Breast Cancer Resistance Protein in Drug Resistance of Primitive CD34+38− Cells in Acute Myeloid Leukemia

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ABSTRACT

Purpose: Acute myeloid leukemia (AML) is considered a stem cell disease. Incomplete chemotherapeutic eradication of leukemic CD34+38− stem cells is likely to result in disease relapse. The purpose of this study was to investigate the role of the breast cancer resistance protein (BCRP/ATP-binding cassette, subfamily G, member 2) in drug resistance of leukemic stem cells and the effect of its modulation on stem cell eradication in AML.

Experimental Design: BCRP expression (measured flow-cytometrically using the BXP21 monoclonal antibody) and the effect of its modulation (using the novel fumitremorgin C analogue KO143) on intracellular mitoxantrone accumulation and in vitro chemosensitivity were assessed in leukemic CD34+38− cells.

Results: BCRP was preferentially expressed in leukemic CD34+38− cells and blockage of BCRP-mediated drug extrusion by the novel fumitremorgin C analogue KO143 resulted in increased intracellular mitoxantrone accumulation in these cells in the majority of patients. This increase, however, was much lower than in the mitoxantrone-resistant breast cancer cell line MCF7-MR and significant drug extrusion occurred in the presence of BCRP blockage due to the presence of additional drug transport mechanisms, among which ABCB1 and multiple drug resistance protein. In line with these findings, selective blockage of BCRP by KO143 did not enhance in vitro chemosensitivity of leukemic CD34+38− cells.

Conclusions: These results show that drug extrusion from leukemic stem cells is mediated by the promiscuous action of BCRP and additional transporters. Broad-spectrum inhibition, rather than modulation of single mechanisms, is therefore likely to be required to circumvent drug resistance and eradicate leukemic stem cells in AML.

INTRODUCTION

Cancer is increasingly recognized as a disease originating from the transformation of normal stem cells, retaining the capacity for self-renewal (1). This emerging concept for the origin of tumorigenesis was stimulated by the postulation of acute myeloid leukemia (AML) as a stem cell disease. Cells with leukemic stem cell characteristics, defined as leukemic engraftment potential and self-renewal capacity, in AML are found in the CD34+CD38− cell population. The involvement of CD34+38− cells in leukemogenesis is suggested by the presence of cytogenetically aberrant cells in the CD34+CD38− compartment as shown by fluorescence in situ hybridization and PCR for leukemia specific translocations (2, 3). Additionally, leukemic cells with long-term proliferative ability both in vitro and in vivo have been identified as CD34+CD38− (4).

Studies using the nonobese diabetic/severe combined immunodeficient mouse model have shown that cells with leukemic engraftment and self-renewal potential in acute myelogenous leukemia are found in the CD34+CD38− subpopulation (5, 6) and not in more differentiated CD34+/CD38+ cells. These studies strongly argue that leukemia-initiating transformation and progression-associated genetic events occur at the level of these primitive cell populations. Importantly, incomplete chemotherapeutic eradication of cancer stem cells is likely to result in disease relapse. Elucidation of the mechanisms conferring resistance to these cells is therefore essential to provide novel targets for stem cell eradication in AML.

The ATP-binding cassette protein family is a large family of highly conserved membrane proteins transporting a wide variety of substrates across the cell membrane (7). Several members, among which multidrug resistance 1 [MDR1; ATP-binding cassette, subfamily B (MDR), member 1 (ABCB1)] and breast cancer resistance protein [BCRP; ATP-binding cassette, subfamily G, member 2 (ABCG2)], extrude a variety of structurally unrelated chemotherapeutic compounds, thereby conferring a multidrug resistance phenotype to cancer cells.

BCRP (ABCG2) is a 655-aa member of the ABCG subfamily of ATP-binding cassette membrane transporters first described in drug-resistant cell lines (8–10). BCRP is a half transporter functioning as a dimer and confers multidrug resistance to topotecan, mitoxantrone, doxorubicin, and related compounds by ATP-dependent drug extrusion (9, 11). BCRP is expressed in placental syncytiotrophoblasts, intestinal epithelium, and liver canalicular membrane (12), suggesting a physiologic role in detoxification. Recently, BCRP has been shown to be highly expressed in a wide variety of stem cells (13) including immature human hematopoietic progenitors (14). BCRP is proposed to protect these long-lived cells from naturally occurring toxic substrates (15).

KO143 is a novel fumitremorgin C analogue which has recently been shown to be an extremely potent BCRP inhibitor (16). KO143 is a specific inhibitor of BCRP and much more potent than currently known inhibitors of BCRP such as fumitremorgin C (17) and GF120918 (18). Importantly, KO143...
is nontoxic at effective in vitro and in vivo concentrations, which makes it one of the most promising compounds for development of clinical modulators of BCRP-mediated efflux. The purpose of the current study was to investigate the role of BCRP in drug resistance of leukemic CD34+38− stem cells and the effect of its modulation on stem cell eradication in AML.

MATERIALS AND METHODS

Bone Marrow Samples and CD34+38− Hematopoietic Cells. Bone marrow was obtained after informed consent from healthy allogeneic bone marrow donors and patients with AML at diagnosis. Patient characteristics are shown in Table 1. Mononuclear cells were isolated by density gradient centrifugation using Ficoll 1.077 g/mL (Pharmacia Biotech, Uppsala, Sweden). Isolation, cryopreservation, and thawing procedures of cells have been previously described (19) and were identical for normal and leukemic bone marrow samples. After thawing, cells were stained with FITC- or CY5-labeled CD34 and CD38-phycoerythrin monoclonal antibodies (Becton Dickinson BV, Etten-Leur, the Netherlands) for 30 minutes at 4°C and washed in HBSS with 1% v/v heat-inactivated FCS (Hyclone, Logan, UT). A Coulter Epics Elite Flow cytometer was used to define cell populations. Gating on forward and right angle scatter was used to exclude dead cells and debris. CD34+CD38− cells of both normal bone marrow and AML were defined as the cells with CD38-phycoerythrin fluorescence within the first decade of emission. CD34+CD38− cells appeared in a consistently restricted light-scattering region confirming the lymphoid appearance of these primitive progenitors (20). The CD34+CD38+ cells were sorted from a gate positioned in the bulk of CD34+ cells and showed more heterogeneous light-scattering properties.

Breast Cancer Resistance Protein Cell Line. The human breast cancer cell line MCF7-MR (gift from R. Scheper, Free University Hospital, Amsterdam, the Netherlands) is resistant to mitoxantrone and overexpresses BCRP but not multiple drug resistance protein (MRP, ABCC1) and ABCB1 (21). MCF7-MR cells were cultured in RPMI 1640 supplemented with 10% FCS in the presence of 80 nmol/L mitoxantrone.

Breast Cancer Resistance Protein Expression. BCRP was detected with the BXP21 monoclonal antibody (gift from R. Scheper), which recognizes an internal epitope of the protein (12) and has been shown to specifically bind BCRP, displaying no cross-reactivity with other proteins (12). In AML patient samples, BXP21 was more sensitive than BXP34, another BCRP-specific monoclonal antibody (22). BXP21 was used in a three-color flow cytometric assay, which in preliminary experiments proved to be much more sensitive than immunocytochemical assessment using immunofluorescence microscopy, as previously recognized for other ATP-binding cassette transporters (23). First, cells (1 × 10^6) were fixed and permeabilized (“Fix & Perm,” Caltag Lab.,

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**Table 1** Patient characteristics

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<td>% CD34</td>
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French-American-British classification

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Fig. 1  BCRP expression and BCRP-mediated mitoxantrone efflux in MCF7-MR cells. A, flow cytometric assessment of BCRP expression using secondary FITC-labeled F(ab)2 fragments against monoclonal antibody BXP21 (dotted line) or isotype control. B, mitoxantrone fluorescence (y axis) in the presence (dotted line) or absence of the BCRP specific inhibitor KO143 (0.1 μmol/L) after 2-hour accumulation (B1) and an additional hour of efflux in drug-free medium (B2).
Burlingame, CA) according to the instructions of the manufacturer, and subsequently washed in HBSS 1% FCS. Following fixation, cells were blocked with pooled human serum for 10 minutes and incubated with BXP21 (2.5 μg/mL) or isotype control IgG2a in the same concentration for 60 minutes at 4°C. BXP21 was detected by FITC-conjugated goat anti-mouse F(ab)2 fragments (5.0 μg/mL) for 30 minutes. A 10-minute incubation with goat serum to block nonspecific binding was followed by staining with CY5-conjugated CD34 and phycoerythrin-conjugated CD38. Appropriate BCRP isotype control at the same protein concentration as the relevant antibody was used as control. BCRP protein expression was quantitated as the median fluorescence channel shift (BXP21/isotype control).

**ABCG2 Gene Expression.** RNA was isolated from subfractions (10^5 cells) of granulocyte colony-stimulating factor–mobilized CD34+ hematopoietic cells using cDNA synthesis and real-time quantitative reverse transcription-PCR was done as described previously (24). ABCG2 target gene expression was quantitated relatively to housekeeping genes gyceraldehye-3-phosphate dehydrogenase (GAPDH) and hydroxymethylbilane synthase (HMBS) as described previously (24). We earlier showed that GAPDH is an appropriate gene for standardization of target gene expression in human CD34+ and CD34+ hematopoietic cell populations (24).

Primer and probe sequences for gene amplification were ABCG2, Taqman gene expression assay (Applied Biosystems, Foster City, CA) no. HS 00184979; GAPDH, Taqman gene expression assay (Applied Biosystems) no. HS 99999905; and HMBS, Taqman gene expression assay (Applied Biosystems) no. HS 00609297.

**Breast Cancer Resistance Protein–Mediated Mitoxantrone Efflux.** BCRP function was tested using mitoxantrone as a substrate and the fumitremorgin C analogue KO143 as an inhibitor for BCRP. Mitoxantrone, rather than an anthracyclin, was used as a substrate because mitoxantrone seems to be a more sensitive substrate to detect BCRP-mediated efflux as suggested by resistance profiles in cell line models (8, 11). The novel fumitremorgin C analogue KO143 (0.1 μmol/L) was used as a specific inhibitor for BCRP-mediated efflux. KO143 was shown to be the most potent inhibitor currently available and, importantly, is BCRP-specific (without inhibition of ABCB1 or ABCC1) at the concentration used in our assay (16). Flow cytometric measurement of fumitremorgin C–sensitive mitoxantrone efflux has been shown to be a sensitive and specific method for measuring the function of BCRP in both selected and unselected cell lines (25).

Cells were stained with CD34-FITC and CD38-phycoerythrin membrane markers, washed in HBSS 1% FCS, and...
preincubated with or without BCRP inhibitor for 20 minutes in Iscove’s modified Dulbecco’s medium supplemented with 1% FCS. Mitoxantrone (10 μmol/L) was added and cells were incubated for 2 hours at 37°C, 5% CO₂, with or without inhibitor. Subsequently, cells were washed in ice-cold HBSS 1% FCS and kept on ice until flow-cytometric analysis or allowed an additional 1-hour efflux in drug-free medium with or without inhibitor to further enhance sensitivity of the assay. Cellular mitoxantrone fluorescence was measured on a FACS-elite cytometer equipped with an argon laser. Fluorescence was assessed at an excitation wavelength of 635 nm through a 670-nm band-pass filter in a three-color protocol with CD34-FITC and CD38-phycoerythrin. At least 200 CD34+38⁻/CD0 cells were analyzed in each sample.

BCRP-mediated efflux was quantitated as the ratio of mitoxantrone fluorescence [mean fluorescence intensity (MFI)] in the presence or absence of KO143 and assessed in CD34+38⁻, CD34+CD38+, and CD34⁻ cells as defined. Interexperimental variability was assessed by performing triplicate, independently done, experiments in a panel of four different normal bone marrow samples. BCRP-mediated efflux, assessed in 200 CD34+38⁻ cells were analyzed in each sample.

**Fluorescence In situ Hybridization.** CD34+38⁻ cells were sorted and lysed in 15 μL KCl (75 μmol/L) on a glass coverslip. Cells were fixed in methanol/acetic acid and stored at 4°C until analysis. Fluorescence in situ hybridization was done using the LSI AML1/ETO Dual Color, Dual Fusion t(8;21) Probe or the LSI EGR1 (5q31) Dual Color Probe (Vysis, Downers Grove, IL) according to the instructions of the

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**Fig. 3** BCRP is preferentially expressed and functionally active in CD34+38⁻ hematopoietic cells in human normal bone marrow. A, BCRP expression indicated as BXP21/IgG2a isotype control. Columns, mean values; lines, individual samples. ABCG2 expression is higher in CD34+38⁻ cells compared with more differentiated CD34+38+ progenitors and CD34⁻ cells [7.95 ± 1.32, 6.81 ± 0.98 (P = 0.05), and 3.36 ± 0.62 (P < 0.001), respectively]. B, BCRP-mediated mitoxantrone efflux indicated as efflux index (EI) in different hematopoietic subpopulations.

**Fig. 4** ABCG2 is highly expressed in normal hematopoietic stem cells. ABCG2 expression was assessed in different subfractions from granulocyte colony-stimulating factor–mobilized peripheral blood in two normal donors and quantitated relative to the housekeeping genes HMBS and GAPDH (y axis; copies ABCG2/copy HMBS/GAPDH).
manufacturer. One-hundred cells were analyzed microscopically for (8;21) or chromosome 5 (5q31) cytogenetic abnormalities, as appropriate.

**Ninety-six-Well Chemosensitivity Assay.** CD34+38/C0 cells were sorted in 96-well, round-bottomed, plates (200 cells/well) containing Iscove’s medium supplemented with 10% FCS without or with mitoxantrone (1.0 μmol/L) and without or with KO143 in different concentrations (0.1, 1.0, and 10.0 μmol/L). Plates were incubated at 37°C, 5% CO2 for 48 hours. All experiments were done in quadruplicate. Cell apoptosis was assessed at 24 and 48 hours using propidium iodide as a cell death marker. Cell images were acquired on a Zeiss Axiovert 35M inverted microscope (Thorwood, NY) equipped with a 40× oil (N.A. 1.3) objective and a cooled 756 × 580 pixel resolution CDD camera (Variocam, PCO computer optics, Kellheim, Germany) coupled with the pixel pipeline in a Macintosh Quadro 800. Cells were excited with a mercury arc lamp using a 510- to 560-nm band-pass filter for propidium iodide. Emission was measured with a 610-nm long-pass filter.

**Statistical Analysis.** The Student’s t test was used to calculate significant differences. Correlations were calculated using correlation coefficients. Data are presented as mean ± SE. P < 0.05 was considered significant.

**RESULTS**

**Breast Cancer Resistance Protein Expression and Breast Cancer Resistance Protein–Mediated Mitoxantrone Efflux in MCF7-MR Cells**

BCRP expression in the MCF7-MR cell line, indicated as BXP21/IgG2a isotype control, and BCRP-mediated mitoxantrone efflux (B), indicated as efflux index, in different hematopoietic subpopulations. Columns, mean values; lines, individual samples.

**Breast Cancer Resistance Protein Is Preferentially Expressed and Functionally Active in Primitive CD34+38– Hematopoietic Cells in Human Normal Bone Marrow**

In order to compare BCRP expression and function in normal and malignant hematopoietic stem cells, first, ABCG2 expression was assessed in hematopoietic subpopulations in seven normal bone marrow samples (Figs. 2 and 3). CD34+38– cells were flow-cytometrically defined as indicated in Fig. 1A. ABCG2-mediated efflux was examined using KO143 as a specific inhibitor of BCRP. KO143 has been shown to be specific for ABCG2 at concentrations of 0.1 to 1.0 μmol/L, with no inhibition of ABCB1 (P-glycoprotein) or ABCC1 at these concentrations (16). The effect of KO143 on cellular mitoxantrone (10 μmol/L) fluorescence in MCF7-MR cells, measured after 2-hour accumulation with or without inhibitor, is shown in Fig. 1B. Mitoxantrone fluorescence (MFI) of M7-MR cells after 2-hour accumulation without mitoxantrone and in the presence of mitoxantrone without and with KO143 were 0.4 ± 0.1 (SD), 9.1 ± 0.3, and 28.5 ± 6.7, respectively (n = 3). The mean BCRP-mediated mitoxantrone efflux, indicated as a fluorescence shift index, was 3.13 ± 0.62. Mitoxantrone efflux during an additional hour in drug-free medium was completely blocked by KO143, illustrating the potency of KO143 as a BCRP inhibitor and the increasing sensitivity of BCRP efflux assessment in the MCF7 cell line (mean efflux index, 5.70 ± 0.35; Fig. 1C). Importantly, these data confirm that KO143 at the concentration used in our assays (0.1 μmol/L) completely blocks BCRP-mediated mitoxantrone efflux, as shown earlier (16).
Table 2  BCRP is expressed and mediates mitoxantrone efflux in leukemic CD34+38− cells

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<th>Patient</th>
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<th>CD34+38− aberrant cells (%)</th>
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<td>t (8;21)</td>
<td>95</td>
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NOTE. BCRP expression (BXP21/IgG2a) and BCRP-mediated mitoxantrone efflux (indicated as efflux index) relative to percentage of cytogenetically aberrant cells in the CD34+38− cell population in AML. Abbreviation: NA, no material available for analysis.

Table 3  Additional drug efflux mechanisms are involved in mitoxantrone efflux from leukemic CD34+38− cells

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<tr>
<td>10</td>
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Mean ± SE 1.14 ± 0.03 1.17 ± 0.02 1.25 ± 0.09

NOTE. ABCB1 and ABCC1-mediated mitoxantrone efflux, indicated as efflux index, in leukemic CD34+38− cells. Abbreviations: NS, not significant (defined as efflux index < 1.05); NA, no material available for analysis.

Using Student’s t test for paired samples) and sharply decreased during further differentiation in CD34− cells (3.36 ± 0.62; P < 0.001; Fig. 3A).

To provide additional evidence that BCRP is preferentially expressed in CD34+38− hematopoietic cells in comparison with committed progenitors and differentiated cells, ABCG2 gene expression was assessed in these cell populations using real-time quantitative reverse transcription-PCR. Because bone marrow samples yielded insufficient cell numbers to reliably perform real-time quantitative reverse transcription-PCR on the relatively low-copy transcript ABCG2, we used granulocyte colony-stimulating factor–mobilized peripheral blood CD34+ cells obtained from two normal controls. ABCG2 expression was assessed relatively to housekeeping genes as previously described (24) in CD34+38−, CD34+38+, and differentiated cells (monocytes; Fig. 4).

These results confirm, in an independent assay, that primitive hematopoietic stem cells express high levels of BCRP, in line with previous reports (14, 26).

Next, we investigated BCRP-mediated efflux in CD34+38−, CD34+38+, and CD34− hematopoietic subpopulations using mitoxantrone as a substrate; cellular mitoxantrone fluorescence (MFI) after 2 hours of mitoxantrone accumulation was low in CD34+38− cells compared with more differentiated CD34+38+ progenitors and CD34− cells (4.4 ± 0.29, 9.29 ± 0.83, and 7.63 ± 0.55, respectively). BCRP-mediated mitoxantrone efflux contributed to the mitoxantrone “dull” appearance of primitive CD34+38− cells as evidenced by the ability of KO143 to increase fluorescence intensity. Significant BCRP-mediated efflux, defined as a mitoxantrone fluorescence (MFI) index without or with KO143 (0.1 μmol/L) ≥ 1.05 (see MATERIALS AND METHODS), was found in CD34+38− cells in 5 of 7 (71%) samples. In 2 of 7 samples no ABCG2-mediated efflux was found in either hematopoietic subpopulation. In those samples displaying BCRP-mediated efflux, this was invariably and significantly higher in CD34+38− cells compared with CD34+38+ and CD34− cells [mean efflux index: 1.22 ± 0.08, 1.13 ± 0.04 (P = 0.03), and 1.11 ± 0.03 (P = 0.03), respectively; Figs. 2 and 3B].

These data show that BCRP is preferentially expressed and functionally active in primitive CD34+38− hematopoietic cells compared with more differentiated cells in human normal bone marrow.

Breast Cancer Resistance Protein Expression and Function Is Conserved in Primitive Leukemic CD34+38− Hematopoietic Cells in Acute Myeloid Leukemia

BCRP expression was assessed in different hematopoietic subpopulations in 22 de novo AML samples (Figs. 2 and 5). Expression was higher in CD34+38− cells compared with more differentiated CD34+38+ and CD34− cell populations in 19 of 22 samples investigated. Mean BCRP expression was 9.00 ± 1.01 (range 1.59-21.4), 6.53 ± 0.68 (range 2.84-14.89, 9.29 ± 0.83, and 7.63 ± 0.55, respectively). BCRP-mediated mitoxantrone efflux contributed to the mitoxantrone “dull” appearance of primitive CD34+38− cells as evidenced by the ability of KO143 to increase fluorescence intensity. Significant BCRP-mediated efflux, defined as a mitoxantrone fluorescence (MFI) index without or with KO143 (0.1 μmol/L) ≥ 1.05 (see MATERIALS AND METHODS), was found in CD34+38− cells in a patient in the presence or absence of mitoxantrone and KO143. Apoptotic (propidium iodide–positive) cells are depicted in black (negative images). (DFM, drug-free medium; MITO, mitoxantrone alone; +KO143, mitoxantrone in the presence of KO143 at different concentrations).

Fig. 6  Specific inhibition of BCRP does not increase mitoxantrone-induced apoptosis in primitive leukemic CD34+38− hematopoietic cells. Effect of KO143 on mitoxantrone-induced apoptosis in leukemic CD34+38− cells. Typical example of microscope images of leukemic CD34+38− cells of a patient in the presence or absence of mitoxantrone and KO143. Apoptotic (propidium iodide–positive) cells are depicted in black (negative images). (DFM, drug-free medium; MITO, mitoxantrone alone; +KO143, mitoxantrone in the presence of KO143 at different concentrations).
normal bone marrow. We next investigated whether the preferential expression of ABCG2 in leukemic CD34+38− cells was reflected by increased ABCG2-mediated transport in these cells. In AML, similar to the situation in normal bone marrow, CD34+38− hematopoietic cells accumulated the lowest amount of mitoxantrone relative to more differentiated CD34+38+ and CD34− cell populations (MF: 6.6 ± 0.49, 10.0 ± 0.66, and 10.34 ± 0.74, respectively). To determine the contribution of BCRP-mediated efflux to the mitoxantrone “null” phenotype of leukemic CD34+38− cells, ABCG2-mediated mitoxantrone efflux was assessed in 22 leukemic bone marrow samples using KO143 (0.1 μmol/L) as an inhibitor. In the majority of samples (14/22 = 64%), BCRP-mediated efflux was detected in CD34+38− cells. The remaining 8 samples displayed no BCRP-mediated efflux in either cell population. In the samples displaying BCRP-mediated efflux, efflux was significantly higher in CD34+38− cells compared with CD34+38+ and CD34− cells [efflux index: 1.20 ± 0.03, 1.08 ± 0.02 (P = 0.003), and 1.06 ± 0.02 (P = 0.001), respectively; Fig. 5B]. The differences remain significant when the overall population (n = 22) is considered [efflux index: 1.11 ± 0.04, 1.03 ± 0.02 (P = 0.005), and 1.02 ± 0.02 (P = 0.003), respectively]. No significant differences were found when BCRP-mediated efflux in different subpopulations in AML was compared with these populations in normal bone marrow. No correlation existed between BCRP expression or ABCG2-mediated mitoxantrone efflux and mitoxantrone accumulation in leukemic CD34+38− cells (r −0.22 and −0.27, respectively).

The CD34+38− cell population in AML harbors residual normal CD34+38− cells (27). To exclude the possibility that the expression and function of ABCG2 is present in residual normal cells, rather than leukemic cells, within the CD34+38− cell compartment in AML, we did fluorescence in situ hybridization analysis on leukemia-associated cytogenetic aberrations in these cells (n = 5). Table 2 shows that either BCRP expression or efflux is present in CD34+38− cells with predominantly leukemic cells within this compartment (samples 2-5). Together, these results show that BCRP is preferentially expressed and functionally active in primitive leukemic hematopoietic CD34+38− cells in the majority of patients. This phenotype reflects a conserved physiologic function, similar to the situation in normal bone marrow.

Additional Transporters Are Involved in Drug Efflux in Leukemic CD34+38− Hematopoietic Cells. The relatively small effect of BCRP blockage by KO143 on cellular mitoxantrone concentration and the lack of correlation between BCRP expression or BCRP-mediated drug efflux and mitoxantrone fluorescence in leukemic CD34+38− cells suggest that BCRP is not the major determinant of the intracellular drug concentration in these cells. To assess the contribution of other transporters to mitoxantrone efflux in leukemic CD34+38− cells, we investigated whether mitoxantrone efflux took place in CD34+38− cells in the presence of BCRP blockage. For this purpose, cells were incubated with mitoxantrone (10 μmol/L) with KO143 (0.1 μmol/L) for 2 hours and subsequently allowed to efflux mitoxantrone in drug-free medium for an additional hour in the presence of KO143 (0.1 μmol/L). In all AML samples (n = 10) mitoxantrone fluorescence (MFI) in CD34+38− cells decreased during the additional hour of efflux with an MFI decrease of 20 ± 12% (mean ± SD). When cells were put on ice after mitoxantrone accumulation to block active transport, no decrease in cellular mitoxantrone was observed. The observation that KO143 in BCRP blocking concentration cannot completely abrogate drug efflux in CD34+38− cells indicates that additional transporters are mediating mitoxantrone efflux in these cells.

To assess the role of other ATP-binding cassette transporters in drug extrusion from leukemic stem cells, ABCB1- and ABCC1-mediated mitoxantrone efflux was assessed in these 10 samples displaying drug efflux in the presence of BCRP inhibition (Table 3). CD34+38− cells in 8 of 10 samples displayed either significant ABCB1- or ABCC1-mediated transport or both. The combined effect of ABCB1 and ABCC1 inhibition on mitoxantrone accumulation exceeded the effect of BCRP blockage in all these samples. Additionally, the observation that drug efflux occurs in the presence of BCRP blockage in a sample with only BCRP-mediated efflux (sample 4) and a sample in which no ATP-binding cassette transporter activity could be shown (sample 2) suggests the presence of yet unidentified drug extrusion mechanisms.

Together these results indicate that BCRP is not the predominant drug efflux mechanism in leukemic CD34+38− stem cells and that other mechanisms, among which ABCB1 and ABCC1, play a role in drug efflux from leukemic stem cells.

Modulation of Breast Cancer Resistance Protein by the Fumitremorgin C Analogue KO143 Does Not Enhance Drug-Induced Apoptosis in Leukemic CD34+38− Cells

Because inhibition of BCRP with KO143 (0.1 μmol/L) increases mitoxantrone concentration only moderately in primitive CD34+38− leukemic cells compared with the drug-resistant MCF-7 cell line, and other drug transporters are active in these leukemic cells, we raised the question whether selective modulation of BCRP could enhance chemosensitivity of these cells. To investigate this, a 96-well cytotoxicity assay was employed on sorted CD34+38− cells in five AML.

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**Fig. 7** Specific inhibition of BCRP does not increase mitoxantrone-induced apoptosis in primitive leukemic CD34+38− hematopoietic cells. Mitoxantrone-induced cell death depicted as relative increase in apoptotic cells in sorted CD34+38− cells in the presence or absence of KO143. Values represent the mean ± SE of five patients. White columns, control experiments with KO143 at different concentrations in the absence of mitoxantrone. No significant increase in mitoxantrone-induced apoptosis is seen when KO143 is added in BCRP specific concentrations (0.1-1.0 μmol/L). Enhanced apoptosis when KO143 was used at high concentration (10 μmol/L) reflects intrinsic toxicity of the compound as shown by increased apoptosis in controls lacking mitoxantrone.
samples that displayed BCRP-mediated drug efflux [median efflux index, 1.25 (1.21-1.37); Figs. 6 and 7]. Mitoxantrone (1.0 \mu M/L) significantly induced apoptosis in leukemic CD34+/38− cells at 48 hours of incubation in all samples (relative increase in apoptotic cells compared with drug-free medium, 1.66 ± 0.23; P = 0.007; Fig. 6). KO143 at concentrations that completely block BCRP-mediated efflux (0.1-1.0 \mu M/L; as shown in the MCF7-MR cell line) did not significantly increase drug-induced apoptosis (relative increase: 1.81 ± 0.32 and 1.87 ± 0.37, respectively). Enhanced apoptosis when KO143 was used at high concentration (10 \mu M/L; average increase, 2.57 ± 0.58; Fig. 6) likely reflected intrinsic toxicity of the compound rather than modulation of drug efflux mechanisms, as shown by increased apoptosis in controls lacking mitoxantrone and the observation that KO143 at 10 \mu M/L did not further increase intracellular mitoxantrone concentration in drug accumulation assays (data not shown).

These results argue that specific modulation of BCRP, although functionally active in these samples, does not significantly enhance drug-induced apoptosis in leukemic CD34+/38− cells.

DISCUSSION

Incomplete eradication of cancer stem cells is likely to result in disease relapse. Elucidation of the mechanisms conferring chemoresistance to these cells is therefore of major importance. In this report, we show that the ATP-binding cassette transporter BCRP/ABCG2 is preferentially expressed in the leukemic CD34+/38− stem cell subpopulation and contributes to mitoxantrone efflux in these cells.

Recently, several studies have addressed the expression of BCRP in leukemic blasts in AML (22, 26). Abbott et al. (26) found relatively low levels of ABCG2 gene expression compared with drug-resistant ABCG2 clones in newly diagnosed adult AML patients. Flow cytometry revealed very small subpopulations of BCRP-expressing cells, suggesting that BCRP expression may be limited to a small cell subpopulation. Similar findings have been reported by Sargent et al. (28) who showed, using immunocytochemistry with the BXP34 antibody, that BCRP is expressed in a minority of leukemic cells in AML. Although the results of these studies suggested the possibility of high BCRP expression in a small subpopulation of cells, until now the subpopulations of BCRP-expressing cells in AML had not been identified. We here show that BCRP is preferentially expressed in a subpopulation of primitive CD34+/38− leukemic cells, which have been previously shown to comprise leukemic stem cells. BCRP expression and efflux in leukemic CD34+/38− cells was not different in expression and function from normal CD34+/38− cells. This shows that the high BCRP expression in leukemic CD34+/38− cells reflects a conserved physiologic function of BCRP in primitive hematopoietic cells rather than being a leukemia-associated phenotype. BCRP has been previously shown to be highly expressed in a wide variety of human normal stem cells (13), including hematopoietic stem cell populations (14), and is believed to protect these long-lived cells against genetic damage induced by naturally occurring xenobiotic toxins (15).

Incomplete chemotherapeutic eradication of leukemia-initiating CD34+/38− cells due to intrinsic chemoresistance is likely to result in disease relapse in AML. The preferential expression in the majority of newly diagnosed AML patients suggested that BCRP might be a target for stem cell eradication in AML. To test this hypothesis, we assessed the effect of BCRP modulation on the chemosensitivity of leukemic CD34+/38− cells. Importantly, selective blockade of ABCG2 by KO143, the most potent BCRP inhibitor currently available (16) and capable of completely blocking BCRP as confirmed in the MCF7-MR cell line, did not increase mitoxantrone-induced apoptosis to leukemic CD34+/38− cells. This lack of a chemosensitizing effect of BCRP modulation by KO143 was anticipated by several observations in this study. First, BCRP expression and ABCG2-mediated efflux were not correlated with intracellular mitoxantrone accumulation in CD34+/38− cells, suggesting that other determinants are involved in the mitoxantrone “dull” phenotype. Second, BCRP expression and the effect of KO143 on mitoxantrone accumulation were moderate in leukemic CD34+/38− cells compared with the drug-resistant MCF7-MR cell line in which BCRP confers resistance to mitoxantrone. Finally, we observed substantial drug efflux in leukemic CD34+/38− cells in the presence of BCRP inhibition, demonstrating that other drug transporters, among which ABCB1 and ABC11, are active in these cells. The presence of these promiscuous drug extrusion mechanisms might provide an explanation for the poor correlation between BCRP expression and function reported in literature (22, 29) and the observation in this study that KO143 increased mitoxantrone accumulation in only 64% of samples expressing ABCG2.

Together, these findings argue that selective modulation of BCRP is not sufficient to circumvent resistance of leukemic CD34+/38− cells. Alternatively, we cannot completely exclude the possibility that KO143 does not completely inhibit BCRP in leukemic cells. The report that KO143 is the most potent BCRP inhibitor known thus far in a panel of tested cell lines and our finding that it completely blocks efflux in the MCF7-MR cell line argue against this possibility.

Simultaneous modulation of several transporters, among which BCRP, could be required to sufficiently increase drug accumulation and eradicate CD34+/38− stem cells in AML. The report of improved clinical outcome in a trial using cyclosporin as a ABCB1 inhibitor in AML (30), in contrast to the other, consistently poor, results on selective ABCB1 modulation in long-term disease outcome, could be in line with this assumption owing to cyclosporin being a more promiscuous ATP-binding cassette transporter inhibitor with effects on both ABCB1 and BCRP (31). Together, these data warrant further research into the usefulness and feasibility of broad-spectrum ATP-binding cassette transporter inhibitors to eradicate leukemic stem cells in AML.

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REFERENCES

Breast Cancer Resistance Protein in Drug Resistance of Primitive CD34+38– Cells in Acute Myeloid Leukemia


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