

Overexpression of the ATP-binding Cassette Half-Transporter, ABCG2 (MXR/BCRP/ABCP1), in Flavopiridol-resistant Human Breast Cancer Cells¹

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ABSTRACT

We sought to characterize the interactions of flavopiridol with members of the ATP-binding cassette (ABC) transporter family. Cells overexpressing multidrug resistance-1 (MDR-1) and multidrug resistance-associated protein (MRP) did not exhibit appreciable flavopiridol resistance, whereas cell lines overexpressing the ABC half-transporter, ABCG2 (MXR/BCRP/ABCP1), were found to be resistant to flavopiridol. Flavopiridol at a concentration of 10 μM was able to prevent MRP-mediated calcein efflux, whereas Pgp-mediated transport of rhodamine 123 was unaffected at flavopiridol concentrations of up to 100 μM . To determine putative mechanisms of resistance to flavopiridol, we exposed the human breast cancer cell line MCF-7 to incrementally increasing concentrations of flavopiridol. The resulting resistant subline, MCF-7 FLV1000, is maintained in 1000 nM flavopiridol and was found to be 24-fold resistant to flavopiridol, as well as highly cross-resistant to mitoxantrone (675-fold), topotecan (423-fold), and SN-38 (950-fold), the active metabolite of irinotecan. Because this cross-resistance pattern is consistent with that reported for ABCG2-overexpressing cells, cytotoxicity studies were repeated in the presence of 5 μM of the ABCG2 inhibitor fumitremorgin C

(FTC), and sensitivity of MCF-7 FLV1000 cells to flavopiridol, mitoxantrone, SN-38, and topotecan was restored. Mitoxantrone efflux studies were performed, and high levels of FTC-reversible mitoxantrone efflux were found. Northern blot and PCR analysis revealed overexpression of the ABCG2 gene. Western blot confirmed overexpression of ABCG2; neither P-glycoprotein nor MRP overexpression was detected. These results suggest that ABCG2 plays a role in resistance to flavopiridol.

INTRODUCTION

Flavopiridol (HMR 1275, L86-8275), an *N*-methylpiperidinyl, chlorophenyl flavone, is the first cdk³ inhibitor used in human clinical trials (1). Flavopiridol can cause both a G₁ and G₂-M cell cycle arrest, which is thought to be attributable to three biochemical effects: (a) it inhibits cdk1, cdk2, cdk4, and cdk7 in a competitive manner with respect to ATP; (b) it inhibits the activity of the cdk7/cyclin H complex, thus preventing necessary activating phosphorylation of cdk5; and (c) it down-regulates cyclin D1 and cyclin D3, which are necessary for cdk4 and cdk6 activation (2, 3). Flavopiridol has also been shown to induce apoptosis in a number of cell lines (4–9).

As the clinical development of flavopiridol is pursued, it becomes important to evaluate mechanisms of cellular resistance. Although a number of resistance mechanisms are possible, we focused on the ABC family of transporter proteins that may mediate efflux of anticancer agents, thereby reducing intracellular drug concentrations. Pgp and MRP are two of the most extensively studied ABC transporters and are known to confer resistance to a wide variety of structurally unrelated cytotoxic agents (10, 11). The newly described mitoxantrone resistance protein, MXR or ABCG2, is an ABC half-transporter that is thought to dimerize to function and has been shown to confer resistance to mitoxantrone, anthracyclines, and to the camptothecins topotecan and SN-38 (12–16). Except for minor sequence differences, MXR is identical to BCRP reported by Doyle *et al.* (13) and to the placental ABC protein (ABCP1), which was reported by Allikmets *et al.* (17) and is expressed in high levels in the placenta. The Human Gene Nomenclature Committee has suggested that MXR/BCRP/ABCP1 be renamed ABCG2,⁴ and such terminology will be used hereafter.

Recent studies have shown that flavopiridol is able to inhibit MRP-mediated transport and increase MRP-related

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³ The abbreviations used are: cdk, cyclin-dependent kinase; ABC, ATP-binding cassette; Pgp, P-glycoprotein; MRP, multidrug resistance-associated protein; BCRP, breast cancer resistance protein; ABCP1, placental ABC protein 1; FTC, fumitremorgin C; 5-FU, 5-fluorouracil.

⁴ Internet address: <http://www.gene.ucl.ac.uk/users/hester/abc.html>.

ATPase activity in membrane vesicles and MRP-overexpressing cells; however, little cross-resistance to flavopiridol attributable to this mechanism was observed (18, 19). Moreover, bladder cancer cells overexpressing Pgp were not found to have increased flavopiridol resistance (20). A flavopiridol-resistant ovarian carcinoma cell line has also recently been described that spontaneously developed resistance to flavopiridol as well as cisplatin after prolonged passage in tissue culture, although no transporter was implicated as a cause for the resistance (21). Schlegel *et al.* (22) also recently reported in abstract form that cells which overexpress ABCG2 were resistant to flavopiridol, and that the dipyrindimol analogue, BIB-E, was able to reverse this resistance.

In the present study, we sought to characterize the interactions of flavopiridol with these three members of the ABC-transporter family. The ability of flavopiridol to block MRP-mediated transport was confirmed, and it was demonstrated that the novel ABC half-transporter, ABCG2, is able to confer resistance to flavopiridol. We also describe a flavopiridol-resistant human breast cancer subline, MCF-7 FLV1000, which is maintained in 1000 nM flavopiridol. This subline was shown to overexpress the ABCG2 gene, thus implicating ABCG2 as a mechanism of resistance for flavopiridol.

MATERIALS AND METHODS

Materials. Flavopiridol (HMR 1275, L86-8275) was supplied by the National Cancer Institute Drug Screen. FTC was synthesized by Thomas McCloud, Developmental Therapeutics Program, Natural Products Extraction Laboratory, NIH (Bethesda, MD). Rhodamine 123 and mitoxantrone were obtained from Sigma Chemical Co. (St. Louis, MO). Calcein AM was purchased from Molecular Probes (Eugene, OR).

Cell Lines. The MCF-7 FLV25, FLV100, FLV250, FLV500, and FLV1000 cells were selected by exposing MCF-7 cells to increasing concentrations of flavopiridol. The Pgp-overexpressing SW620 Ad300 cell line was derived from SW620 colon cancer cells and is maintained in 300 ng/ml Adriamycin (23). The human colon carcinoma cell line S1 and its ABCG2-overexpressing subline S1-M1-3.2 were obtained from Dr. Lee M. Greenberger (Wyeth-Ayerst; Ref. 24). The S1-M1-80 subline was generated by exposing the S1-M1-3.2 subline to increasing concentrations of mitoxantrone (12). The S1-M1-3.2 and S1-M1-80 subline were maintained in 3.2 and 80 μ M of mitoxantrone, respectively. The Pgp-overexpressing subline S1-B1-20, which is maintained in 20 μ M bisantrene, was also obtained from Dr. Greenberger (25). The ABCG2-overexpressing MCF-7 subline MCF-7 AdVp3000 was maintained in 3000 ng/ml Adriamycin and 5 μ g/ml verapamil (26). The MRP-overexpressing subline MCF-7/VP was provided by Dr. Kenneth Cowan (University of Nebraska Medical Center, Omaha, NE) and is maintained in 4 μ M etoposide (27). The ABCG2-transfected cell line, MCF-7/BCRP, and the empty vector-transfected cell line, MCF-7/pcDNA3, were kindly provided by Dr. Douglas Ross and have been described previously (13). The MCF-7 and derivative sublines were cultured in Iscove's modified Eagle's medium, and the SW620 and S1 cells and derivative lines were cultured in RPMI 1640. Both were supplemented with 2 mM L-glutamine, 100 units/ml penicillin,

and 100 μ g/ml streptomycin. All cells were maintained in the selecting drug at the indicated concentrations at 37°C in 5% CO₂. Cells were placed in drug-free medium 7–14 days prior to assay.

Cytotoxicity Assays. Assays were performed in 96-well plates using the colorimetric method described by Skehan *et al.* (28). Cells were seeded in 96-well plates at a density of 2000 cells/well and allowed to attach overnight. Subsequently, drug was added at the desired concentrations, and the cells were incubated 4 days at 37°C, fixed in 50% trichloroacetic acid, then stained in 0.4% sulforhodamine B dissolved in 1% acetic acid. After washing, bound dye was solubilized with 10 mM unbuffered Tris base (pH 10.5). Cell density was determined by measuring the absorbance at 570 nm.

Efflux Assays. The rhodamine 123, calcein, and mitoxantrone efflux assays were performed as described previously (29, 30). Briefly, a suspension of log-phase cells was obtained by trypsinization. Cells were incubated in 0.5 μ g/ml rhodamine 123, 0.5 μ M calcein AM, or 20 μ M mitoxantrone with or without FTC, flavopiridol, probenecid, or PSC 833 at the desired concentration in complete medium (phenol red-free Iscove's modified Eagle's medium with 10% FCS) at 37°C in 5% CO₂ for 30 min. The cells were washed once with cold complete medium and then resuspended in complete medium with or without FTC, flavopiridol, probenecid, or PSC 833 for a 60-min efflux period at 37°C. Cells were then washed in ice-cold Dulbecco's PBS, placed in Dulbecco's PBS at 4°C, and kept in the dark until flow cytometric analysis. A FACSort flow cytometer with a 488-nm argon laser and 530 nm bandpass filter was used to read the fluorescence of rhodamine and calcein, whereas a FACSCalibur flow cytometer equipped with a 635-nm red diode laser and 670-nm bandpass filter was used to read the fluorescence of mitoxantrone. At least 10,000 events were collected. Debris was eliminated by gating on forward *versus* side scatter, and dead cells were excluded based on propidium iodide staining.

Northern Blot and PCR Analysis. RNA was prepared using RNA STAT-60 according to the manufacturer's instructions (Tel-Test, Inc., Friendswood, TX). Northern blotting was performed as described previously (12). Quantitative PCR analysis was performed as described previously (31) using the following primers: MDR1 5' primer, 5'-GCC TGG CAG CTG GAA GAC AAA TAC ACA AAA TT-3'; MDR1 3' primer, 5'-CAG ACA GCA GCT GAC AGT CCA AGA ACA GGA CT-3'; MRP1 5' primer, 5'-CGG AAA CCA TCC ACG ACC CTA ATC C-3'; MRP1 3' primer, 5'-ACC TCC TCA TTC GCA TCC ACC TTG G-3'; ABCG2 3' primer, 5'-TGC CCA GGA CTC AAT GCA ACA G-3'; and ABCG2 5' primer, 5'-GAC TGA AGG GCT ACT AAC C-3'.

Western Blot Analysis. Microsomal membrane fractions were prepared by nitrogen cavitation, subjected to electrophoresis on a premade 8% SDS-polyacrylamide gel, and electrotransferred onto polyvinylidene difluoride membranes. The blots were blocked with 5% nonfat dry milk and probed with a polyclonal anti-ABCG2 antibody, 87405, which was raised against a region of the ATP-binding site of ABCG2, a polyclonal anti-Pgp antibody (Calbiochem, San Diego, CA), or a monoclonal anti-MRP antibody, MRPm5 (Kamiya Biomedical, Seattle, WA). The blots were then incubated with a secondary antirabbit or antimouse horseradish peroxidase-conjugated anti-

Table 1 Flavopiridol resistance in Pgp-, MRP-, and ABCG2-overexpressing cell lines

Cell line	Resistance mechanism	IC ₅₀ ^a (nM)	RR ^b
SW620		96.7 ± 6.33	
SW620 Ad300	Pgp	142 ± 3.21	1.5
S1		74.4 ± 5.88	
S1-B1-20	Pgp	64.7 ± 6.77	0.87
S1-M1-80	ABCG2	460 ± 23.1	6.2
MCF-7		87.2 ± 13.3	
MCF-7/VP	MRP	97.3 ± 12.6	1.1
MCF-7 AdVp3000	ABCG2	3100 ± 298	35.6

^a IC₅₀ values were determined by the sulforhodamine B assay.

^b Relative resistance values were determined by dividing the IC₅₀ for the resistant cell line by the IC₅₀ for the parental cell line. Experiments were performed at least three times.

body (Amersham Pharmacia Biotech, Piscataway, NJ), followed by enhanced chemiluminescence detection (DuPont NEN, Boston, MA) and subsequent exposure to Kodak X-OMAT AR film.

Fluorescence Immunohistochemistry. Cells were grown on eight-well chamberslides (Nunc, Roskilde, Denmark) for 3 days before analysis. The samples were fixed in methanol: ethanol (1:1) for 1 min and then washed in PBS with 2% human type AB serum (Sigma) three times for 10 min. The primary antibody, anti-ABCG2 polyclonal rabbit, 87405, was added at a 1:3000 dilution and incubated for 2 h at room temperature. After washing three times for 10 min with PBS with 2% human AB serum, the secondary antibody, FITC-conjugated pig antirabbit (DAKO, Glostrup, Denmark) at a dilution of 1:50 was added for 1 h. The samples were washed four times for 10 min in PBS, and a coverslip was mounted with a drop of Antifade (Molecular Probes, Leiden, Denmark). Antibody binding was detected by confocal microscopy with a Zeiss LSM410, exciting FITC at 488 nm (Ar-Kr laser), and measuring emission through a 515–540 nm bandpass filter.

RESULTS

Cross-Resistance to Flavopiridol in Cell Lines with Pgp-, MRP-, and ABCG2-mediated Resistance. The results of 4-day cytotoxicity assays performed with flavopiridol are summarized in Table 1. The MCF-7 AdVp3000 and S1-M1-80 cells, which express the newly identified ABC half-transporter, ABCG2, were approximately 36- and 6-fold resistant to flavopiridol, respectively. Little cross-resistance was observed in the two Pgp-overexpressing lines, S1-B1-20 or SW620 Ad300 (0.87- and 1.5-fold, respectively), or in the MRP-overexpressing MCF-7/VP cells (1.1-fold).

Antagonism of MRP- or Pgp-mediated Transport by Flavopiridol. The intracellular fluorescence of calcein or rhodamine was measured by flow cytometry as an assay for MRP- or Pgp-mediated transport, respectively. We confirmed previous reports that flavopiridol is able to antagonize MRP-mediated transport by examining the effect of flavopiridol on MRP-mediated calcein efflux (19). Fig. 1A shows 10 μM flavopiridol (*dashed line*) was almost as effective as 5 mM probenecid (*dotted line*) in preventing MRP-mediated calcein efflux (*solid*

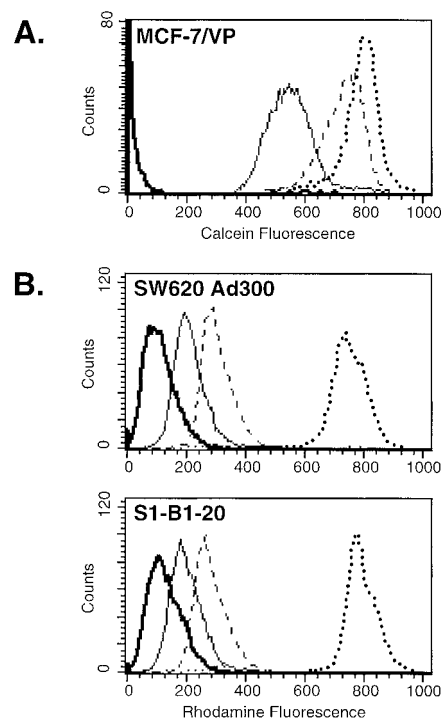


Fig. 1 Flavopiridol antagonizes MRP-mediated calcein efflux but not Pgp-mediated rhodamine efflux. Autofluorescence of cells incubated with media alone is shown as a heavy solid line. A, calcein efflux in MRP-overexpressing MCF-7/VP cells. Solid line, cells incubated for 30 min with medium containing 0.5 μM calcein AM alone, washed, then incubated 60 min with calcein-free complete medium. Dashed line, cells incubated for 30 min with complete medium containing 0.5 μM calcein AM with 10 μM flavopiridol, washed, then incubated 60 min with calcein AM-free medium with 10 μM flavopiridol. Dotted line, cells incubated for 30 min with complete medium containing 0.5 μM calcein AM with 5 mM probenecid, washed, then incubated 60 min with calcein-free complete medium with 5 mM probenecid. B, rhodamine efflux in SW620 Ad300 and S1-B1-20 cells. Solid line, cells incubated for 30 min with medium containing 0.5 μg/ml rhodamine 123 alone, washed, then incubated 60 min with rhodamine-free complete medium. Dashed line, cells incubated 30 min with complete medium containing 0.5 μg/ml rhodamine with 100 μM flavopiridol, washed, then incubated 60 min in rhodamine-free medium with 100 μM flavopiridol. Dotted line, cells incubated 30 min with medium containing 0.5 μg/ml rhodamine with 3 μg/ml PSC 833, washed, then incubated 60 min with rhodamine-free complete with 3 μg/ml PSC 833.

line) in the MRP-overexpressing MCF-7/VP cell line. Probenecid has been shown previously to prevent MRP-mediated transport in MRP-overexpressing cell lines (32).

Although neither of the Pgp-expressing cell lines was cross-resistant to flavopiridol, the ability of flavopiridol to inhibit Pgp-mediated rhodamine transport was evaluated. Pgp-overexpressing SW620 Ad300 and S1-B1-20 cells were incubated in rhodamine 123 in the presence of increasing concentrations of flavopiridol. At concentrations up to 100 μM, flavopiridol (*dashed line*) had little effect on Pgp-mediated transport of rhodamine (*solid line*) in either of the cell lines shown in Fig. 1B. The effect of the Pgp inhibitor PSC 833 is shown for comparison (*dotted line*). With PSC 833, intracellular accumulation of rhodamine was restored to the levels seen in parental cells in both sublines.

Table 2 Cross-resistance profile of the MCF-7 FLV1000 cell line

Drug	MCF-7 IC ₅₀ ^a (μM)	MCF-7 FLV1000 IC ₅₀ ^a (μM)	RR ^b
Flavopiridol	0.18 ± 0.04	4.3 ± 2.4	24
Topotecan	0.03 ± 0.005	12.7 ± 3.8	423
Mitoxantrone	0.004 ± 0.002	2.7 ± 0.04	675
SN-38	0.002 ± 0.001	1.9 ± 0.3	950
Taxol	0.005 ± 0.0007	0.018 ± 0.009	4
5-FU	0.83 ± .021	8.3 ± 1.5	10

^a IC₅₀ values were determined by the sulforhodamine B assay.

^b Relative resistance values were determined by dividing the IC₅₀ for the resistant cell line by the IC₅₀ for the parental cell line. Experiments were performed at least three times.

Characterization of a Flavopiridol-resistant Subline.

To explore the potential contribution of ABC transporters to flavopiridol resistance, MCF-7 human breast cancer cells were exposed to increasing concentrations of flavopiridol. The resulting sublines, MCF-7 FLV25, FLV100, FLV250, FLV500, and FLV1000, were maintained in 25, 100, 250, 500, and 1000 nM flavopiridol, respectively. The cross-resistance profile of the MCF-7 FLV1000 subline was evaluated, and the results are summarized in Table 2. The MCF-7 FLV1000 cells were found to be 24-fold resistant to flavopiridol and were highly cross-resistant to topotecan (423-fold), mitoxantrone (675-fold), and SN-38 (950-fold), the active metabolite of irinotecan, and less so to paclitaxel (3.6-fold) and 5-FU (10-fold). As the mitoxantrone and camptothecin resistance profile was similar to that of cells that overexpress the ABCG2 protein, cytotoxicity assays with and without 5 μM FTC were performed. FTC, an extract of *Aspergillus fumigatus*, is known to inhibit transport mediated by ABCG2 (24, 33). As seen in Fig. 2A, FTC was able to sensitize the cells to flavopiridol as well as to topotecan, mitoxantrone, and SN-38. FTC was not able to restore sensitivity to 5-FU or paclitaxel in the MCF-7 FLV1000 cells, suggesting that another resistance mechanism is responsible for the low levels of cross-resistance observed in these cells. In Fig. 2B, the ABCG2-transfected cell line, MCF-7/BCRP, was also found to be resistant to flavopiridol (3.75-fold, average of two independent experiments), again confirming the role of ABCG2 in flavopiridol resistance.

Mitoxantrone efflux was examined in all of the flavopiridol-resistant MCF-7 sublines by flow cytometry. In separate studies, we have demonstrated that FTC inhibition of mitoxantrone transport reflects the expression level of ABCG2 (data not shown). As Fig. 3 shows, FTC (dashed line) is able to increase intracellular mitoxantrone fluorescence, which reflects the intracellular mitoxantrone concentration, in the MCF-7 FLV250, MCF-7 FLV500, and MCF-7 FLV1000 cells. Mitoxantrone fluorescence in MCF-7 FLV1000 cells is reduced to levels comparable with those in the ABCG2-overexpressing MCF-7 AdVp3000 cells, which are shown for comparison. In early steps of selection, small decreases in mitoxantrone fluorescence were also noted, suggesting that low levels of ABCG2 may be present in these cells.

The rhodamine and calcein efflux assays were also performed on the MCF-7 FLV1000 cell line to detect the presence of Pgp- or MRP-mediated transport in these cells. No efflux of

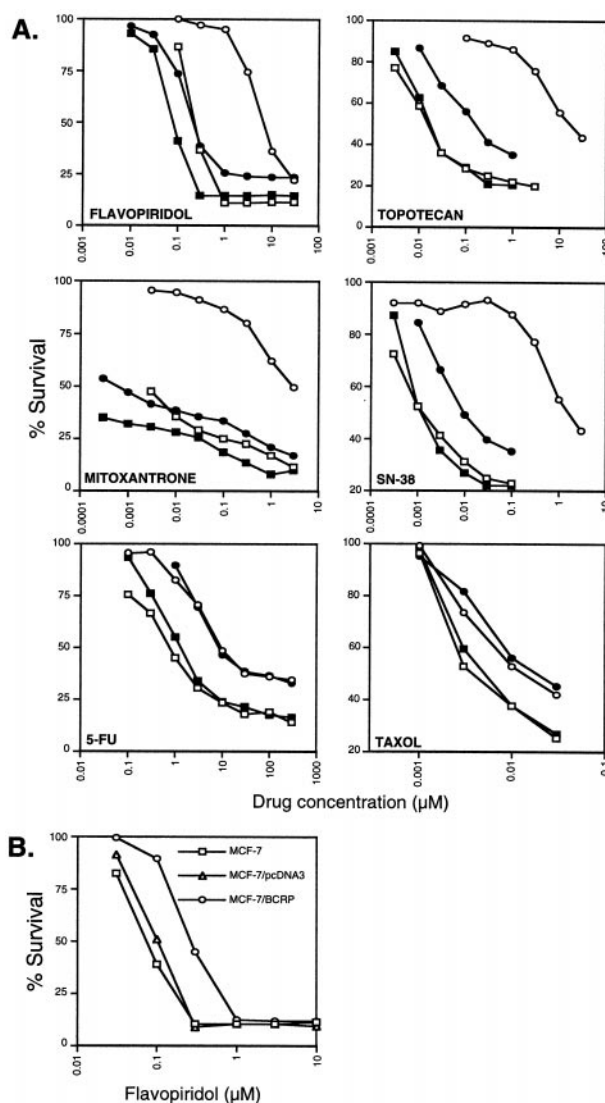


Fig. 2 Reversal of resistance by FTC. A, MCF-7 parental (□, ■) and MCF-7 FLV1000 cells (○, ●) were incubated for 4 days with flavopiridol, topotecan, mitoxantrone, SN-38, 5-FU, and paclitaxel in the presence (●, ■) or absence (□, ○) of 5 μM FTC. IC₅₀s were determined by the sulforhodamine B assay. Assays were repeated at least three times. Representative curves are shown. B, MCF-7 parent, empty vector-transfected MCF-7/pcDNA3, and ABCG2-transfected MCF-7/BCRP cells were incubated for 4 days with flavopiridol. Assays were performed twice. Representative curves are shown.

rhodamine 123 or calcein was detected in the flavopiridol-selected cell line, suggesting that neither Pgp nor MRP plays a role in flavopiridol resistance in these cells (data not shown). This result is consistent with the observation in Table 1 that neither Pgp nor MRP overexpression confers flavopiridol resistance.

Northern analysis was performed to evaluate ABCG2 expression levels in the flavopiridol-resistant sublines. Fig. 4A shows a dose-dependent increase in the expression of ABCG2 at the RNA level in MCF-7 FLV250, MCF-7 FLV500, and MCF-7 FLV1000 cells. High levels were found in the MCF-7 FLV1000

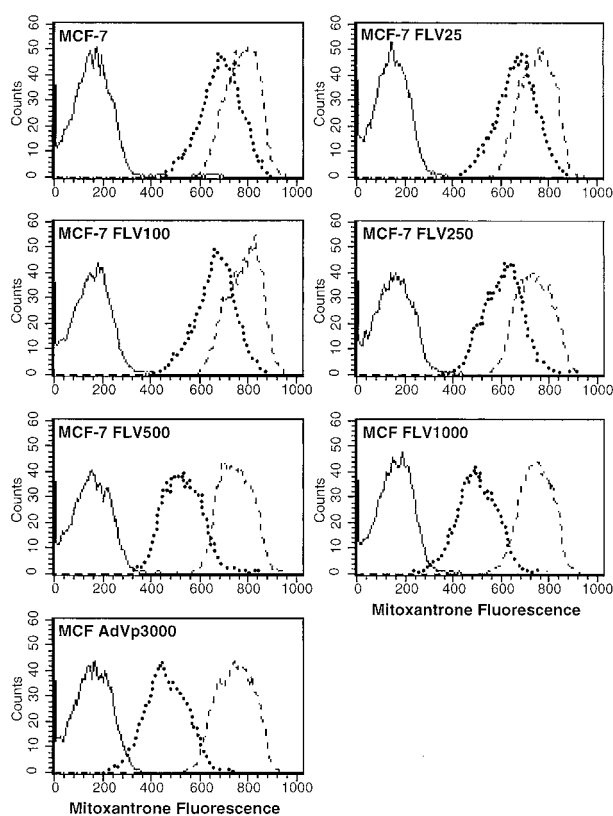


Fig. 3 Mitoxantrone efflux in flavopiridol-selected cell lines. The mitoxantrone efflux assay was performed on the MCF-7 parent, FLV25, FLV100, FLV250, FLV500, and FLV1000 cell lines. Representative histograms are shown. Autofluorescence of cells incubated with medium alone is shown as a *solid line*. Efflux histograms: cells incubated for 30 min with complete media containing 20 μM mitoxantrone alone or with 10 μM FTC, washed, then incubated 60 min with mitoxantrone-free complete medium alone (*dotted line*) or with 10 μM FTC (*dashed line*). ABCG2-overexpressing MCF-7 AdVp3000 cells are shown for comparison.

cells but were slightly lower than those in the S1-M1-80 cells, which are shown for comparison.

Immunoblot analysis also confirmed the presence of the ABCG2 protein as seen in Fig. 4B. Additionally, the cells were found not to overexpress MRP or Pgp, which correlates with results obtained from flow cytometry analysis. MCF-7 AdVp30000 cells served as a positive control for ABCG2, MCF-7/VP cells for MRP, and S1-B1-20 cells for Pgp in Fig. 4B. Immunofluorescence studies with the polyclonal antibody 87405 also showed increased ABCG2 expression in MCF-7 FLV1000 cells. Confocal images of MCF-7 parental cells incubated with 87405 show that the level of ABCG2 is below the limits of detection (Fig. 4C), whereas increased expression is detected in the MCF-7 FLV1000 cell line (Fig. 4D).

PCR analysis revealed a 48-fold increase in ABCG2 expression and a 1.3-fold increase in MRP expression in MCF-7 FLV1000 cells versus MCF-7 parental cells; MDR-1 levels were undetectable in both the parental and resistant line (data not shown).

Antagonism of ABCG2-mediated Mitoxantrone Transport by Flavopiridol.

Because flavopiridol appears to be a substrate for ABCG2, its ability to inhibit ABCG2-mediated drug efflux was assessed, because many transporter substrates are known to act as inhibitors as well (34). We incubated S1-M1-80, MCF-7 FLV1000, MCF-7 AdVp3000, and MCF-7/BCRP cells in mitoxantrone with 1, 10, and 100 μM flavopiridol. Flavopiridol at a concentration of 100 μM (*dashed line*) was indeed able to antagonize mitoxantrone transport from all four cell lines, as seen in Fig. 5. The ABCG2 blocker FTC at a concentration of 10 μM (*dotted line*) is also shown for comparison. In the ABCG2-transfected cell line MCF-7/BCRP, flavopiridol was as effective as 10 μM FTC in preventing mitoxantrone efflux, but this may be attributable to the fact that this cell line expresses a low level of ABCG2. Flavopiridol fluorescence was found to be negligible under these conditions (data not shown).

DISCUSSION

The present report describes a flavopiridol-resistant breast cancer subline, MCF-7 FLV1000, which is 24-fold resistant to flavopiridol as well as 675-fold resistant to mitoxantrone, 426-fold resistant to topotecan, and 950-fold resistant to SN-38. In cytotoxicity assays with the ABCG2 inhibitor FTC, cellular resistance to flavopiridol, mitoxantrone, topotecan, and SN-38 was abrogated, thus implicating ABCG2 as an important mechanism of resistance to flavopiridol in the MCF-7 FLV1000 cell line. Pgp and MRP do not appear to contribute to flavopiridol resistance in MCF-7 FLV1000 cells based on flow cytometry results, Western blotting, and PCR analysis.

The interactions of flavopiridol with members of the ABC-transporter family were also characterized. Pgp was not found to confer resistance to flavopiridol in Pgp-overexpressing cell lines, nor was flavopiridol able to appreciably prevent Pgp-mediated rhodamine transport in these cells. This is in agreement with Chien *et al.* (20), who also demonstrated little cross-resistance to flavopiridol in Pgp-overexpressing, drug-selected bladder cancer cells. In contrast, flavopiridol was able to prevent MRP-mediated calcein efflux from MRP-overexpressing cells, which is consistent with the findings of Hooijberg *et al.* (19), who demonstrated increased daunorubicin accumulation in MRP-overexpressing cells in the presence of flavopiridol. Our observation that ABCG2 is able to confer resistance to flavopiridol parallels that of a preliminary report by Schlegel *et al.* (22), who demonstrated increased resistance to flavopiridol in ABCG2-overexpressing cells.

Another flavopiridol-resistant subline was recently reported by Bible *et al.* (21). The ovarian carcinoma cell line, OV202 hp, spontaneously developed drug resistance after long-term culture and was found to be 5-fold resistant to flavopiridol as well as 3-fold resistant to cisplatin. Although decreased intracellular flavopiridol concentrations were noted, Bible *et al.* (21) conclude that neither Pgp nor MRP were responsible for drug transport based on the fact that no cross-resistance to known substrates was observed. They did not test for the presence of ABCG2. However, Bible *et al.* (21) did not find enhanced resistance to mitoxantrone or topotecan, two cytotoxics that are readily transported by ABCG2 (12–16). Thus, it does

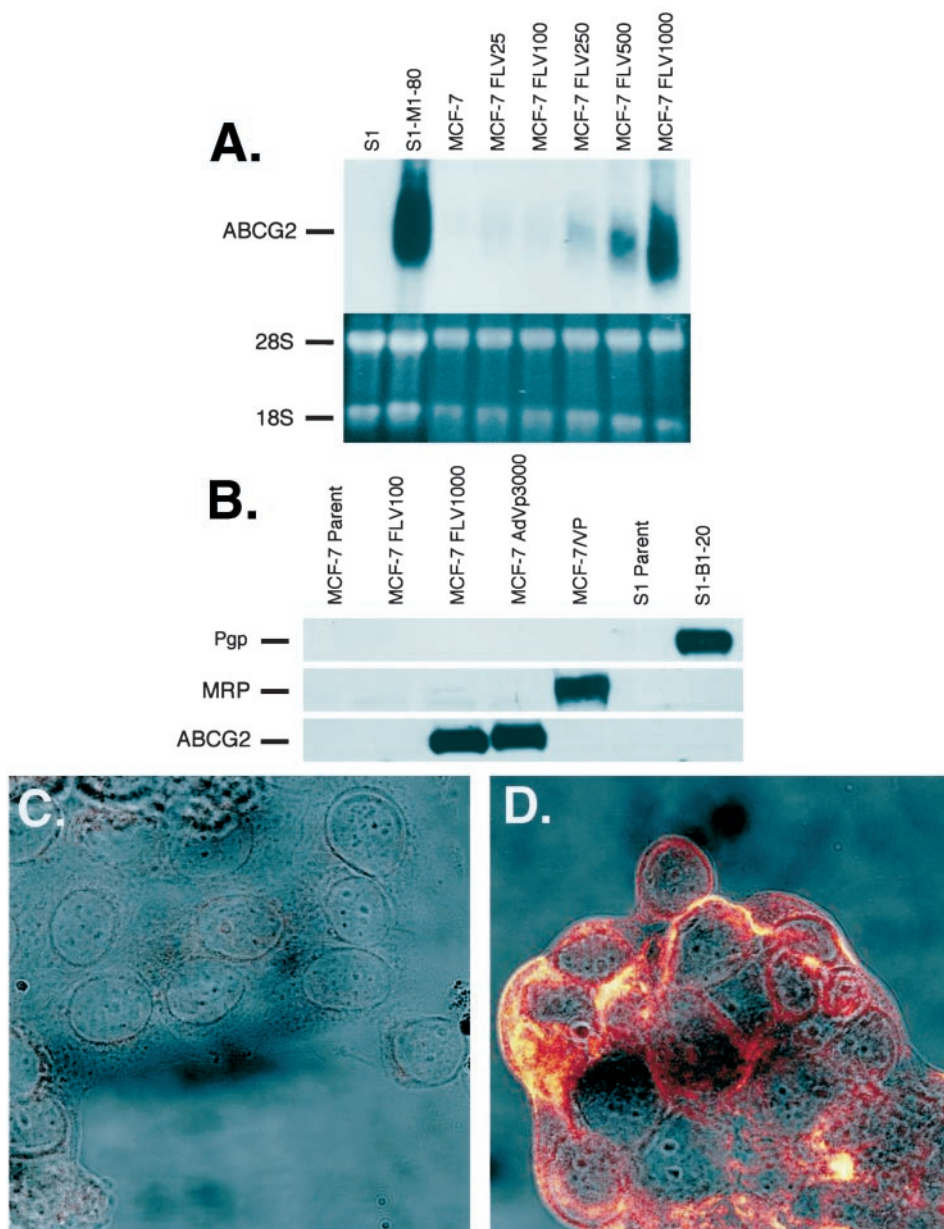


Fig. 4 ABCG2 expression in flavopiridol-resistant cells. *A*, ABCG2 expression was determined by Northern blot in MCF-7 parent, FLV25, FLV100, FLV250, FLV500, and FLV1000 cell lines. Parental S1 and ABCG2-overexpressing S1-M1-80 cells are shown as negative and positive controls, respectively. *B*, Western Blot of ABCG2, MRP, and Pgp expression in MCF-7 parental, MCF-7 FLV100, MCF-7 FLV1000, MCF-7 Advp3000, MCF-7/VP, S1 parental, and S1-B1-20 cells. Immunofluorescence micrographs using the 87405 antibody show ABCG2 expression in the MCF-7 parental cells (*C*) compared with the MCF-7 FLV1000 cells (*D*). ABCG2 localization mainly to the plasma membrane is observed.

not appear that resistance to flavopiridol is attributable to ABCG2 in the OV202 hp cell line.

Interestingly, we found the S1-M1-80 cell line to be 6-fold resistant to flavopiridol, whereas the MCF-7 AdVp3000 cell line was 36-fold resistant. This difference could be explained by the structure of the ABCG2 protein. Because it is thought to dimerize for function, it is possible that ABCG2 homodimerizes in one cell line and heterodimerizes in the other cell line, leading to differing substrate specificity, much as in the case of the *Drosophila* white protein (35). Alternatively, polymorphisms in ABCG2 could result in differing substrate affinities. A splice variant of the human *Tap2* gene, *Tap2iso*, was shown recently to encode for a protein having differing substrate specificities from the normal *Tap2* gene product (36).

Flavopiridol was also found to prevent ABCG2-mediated mitoxantrone efflux in ABCG2-overexpressing cells, albeit at a relatively high concentration. The mechanism of inhibition is unknown. This could be attributable to competitive inhibition, much like the classic paradigm for Pgp where a Pgp substrate may also function as an antagonist if used at sufficiently high concentrations (34). Alternatively, as flavopiridol does compete for the ATP-binding site of cdk2 (2), it is possible that flavopiridol is able to compete for the ATP-binding site of ABCG2.

We conclude from these studies that ABCG2 is able to confer resistance to flavopiridol. One solution that has been proposed to the problem of drug resistance is to develop agents that are not substrates for P-glycoprotein and thereby circumvent drug resistance. However, it could be argued that this

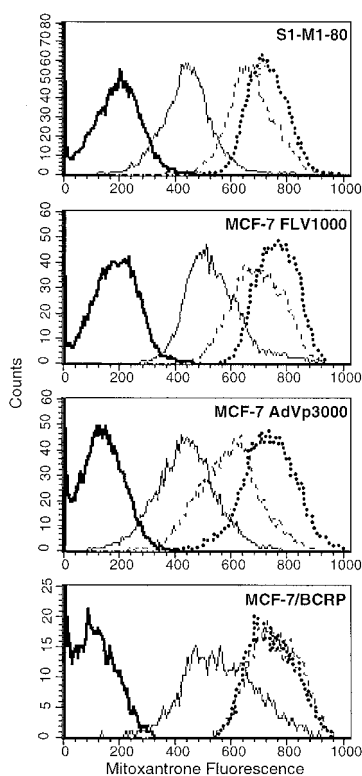


Fig. 5 Antagonism of ABCG2-mediated mitoxantrone efflux by flavopiridol. A mitoxantrone efflux assay was performed on the S1-M1-80, MCF-7 FLV1000, MCF-7 AdVp3000, and MCF-7/BCRP cell lines. Representative histograms are shown. Efflux histograms: cells incubated for 30 min with complete medium containing 20 μM mitoxantrone alone or with 100 μM flavopiridol or 10 μM FTC, washed, then incubated 60 min with mitoxantrone-free complete medium alone (solid line) or with 100 μM flavopiridol (dashed line) or with 10 μM FTC (dotted line). Autofluorescence of cells incubated with medium alone is shown as a heavy solid line.

strategy is flawed because there appears to be enough genetic diversity among transporters to suggest that most compounds may be a substrate for some transporter. As flavopiridol enters Phase II trials, it becomes important to examine which mechanisms of resistance may emerge as a result of drug treatment. With the expanding number of ABC transporters described, it will become important to identify which transporters may play a role in resistance to drugs in the clinic and developing strategies to prevent or thwart this resistance.

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