their effects on BCRP-mediated efflux and the cytotoxicity of the BCRP substrate and chemotherapeutic agent mitoxantrone. DHP-014 (Compound Im), a new dihydropyridine compound shown to be a potent BCRP and P-glycoprotein inhibitor in vitro, was selected to investigate its effect on topotecan pharmacokinetics in Sprague-Dawley rats. Topotecan is a good BCRP substrate (Jonker et al., 2000), as well as a moderate to poor P-glycoprotein substrate (Chen et al., 1991). Pronounced changes of topotecan bioavailability due to BCRP inhibition have been reported both in mice (Jonker et al., 2000), humans (Kruijtzer et al., 2002) and rats (Zhang et al., 2004a). Topotecan undergoes very limited metabolism in rats (Platzer et al., 1998) and humans (Rosing et al., 1997), making it a suitable model BCRP substrate for the investigation of BCRP-mediated drug-drug interactions in vivo, since interactions between inhibitors and metabolizing enzymes will not confound the results.
MATERIALS AND METHODS

Compounds and cell lines

Mitoxantrone, nicardipine nifedipine, niguldipine and nitrendipine were purchased from Sigma (St. Louis, MO). Compounds Ia – Io and IIa – IIo were synthesized in Dr. Robert Coburn’s laboratory, Department of Chemistry, University at Buffalo (Zhou et al., 2005). The chemical structures of these test compounds are shown in Figure 1. Series I are 1,4-dihydropyridines and Series II are corresponding aromatized pyridines. Human breast cancer MCF-7 sensitive and MCF-7/MX100, human large cell lung carcinoma NCI-H460 and NCI-H460/MX20, and fumitremorgin C were kind gifts from Dr. Susan E. Bates (National Cancer Institute, Bethesda, MD). Topotecan was purchased from ChemPacific (Baltimore, MD). Injectable anesthetics and suture materials were obtained from Henry Schein Incorporation (Melville, NY). Acridine was purchased from Sigma (St. Louis, MO).

Cell Culture

Parental human breast cancer cell MCF/s and human non-small cell lung cancer cell H460/s were grown in RPMI 1640 medium (Life Technologies, Inc., Grand Island, NY) supplemented with 5% heat-inactivated fetal bovine serum (Life Technologies). Resistant MCF-7/MX100 cells and H460/MX20 were cultured in the above-mentioned media with addition of 100 nM and 20 nM mitoxantrone, respectively. All cells were incubated at 37°C in 5% CO₂/95% air.
Animals

Female Sprague-Dawley rats (220 – 260 g in body weight) were obtained from Harlan (Indianapolis, IN) and housed according to institutional guidelines. The rats were kept in a temperature-controlled environment with a 12-hour-light-12-hour-dark cycle and given a standard diet with water ad libitum. Rats were fasted overnight before oral administration of drug. The animal protocol was approved by the Institutional Animal Use and Care Committee at the University at Buffalo.

Western Blotting Assay

Cells grown in 100 × 15 mm culture dishes were washed with PBS, and harvested using a cell scraper. Total cell lysates were prepared by adding a lysis buffer (20 mM Tris pH 7.5, 120 mM sodium chloride, 100 mM sodium fluoride, 1% octylphenoxy polyethoxy ethanol, 200 µM sodium orthovanadate, 50 mM β-glycerolphosphate, 10 mM sodium pyrophosphate, 4 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 10 µg/ml leupeptin, and 10 µg/ml aprotinin) to the harvested cells and keeping on an ice bath for 30 minutes. Soluble extracts were obtained by centrifuging cell lysates at 13,000 x g for 20 minutes. The protein concentration of the supernatant was determined by the Bradford method (Bradford, 1976). Lysates were subjected to electrophoresed on 7.5% SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes (Invitrogen, Grand Island, NY). Membranes were then blocked overnight at 4°C in Tris-buffered saline containing 0.2% (v/v) Tween-20 and 5% (w/v) fat-free dry milk (Bio-Rad, Hercules, CA) with a 1:750 dilution of the BXP-21 monoclonal antibody against BCRP (Maliepaard et al., 2001a). The blots were then incubated at room temperature for 1 hour.
with a horseradish peroxidase-conjugated sheep anti-mouse IgG secondary antibody (Amersham Biosciences, Piscataway, NJ), followed by enhanced chemiluminescence (ECL) detection (Amersham Biosciences, Piscataway, NJ).

*Mitoxantrone Cellular Accumulation Studies*

Mitoxantrone cellular accumulation studies with analysis by flow cytometry followed the protocols described by Minderman et al (Minderman et al., 2002) and Zhang et al. (Zhang et al., 2004b). Briefly, cells were incubated at a density of 1×10^6 cells/ml for 30 minutes at 37°C in RPMI 1640 medium with 3 μM mitoxantrone. At the end of the 30-minute incubation period, cells were washed once with ice-cold phosphate-buffered saline and then resuspended in ice-cold phosphate-buffered saline. An aliquot of cells was kept on ice until flow cytometry analysis for drug retention. Samples were analyzed on a FACScan flow cytometer (Becton Dickinson Immunocytometry System, San Jose, CA) equipped with a standard argon laser for 488 nm excitation and with 530/30 nm band pass (FL1), 585/42 band pass (FL2), and 670 nm long pass (FL3) filters for emission collection. All flow cytometric data were analyzed with the WinList software program (Verity Software House, Topsham, ME).

*Mitoxantrone Cytotoxicity Assay*

One hundred µl of MCF-7/MX100 cells were seeded in 96 well plates at a density of 4000 cells per well. The cells were allowed to attach for 24 hours at 37°C, following which additional 100 µl media was added to each well containing the desired final concentration of mitoxantrone (0.1 μM to 1000 μM) with or without the
modulator. After a six-hour exposure to the drug, the cells were washed twice with sterile 1X phosphate-buffered saline and fresh media was added to each well. The cells were allowed to grow for four more days. After four days, the total protein was measured by a sulforhodamine B staining assay (Skehan et al., 1990). Briefly, 10% trichloracetic acid was added to the cells for an hour on ice in order to fix cellular protein to the wells, rinsed five times with water and allowed to air dry. Sulforhodamine B (0.4% w/v in 1% glacial acetic acid) was added to each well for 15 minutes, and washed four times with 1% acetic acid. After drying the plates, protein bound dye was solubilized in 10 mM Tris base and quantitated by measuring the absorbance at 570 nm.

**DHP-014 (Compound Im)-Topotecan Interaction Study in Rats**

Topotecan was freshly prepared in 5% D-glucose for oral dosing. Three groups of 4 animals each were given topotecan 2 mg/kg orally alone or co-administered with DHP-014 10 mg/kg or 20 mg/kg by intraperitoneal injection. Blood samples (150 µl) taken from the jugular vein cannula were collected in heparinized tubes prior to drug administration and at 2, 7, 15, 30, 60, 120, 240, 480 and 720 min after dosing. Plasma samples were obtained by centrifugation at 1000 g for 10 min and analysed by HPLC (Chen and Balthasar, 2002; Zhang et al., 2004a). The HPLC assay utilized a mobile phase of methanol-10 mM potassium phosphate (KH₂PO₄) (25:75, v/v) containing 2% triethylamine with pH of 3.72 and a flow rate of 1.0 ml/min. For sample preparation, 40 µl plasma was mixed with 4 µl acridine (1.5 µg/ml as the internal standard), 120 µl methanol and 40 µl 100 mM phosphoric acid. The mixture was then centrifuged at 2000 g for 8 min. A clear supernatant was collected and 100 µl of the sample solution was
injected into the HPLC system. Topotecan was detected with excitation and emission wavelengths set at 361 and 527 nm, respectively. The lower limit of quantitation of this method was 0.02 ng. Standard curves are linear over the concentration range of 1-500 ng/ml. The intra-day and inter-day variabilities were 9.48% and 14.2%, respectively.

Data Analysis

For mitoxantrone accumulation studies, mitoxantrone fluorescence in modulator-treated cells and vehicle (0.1% DMSO)-treated cells were compared. Statistical significance was determined using a one-way analysis of variance followed by Dunnett’s post hoc test. Differences were considered to be significant when p < 0.05. In the concentration-dependent study, the EC50 values of compounds for increasing mitoxantrone accumulation in MCF-7/MX100 and H460/MX20 cells were obtained by fitting the fraction of maximal increase (F) by Equation (1) using the computer program WinNonlin (Pharsight, Mountain View, CA)

\[ F = \frac{C^\gamma}{EC_{50} + C^\gamma} \]  

(1)

C is the concentration of the test compound. \( \gamma \) represents the curve fitting coefficient. F was calculated as the ratio of the net increase of mitoxantrone accumulation in the presence of the test compound (A-A0) to the maximal net increase, in this study, represented by the net increase of mitoxantrone accumulation in the presence of 40 µM nicardipine (A_{nicardipine} – A0). A0, A and A_{nicardipine} are the mitoxantrone accumulation in the presence of the vehicle control (0.1% DMSO), the test compound, and 40 µM nicardipine, respectively.
In mitoxantrone cytotoxicity studies, the IC\textsubscript{50} values were obtained by fitting the growth inhibition data to an inhibitory sigmoidal model of a Hill Equation (Equation 2) using WinNonlin (Pharsight, Mountain View, CA).

\[
SF = 1 - \frac{I_{\text{max}} \times C^\gamma}{IC_{50} + C^\gamma}
\]  

(2)

SF represents the survival fraction of cells after treatment. \(I_{\text{max}}\) is the maximal percentage of inhibition and in the present study was fixed as 1. IC\textsubscript{50} is the concentration of mitoxantrone that causes 50% inhibition of cell growth. \(\gamma\) represents the curve fitting coefficient, or Hill coefficient.

The pharmacokinetic parameters of topotecan were obtained by noncompartmental analysis using WinNonlin version 2.1 (Pharsight, Mountain View, CA). The area under the plasma concentration-time curves (AUC) was calculated using the trapezoidal method. Analysis of variance was applied to assess the statistical significance of pharmacokinetic parameters of topotecan among different dosing regimens. Differences were considered to be statistically significant when \(p < 0.05\).
RESULTS

Expression of BCRP in H460/MX20 and MCF7/MX100 Cells

Total cell lysate was prepared from H460/wt, H460/MX20, MCF7/wt and MCF7/MX100 cells and subjected to an immunoblotting assay for BCRP expression. The results shown in Figure 2 demonstrate that BCRP was expressed in the mitoxantrone resistant H460/MX20 and MCF7/MX100 cells but was undetectable in the drug-sensitive parental cells when using the same amount of lysate protein per lane. Since dihydropyridines and pyridines are known as P-glycoprotein and MRP1 inhibitors, to rule out the possible contributions of these two transporters, Western blotting assays for P-glycoprotein and MRP1 expression were also performed in the same protein samples. No MRP1 or P-glycoprotein protein bands were detected in these cell lines (Zhang et al., 2004b).

Mitoxantrone Retention Studies

Figure 3 and 4 show the effect of the modulators on mitoxantrone retention in BCRP-overexpressing H460/MX20 and MCF7/MX100 cells, respectively. To exclude the possibility that the dihydropyridine/pyridine compounds may contribute to the fluorescence intensity, H460/MX20 and MCF7/MX100 cells were incubated in medium with 10 µM of each test compound. No test compound-associated fluorescence was detected with the FacScan instrument settings used to detect mitoxantrone (data not shown). The 30-minute accumulation of 3 µM mitoxantrone in MCF-7/adr cells was measured in the presence of 2.5 µM of synthesized dihydropyridines and pyridines. Fumitremorgin C 10 µM served as the positive control in these experiments. As shown in
Figure 3, all synthesized dihydropyridine compounds demonstrated a significant increase of intracellular mitoxantrone concentration at \( p < 0.001 \) or \( p < 0.05 \) except for compound I\(_h\) (no significant difference vs. control). All of the corresponding pyridine analogues also significantly increased the intracellular mitoxantrone concentrations in the drug resistant breast cancer cells.

Similar results were obtained in MCF-7/MX100 cells (Figure 4). All of the synthesized dihydropyridines except for compound I\(_h\) and all of the pyridine compounds demonstrated a significant increase of intracellular mitoxantrone concentration. None of the synthesized compounds significantly affected mitoxantrone accumulation in the parental MCF-7 and H460 cells (data not shown).

Figure 5 represents the relationship between mitoxantrone accumulation in the two BCRP-overexpressing cell lines after treatment with modulators. A statistically significant correlation for the inhibition of mitoxantrone efflux by the modulators in MCF7/MX100 and H460/MX20 cells was observed \( (r^2 = 0.71, \ p < 0.01) \).

Four commercially available dihydropyridines, nicardipine, nifedipine, niguldipine, nitrendipine along with compounds I\(_m\) and II\(_b\), two compounds selected from our new series of compounds due to their potent effect on modulating mitoxantrone retention in resistant cells, were investigated for their concentration-dependent effects on mitoxantrone accumulation in MCF7/MX100 and H460/MX20 cells (Figure 6). Nicardipine, niguldipine, nitrendipine, compound I\(_m\) and compound II\(_b\) elicited concentration-dependent increases in mitoxantrone retention in two resistant cell lines. No significant effect of nifedipine was observed at concentrations ranging from 1 to 20 \( \mu \text{M} \). The maximal accumulation was based on that observed with nicardipine at
concentrations of 10, 20 and 40 µM, and was similar to that observed at the 40 µM concentrations of niguldipine, nitrendipine, compound I_m and compound II_b, although compound II_b at a 40 µM concentration demonstrated somewhat higher values in H460/MX20 cells. It should be noted that the maximal accumulation values were approximately 2-fold higher than observed with FTC 10 µM. The estimated EC_{50} values of nicardipine, niguldipine, nitrendipine, compound I_m and compound II_b for increasing mitoxantrone accumulation in MCF-7/MX100 and H460/MX20 cells are summarized in Table 1.

**Mitoxantrone Cytotoxicity Studies**

Nicardipine, niguldipine, nitrendipine, compound I_m and compound II_b restored the sensitivity of resistant MCF-7/MX100 cells to mitoxantrone cytotoxicity in a concentration-dependent manner. As shown in Table 2, the IC_{50} values of mitoxantrone in MCF-7/MX100 were significantly reduced by treatment with compounds I_m and II_b at concentrations ranging from 1 to 40 µM. Nicardipine demonstrated the most potent effect on mitoxantrone cytotoxicity compared with the other commercially available dihydropyridines. At 2.5 µM, nicardipine potentiated mitoxantrone cytotoxicity by nearly 2-fold compared with the vehicle control, an effect comparable to that of the positive control fumitremorgin C at 10 µM. However, nifedipine showed no significant effect on mitoxantrone cytotoxicity in BCRP-overexpressing MCF-7/MX100 cells.

**Co-administration of Topotecan with DHP-014 (Compound I_m) in Rats**

Topotecan was administered to rats (2 mg/kg orally) either alone or with DHP-014 (10 or 20 mg/kg i.p.). DHP-014 was selected for further study based on the following criteria: good inhibitory activity for BCRP, negligible calcium channel binding
activity, and predicted low systemic clearance based on quantitative structure-pharmacokinetic relationship analysis (Zhou et al., 2003; Zhou et al., 2005). The plasma concentration-time profiles of topotecan are given in Figure 7 and the pharmacokinetic parameters are summarized in Table 3. The area under the curve (AUC) and peak concentration (C_{max}) of topotecan were significantly increased (p < 0.05) by co-administration with DHP-014 at a dose of 20 mg/kg, while the oral clearance (CL/F) of topotecan was markedly decreased (p < 0.05) compared to topotecan given alone. No statistically significant differences of topotecan terminal half-life (t_{1/2}) were observed among the three treatments.
DISCUSSION

ABC transporters play important roles in the absorption, distribution and elimination of many substrate drugs, including anticancer drugs, and in the multidrug resistance observed in tumor cells. The development of novel agents to inhibit ABC transporters for clinical application thus represents an active area of research, with P-glycoprotein being the most extensively studied ABC transporter (Lin and Yamazaki, 2003).

BCRP is a recently identified ABC transporter and is highly expressed in the placenta (Maliepaard et al., 2001a), with lesser expression in brain, prostate, small intestine, testis, ovary, colon and liver (Doyle et al., 1998). Several substances have been reported as BCRP inhibitors: GF120918 (de Bruin et al., 1999), the mycotoxin fumitremorgin C (Rabindran et al., 2000), its derivatives demethoxyfumitremorgin C (van Loevezijn et al., 2001), Ko132, Ko134 and Ko143 (Allen et al., 2002), the HER tyrosine kinase inhibitor CI1033 (Erlichman et al., 2001), experimental camptothecin analogues (Maliepaard et al., 2001b; Perego et al., 2001) and estrogens such as estrone and 17β-estradiol (Imai et al., 2002).

We have previously reported that our new series of dihydropyridines and pyridines were synthesized based on the structural modification of dexniguldipine (Zhou et al., 2005). Most compounds from this series have been shown to be potent P-glycoprotein inhibitors, with little or no calcium channel blocking activity (Zhou et al., 2005). Moderate effects on MRP1-mediated drug efflux were also observed (unpublished data). In the present study, we have demonstrated that these compounds can effectively increase the intracellular accumulation of mitoxantrone in BCRP-overexpressing...
mitoxantrone resistant cell lines. To the best of our knowledge, effects of dihydropyridines on BCRP have not been previously reported. Four commercially available dihydropyridines nicardipine, niguldipine, nifedipine and nitrendipine were also evaluated for their effects on BCRP-mediated drug transport. All compounds, except nifedipine, demonstrated a BCRP modulation effect by enhancing intracellular mitoxantrone accumulation and sensitizing resistant cells to mitoxantrone cytotoxicity in a concentration-dependent manner. In the mitoxantrone accumulation studies in H460/MX20 cells, compound IIb exhibited more than a 4-fold increase of drug accumulation in BCRP-expressing cells at the concentration of 2.5 µM. Compound Im also showed a nearly 4-fold enhancement of mitoxantrone accumulation at the same concentration. These increases are larger than that observed with the positive control, 10 µM fumitremorgin C. Compounds Im and IIb were selected for the mitoxantrone cytotoxicity study in MCF7/MX100 cells, and they were able to substantially reduce the IC₅₀ values of mitoxantrone in a concentration-dependent manner. Since H460/MX20 and MCF-7/MX100 cells do not express P-glycoprotein or MRP1, and the dihydropyridines/pyridines do not affect transport of mitoxantrone in the parental cell line, our results suggest that the effects of the dihydropyridines/pyridines are mediated through BCRP.

Our in vivo studies with topotecan confirmed the results of the in vitro studies. Topotecan is a BCRP substrate that also demonstrates moderate to weak affinity for P-glycoprotein. Moderate to large inter-patient variability was noted in topotecan pharmacokinetics and 30% to 44% oral bioavailability was reported in clinical studies (Schellens et al., 1996; Zamboni et al., 1999). The absolute bioavailability of topotecan in
rats was approximately 29.7% (Zhang et al., 2004a). In humans, renal clearance accounts for approximately 40% of the clearance of topotecan (Herben et al., 1996). GF120918 is a potent inhibitor both for P-glycoprotein (den Ouden et al., 1996) and BCRP in humans and mice (Allen et al., 1999; de Bruin et al., 1999; Maliepaard et al., 2001b). The preclinical study by Jonker et al. demonstrated that treatment with GF120918 increased the systemic exposure of oral topotecan approximately 7-fold in mdr1a/1b(-/-) P-glycoprotein knock-out mice and 10-fold in wild-type mice (Jonker et al., 2000), suggesting the importance of BCRP in topotecan disposition. Treatment with oral GF120918 in combination with intravenous topotecan in mdr1a/1b (-/-) mice decreased the plasma clearance and hepatobiliary excretion of topotecan (Jonker et al., 2000). Recently, Zhang et al. demonstrated for the first time that GF120918 could significantly increase the AUC and bioavailability of topotecan in Sprague-Dawley rats by more than 4-fold after oral co-administration (Zhang et al., 2004a). In the study reported here, co-administration of DHP-014 (Compound Im) 20 mg/kg produced a 2-fold increase of the systemic exposure and significantly enhanced the peak concentration of oral topotecan in female Sprague-Dawley rats. Tanaka and co-workers demonstrated that BCRP mRNA in rats is predominantly expressed in kidney and intestine (Tanaka et al., 2004). We speculate that the observed increase of the systemic exposure levels of topotecan is due to inhibition of BCRP in rat small intestine, increasing the amount of topotecan entering the circulation. Inhibition of topotecan metabolism by DHP-014 is unlikely to contribute significantly to the increased systemic availability of topotecan because topotecan metabolism in rat liver is low (Platzer et al., 1998).
P-glycoprotein inhibition by DHP-014 will also contribute to the enhanced topotecan systemic exposure observed in this investigation. P-glycoprotein is expressed in the apical membrane of intestinal epithelial cells and associated with a decreased bioavailability for several drugs (Lin and Yamazaki, 2003). DHP-014 is a potent P-glycoprotein inhibitor, capable of substantially enhancing the intracellular accumulation of vinblastine in P-glycoprotein-overexpressing human breast cancer MCF-7/adr cells (Zhou et al., 2005). However, topotecan is a moderate to weak P-glycoprotein substrate (Chen et al., 1991); therefore the effect of P-glycoprotein inhibition on the bioavailability of topotecan is likely to be less important than that of BCRP inhibition.

The mechanism underlying the interaction of dihydropyridines and pyridines with BCRP has not been elucidated. It has been reported that the dihydropyridine derivative denuiguldipine can inhibit P-glycoprotein-mediated drug extrusion through a direct interaction with P-glycoprotein (Borchers et al., 2002). Dihydropyridines and pyridines may act on BCRP in a similar manner because these compounds have a planar, multiring structure, like mitoxantrone; thus they may compete with mitoxantrone for binding sites on BCRP. Pascaud et al. characterized the modulation of P-glycoprotein ATPase activity by five dihydropyridines: nicardipine, nimodipine, nitrendipine, nifedipine and azidopine (Pascaud et al., 1998). They reported that P-glycoprotein ATPase was activated by approximately 2-fold in the presence of 3 – 4 µM nicardipine. Nifedipine had no effect on P-glycoprotein ATPase. In the present study, nicardipine was the most potent BCRP inhibitor, among the four commercially available dihydropyridines, while nifedipine showed no inhibitory effect on BCRP-mediated mitoxantrone efflux. It is possible that
nicardipine and other dihydropyridines may interact with BCRP by a similar mechanism, i.e., activation of BCRP ATPase activity.

The extrapolation of drug-transporter interactions from animals to humans should be done cautiously due to potential species differences in the expression and activity of these transporters. While Jonker and colleagues have demonstrated a substantial increase in AUC and a decreased plasma clearance in mdr1a/1b(-/-) knockout and wild-type mice when topotecan was administered intravenously in combination with oral GF120918 (Jonker et al., 2000), the effect was less pronounced in humans than mice (Kruijtzer et al., 2002). One possible explanation for this difference is that the expression of BCRP in kidneys is higher in rats and mice than humans (Tanaka et al., 2004), although other possibilities such as differences in topotecan/GF120918 affinity for human versus murine BCRP may also be important.

In summary, the present study indicates that most of the dihydropyridines and pyridines in our new series of compounds reverse resistance against mitoxantrone in MCF7/MX100 and H460/MX20 cell lines. All these compounds were synthesized based on the structure optimization of dexniguldipine in order to maximize P-glycoprotein binding affinity and decrease calcium channel binding activity. Therefore, these dihydropyridines/pyridines may be promising agents for clinical application due to their potent inhibition of both BCRP and P-glycoprotein. The commercially available dihydropyridines, nicardipine, nigudipine and nitrendipine were also demonstrated to be potent BCRP inhibitors; nifedipine, on the other hand, did not show any significant effect on BCRP function. This study represents the first report that dihydropyridines and pyridines are potent inhibitors of BCRP.
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Footnotes

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FIGURE LEGENDS

Figure 1. Chemical Structures of Dihydropyridines and Pyridines

Figure 2. Western Blotting of BCRP Expression. The cellular expression of BCRP was determined as described in Materials and Methods. MCF-7/sensitive and H460/sensitive cell lysates were used as negative controls for BCRP detection. The protein loading for each lane was 30 µg.

Lane 1: H460/sensitive cells
Lane 2: H460/MX20 cells
Lane 3: MCF7/sensitive cells
Lane 4: MCF7/MX100 cells.

Figure 3. Mitoxantrone Accumulation in H460/MX20 Cells. The 30-min accumulation of mitoxantrone in H460/MX20 cells in the presence of 2.5 µM of the newly synthesized compounds or the vehicle (0.1% DMSO) was determined using flow cytometry, as described in Materials and Methods. Fumitremorgin C (FTC, 10 µM) was included as a positive control. The absolute value for the mean mitoxantrone fluorescence intensity in the vehicle (0.1%DMSO)-treated H460/MX20 cells (control) was 5.2 ± 0.6 (n = 9)

A: Synthesized Dihydropyridines (Ia – Io)
B: Synthesized Pyridines (IIa – IIo)

* p < 0.05, ** p < 0.001 (n = 9 – 12) compared with the vehicle control.

Figure 4. Mitoxantrone Accumulation in MCF-7/MX100 Cells. The 30-min accumulation of mitoxantrone in MCF-7/MX100 cells cells in the presence of 2.5 µM of the newly synthesized compounds or the vehicle (0.1% DMSO) was determined using flow cytometry, as described in Materials and Methods. The absolute value for the mean
mitoxantrone fluorescence intensity in the vehicle (0.1% DMSO)-treated MCF-7/MX100 cells (control) was 4.1 ± 0.4 (n = 9)

A: Synthesized Dihydropyridines (Iₐ – Iₒ)

B: Synthesized Pyridines (IIₐ – IIₒ)

FTC: fumitremorgin C

* p < 0.05, ** p < 0.001 (n = 9 – 12) compared with the vehicle control.

Figure 5. Correlation of Mitoxantrone Accumulation in MCF-7/MX100 and H460/MX20 Cells after Treatment with Modulators (Iₐ – IIₒ), r² = 0.71, p < 0.01. The accumulation of mitoxantrone in both MCF-7/MX100 cells and H460/MX20 cells in the presence of 2.5 µM of the modulators (Iₐ – IIₒ) was determined as described in *Materials and Methods*. Regression analysis was performed using the mean values (n = 9 - 12) of mitoxantrone accumulation in MCF-7/MX100 cells and those in H460/MX20 cells.

Figure 6. Concentration-Dependent Effects on Mitoxantrone Accumulation in H460/MX20 ( ) cells and MCF-7/MX100 ( ) cells. The 30-min accumulation of mitoxantrone in MCF-7/MX100 cells and H460/MX20 cells in the presence of various concentrations (1 - 40 µM) of test compounds (nicardipine, nifedipine, niguldipine, nitrendipine, Iₐ and IIₒ) or the vehicle control (0.1% DMSO) was performed as described in *Materials and Methods*. Fumitremorgin C (FTC) was used as a positive control at a concentration of 10 µM. Data are expressed as mean ± S.D. (n = 9). The absolute value of mean mitoxantrone fluorescence intensity in the vehicle (0.1% DMSO)-treated H460/MX20 cells and MCF-7/MX100 cells (controls) were 5.9 ± 0.6 (n = 9) and 4.3 ± 0.5 (n = 9), respectively.
* p < 0.05, *** p < 0.001 compared with the vehicle control.

Figure 7. Plasma Concentration-Time Profiles of Topotecan (TPT) after Oral Dosing alone or with the Coadministration of DHP-014 (Compound I_m) The plasma concentration-time profile of topotecan after an oral dose of 2 mg/kg in SD rats alone (●), in combination with 10 mg/kg DHP-014 (○) or 20 mg/kg DHP-014 (■), administered 3 minutes before topotecan, was determined by HPLC as described in Materials and Methods. Data are expressed as mean ± SD. For all three treatment groups, the animal number n = 4.
Table 1. EC<sub>50</sub> Values of Selected Dihydropyridines for Increasing Mitoxantrone Accumulation in MCF-7/MX100 and H460/MX20 Cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>MCF-7/MX100</th>
<th>H460/MX20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicardipine</td>
<td>6.97 ± 1.25</td>
<td>7.81 ± 0.96</td>
<td></td>
</tr>
<tr>
<td>Niguldipine</td>
<td>9.82 ± 1.39</td>
<td>6.16 ± 1.16</td>
<td></td>
</tr>
<tr>
<td>Nitrendipine</td>
<td>22.1 ± 10.2</td>
<td>21.1 ± 10.5</td>
<td></td>
</tr>
<tr>
<td>I&lt;sub&gt;m&lt;/sub&gt;</td>
<td>3.71 ± 0.85</td>
<td>4.28 ± 0.61</td>
<td></td>
</tr>
<tr>
<td>I&lt;sub&gt;l&lt;/sub&gt;</td>
<td>3.80 ± 0.64</td>
<td>3.56 ± 0.86</td>
<td></td>
</tr>
</tbody>
</table>

The EC<sub>50</sub> values of compounds for increasing mitoxantrone accumulation in MCF-7/MX100 and H460/MX20 cells were obtained by fitting the fraction of maximal increase to a Hill equation using the computer program WinNonlin (Pharsight, Mountain View, CA), as described in Material and Methods. The data are expressed as mean ± SD from three independent experiments.
Table 2. IC$_{50}$ (µM) Values of Mitoxantrone Cytotoxicity in Resistant MCF-7/MX100 Cells in the Absence or Presence of Various Concentrations of Modulators

<table>
<thead>
<tr>
<th></th>
<th>40 µM</th>
<th>20 µM</th>
<th>10 µM</th>
<th>5 µM</th>
<th>2.5 µM</th>
<th>1.0 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicardipine</td>
<td>6.29±0.35</td>
<td>5.68±0.80</td>
<td>10.07±1.16</td>
<td>28.7±3.66</td>
<td>80.1±7.4</td>
<td>166.9±19.0*</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>143.1±17.8*</td>
<td>147±22.2*</td>
<td>159.6±7.1*</td>
<td>174.2±19.4*</td>
<td>153.1±32.1*</td>
<td>--</td>
</tr>
<tr>
<td>Niguldipine</td>
<td>6.23±1.20</td>
<td>14.6±4.32</td>
<td>43.0±6.56</td>
<td>54.7±5.6</td>
<td>58.6±16.5</td>
<td>149.3±36.1*</td>
</tr>
<tr>
<td>Nitrendipine</td>
<td>5.9±0.69</td>
<td>8.66±1.29</td>
<td>28.5±11.0</td>
<td>68.1±10.5</td>
<td>132.1±24.3*</td>
<td>162.7±24.4*</td>
</tr>
<tr>
<td>Compound I$_m$</td>
<td>4.65±0.39</td>
<td>4.61±0.99</td>
<td>7.82±2.00</td>
<td>11.7±2.1</td>
<td>18.8±3.49</td>
<td>24.4±4.34</td>
</tr>
<tr>
<td>Compound I$_b$</td>
<td>4.79±0.20</td>
<td>5.56±0.12</td>
<td>6.03±1.38</td>
<td>6.47±1.55</td>
<td>11.5±2.13</td>
<td>32.8±15.2</td>
</tr>
<tr>
<td>Control</td>
<td>142.5±19.9</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

The cytotoxicity of mitoxantrone in resistant MCF-7/MX100 cells was determined in the presence of specified concentrations of test compounds (nicardipine, nifedipine, niguldipine, nitrendipine, I$_m$ and I$_b$) or the vehicle (0.1% DMSO, control) as described in Materials and Methods. Data are the mean ± S.D. from three independent quadruplicate experiments.

* p > 0.05, indicating no statistically significant difference compared to the control.
Table 3. Pharmacokinetic Parameters of Topotecan in Rat Plasma after Oral Administration Alone (2 mg/kg) or in Combination with DHP-014

<table>
<thead>
<tr>
<th></th>
<th>Topotecan with DHP-014</th>
<th>Topotecan with DHP-014</th>
<th>Topotecan without DHP-014</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 mg/kg</td>
<td>10 mg/kg</td>
<td></td>
</tr>
<tr>
<td>AUC (ng*min/ml)</td>
<td>48,619±14,908 *</td>
<td>25,995±6,316</td>
<td>16,642±3,794</td>
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<tr>
<td>Cmax (ng/ml)</td>
<td>160.4±34.5 *</td>
<td>93.3±10.3</td>
<td>78.3±5.0</td>
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<tr>
<td>t1/2 (min)</td>
<td>130±4.8</td>
<td>139±3.5</td>
<td>137±5.5</td>
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<tr>
<td>Clearance/F (ml/min/kg)</td>
<td>41±12.6 *</td>
<td>76.5±20.6</td>
<td>120±27.3</td>
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</tbody>
</table>

The plasma concentration-time profile of topotecan after an oral dose of 2 mg/kg in SD rats alone or in combination with DHP-014 was determined as described in Materials and Methods. The pharmacokinetic parameters were obtained by noncompartmental analysis using WinNonlin. Data are expressed as mean ± SD. For all three treatment groups, the animal number \( n = 4 \). * \( p < 0.05 \) compared with the treatment group that was given topotecan 2 mg/kg alone.
Fig 1

<table>
<thead>
<tr>
<th>Series I Compound</th>
<th>R</th>
<th>Series II Compound</th>
<th>R</th>
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<tbody>
<tr>
<td>$I_a$</td>
<td>3-NO₂</td>
<td>$I_a$</td>
<td>3-NO₂</td>
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<td>$I_b$</td>
<td>4-OMe</td>
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<td>4-OMe</td>
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<td>$I_c$</td>
<td>4-OEt</td>
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<td>$I_d$</td>
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<td>4-OMe, 3-OMe</td>
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<tr>
<td>$I_n$</td>
<td>3,4-OCH₂CH₂O</td>
<td>$I_n$</td>
<td>3,4-OCH₂CH₂O</td>
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<tr>
<td>$I_o$</td>
<td>3,4-OCH₂O⁻</td>
<td>$I_o$</td>
<td>3,4-OCH₂O⁻</td>
</tr>
</tbody>
</table>

----------Dihydropyridines----------  ----------Pyridines----------
Fig. 3B

[Bar chart showing the percentage control for different compounds.]

% Control

Compounds

control FTC Ila IIb IIC Ild Ile IIIf Ili IIIm IIn Ilo
Mitoxantrone Accumulation (% of Control)

- **Control**
- **1 μM**
- **2.5 μM**
- **5 μM**
- **10 μM**
- **20 μM**
- **40 μM**
- **FTC**

Nicardipine
Fig. 6B

Mitoxantrone Accumulation (% of Control)

Nifedipine

control  1 µM  2.5 µM  5 µM  10 µM  20 µM  40 µM  FTC

***  ***  *
Fig. 6C

Mitoxantrone Accumulation (% of Control)

Niguldipine

- control
- 1 uM
- 2.5 uM
- 5 uM
- 10 uM
- 20 uM
- 40 uM
- FTC

* *** ****
Fig. 6D

Nitrendipine

Mitoxantrone Accumulation (% of Control)

control  1 uM  2.5 uM  5 uM  10 uM  20 uM  40 uM  FTC

***  ***  ***  ***  ***  ***  ***  ***
Fig. 6E

The graph shows the effect of various concentrations of Compound 1m on Mitoxantrone Accumulation (% of Control). The x-axis represents the different concentrations (control, 1 uM, 2.5 uM, 5 uM, 10 uM, 20 uM, 40 uM, FTC) and the y-axis represents the Mitoxantrone Accumulation (% of Control). The bars are labeled with asterisks indicating statistical significance: ***, ***, and ***. The graph includes error bars to indicate variability.
**Fig. 7**

Plasma topotecan (TPT) concentration (ng/ml) over time (min) with different treatments:
- TPT 2mg/kg (p.o.)
- TPT 2mg/kg (p.o.) + DHP-014 10mg/kg (i.p.)
- TPT 2mg/kg (p.o.) + DHP-014 20mg/kg (i.p.)

The graph shows the concentration of plasma topotecan over time for different treatment conditions, with error bars indicating variability.