Quantitative Analysis of Breast Cancer Resistance Protein and Cellular Resistance to Flavopiridol in Acute Leukemia Patients

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ABSTRACT

Purpose: Flavopiridol is a cyclin-dependent kinase inhibitor currently undergoing human clinical trials. As clinical development is pursued, it becomes important to evaluate resistance mechanisms to flavopiridol. To elucidate the contribution of breast cancer resistance protein (BCRP) to cellular resistance to flavopiridol in acute myeloid leukemia, we studied the relationship between cellular resistance to flavopiridol and mRNA expression of BCRP or P-glycoprotein (P-gp, product of MDR1 gene) in blast cells from adult patients with acute leukemia.

Experimental Design: Twenty-one blast cell samples from 20 patients were studied. The expression of BCRP, P-gp, or β-actin mRNA was determined by real-time reverse transcription-PCR, using fluorescent hybridization probes to evaluate codon 482, a known site of mutations in BCRP mRNA. In vitro cell viability and apoptosis were examined after 24 h exposure to flavopiridol.

Results: BCRP mRNA expression varied over a 200-fold range. In the blast cell samples with BCRP mRNA expression > 10,000 copies/pg β-actin (n = 9), BCRP mRNA correlated proportionally with cell viability in the presence of 250 nM flavopiridol (r = 0.86, P = 0.003) and with apoptosis induced by flavopiridol (r = 0.71, P = 0.031). In contrast, MDR1 mRNA expression did not correlate with either flavopiridol cytotoxicity or induction of apoptosis. Melting point analysis of the hybridization probes determined that all 21 patient samples had arginine at codon 482 of BCRP mRNA, the wild-type form.

Conclusions: These results suggest that unlike P-gp, BCRP may play a role in leukemia cellular resistance to flavopiridol. No mutations at codon 482 were observed in BCRP mRNA in this group of patients.

INTRODUCTION

Drug resistance is a major obstacle to curing cancer. To improve treatment outcome, a number of studies have focused on relevant mechanisms of chemotherapeutic drug resistance in AML. Of several possible biological factors, increased expression of multidrug resistance transporters has been associated with drug resistance to cancer chemotherapy because of their ability to export drugs from cells. The first transporter to be identified and characterized, P-gp (product of MDR1 gene), is a member of the ABC family of transport proteins. P-gp has been described to be a poor prognostic factor in the treatment of AML. In contrast, the relation of expression of another multidrug resistance-associated ABC transporter, MRPI, to prognosis in acute leukemia is less clear (1–4).

BCRP was isolated from multidrug-resistant human breast cancer MCF-7/AdrVp cells (5). This transporter belongs to the G subfamily of ABC transporters (ABC1) and is also known as the placental ABC transporter, ABCP (6), or the mitoxantrone resistance gene (7). Enforced expression of BCRP in drug-sensitive MCF-7 cells results in resistance to cancer chemotherapeutic agents, including daunorubicin, mitoxantrone, and topoisomerase I inhibitors (5, 8). Because previous studies of AML indicated that the overexpression of P-gp or MRPI in patient-derived blast cells was insufficient to account for the observed frequency of a drug efflux-based resistance phenotype (9, 10), BCRP has been proposed as an independent efflux mechanism in AML (4). Recently, BCRP mRNA expression has been found in AML patient-derived blast cells at a relatively high level, suggesting that BCRP may be involved in drug resistance (10–12).

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3 The abbreviations used are: AML, acute myelogenous leukemia; BCRP, breast cancer resistance protein; MDR1, multidrug resistance gene 1; ABC, ATP-binding cassette; P-gp, P-glycoprotein, the product of MDR1; MRPI, multidrug resistance protein-1, cdk, cyclin-dependent kinase; ALL, acute lymphoblastic leukemia; MDS, myelodysplastic syndrome; Dx, diagnosis; RT-PCR, reverse transcription-PCR; cRNA, copy RNA; Tm, melting temperature; FDA, fluorescein diacetate; PI, propidium iodide.
Flavopiridol is a cdk inhibitor that recently entered clinical trials as a potential anticancer agent (13, 14). The cytotoxic activity of flavopiridol is associated with arrest of cells in G₁ or G₂ phases of the cell cycle (15). Subsequently, studies have shown flavopiridol inhibits cdk1, cdk2, cdk4, cdk7, and cdk8 (16). The antitumor activity of flavopiridol has been evaluated in a variety of cell lines (17, 18), including B-cell malignancies (19, 20). Cells treated with flavopiridol undergo apoptosis independent of p53 status (21). Recent studies in malignant B-cell populations suggest that this apoptotic activity is mediated by down-regulation of multiple antiapoptosis proteins (19, 20, 22), including reduction in the level of Mcl-1 in myeloma cells (23).

Flavopiridol is currently undergoing Phase I and II clinical trials, alone and in combination with traditional cytotoxic agents, to define its antitumor potential against solid tumors, leukemia, and lymphomas (14, 24). Despite the clinical interest, however, little is known about mechanisms of resistance to flavopiridol. Although flavopiridol is not a substrate for P-gp or MRP1 (25–28), Schlegel et al. (25) found BCRP expression associated with resistance to flavopiridol, and Robey et al. (28) found that resistance to flavopiridol was blocked by the BCRP inhibitor fumitremorgin C and that flavopiridol induced BCRP expression without concomitant induction of P-gp and MRP1 overexpression. These findings suggested that BCRP might export flavopiridol from cells, resulting in decreased intracellular flavopiridol accumulation.

Sequencing of the BCRP gene in drug-selected cancer cell lines has revealed mutations of BCRP at codon 482 (29, 30). The wild-type amino acid at codon 482 is arginine (R482). Wild-type BCRP transports methotrexate but not anthracyclines or rhodamine 123. The codon 482 mutations observed have substitutions of threonine (R482T) or glycine (R482G), which results in the ability of the mutant protein to transport anthracyclines and rhodamine 123 but loss of ability to transport methotrexate (29–31). Because anthracyclines and methotrexate are widely used in the treatment of acute leukemias, it is reasonable not only to quantify BCRP mRNA expression but also to determine whether a mutation of BCRP occurs at codon 482 in blast cell samples from acute leukemia patients.

Our previous work (10) found a wide range of expression of BCRP among blast cell samples obtained from acute leukemia patients. The present studies were designed to elucidate the contribution of BCRP to acquired resistance to flavopiridol in acute leukemia. We studied the relationship between cellular resistance to flavopiridol and BCRP mRNA expression in leukemic blast cells obtained from patients with acute leukemias before treatment with a flavopiridol-containing regimen. The expression of BCRP and P-gp mRNA was quantitatively determined by real-time RT-PCR using specific hybridization probes. The amino acid at position 482 of the BCRP gene was estimated by determining the melting point (Tₘ) of the PCR product with the hybridization probes. Here, we present data suggesting that cellular resistance to flavopiridol in a subset of blast cell samples from acute leukemia patients correlates with BCRP mRNA expression, not P-gp expression.

**MATERIALS AND METHODS**

**Cell Culture.** MCF-7 human breast carcinoma and HL-60 human acute promyelocytic leukemia cells were cultured in supplemented IMEM or RPMI 1640 (Biofluids, Camarillo, CA) in 10% fetal bovine serum as described previously (5, 32).

**Patient Characteristics.** Bone marrow aspirates (21 samples) were collected from 20 consecutive adult patients with aspirable marrows who had poor risk acute leukemia (17 AML and 3 ALL). The samples were collected between March 2001 and February 2002. One patient with refractory AML was studied twice, once before a flavopiridol-containing regimen (sample no. 15) and again at time of progressive disease after treatment (sample no. 19). The median age of the patients was 64 years old (range, 20–78 years); 14 were male, and 6 were female. Patient characteristics are described in Table 1.

**Collection and Preparation of Patient-derived Blast Cells.** All bone marrow specimens were collected after informed consent, as approved by the University of Maryland Baltimore Institutional Review Board. Blast cells were enriched from the marrow samples by discontinuous ficoll-hypaque density gradient (33) as reported previously (34, 35). This procedure yields marrow mononuclear cells that have the appearance of myeloid or lymphoid blast cells when Wright-stained specimens are viewed by light microscopy. The blast cell yield by this procedure for the samples included in this study ranged from 85 to 95%. The cellular viability by trypan blue staining was routinely 80–100%.

**Isolation of Wild-Type BCRP (R482 BCRP).** To obtain a full-length R482 BCRP cDNA, total cellular RNA was prepared from MCF-7 cells, then subjected to reverse-transcription at 37°C for 1 h in the presence of random hexamer and dT primers. This was followed by PCR using Pfx DNA polymerase I (Life Technologies, Inc., Rockville, MD) using PCR primers, which were designed to span the open-reading frame of BCRP mRNA. The PCR primers used were as follows: sense, 5'-CTGAGCCCTTTGATGTTAAGACCCG-3' and antisense, 5'-GCTGTGCAACAGTGATGG-3'. The PCR-product was subcloned into pcDNA Blunt TOPO II vector (Invitrogen, Carlsbad, CA), then the sequence was confirmed by an automated dideoxynucleotide method.

**Total RNA Isolation from Blast Cells.** Total cellular RNA was isolated from 0.9 × 10⁸ to 1.5 × 10⁹ AML blast cells using RNeasy (Qiagen, Valencia, CA). According to manufacturer's protocol, total RNA was immediately stabilized after lysis and separated on a silica-gel column. Additionally, the total RNA was treated with DNase I (Qiagen) on the same column. Finally, the RNA was dissolved in water. As a control, total RNA was also isolated from MCF-7 and HL-60 cells.

**Quantitative Detection of BCRP, MDR1, and β-Actin mRNA by Real-Time RT-PCR.** Real-time, one-step RT-PCR for mRNA of BCRP (product length 186 bp), MDR1 (product length 190 bp), and β-actin (product length 238 bp) was performed using the LightCycler thermal cycler system according to the manufacturer’s instructions (Roche Diagnostics, Indianapolis, IN). The LightCycler allows the annealing and denaturation of nucleic acid to be followed in real time, using double-stranded DNA dyes (e.g., Sybr Green I) or fluorescently labeled oligonucleotides (hybridization probes). Dur-
Quantification of BCRP mRNA in AML

Hybridization probes labeled with donor and acceptor fluorophores were used for determination of PCR products of BCRP or MDR1. In this case, separate 3'-FITC and 5'-LCRed705 labeled probes were designed to hybridize adjacent (within 2 bp) to the expected PCR product to obtain adequate fluorescence resonance energy transfer. After completion of the PCR reaction, the melting temperature (Tm) of the PCR product and the hybridization probes is obtained. A mutation of the BCRP gene within the span of the hybridization causes a lowering of the Tm of the hybridization probe. For authentic BCRP, the hybridization probes used were: donor, 5'-TCAACTCG-TAGGAGTGGCGT-3' (2423–2443) and acceptor, 5'-LCRed705-ATCAACGCCCCAGACGAAA-phosphate 3' (2446–2467). To quantify the mRNA copy number of the target gene, fluorescence data were calibrated using standards containing various copy numbers (10^2–10^9) of cRNA of the target gene. cRNA was prepared using mMessage mMachine (Ambion, Austin, TX). For BCRP quantification, R482 BCRP was used. A full-length MDR1 cDNA was obtained from the plasmid PI-4 (36). β-Actin CDNA was purchased from American Type Culture Collection (Manassas, VA). Finally, the copy numbers of BCRP and MDR1 mRNA were normalized to the amount (pg) of β-actin in the sample. Generally, RT-PCR was performed in the presence of 6 mM MgCl2, 0.5 μM primer (each), 2 μM hybridization probe (each), and ~200 ng of total RNA. In the case of β-actin, MgCl2 was reduced to 4.5 mM to decrease the chance of primer dimerization. Reverse transcription was done at 55°C for 30 min, with a denaturation step at 95°C for 30 s followed by 45 cycles with 95°C denaturation for a moment, followed by 45 cycles with 95°C denaturation for 30 s, 72°C extension for 13 s. Negative controls were run concomitantly to assure that the samples were not cross-contaminated.

Cytotoxicity Assay. Flavopiridol was obtained from the Developmental Therapeutics Program, Division of Cancer Treatment and Dx, NCI, and Aventis Pharmaceuticals. Flavopiridol stock solutions (22.8 mm) were stored at −20°C before use. The ficol-hypaque-purified marrow blast cells were cultured in RPMI 1640 containing 10% autologous patient serum at an initial cell concentration of 5 × 10^5 cells/ml. Cells were exposed to vehicle (PBS) or 250 nM flavopiridol for 24 h, then cultured in drug-free medium for another 96 h, at which time the number of surviving viable cells/ml culture medium

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Age/Sex</th>
<th>Diagnosis</th>
<th>Stage of disease</th>
<th>Cytogenetics</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>36 M</td>
<td>AML, M5</td>
<td>Refractory</td>
<td>46XY, +6(qter), inv (3)</td>
</tr>
<tr>
<td>2</td>
<td>71 F</td>
<td>AUL, M5</td>
<td>New Dx</td>
<td>46XX, −12p11.2</td>
</tr>
<tr>
<td>3</td>
<td>73 M</td>
<td>AML, M1</td>
<td>Refractory</td>
<td>46XY</td>
</tr>
<tr>
<td>4</td>
<td>54 M</td>
<td>MDS/AML, M7</td>
<td>Refractory</td>
<td>43–47XY, t(5;9), −7, −9p13, +9p22, +18(pter)</td>
</tr>
<tr>
<td>5</td>
<td>56 F</td>
<td>AML, M2</td>
<td>Refractory</td>
<td>46–47XX, −3, +21, + marker</td>
</tr>
<tr>
<td>6</td>
<td>75 F</td>
<td>AML, M1</td>
<td>Relapse I</td>
<td>47–48XX, +8, +8, −6q13–23</td>
</tr>
<tr>
<td>7</td>
<td>69 M</td>
<td>AML, M4</td>
<td>Refractory</td>
<td>47XY, +11, −20q</td>
</tr>
<tr>
<td>8</td>
<td>78 M</td>
<td>MDS/AML, M5</td>
<td>New Dx</td>
<td>46XY, t(1;14), −4q31, −5q32, der (7), t(7;12), +der (8), t(8;17), −12</td>
</tr>
<tr>
<td>9</td>
<td>46 M</td>
<td>AML, M2</td>
<td>Refractory</td>
<td>46XY</td>
</tr>
<tr>
<td>10</td>
<td>54 F</td>
<td>ALL, L2</td>
<td>Relapse I</td>
<td>46XX, t(11;19)</td>
</tr>
<tr>
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<td>64 M</td>
<td>ALL, L3</td>
<td>Refractory</td>
<td>47–61XY, −1p34, t(5;12), +1, +2, +6, +11, +13, +21, +22, +X</td>
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<td>73 M</td>
<td>MDS/AML, M4</td>
<td>New Dx</td>
<td>46XY</td>
</tr>
<tr>
<td>13</td>
<td>68 M</td>
<td>MDS/AML, M1</td>
<td>Relapse I</td>
<td>46XY</td>
</tr>
<tr>
<td>14</td>
<td>49 F</td>
<td>AML, M5</td>
<td>New Dx</td>
<td>46XX, t(9;11)</td>
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<tr>
<td>15a</td>
<td>57 M</td>
<td>AML, M4</td>
<td>Refractory</td>
<td>46XY</td>
</tr>
<tr>
<td>16</td>
<td>64 F</td>
<td>AML, M1</td>
<td>Relapse I</td>
<td>46XY</td>
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<td>17</td>
<td>78 M</td>
<td>MDS/AML, M5</td>
<td>New Dx</td>
<td>46Y, t(X;1), +6, −10, 2–20 double minutes</td>
</tr>
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<td>MDS/AML, M6</td>
<td>New Dx</td>
<td>45–46XY, −3p12, −5, −6p23, −7, t(5;12), +1–2 markers</td>
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<td>19b</td>
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<td>AML, M4</td>
<td>Refractory, postflavopiridol</td>
<td>46XY</td>
</tr>
<tr>
<td>20</td>
<td>71 M</td>
<td>AML, M0</td>
<td>Refractory</td>
<td>47XY, +11, + marker</td>
</tr>
<tr>
<td>21</td>
<td>20 M</td>
<td>ALL, L2</td>
<td>Relapse I</td>
<td>47XY, +19, +19, −22, −22, (iso)22</td>
</tr>
</tbody>
</table>

a AUL, acute undifferentiated leukemia.
b Sample nos. 15 and 19 are from the same patient. Sample no. 15 was obtained before, and sample no. 19 was obtained at time of progressive leukemia after treatment with a regimen containing flavopiridol (24).
was determined by FDA and PI staining (37), using a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA). FDA/PI staining measures viability based on membrane integrity.

**Determination of Apoptosis in Blast Cells.** The percentage of apoptotic cells in blast cell samples cultured for 24 h in either flavopiridol treated or control conditions was obtained by flow cytometric determination of nuclear DNA content. To measure DNA content, cells were removed from culture and suspended in ice-cold lysis buffer, which contained PI (0.05 mg/ml) and 0.05% sodium citrate (38). The nuclei in the PI/citrate solution were analyzed by flow cytometry, using laser excitation of 488 nm, and detecting red fluorescence emission using a FACScan flow cytometer. To evaluate the effect of flavopiridol on apoptosis in the blast cells, the relative flavopiridol-induced apoptosis was calculated by the following formula:

Relative flavopiridol-induced apoptosis =

\[
\frac{\text{percentage of apoptosis}_{\text{flavopiridol treated}} - \text{percentage of apoptosis}_{\text{control}}}{(100\% - \text{percentage of apoptosis}_{\text{control}})}
\]

This formula results in a number between +1 and −1, with a positive value indicating a specific increase in apoptosis in the flavopiridol-treated cells.

**Statistical Analysis.** Statistical analyses were performed for patients whose BCRP mRNA expression level was >10,000 copies/pg β-actin, which was 30% above the median. The association of cell survival rate and relative apoptosis with BCRP mRNA expression levels was assessed using a multiple regression analysis. The significance test was based on t-value. The R value from the linear regression was used to measure the linear association between the cell survival rate or relative apoptosis and BCRP mRNA expression levels. The Spearman correlation coefficient was used to assess the increasing (decreasing) relationship between these levels. SAS was used for statistical analyses. No adjustments were made for multiple comparisons in these exploratory analyses.

**RESULTS**

**Expression of BCRP and MDRI mRNA in the Blast Cell Samples.** To explore the influence of multidrug resistance transporters on the cellular toxicity of flavopiridol in the patient-derived blast cells, BCRP and MDRI mRNA expression were quantified by real-time RT-PCR and normalized for the expression of β-actin. The specificity of the method was enhanced by detection of the PCR products with product-specific fluorescent hybridization probes, with additional confirmation of product fidelity by Tm analysis following PCR. Table 2 displays the expression of BCRP and P-gp mRNA in the 21 blast cell samples, as well as flavopiridol-induced apoptosis and cytotoxicity. The expression of BCRP in the 21 patient samples is arranged in order of magnitude in Fig. 1A. As we previously observed (10), there was considerable variation in BCRP expression among the blast cell samples, with approximately one-third of the samples displaying more than twice of the median value for BCRP expression. Although BCRP mRNA expression

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Patient diagnosis</th>
<th>Relative in vitro flavopiridol-induced apoptosis after 250 nm flavopiridol (copies/pg)</th>
<th>Cell survival in vitro after 250 nm flavopiridol (% control)</th>
<th>BCRP mRNA expression (copies/pg β-actin)</th>
<th>MDRI mRNA expression (copies/pg β-actin)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>AML, M5</td>
<td>+0.001</td>
<td>75.5</td>
<td>3,507</td>
<td>4,709,520</td>
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<td>2</td>
<td>AUL (2)</td>
<td>−0.042</td>
<td>84.5</td>
<td>74,358</td>
<td>191,115</td>
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<td>3</td>
<td>AML, M1</td>
<td>+0.061</td>
<td>47.1</td>
<td>732</td>
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<td>MDS, M7</td>
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<td>111.1</td>
<td>6,434</td>
<td>43,774</td>
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<td>81,669</td>
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<td>8</td>
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<td>708</td>
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<td>27,013</td>
<td>10,733</td>
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<td>19°</td>
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<tr>
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<td>ALL, L2 (T cell)</td>
<td>+0.269</td>
<td>10.3</td>
<td>8,308</td>
<td>247,587</td>
</tr>
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</table>

* Relative flavopiridol-induced apoptosis = (% Apoptosisflavopiridol treated − % Apoptosiscontrol) / (100% − % Apoptosiscontrol).
*° Sample nos. 15 and 19 are from the same patient. Sample no. 15 was obtained before and sample 19 was obtained at time of progressive leukemia after treatment with a regimen containing flavopiridol (24).
varied >200-fold overall, 16 blast cell samples (76%) expressed BCRP at a level greater than the HL-60 human AML cell line (HL-60 BCRP expression was 1900 copies/pg β-actin). Consistent with our previous study (10), the blast cell samples with the highest BCRP expression were comparable with the endogenous expression of BCRP mRNA by MCF-7 cells (MCF-7 BCRP expression was 170,000 copies/pg β-actin).

MDR1 mRNA expression in the blast cell samples is shown in Fig. 1B. Similar to BCRP expression, variations were also found for MDR1 mRNA expression. Overall, there was a poor correlation (r = 0.11; P = 0.628) between the expression of BCRP and MDR1 mRNA for all patients (Fig. 1C). This poor correlation suggests that both transporters might be expressed independently in the blast cell samples.

Correlation of Blast Cell Survival after in Vitro Flavopiridol Exposure and Expression of BCRP or P-gp mRNA. The in vitro survival of the 21 blast cell samples was determined by FDA/PI staining 96 h after a 24-h cellular exposure to flavopiridol (250 nM), and the relationship between cell viability and multidrug resistance transporter mRNA expression was determined. Overall, exposure to 250 nM flavopiridol reduced survival of the blast cell samples to 56.1 ± 15.5%, compared with controls (no treatment with flavopiridol). When cell survival after flavopiridol is plotted against BCRP mRNA expression (Fig. 2A), two distinct patterns are observed. In the 9 blast cell populations with BCRP mRNA expression > 10,000 copies/pg β-actin, cell survival after flavopiridol correlated directly with BCRP mRNA expression (r = 0.86, P = 0.003; regression line is shown in Fig. 2A). Furthermore, the Spearman correlation coefficient was 0.88 (P = 0.002), indicating a sta-
However, in the remaining 12 samples with BCRP mRNA expression, the ability to survive flavopiridol treatment may be ascribed to factors other than BCRP. Thus, in a subset of blast cell samples where BCRP was expressed at low levels. In contrast, such relationship was observed in the remaining 12 blast cell samples, where BCRP was expressed at low levels. In contrast, the expression level of MDR1 mRNA did not correlate with decreased apoptosis induced by flavopiridol, as shown in Fig. 3B.

Resampling of Patient Bone Marrow after in Vivo Treatment with a Flavopiridol-containing Regimen (24). One patient donated two specimens of bone marrow for these studies (samples no. 15 and no. 19). Sample no. 15 was obtained before treatment, and sample 19 was obtained at time of progressive leukemia after treatment with a regimen containing flavopiridol (24). This patient’s leukemia was characterized by a high degree of clinical drug resistance, with multiple chemotherapeutic regimens failing to produce complete remission. Table 2 shows the flavopiridol-induced apoptosis, cytotoxicity, as well as BCRP and MDR1 mRNA expression in these samples. Both BCRP and MDR1 mRNA expression rose considerably in sample no. 19, taken after marrow recovery from the flavopiridol-containing regimen at a time when the marrow had repopulated with leukemic blast cells. Similarly, in vitro flavopiridol-induced apoptosis fell from +0.657 to +0.145, and blast cell survival after in vitro treatment with 250 nM flavopiridol increased from 14.2 to 57.6% in sample no. 19, compared with sample no. 15, consistent with an increase in measurable in vitro flavopiridol resistance after in vivo exposure to flavopiridol.

After obtaining the second sample (no. 19), this patient was re-treated with the same flavopiridol-containing regimen. In parallel with the in vitro findings, there was a notable difference in the clinical cytoreductive response to flavopiridol between the first and second exposures to drug. The initial cycle of flavopiridol induced rapid tumor lysis, as evidenced by an abrupt fall in peripheral blood WBC count from 19.2 × 10^9/liter to 5.4 × 10^9/liter at 12 h and 3.2 × 10^9/liter at 24 h after the first dose and 1.2 × 10^9/liter immediately after the third and final dose and 0.6 × 10^9/liter 72 h thereafter. This rapid cytoreduction was accompanied by acute and transient rises in serum phosphate (4.5–7.4 mg/dl), uric acid (4.2–7.7 mg/dl), and lactate dehydrogenase (LDH, 2768–6108 units/liter) within 24 h of the first drug dose. In contrast, the second cycle of flavopiridol drug administration was accompanied by much less cytoreduction. Specifically, WBC fell in the first 24 h from 7.8 to 4.7 × 10^9/liter, reached a nadir of 2.3 × 10^9/liter after the third dose, and was back up to 4.6 × 10^9/liter 72 h after the third and final dose of flavopiridol. This attenuated cytoreductive response to flavopiridol was accompanied by lesser degrees of drug-induced
hyperphosphatemia (peak 6.0 mg/dl), uric acid (peak 6.3 mg/dl), and LDH (2406 units/liter).

Investigation of Possible Mutations in the Region of BCRP mRNA Containing Codon 482. The possibility of a single or multiple base mutations in the area corresponding to codon 482 of BCRP, which has been characterized as a mutational hot spot (30, 39), was estimated by the use of melting point analysis of hybridization probes and the PCR product formed in the BCRP RT-PCR reaction. In all samples, the BCRP PCR product from patient-derived blast cells and the wild type BCRP-specific hybridization probes had a $T_m$ equal to that expected for the wild-type sequence. This indicates that in these leukemic blast cell samples, no mutations of BCRP exist in the region of the mRNA that corresponds to the hybridization probes used.

DISCUSSION

Our study in this small group of patients describes a relationship between acute leukemia blast cell resistance to flavopiridol and mRNA levels of BCRP, which were quantitatively determined by real-time RT-PCR. The data suggest that BCRP may contribute to flavopiridol resistance in a subset of leukemia blast cell populations.

Reports of BCRP mRNA expression in AML blast cells are beginning to appear in the literature. Some studies, to date, have detected BCRP expression in a significant proportion of AML populations, with BCRP expression being independent of P-gp expression (10–12). In our current series, approximately one-third of the samples displayed BCRP expression more than twice the median value, and the correlation of BCRP mRNA expression with that of MDR1 was poor. The observation that BCRP is independent of P-gp expression suggests that BCRP might play a role by itself in resistance to flavopiridol in AML. Furthermore, P-gp itself is not a strong transporter of flavopiridol.

Cellular viability correlated proportionally with BCRP mRNA expression levels in the 43% (9 of 21) of the leukemia blast cell samples with the highest BCRP mRNA expression. This proportion is similar to another study in which 30% of AML blast cell samples exhibited a good correlation between BCRP protein expression and increased resistance to daunorubicin (11). Although we did not examine BCRP protein expression in our study, others have shown good correlations between MDR1 mRNA and P-gp function and between MRPI mRNA and its function (40). It is therefore a reasonable assumption that BCRP mRNA levels are an indicator of its protein and functional expression. In this regard, a high correlation was seen between BCRP mRNA level and both flavopiridol-induced apoptosis and cell survival after flavopiridol treatment. These two complementary measures of flavopiridol toxicity could be a consequence of a decreased drug accumulation by BCRP-mediated flavopiridol transport. Indeed, in the patient from whom we examined leukemic blasts before and after in vivo flavopiridol exposure and demonstrated a significant increase in blast cell BCRP expression, we noted a marked decrease in flavopiridol-induced cytotoxicity in vivo in terms of both WBC response and related tumor lysis parameters.

Marked variation in cell apoptosis and survival after flavopiridol treatment was observed in the population exhibiting BCRP mRNA levels <10,000 copies/pg β-actin (12 patient samples). This observation indicates that in the cases with low BCRP expression and resistance to flavopiridol-induced cytotoxicity in vitro (low apoptosis + high survival), mechanisms of resistance other than efflux by BCRP are operative. Recently, Smith et al. (41) found that cyclin E protein levels and cyclin E-associated kinase activity were increased in a flavopiridol-resistant cell line, suggesting cyclin E overexpression may be involved in resistance to flavopiridol. Interestingly, they found that decreased drug accumulation did not play a role in resistance to this agent in their experimental model, in contrast to a previous study (26) where resistance to flavopiridol was associated with reduced intracellular drug accumulation. Hence, our study illustrates two distinct mechanisms of resistance to flavopiridol operative in these blast cell samples: one involving BCRP, and another involving mechanisms independent of BCRP. It is tempting to speculate that one mechanism of resistance could relate to cyclin E activity, independent of drug transport and net intracellular drug levels. Additional study of cell cycle regulatory proteins, including cyclin E will help to elucidate the diverse mechanisms by which malignant cells can circumvent flavopiridol cytotoxicity.

Compared with BCRP, MDR1 mRNA levels did not correlate with leukemic blast cell viability or apoptosis in the presence of flavopiridol. Previously, Elgie et al. (42) could not establish an overall, statistically significant correlation of in vitro chemotherapy with functional expression of P-gp in AML. Moreover, Sargent et al. (11) emphasized that BCRP might be more important than P-gp in AML as a xenobiotic transporter. Our data support this connection with regard to cytotoxicity of flavopiridol, which appears to be more susceptible to BCRP-mediated transport than to P-gp.

Recently, the R482T and R482G mutations of BCRP have been described. In contrast to the wild-type R482 form, these mutant versions of BCRP are able to transport anthracyclines and rhodamine 123 (29, 30). Additionally, a recent study (31) indicates that the R482T and R482G mutations result in loss of the ability to transport methotrexate. Our data indicate that the frequency of mutation of the BCRP gene in the region encompassing codon 482 is low in blast cells from the group of poor risk leukemia patients studied because no mutations were detected in BCRP mRNA in the 21 patient samples evaluated. This finding has relevance to acute leukemia, where anthracyclines—particularly daunorubicin and idarubicin—are key members of the therapeutic armamentarium. If BCRP is expressed predominantly as the wild-type form in acute leukemias, this would be expected to have no or only a minor impact on resistance to the anthracyclines. On the other hand, the predominant expression of wild-type BCRP in leukemia may have impact on leukemias where methotrexate plays a vital therapeutic role such as ALL. In summary, this study demonstrates varying expression of BCRP mRNA among blast cell samples from patients with acute leukemia. In certain leukemias, resistance to the cytotoxic effects of flavopiridol on the leukemic blasts may be linked to BCRP mRNA expression. In contrast, P-gp expression does not appear to explain flavopiridol resistance in any of the patients studied. Additional studies are needed to confirm these findings.
and establish the clinical implications of BCRP expression in the net cellular metabolism of flavopiridol.

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REFERENCES


18. Honjo, Y., Hrycyna, C. A., Yan, Q. W., Medina-Perez, W. Y., Robey, R. W., van de Laar, A., Litman, T., Dean, M., and Bates, S. E. Acquired mutations in the MXR/ABCP gene alter substrate spec-


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