Abstract

**Purpose:** Acute myelogenous leukemia (AML) is a disease originating from normal hematopoietic CD34⁺CD38⁻ progenitor cells. Modulation of the multidrug ATP-binding cassette transporter ABCB1 has not resulted in improved outcome in AML, raising the question whether leukemic CD34⁺CD38⁻ cells are targeted by this strategy.

**Experimental Design:** ABCB1-mediated transport in leukemic CD34⁺CD38⁻ cells compared with their normal counterparts was assessed by quantitating the effect of specific ABCB1 modulators (verapamil and PSC-833) on mitoxantrone retention [defined as efflux index (EI), intracellular mitoxantrone fluorescence intensity in the presence/absence of inhibitor].

**Results:** ABCB1 was the major drug transporter in CD34⁺CD38⁻ cells in normal bone marrow (n = 16), as shown by the abrogation of mitoxantrone extrusion by ABCB1 modulators (EI, 1.99 ± 0.08). Surprisingly, ABCB1-mediated drug extrusion was invariably reduced in CD34⁺CD38⁻ cells in AML (n = 15; EI, 1.21 ± 0.05; P < 0.001), which resulted in increased intracellular mitoxantrone retention in these cells (mitoxantrone fluorescence intensity, 4.54 ± 0.46 versus 3.08 ± 0.23; P = 0.004). Active drug extrusion from these cells occurred in the presence of ABCB1 modulators in the majority of samples, pointing in the direction of redundant drug extrusion mechanisms. Residual normal CD34⁺CD38⁻ cells could be identified by their conserved ABCB1-mediated extrusion capacity.

**Conclusion:** ABCB1-mediated drug extrusion is reduced in leukemic CD34⁺CD38⁻ progenitor cells compared with their residual normal counterparts. Redundant drug transport mechanisms confer mitoxantrone transport from leukemic progenitors. These data argue that ABCB1 modulation is not an effective strategy to circumvent drug extrusion from primitive leukemic progenitor cells and may preferentially target residual normal progenitors in AML.

Acute myelogenous leukemia (AML) is considered a disease originating from CD34⁺CD38⁻ hematopoietic stem cells (1). This has been conclusively shown in studies using the nonobese diabetic-severe combined immunodeficient mouse model showing that cells with leukemic engraftment and self-renewal potential in AML are found in the CD34⁺CD38⁻ subpopulation (2). This CD34⁺CD38⁻ phenotype of leukemia-initiating cells was observed regardless of the lineage markers expressed by the leukemic blasts, percentage of cells expressing the CD34 surface antigen, or the French-American-British subtype. These studies stress that leukemia-initiating transformation and progression-associated genetic events occur at the level of these primitive CD34⁺CD38⁻ cells. Consequently, incomplete chemotherapeutic eradication of these cells may ultimately result in disease relapse. Therefore, elucidation of the mechanisms conferring resistance against chemotherapy to these cells is of crucial importance (3).

The ABCB1-encoded P-glycoprotein is a highly conserved membrane-bound ATP-binding cassette (ABC) transporter, which extrudes a wide variety of structurally unrelated chemotherapeutic compounds across the cell membrane (4), conferring the multidrug resistance phenotype in cancer cells. ABCB1 is highly expressed in normal hematopoietic CD34⁺CD38⁻ stem cells (5, 6).

The observation that it is frequently expressed in AML and that expression is associated with adverse treatment outcome (7) has led to the introduction of ABCB1 modulators to enhance chemotoxicity of various chemotherapeutic agents in clinical trials. Phase III clinical trials using PSC-833 (valspodar), the most potent and specific inhibitor of ABCB1 function today, however, failed to reach their intended end points of improved complete remission and survival and have been complicated by increased toxicity (8–12). In contrast, a clinical trial using cyclosporin A, which is a broad-spectrum ABC
transporter inhibitor, showed clinical benefit (13). The reasons for the lack of benefit of ABCB1 modulation in AML remain to be elucidated. In interpreting these negative results, it is important to know whether the leukemia-initiating CD34+CD38+ hematopoietic subpopulation is targeted by ABCB1 modulation and whether additional transporters confer drug extrusion from these cells, which may explain the beneficial effect of broad-spectrum inhibition. The vast amount of studies on ABCB1 expression and function in AML has been done on general blast populations. The goal of the current study was to investigate ABCB1 expression and function in leukemic CD34+CD38+ cells and the effect of ABCB1 modulators on drug retention in these cells compared with their normal counterparts.

Materials and Methods

Bone marrow samples. Bone marrow was obtained after informed consent from healthy bone marrow donors and patients with AML at diagnosis. Mononuclear cells were isolated by Ficoll (1.077 g/mL density centrifugation; Pharmacy Biotech, Uppsala, Sweden). Isolation, cryopreservation, and thawing procedures of cells have been described previously (14) and were identical for normal and leukemic bone marrow samples. CD34+CD38- hematopoietic cells. CD34+CD38- cells were defined flow cytometrically as described previously (15) and are shown in Fig. 1A. The flow cytometric assessment of ABCB1 expression. ABCB1 expression was analyzed using the ABCB1-specific antibody MRK16, recognizing an external epitope of the protein in three-color flow cytometric assays as described previously (7). ABCB1 protein expression was quantitated as the median fluorescence channel shift (MRK16/IgG2a isotype control) in CD34+CD38-, CD34+CD38+, and CD34- cells populations. Differences in fluorescence are assessed using the Kolmogorov-Smirnov test, denoted as D. This method accurately identifies small differences in fluorescence and is useful in detection of low-level ABCB1 expression (7). MRK16 staining intensity is categorized as follows: negative (D < 0.10), dim (0.10 < D < 0.15), moderate (0.15 < D < 0.25), and bright (D > 0.25).

Flow cytometric assessment of ABCB1-mediated mitoxantrone transport. Cells were stained with CD34-FITC and CD38-PE membrane markers, washed in HBSS 1% FCS, and preincubated with or without verapamil (20 μg/mL; Knoll AG, Ludwigshafen, Germany; ref. 16) or PSC-833 (2 μmol/L; Novartis Pharma AG, Basel, Switzerland; ref. 17) as inhibitors for ABCB1-mediated transport for 20 minutes in Iscove’s modified Dulbecco’s medium supplemented with 1% FCS. Verapamil at the concentration used in this study is regarded ABCB1 specific without inhibition of ABCG2 [breast cancer resistance protein (BCRP); ref. 18] and ABCG1 (multidrug resistance protein; ref. 19). Although inhibition of ABCG2 by verapamil has been described at higher concentrations (20). Mitoxantrone (Novantrone; 10 μmol/L; Lederle, Ettten-Leur, the Netherlands) was added, and cells were incubated for 2 hours at 37°C, 5% CO2, with or without verapamil or PSC-833. Subsequently, cells were allowed an additional 1-hour efflux in drug-free medium with or without inhibitor. The combined uptake and efflux with or without inhibitor assay was chosen because it has higher sensitivity for ABC transporter function compared with assays using only uptake with or without inhibitor (15).

Cellular mitoxantrone fluorescence was measured at a single time point after the additional hour of efflux in drug-free medium on a flow cytometer (Coulter Elite, Beckman Coulter, Fullerton, CA) equipped with an argon laser. Fluorescence was assessed at an excitation wavelength of 635 nm through a 670-nm bandpass filter in a three-color protocol with CD34-FITC and CD38-PE. At least 200 CD34+CD38- cells were analyzed in each sample. ABCB1-mediated transport was depicted as the ratio of intracellular mitoxantrone fluorescence intensity (MFI) in the presence or absence of inhibitor [defined as efflux index (EI)]. To assess interexperimental reproducibility, ABCB1-mediated transport in CD34+CD38- cells was assessed in a normal bone marrow sample in completely independent experiments done on separate days (n = 7). ABCB1-mediated EI was 2.10 ± 0.09 (mean ± SE), indicating good reproducibility of the assay. This sample was subsequently included in other experiments as a control. Preliminary experiments showed similar ABCB1-mediated efflux in cryopreserved samples when compared with fresh cells in normal bone marrow and AML, in line with a previous report addressing this issue (21).

Figure 1. ABCB1-mediated drug extrusion is reduced in CD34+CD38- progenitor cells in AML compared with their normal counterparts. A, representative examples of normal bone marrow (NBM; top) and AML (bottom). CD34+CD38- cells were defined flow cytometrically as the CD34-FITC + cells (blue) with CD38-PE expression within the first decade of fluorescence emission (red) and compared with CD34+CD38- cells (blue gated) with exclusion of a decade between CD38- and CD38+ cells. Gray CD34+CD38- cells exhibited restricted light-scattering characteristics (SSC), confirming the lymphoid appearance of these cells (inset). The median frequency of CD34+CD38- cells was 0.1% of mononuclear cells (range, 0.1-0.3%) in normal bone marrow and 0.2% of mononuclear cells (range, 0.1-1.0%) in AML. No difference existed in average CD38 density between normal and leukemic CD34+CD38- cells (MFI, 0.57 ± 0.05 SD; range, 0.40-0.70; MFI, 0.53 ± 0.12 SD; range, 0.30-0.70). B, representative examples of flow cytometric assessment of ABCB1-mediated mitoxantrone transport in CD34+CD38- cells in normal bone marrow (top) and AML (bottom). Mean intracellular MFI was assessed in CD34+CD38- cells as described in Materials and Methods. Normal CD34+CD38- cells display low intracellular mitoxantrone fluorescence compared with CD34+CD38- cells (inset), which can be increased significantly by blockade of ABCB1-mediated transport by verapamil/PSC-833 (EI, 2.29). CD34+CD38- cells in AML display increased intracellular mitoxantrone retention compared with CD34+CD38- cells from normal bone marrow due to reduced ABCB1-mediated transport as illustrated by a lack of effect of verapamil/PSC-833 on intracellular mitoxantrone fluorescence (EI, 0.98). C, representative examples of the effect of ABCB1 inhibition by verapamil on rhodamine 123 fluorescence in CD34+CD38- cells assessed by single-cell image analysis. For experimental procedure, see Materials and Methods. Encircled, cells. Normal CD34+CD38- cells (top) display low retention of rhodamine 123, which can be significantly increased when verapamil is added (EI, 3.24). ABCB1-mediated rhodamine 123 extrusion from CD34+CD38- cells is reduced in AML (bottom), illustrated by the lack of effect of ABCB1 inhibition by verapamil on intracellular rhodamine 123 fluorescence (EI, 1.00).

www.aacrjournals.org
Clin Cancer Res 2006;12(11) June 1, 2006 3453
CancerResearch.

Downloaded from clincancerres.aacrjournals.org on April 20, 2017. © 2006 American Association for Cancer Research.
Single-cell image analysis for assessment of ABCB1-mediated transport of rhodamine 123 in CD34+CD38- cells. ABCB1-mediated transport of rhodamine 123 was assessed in CD34+CD38- cells sorted on adhesive biolayers as comprehensively described elsewhere (22). Briefly, 500 CD34+CD38- cells were sorted in 8 μL washing buffer solution containing 0.9% NaCl supplemented with sodium dihydrogen phosphate (6.6 mM), albumin (1%, v/v), and glucose (1%, v/v) applied on a glass coverslip coated with mussel adhesive protein (10 μL/cm²). Cells were allowed to adhere for 10 minutes in a perfusion chamber constructed to enable cell environment manipulation (incubation and washing steps) within the microscope environment. The microscope was placed in an incubator to allow all procedures to run at 37°C, which is essential for physiologic efflux studies.

After immobilization, cells were incubated with rhodamine 123 dissolved in Iscove’s modified Dulbecco’s medium containing 0.5% (v/v) heat-inactivated FCS (HyClone, Logan, UT) with or without efflux modulator during 30 minutes. Cells were washed with washing buffer at 37°C, again in the presence or absence of efflux modulators, and allowed to efflux the dye for an additional 15 minutes. Fluorescence images were acquired after 15 minutes of efflux. Cells were excited with a mercury arc lamp using bandpass filter of 440 to 490 nm for rhodamine 123 or bandpass filter of 510 to 560 nm for propidium iodide. Emission was measured with bandpass filter of 515 to 565 nm for ABCB1-mediated efflux and 585 to 610 nm for propidium iodide. Cell viability typically exceeded 90% of cells. Dead cells were excluded from analysis.

Fluorescence intensity analysis of individual cells was done on TCell Image 4.6 software package (TNO, Delft, the Netherlands). At least 30 cells were evaluated per sample. Fluorescence intensity of the overall cell population is expressed as MFI per 10,000 cells and depicted as “EI.”

Fluorescence in situ hybridization. CD34+CD38- 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 (8:21) probe, or LSI EGR1 (5q31) dual-color probe (Vysis, Downsview, IL) according to the manufacturer’s instructions. Cells (100) were analyzed microscopically for (8:21) or chromosome 5 (5q31) cytogenetic abnormalities as appropriate.

Adapted real-time quantitative reverse transcription-PCR for assessment of CBFB-MYH11 gene expression in CD34+CD38- cells. A linear real-time quantitative reverse transcription-PCR approach has been developed for quantitation of gene expression in low-frequency hematopoietic stem cells and was described comprehensively elsewhere (6). Primer and probe sequences were as follows: glyceraldehyde-3-phosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase control reagents (Applied Biosystems, Foster City, CA; part 402869); CBFB-MYH11 (type D) fusion gene, 5'-CATTAGCACACAGGCCTTTGA-3' (forward), 5'-CTCCGTITAGCCTCCCTGTGA-3' (reverse), and TET-ATAGACAGGCTCATCG (probe).

Normalized gene expression to the internal standard glyceraldehyde-3-phosphate dehydrogenase is given by the following equation: To/Ro(Xn) = K (1 + E) (CTn – CT), where To is the initial number of target gene copies, Ro is the initial number of standard gene copies, E is the efficiency of amplification, CTn is the threshold cycle of target gene, CT is the threshold cycle of standard gene, and K is constant.

Statistical analysis. Differences in ABCB1 protein expression (D of MRK16/isotype control) and function (EI) between different normal and leukemic cell populations were calculated using the Student’s t test with a level of significance of P < 0.05.

Results

ABCB1 is the major determinant of mitoxantrone extrusion in CD34+CD38- cells in normal bone marrow. CD34+CD38- hematopoietic cells were flow cytometrically defined as shown in Fig. 1A. ABCB1 protein expression was assessed in hematopoietic cell populations using the monoclonal antibody MRK16. ABCB1 was differentially expressed in CD34+CD38- cells compared with more differentiated cell populations (Fig. 2). A decrease in ABCB1 protein expression in CD34+CD38- and CD34+ cells was observed in all samples examined.

CD34+CD38- cells in normal bone marrow were mitoxantrone “null” compared with CD34+CD38+ cells (MFI, 3.08 ± 0.23 versus 7.29 ± 0.50; n = 16; P < 0.001). Modulation of ABCB1-mediated transport by verapamil significantly increased mitoxantrone fluorescence in CD34+CD38- cells (Fig. 1B). In line with expression profiles, ABCB1-mediated efflux was invariably higher in CD34+CD38- cells (mean EI, 1.99 ± 0.08; range, 1.64-2.92) compared with more differentiated CD34+CD38+ cells (1.45 ± 0.06; Fig. 3A). Serial measurements of mitoxantrone fluorescence in CD34+CD38- cells both after 2-hour exposure with mitoxantrone and after an additional hour of efflux in drug-free medium done in a subpanel of samples (n = 7) showed complete abrogation of mitoxantrone extrusion by ABCB1 modulation (Fig. 4A).

Verapamil is regarded ABCB1-specific, although inhibition of ABCG2 (BCRP) has been observed at concentrations considerably higher than used in our experiments. To definitely confirm that the drug efflux from normal CD34+CD38- cells was indeed ABCB1 mediated, additional experiments were done. First, similar results were found when the ABCB1-specific inhibitor PSC-833 was used in a subgroup of samples (n = 4; mean EI ± SE, 2.03 ± 0.12 and 1.38 ± 0.09 for CD34+CD38- and CD34+CD38+ cells, respectively; Fig. 1B), showing that the observed efflux is ABCB1 rather than BCRP mediated because PSC833 is not an inhibitor of BCRP. This was further confirmed using rhodamine 123 as a substrate, which is not a substrate for BCRP (23), in an independent assay in single CD34+CD38- cells sorted on an adhesive biolayer (Fig. 1C). Rhodamine 123 retention was significantly lower in CD34+CD38- cells compared with CD34+CD38+ cells in normal bone marrow (P < 0.001), similar to the mitoxantrone null phenotype of CD34+CD38- cells. Blocking of ABCB1-mediated transport by verapamil increased rhodamine 123 fluorescence significantly.
Reduced ABCB1 Function in Leukemic Progenitor Cells

ABC21-mediated efflux was found in all examined (n = 5) CD34+CD38+ samples (mean EI, 3.74 ± 1.81; Fig. 3B). Together, these results show that ABCB1-mediated transport is the major determinant of the mitoxantrone dull phenotype of CD34+CD38+ cells in human normal bone marrow and that its modulation completely abrogates mitoxantrone extrusion from these cells.

**ABC21-mediated transport is reduced in CD34+CD38+ hematopoietic cells in AML.** Conserved ABCB1-mediated drug extrusion from CD34+CD38+ cells after malignant transformation would be an important obstacle for chemotherapeutic eradication of these cells in AML. We therefore investigated ABCB1 expression and function of this cell population in AML compared with their counterparts in normal bone marrow.

Investigations were done on a panel of bone marrow samples of 20 untreated AML patients (Table 1). The panel consisted predominantly of CD34+ leukemias, with CD34+ cells median percentage of 31% (range, 1-91). CD34+CD38+ cells in AML were immunophenotypically defined as indicated in Fig. 1A. Fluorescence in situ hybridization analyses in AML patients who carried a cytogenetic abnormality and from whom sufficient cells were available for analysis showed a predominantly leukemic character of CD34+CD38+ cells in these samples (n = 5; Table 2) as described previously (24).

ABC21 expression in CD34+CD38+ cells in AML as assessed by the MRK16 monoclonal antibody was comparable with expression of their counterparts in normal bone marrow (Fig. 2). Similar to the expression in normal bone marrow, decreased protein expression in CD34+CD38+ and CD34- subsets was observed in all samples.

Surprisingly, ABCB1-mediated mitoxantrone transport was invariably reduced in CD34+CD38+ cells in AML compared with their counterparts in normal bone marrow as shown by a decreased effect of ABCB1 modulation by verapamil on intracellular mitoxantrone fluorescence (mean EI, 1.21 ± 0.05; range, 0.93-1.51; P < 0.0001; Figs. 1B and 3A). Similar results were found when PSC-833 was used as an inhibitor (n = 4; mean EI in CD34+CD38+ cells, 1.23 ± 0.16; Fig. 1B). Reduced ABCB1 function was not restricted to CD34+CD38+ cells in AML; reduced ABCB1 function compared with normal counterparts was also found for CD34+CD38- cells (mean EI, 1.17 ± 0.03; range, 1.05-1.31; P < 0.001). Reduced ABCB1-mediated transport in CD34+CD38- cells in AML was confirmed using rhodamine 123 as a substrate in single cells (Figs. 1C and 3B). CD34+CD38- cells in AML (n = 8) were rhodamine 123 dull compared with the more differentiated CD34+CD38+ cells, but ABCB1 modulation by verapamil did not increase intracellular retention (mean EI, 0.99 ± 0.07; Fig. 3B). Of interest, analysis of mitoxantrone efflux mediated by the ABC transporters ABCG2 (BCRP), done in a series of normal and leukemic bone marrow samples, including those described in this article, using the ABCG2-specific fumitremorgin C analogue KO143 as inhibitor, showed no difference between normal and leukemic CD34+CD38- cells (15), further showing that the observed decrease in mitoxantrone efflux is indeed due to deficient ABCB1 rather than ABCG2 activity.

Serial measurements both after 2 hours of drug exposure and after an additional hour in drug-free medium in a subpanel of AML samples (n = 10; Fig. 4B) showed significant extrusion of mitoxantrone in the presence of verapamil in the majority of patients, pointing in the direction of redundant drug extrusion mechanisms.

These redundant drug transport mechanisms, however, did not compensate for the reduced ABCB1-mediated transport from leukemic CD34+CD38- cells as suggested by the significantly higher mitoxantrone retention in leukemic CD34+CD38- cells compared with their counterparts in normal bone marrow after drug exposure and subsequent efflux in drug-free medium (MFI, 4.54 ± 0.46 versus 3.08 ± 0.23; P = 0.004). These findings strongly suggest that the decreased efficacy of ABCB1 modulation on mitoxantrone retention of leukemic CD34+CD38- cells is indeed due to reduced ABCB1 activity rather than increased activity of other transport mechanisms, which would have resulted in similar or lower retention of mitoxantrone compared with their normal CD34+CD38- counterparts.

Translated to the clinical context, these data indicate that modulation of ABCB1 has limited effect on mitoxantrone retention in leukemic CD34+CD38- cells due to reduced activity of ABCB1 in leukemic CD34+CD38- cells. Drug efflux from these cells occurs in the presence of ABCB1 modulation due to the activity of promiscuous transport mechanisms.
**Discussion**

In this report, we show that ABCB1-mediated drug transport is reduced in leukemic CD34+CD38+ progenitor cells compared with their normal counterparts in AML.

This finding was initially unanticipated because AML is generally considered to be an ABCB1-overexpressing malignancy with conserved physiologic function (25). This assumption, however, is based on the observation that ABCB1 is preferentially expressed in CD34+ blasts in AML (26) similar to preferential expression of ABCB1 in these cells in normal bone marrow (27), thus reflecting similar patterns of expression rather than direct comparison of ABCB1 expression and function of normal and malignant cell subpopulations. Expression data in our study confirm the preferential expression of ABCB1 in CD34+ cells in AML (26) at levels comparable with those observed in normal bone marrow. ABCB1 transport capacity, however, was markedly impaired in both CD34+CD38+ and CD34+CD38− cells in AML.

The occurrence of ABCB1− protein− efflux− leukemic blasts has been observed in a minority of AML cases (28) earlier, pointing in the direction of the presence of nonfunctional forms of the ABCB1 protein, but the current report, aimed at directly comparing ABCB1 function between normal and leukemic primitive progenitor cells, shows that impaired ABCB1 function is a biological commonality rather than an occasional finding in AML.

The molecular determinants behind impaired ABCB1-mediated transport in hematopoietic cells in AML remain to be elucidated and may comprise both downstream event of oncoproteins central in leukemogenesis, such as AML1-ETO (29) and TEL-AML1 (30), and factors governed by the cellular environment, such as disruption of cellular ATP metabolism, membrane integrity, or cell cycle regulation (31).

**Table 1.** Patient characteristics

<table>
<thead>
<tr>
<th>N</th>
<th>Sex</th>
<th>French-American-British</th>
<th>% CD34</th>
<th>Cytogenetics</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>Male</td>
<td>9</td>
<td>31 (1-91)</td>
<td>9</td>
</tr>
<tr>
<td>11</td>
<td>Female</td>
<td>8</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>50 (15-71)</td>
<td>M1</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M2</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M4</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M5</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>t(8;21)</td>
<td>4 (+2 complex)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(6;11)</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>45XO</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Complex</td>
<td>4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Regardless of the underlying mechanism, the finding that ABCB1-mediated transport is impaired in CD34+CD38−/− cells in AML has important clinical implications. The recognition of AML as a disease originating from these cells dictates a paradigm shift in the treatment toward the eradication of this crucial stem cell population in AML.

Our results indicate that ABCB1 modulation does not circumvent drug extrusion from these cells and that additional, redundant drug extrusion mechanisms determine the relatively dull mitoxantrone appearance of these cells. We have recently documented ABCG2/BCRP as one of these additional drug transporters in leukemic CD34+CD38− cells (15). Additionally, using low-density real-time PCR arrays, we recently showed differential expression of all 13 ABC transporters currently associated with drug extrusion in CD34+CD38− cells in AML (32). These studies, however, did not show higher expression or function of these transporters in leukemic CD34+CD38− cells compared with their normal counterparts. This may explain the observation in this study that intracellular retention of mitoxantrone is increased in leukemic CD34+CD38− cells compared with their normal counterparts, suggesting that redundant mechanisms confer drug extrusion from leukemic

### Table 2. CD34+CD38− cells in AML are predominately of leukemic origin

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Cytogenetic aberrancy</th>
<th>% Cyto. aberrant CD34+CD38− cells</th>
<th>Rhodamine 123 (SD)</th>
<th>Mitoxantrone (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>−5; −7; −15; −17; t(2;3)</td>
<td>56</td>
<td>1.32</td>
<td>0.84</td>
</tr>
<tr>
<td>2</td>
<td>t(8;21)</td>
<td>90</td>
<td>NA</td>
<td>0.92</td>
</tr>
<tr>
<td>3</td>
<td>t(8;21)</td>
<td>95</td>
<td>1.15</td>
<td>0.81</td>
</tr>
<tr>
<td>4</td>
<td>t(8;21)</td>
<td>90</td>
<td>1.28</td>
<td>1.39</td>
</tr>
<tr>
<td>5</td>
<td>t(8;21)</td>
<td>100</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Abbreviation: NA, material not available for analysis.

*Values normal bone marrow: mean (range).

Figure 5. ABCB1-mediated mitoxantrone transport identifies a subpopulation that harbors residual normal CD34+CD38− cells in a patient with CBFB-MYH11 AML. A patient was identified in which mitoxantrone discriminated a population of CD34+CD38− mitoxantrone dull cells. The dull phenotype was determined by strong ABCB1-mediated efflux (CD34+CD38− ABCB1 efflux ++ ) within the range observed in CD34+CD38− cells observed in normal bone marrow (El 2.50). Real-time PCR confirmed the residual normal character of these cells.
cells but that this redundant action does not completely compensate for the reduced ABCB1 function in these cells.

The combined data suggest that the prognostic value of ABCB1, one of the main rationales for ABCB1 targeting in AML, may be pleiotropic, identifying leukemias with a primitive CD34+ (CD38−) character and innate drug resistance due to differential expression of many, promiscuous ABC transporters and possibly other mechanisms protecting primitive progenitor cells. Modulation of additional transporters is therefore likely to be required to abrogate drug extrusion and to enhance drug-induced eradication of leukemia stem cells in AML. These laboratory findings provide an alternative explanation for the poor results of ABCB1 modulation by the highly potent ABCB1 inhibitor PSC-833 and other inhibitors on long-term disease outcome in clinical trials (8–10). The report of improved clinical outcome in a trial using cyclosporin as an ABCB1 inhibitor in AML (13) may reflect these findings because cyclosporin is a more promiscuous ABC transporter inhibitor with effects on ABCB1 and other ABC transporters (33).

Additionally, the current study shows that residual normal CD34+/CD38− cells are likely to harbor within the ABCB1 efflux ++ subpopulation. This seems congruent with the report (34) that the capacity to efflux Hoechst 33342 (side population identifies a subpopulation of CD34+/CD38− residual normal cells in AML, whereas CD34+/CD38− Hoechst bright cells were leukemic. Although these authors could not relate Hoechst 33342 efflux with ABCB1 protein expression in the overall side population of cells, this was not investigated in the CD34+/CD38− subset. The current report shows that differences in ABCB1-mediated efflux discriminate normal from leukemic CD34+/CD38− cells. This finding implies that ABCB1 modulation may preferentially target residual normal hematopoietic cells, whereas drug extrusion from leukemic CD34+/CD38− cells occurs through the activity of redundant mechanisms. Together, these data argue against the use of ABCB1 modulation as a strategy to eradicate leukemic stem cells in AML but prompt further research to elucidate the mechanisms involved in the extrusion of drugs from leukemic CD34+/CD38− cells to provide novel targets for the eradication of this crucial cell population in AML.

Acknowledgments

We thank L. Heuer, A. Pennings, and G. Vierwinden for technical assistance; Hematology Data Base Center Nijmegen for providing material and clinical data; and F. Russel for critically reviewing the article.

References

2. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primi-
cular, cellular, and pharmacological aspects of the multi-
8. van der Holt B, Louwenberg B, Burnett AK, et al. The value of the MDR1 reversal agent PSC-833 in addition to daunorubicin and cytarabine in the treatment of eld-
erly patients with previously untreated acute myeloid leukemia (AML, in relation to MDR1 status at diagno-
9. Baer MR, George SL, Dodge RK, et al. Phase 3 study of the multidrug resistance modulator PSC-833 in previously untreated patients 60 years of age and older with acute myeloid leukemia: Cancer and Leuke-
10. Greenberg PL, Lee SJ, Advani R, et al. Mitoxantrone, etoposide, and cytarabine with or without valspodar in patients with relapsed or refractory acute myeloid leu-
11. Mahadevan D, List AF. Targeting the multidrug resis-
16. Uyts K, Tsuro T. Reversal mechanism of multidrug resistance by verapamil: direct binding of verapamil to P-glycoprotein on specific sites and transport of ve-
cumvention of P-glycoprotein-mediated multidrug re-
19. Lauepezo B, Ansell L, Puyen L, et al. Multidrug resis-
tance protein (MRP) activity in normal mature leuko-
25. List AF, Spier CM. Multidrug resistance in acute leu-
kemia: a conserved physiologic function. Leuk Lym-
27. Drach D, Zhao S, Drach J, et al. Subpopulations of normal peripheral blood and bone marrow cells ex-
29. Lutterbach B, Sun D, Schuett J, Hiebert SW. The MYND motif is required for repression of basal tran-
scription from the multidrug resistance 1 promoter by the 1(8;21) fusion protein. Mol Cell Biol 1998;18:3604–11.
30. Miyazaki K, Uchida H, Miyachi H, et al. TEL/AML1, the leukemia-related chimeric protein overcomes drug resistance through transcriptional repression of multi-
drug resistance-1 (MDR1) gene expression [abstract 242]. Blood Suppl 2002;100:
31. Smeets ME, Raymakers RA, Vierwinden G, et al. Triggering noncyling hematopoietic progenitors and leukemic blasts to proliferate increases anthracline resistance and toxicity by downregulating multidrug re-
32. de Gouw EP, Raaijmakers MHPG, Boezemans J, et al. Preferential expression of a high number of ATP binding cassette transporters in both normal and leuke-
33. Ould M, O’Loughlin KL, Fricke SM, et al. Cyclo-
34. Feuring-Buske M, Hogge DE. Hoechst 33342 ef-
ABCB1 Modulation Does Not Circumvent Drug Extrusion from Primitive Leukemic Progenitor Cells and May Preferentially Target Residual Normal Cells in Acute Myelogenous Leukemia


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/12/11/3452

Cited articles
This article cites 33 articles, 20 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/12/11/3452.full.html#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
/content/12/11/3452.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.