Abstract

Purpose: Overexpression of the transporter ABCG2, also known as breast cancer resistance protein and mitoxantrone resistance protein, can confer resistance to a variety of cytostatic drugs, such as mitoxantrone, topotecan, doxorubicin, and daunorubicin. This study analyzes the ABCG2 expression and activity in 46 human de novo acute lymphoblastic leukemia B- and T-lineage (ALL) samples.

Experimental Design: ABCG2 expression was measured flow cytometrically with the BXP-34 monoclonal antibody. ABCG2 functional activity was determined flow cytometrically by measuring mitoxantrone accumulation in combination with the ABCG2 inhibitor fumitremorgin C (FTC). To determine a possible effect of the transporters P-glycoprotein and multidrug resistance-associated protein (MRP1 and MRP2) on mitoxantrone accumulation, the accumulation was investigated in the presence of the P-glycoprotein inhibitor PSC 833 and MRP inhibitor MK-571. The ABCG2 gene was sequenced to investigate the amino acid at position 482.

Results: In B-lineage ALL (n = 23), the median BXP-34/IgG1 ratio was higher, namely 2.4 (range, 1.7–3.7), than in T-lineage ALL (n = 23; 1.9; range, 1.2–6.6; P = 0.003). The addition of FTC to mitoxantrone treatment caused a median increase in mitoxantrone accumulation of 21% (range, 0–140%) in B-lineage ALL. In T-lineage ALL, this FTC effect was less pronounced (5%; range, 0–256%; P = 0.013). The influence of FTC on mitoxantrone accumulation correlated with ABCG2 protein expression (r = 0.52; P < 0.001; n = 43). The increase in mitoxantrone accumulation, when FTC was added to cells treated with both PSC 833 and MK-571, correlated with the ABCG2 expression in B-lineage ALL but not in T-lineage ALL. Sequencing the ABCG2 gene revealed no ABCG2 mutation at position 482 in patients who accumulated more rhodamine after FTC.

Conclusions: This study shows that ABCG2 is expressed higher and functionally more active in B-lineage than in T-lineage ALL.

Introduction

The prognosis of ALL has improved over the last decades, in children even more than in adults. Still, many ALL patients do not achieve a complete remission or develop a relapse (1, 2). Resistance to chemotherapeutic agents plays an important role. MDR is defined as resistance to multiple, structurally and functionally unrelated natural product drugs. One mechanism of MDR is the overexpression of members of the ABC superfamily of membrane transporters. This family of transporters consists of a number of proteins, including P-gp and the MRPs (MRP1 and MRP2). Contradictory results have been observed with respect to the prognostic impact of P-gp expression and functional activity (3–8). For MRP1 protein and mRNA expression, neither a difference has been found between initial and relapse samples, nor a correlation with complete response or survival (3). We have studied previously the functional activity of MRP1 and MRP2 in children and adults with ALL, and observed no correlation between functional activity and clinical outcome (9).

Another ATP-binding cassette transporter has been identified in a MDR human breast cancer cell line MCF-7/AdrVp (10, 11). This cell line shows an ATP-dependent reduction in the intracellular accumulation of anthracycline anticancer drugs, in the absence of overexpression of known MDR transporters such as P-gp or MRP. This transporter protein was named ABCG2, and is also known as breast cancer resistance protein, placental ABC transporter, or mitoxantrone resistance protein. Several human cancer cell lines selected for resistance to mitoxantrone or topotecan have shown a marked ABCG2 overexpression. In addition, enforced expression of the full-length ABCG2 cDNA in MCF-7 breast cancer cells confers resistance to mitoxantrone, bisantrene, topotecan, and doxorubicin or daunorubicin in the presence of verapamil (11–14). The physiological function of ABCG2 may be similar to P-gp, because it is also located in the plasma membrane.
blood-brain barrier, the placenta, the liver, and intestinal tract (15). The spectrum of ABCG2 substrates shows an overlap with that of P-gp and MRP. All three of the transporters can extrude the anthracyclines, such as daunorubicin, doxorubicin, and epirubcin. P-gp (16), ABCG2, and probably MRP1 (17, 18) can also transport mitoxantrone. There are indications that the spectrum of drugs transported by ABCG2 might differ in cell lines because of a mutation in the ABCG2 gene at amino acid position 482 (19). When a mutation is present in the ABCG2 gene with threonine or glycine situated at position 482 instead of arginine, ABCG2 is able to transport rhodamine and doxorubicin additionally (19) but not methotrexate (20).

Mitoxantrone, methotrexate, daunorubicin, and doxorubicin are used frequently during the induction or consolidation phase in ALL, indicating that ABCG2 might have clinical impact in this patient group. The present study demonstrates that the ABCG2 protein is expressed in ALL. The functional activity was measured by mitoxantrone accumulation in the absence or presence of the ABCG2 inhibitor FTC (21), and FTC in combination with the inhibitors of the other transporters P-gp and MRP1, which might obscure the ABCG2 function (12, 22). The ABCG2 gene was sequenced in patients who accumulated more rhodamine after FTC, to investigate the amino acid at position 482. Because ABCG2 expression is correlated with an immature phenotype in normal human hematopoietic cells (15, 23, 24) and AML blasts (21), we also determined the immunophenotype of the patient samples.

Materials and Methods

Cell Lines. The drug-sensitive human breast cancer cell line MCF-7 and its mitoxantrone-resistant counterpart MCF-7 MR, which overexpresses ABCG2 but not P-gp or MRP-1, were cultured in RPMI 1640 (Bio Whittaker, Brussels, Belgium) supplemented with 10% FCS (HyClone, Logan, UT). The MCF-7 MR cell line was cultured in the presence of 80 nM mitoxantrone. The human small cell lung cancer cell line GLC4, the in vitro doxorubicin-selected MRP1-overexpressing subline GLC4/ADR, and the MDR-1 transfected cell line GLC4/P-gp were also cultured in RPMI 1640 with 10% FCS. The doxorubicin-selected cell line was cultured in the presence of doxorubicin (2 μM; Pharmacia and Upjohn, Woerden, the Netherlands) and the MDR-1 transfected cell line in the presence of 50 nM vincristine (Teva Pharma BV, Mijdrecht, the Netherlands). Cells were cultured in drug-free medium at least 7 days before the analyses. The cell lines served as controls for the ABCG2 expression and functional assay.

Patient Samples. From all of the patients newly diagnosed with ALL at the University Hospital Groningen during the period of 1989–2001, bone marrow and peripheral blood samples were collected for diagnostic evaluation and cryopreserved. Patients were included in the study if a sufficient number of preserved bone marrow or peripheral WBCs (>10 x 10^6) was available. The Dutch Childhood Leukemia Study Group performed the immunophenotyping and cytomorphology of pediatric samples. Adult patient samples were immunophenotyped by the University Hospital Groningen. The children were treated according to the Dutch Childhood Leukemia Study Group protocols ALL-8 or -9 (25), containing the MDR drugs dexamethasone, daunorubicin, doxorubicin, methotrexate, and vincristine. The adult patients received a regimen including the MDR drugs prednisone, doxorubicin, vincristine, methotrexate, and etoposide (26).

Mononuclear cells from bone marrow or peripheral blood were isolated on a Ficoll-Isoopaque (Nycomed, Oslo, Norway) density gradient by centrifugation for 20 min at 800 x g. The cells were cryopreserved in RPMI 1640 supplemented with 10% FCS and 10% DMSO (Merck, Amsterdam, the Netherlands) and stored at −196°C. Upon analysis cells were thawed, centrifuged in newborn calf serum (Life Technologies, Inc., Breda, the Netherlands), treated with DNase (Boehringer Mannheim, Mannheim, Germany), and washed with RPMI 1640. Immunophenotyping showed that all of the samples contained >90% of leukemic cells. For immunophenotyping, 5 x 10^6 cells were incubated with 5 μL of FITC- or phycoerythrin-labeled mouse monoclonal antibodies to CD117, CD3, CD7 (all IQ Products, Groningen, the Netherlands), CD2, CD10, CD19, CD20, CD33, CD34, or IgG isotype-matched controls (all Becton Dickinson, Woerden, the Netherlands) for 30 min at 4°C. Subsequently, cells were washed with RPMI 1640 and analyzed with a FACStar flow cytometer (Becton Dickinson Medical Systems, Sharon, MA). The immature markers CD34 and CD117, expressed only on progenitor cells, were analyzed in both B-lineage and T-lineage ALL. For B-lineage ALL samples, the B-lineage markers CD10, CD19, and CD20 were used. The T-lineage markers CD3 and CD7 were investigated in T-lineage ALL samples. Because the myeloid marker CD33 correlated with P-gp functional activity in an earlier study (9), CD33 expression was also measured.

Flow Cytometric Detection of ABCG2 Protein Expression. ABCG2 protein expression was measured with the monoclonal antibody BXP-34 (27), which recognizes an internal epitope of the ABCG2 protein. Cells (0.5 x 10^6) were incubated with FACS lysing solution (Becton Dickinson) to permeabilize the cell membranes for 10 min at room temperature. Then the cells were incubated for 15 min at room temperature, with 1% goat serum in PBS [PBS: 140 mM NaCl, 9.0 mM Na2HPO4·2H2O, and 1.3 mM NaH2PO4·2H2O (pH 7.4)] containing 2% BSA. Subsequently, the cells were incubated for 60 min at room temperature with 4 μL (1.0 μg) of BXP-34 mouse antibody or with 20 μL (1.0 μg) IgG1 isotype control. Cells were washed with PBS and incubated for 20 min with PE-conjugated rabbit-antimouse F(ab’)2 fragments. PE fluorescence was measured on a FACScan flow cytometer and expressed as MFI. ABCG2 protein expression was measured as adjusted for IgG1 control, i.e., the ratio of BXP-34-IgG1 antibody MFI. Control cell lines used to standardize the ABCG2 protein expression assay included the MCF-7 cell line, expressing low levels of ABCG2, and the mitoxantrone-resistant subline MCF-7 MR, expressing high levels of ABCG2.

Flow Cytometric Detection of Mitoxantrone Accumulation. The capacity of ALL blasts to extrude mitoxantrone in the absence or presence of the ABCG2 inhibitor FTC (12), the P-gp inhibitor PSC 833 (provided by Novartis Pharma Inc., Basel, Switzerland), the MRP inhibitor MK-571 (Merck Sharp, Kirkland, PG, Quebec, Canada; Ref. 28), or combinations of these inhibitors, was measured in a flow cytometric assay (21). The cell lines MCF-7 and MCF-7 MR served as controls. To
mitoxantrone accumulation in combination with FTC was studied in the P-gp and MRP overexpressing cell lines GLC4/ADR and GLC4/P-gp. Cells (0.5 × 10⁶) were preincubated with the inhibitors for 20 min at 37°C, 5% CO₂, in the following combinations: RPMI 1640 alone (0.5 ml), RPMI 1640 plus FTC (10 μM), the maximum nontoxic doses of PSC 833 (2 μg/ml) and MK-571 (10 μM), FTC plus PSC 833, FTC plus MK-571, PSC 833 plus MK-571, or FTC plus PSC 833 plus MK-571. Thereafter, mitoxantrone (10 μM) was added, and the cells were incubated for 60 min at 37°C, 5% CO₂. Cells were washed with ice-cold RPMI 1640. Mitoxantrone fluorescence was analyzed with a FACSCalibur flow cytometer equipped with an argon laser. The blast population was gated by forward and side scatter characteristics. The mitoxantrone fluorescence of 5000 gated events was logarithmically measured at a laser excitation wavelength of 635 nm through a 670-nm band-pass filter. Before assessing the mitoxantrone accumulation, the flow cytometer was calibrated for the specific wavelength using spheroparticles (Spherotech Inc., Libertyville, IL). Mitoxantrone accumulation was expressed as MFI. The effects of the inhibitors were calculated as the ratio of the shift of MFI of the mitoxantrone accumulation divided by the mitoxantrone accumulation without the inhibitors, multiplied by 100%. When an inhibitor caused a decrease in mitoxantrone accumulation, the effect of the inhibitor was equated to zero. The mitoxantrone accumulation was determined in normal B and T lymphocytes from healthy donors in combination with CD2-FITC and CD19-PE monoclonal antibodies.

Flow Cytometric Detection of P-gp and MRP Functional Activity. Functional activity of the P-gp and MRP transporter proteins was demonstrated as described previously (29). Shortly, P-gp activity was analyzed with Rh123 (Sigma Chemical, Bornem, Belgium) in combination with PSC 833. Because a mutation in the ABCG2 gene can render the protein capable of transporting Rh123, the effect of FTC on Rh123 accumulation was also studied (19). To determine MRP (MRP1 and homologue MRP2) activity the compound CF (Sigma Chemical Co.) was used, in combination with MK-571. Rh123 and CF fluorescence of 5000 events was measured with a FACSCalibur flow cytometer. The effects of the inhibitors were expressed as a ratio of the shift of MFI divided by the MFI in unblocked cells, multiplied by 100%.

RNA Isolation, cDNA, and Genomic DNA Sequencing. The ABCG2 gene was sequenced to investigate the amino acid at position 482 (arginine, threonine, or glycine; Ref. 19). Total cellular RNA was extracted from leukemic cells using 1 ml of TRIzol reagent (Life Technologies). RNA was extracted, precipitated, and washed according to the manufacturer’s protocol. Subsequently, RNA was reverse transcribed in 20 μl reverse transcriptase buffer containing 10 mU RT, 0.5 mU of each dATP, dGTP, dCTP, and dTTP, 200 units of Moloney murine leukemia virus reverse transcriptase, 5 units of RNase inhibitor, and 10 ng/μl dN(6) random primers. Sequencing was performed using the ABI PRISM Rhodamine Terminator Cycle Sequencing Ready Reaction kit with an ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA).

Statistical Analysis. The Mann-Whitney U and Wilcoxon nonparametric tests were performed to test for differences in variables between children and adults, B-lineage ALL and T-lineage ALL, and effects of combination of inhibitors on mitoxantrone accumulation. The Spearman rank correlation was used to determine correlation among ABCG2, P-gp, and MRP activities and continuous variables. Ps <0.05 were considered significant.

Results

Patient Characteristics. Samples from 46 patients with de novo ALL were studied, including 23 patients with B-lineage and 23 patients with T-lineage ALL. The patient characteristics are summarized in Table 1.

ABCG2 Protein Expression. To standardize the ABCG2 protein expression assay, the ABCG2 expression was studied in the ABCG2 low-expressing cell line MCF-7 and the ABCG2 overexpressing cell line MCF-7 MR. The monoclonal antibody BXP-34 was used in combination with the IgG1 isotype control in a flow cytometric assay. A median BXP-34:IgG1 ratio of 8.3 (range, 5.4–11.1) was observed in the MCF-7 cell line versus a high median expression of 86.4 (range, 59.8–112.7) in the MCF-7 MR cell line. Subsequently, the ABCG2 protein expression was investigated in ALL blasts (Table 2). In B-lineage ALL samples, the median BXP-34:IgG1 ratio was 2.4 (range, 1.7–3.7). The T-lineage ALL samples showed a median expression of 1.9 (range, 1.2–6.6). The BXP-34:IgG1 ratios in all of the ALL samples were lower than the observed ratios in

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Characteristics of ALL patients</th>
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<tbody>
<tr>
<td></td>
<td>Unfavorable cytogenetics include the translocations t(9;22), t(4;11) and 11q23 rearrangements.</td>
</tr>
<tr>
<td></td>
<td>Number</td>
</tr>
<tr>
<td>B-lineage ALL</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
</tr>
<tr>
<td>Children</td>
<td>12</td>
</tr>
<tr>
<td>Adults</td>
<td>11</td>
</tr>
<tr>
<td>T-lineage ALL</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
</tr>
<tr>
<td>Children</td>
<td>10</td>
</tr>
<tr>
<td>Adults</td>
<td>13</td>
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* The results represent median values with range. 

a WBC, white blood cell count at diagnosis.
**Table 2** ABCG2 protein expression and mitoxantrone accumulation in ALL patients

ABCG2 protein expression calculated as the BXP-34:IgG1 ratio. Shifts calculated as percentage of accumulation without inhibitors. The results represent median values with range.

<table>
<thead>
<tr>
<th></th>
<th>B-lineage ALL</th>
<th>T-lineage ALL</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCG2 protein expression</td>
<td>2.4 (1.7–3.7)</td>
<td>1.9 (1.2–6.6)</td>
</tr>
<tr>
<td>Mito accumulation (MFI)</td>
<td>148 (77–555)</td>
<td>108 (65–281)</td>
</tr>
<tr>
<td>FTC shift</td>
<td>21 (0–140)</td>
<td>5 (0–256)</td>
</tr>
<tr>
<td>PSC 833 shift</td>
<td>22 (6–70)</td>
<td>24 (0–81)</td>
</tr>
<tr>
<td>MK-571 shift</td>
<td>16 (0–173)</td>
<td>18 (0–141)</td>
</tr>
<tr>
<td>Rh123 accumulation</td>
<td>0 (0–197)</td>
<td>14 (0–362)</td>
</tr>
<tr>
<td>PSC 833 shift</td>
<td>192 (23–639)</td>
<td>239 (68–500)</td>
</tr>
</tbody>
</table>

In normal B- and T-lymphocytes from healthy donors, the MCF-7 parent cell line. In B-lineage ALL, ABCG2 expression was higher compared with T-lineage ALL ($P = 0.003$). The ABCG2 expression in B-lineage ALL showed also a trend toward a higher expression in children than adults ($P = 0.071$). In T-lineage ALL, no difference was observed between the two age groups ($P = 0.574$).

**Mitoxantrone Accumulation.** The functional activity of ABCG2 was examined by measuring the intracellular mitoxantrone accumulation, expressed as MFI, in the absence or presence of the ABCG2 inhibitor FTC.

In the MCF-7 MR cell line, the mean mitoxantrone accumulation was lower than in the MCF-7 cell line (465 versus 1004), which was expected, because the overexpression of ABCG2 causes a decrease in mitoxantrone accumulation.

Next, the mitoxantrone accumulation was determined in ALL blasts. In the B-lineage ALL samples, the median mitoxantrone accumulation was 148 (range, 77–555 MFI). The median mitoxantrone accumulation in T-lineage ALL was 108 (range, 65–281 MFI; Table 2).

To investigate whether the efflux of mitoxantrone was ABCG2-mediated, the ABCG2 inhibitor FTC was added. In the MCF-7 cell line, FTC caused an increase in mitoxantrone accumulation of 18% (range, 15–38%). In the ABCG2 overexpressing cell line MCF-7 MR an increase of 168% was observed (range, 141–227%). FTC did not affect mitoxantrone accumulation in P-gp- and MRP-overexpressing cell lines (data not shown).

The addition of the ABCG2 inhibitor FTC caused a higher median increase in mitoxantrone accumulation in B-lineage ALL, namely 21% (range, 0–140%), than in T-lineage ALL (5%; range, 0–256%; $P = 0.013$). From the ALL samples, 27 (59%) patient samples showed a smaller effect of FTC on mitoxantrone accumulation than the MCF-7 cell line. Eighteen (39%) patient samples showed a FTC effect between the MCF-7 and MCF-7 MR cell lines, and 1 (2%) patient sample showed a larger increase in mitoxantrone accumulation by FTC than the MCF-7 MR cell line. These results indicate that ABCG2 is functionally active on a low but significant level in B-lineage ALL.

In normal B- and T-lymphocytes from healthy donors, the effect of FTC on mitoxantrone accumulation was also studied. No effect of FTC could be observed in both B and T lymphocytes.

The FTC effect on mitoxantrone accumulation correlated positively with ABCG2 expression ($r = 0.52; P < 0.001; n = 43$; Fig. 1). Even when the outlier is removed, the correlation remains present ($r = 0.49; P = 0.001; n = 42$). No difference was observed between children and adults in mitoxantrone accumulation or in the effect of FTC (B-lineage ALL $P = 0.268$ and $P = 0.566$; T-lineage ALL $P = 0.352$ and $P = 0.226$).

**P-gp Functional Activity.** Mitoxantrone can also be transported by P-gp and MRP. To get an impression of the impact of ABCG2 on mitoxantrone transport, P-gp and MRP activity, and the effect of their blockers on mitoxantrone accumulation were studied as well. In B-lineage ALL, there was no median effect of PSC 833 on Rh123 accumulation (range, 0–197%; Table 2), whereas PSC 833 gave a median increase of 14% in T-lineage ALL (range, 0–362%; $P = 0.001$; Table 2). In brief, P-gp activity was observed only in T-lineage ALL.

To study the specific transport of mitoxantrone by P-gp, the effect of PSC 833 on mitoxantrone was studied. Surprisingly, PSC 833 increased the mitoxantrone accumulation in all of the B-lineage and most T-lineage ALL patients (Table 2).

**MRP Functional Activity.** The functional activity of MRP was determined by assessing the effect of MK-571 on CF accumulation. In B-lineage ALL, MK-571 increased the CF accumulation with 192% (range, 23–639%) and in T-lineage ALL with 239% (range, 68–500%), indicating that MRP is functionally active in both B- and T-lineage ALL (Table 2). To investigate the role of MRP in mitoxantrone transport in ALL, mitoxantrone accumulation was studied in the presence of MK-571. MK-571 increased the mitoxantrone accumulation in 20 of 21 B-lineage samples and 18 of 22 T-lineage samples (Table 2).

**Mutation Analysis of the ABCG2 Gene and Substrate Spectrum.** The ability of ABCG2 to transport Rh123 was studied by measuring the effect of FTC on Rh123 accumulation. In three of the 46 samples (1 B-lineage and 2 T-lineage ALL) the addition of FTC showed an increase in Rh123 accumulation. These three samples, as well as 5 control samples, were se-
out inhibitors. The results represent median values with range.

Table 3 Effect of the combination of inhibitors on mitoxantrone accumulation in ALL patients

<table>
<thead>
<tr>
<th></th>
<th>B-lineage ALL</th>
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</tr>
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<td>22 (6–70)</td>
<td>24 (0–81)</td>
</tr>
<tr>
<td>MK-571 shift</td>
<td>16 (0–173)</td>
<td>18 (0–141)</td>
</tr>
<tr>
<td>FTC+PSC 833 shift</td>
<td>27 (0–156)</td>
<td>43 (0–308)</td>
</tr>
<tr>
<td>FTC+MK-571 shift</td>
<td>39 (0–226)</td>
<td>22 (0–281)</td>
</tr>
<tr>
<td>PSC 833+MK-571 shift</td>
<td>32 (8–166)</td>
<td>50 (0–225)</td>
</tr>
<tr>
<td>FTC+PSC 833+MK-571 shift</td>
<td>44 (6–217)</td>
<td>46 (0–340)</td>
</tr>
</tbody>
</table>

In summary, a number of correlations were found between more mature markers in B-lineage ALL and ABCG2. No relation was observed between the protein expression and functional activity of ABCG2, and an immature phenotype in our patient samples.

Table 4 Wilcoxon rank test calculating the effect of the addition of one inhibitor to a combination of inhibitors on mitoxantrone accumulation in ALL patients

<table>
<thead>
<tr>
<th>Combination of inhibitors</th>
<th>Shift</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>FTC+PSC833 vs. PSC833</td>
<td>27 vs. 22</td>
<td>0.002</td>
</tr>
<tr>
<td>FTC+MK571 vs. MK571</td>
<td>39 vs. 16</td>
<td>0.002</td>
</tr>
<tr>
<td>FTC+PSC833+MK571 vs. PSC833+MK571</td>
<td>44 vs. 32</td>
<td>0.351</td>
</tr>
<tr>
<td>FTC+PSC833 vs. PSC833</td>
<td>43 vs. 24</td>
<td>0.016</td>
</tr>
<tr>
<td>FTC+MK571 vs. MK571</td>
<td>22 vs. 18</td>
<td>0.170</td>
</tr>
<tr>
<td>FTC+PSC833+MK571 vs. PSC833+MK571</td>
<td>46 vs. 50</td>
<td>0.408</td>
</tr>
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</table>

Combination of Inhibitors on Mitoxantrone Accumulation. Finally, the inhibitors were combined to analyze whether their effects are the result of the activity of one transporter or a combined action of transporters. First, the addition of FTC to PSC 833 on mitoxantrone accumulation was studied, to explain the effect of PSC 833 in samples without P-gp activity, as assessed by the Rh123 efflux assay. Our results demonstrated an increased effect of the combination of FTC and PSC 833 on mitoxantrone accumulation in comparison with just PSC 833 or FTC, in both B-lineage and T-lineage ALL samples (Tables 3 and 4).

By adding FTC to MK-571, the role of ABCG2 in mitoxantrone transport in patients with MRP activity was studied. The addition of FTC to MK-571 caused an increase in mitoxantrone accumulation in only B-lineage ALL (Tables 3 and 4).

The addition of FTC to the combination of PSC 833 and MK-571 gives a reflection of the ABCG2 activity not biased by P-gp and MRP functional activity. In the B- and T-lineage ALL samples, this did not cause an overall increase in mitoxantrone accumulation (B-lineage $P = 0.351$ respectively. T-lineage ALL $P = 0.408$; Tables 3 and 4). However, in B-lineage ALL samples in which the addition of FTC caused an increase in mitoxantrone accumulation, this increase correlated with the ABCG2 protein expression ($r = 0.57$; $P = 0.009$; $n = 20$).

Immunophenotype. ABCG2 expression has been associated with an immature phenotype in normal hematopoietic cells (15, 23, 24) and AML blasts (21). Therefore, the immunophenotype of the patient blasts was determined and correlated with the maturation stage.

In B-lineage ALL, CD10 (“common” ALL antigen) expression correlated with ABCG2 protein expression and ABCG2 functional activity ($r = 0.51$; $P = 0.021$; $n = 20$ resp. $r = 0.52$; $P = 0.018$; $n = 20$). The CD19 expression was also associated with ABCG2 activity ($r = 0.48$; $P = 0.039$; $n = 19$). No correlation was observed between the markers CD20 and CD33, the immature markers CD34 or CD117 (c-kit), and the ABCG2 protein expression and ABCG2 functional activity (i.e., the effect of FTC on mitoxantrone accumulation). In T-lineage ALL, the expression of the CD markers CD34, CD117, CD3, CD7, and CD33 did not correlate with ABCG2 expression and functional activity. Of all of the investigated markers, CD33 and CD117 were scarcely expressed on ALL blasts.

In normal hematopoietic cells, it was shown that ABCG2 is expressed in pluripotent stem cells and sharply down-regulated at the point of commitment to lineage-specific development (15, 23, 24). Also in AML, an immature phenotype, as determined by the expression of CD34, correlated with ABCG2 expression (21). In ALL, we did not observe an association between the immature markers CD117 or CD34 and ABCG2. The expression of other transporters, such as P-gp and MRP, is also associated with an immature phenotype in normal hematopoietic cells (31, 32) and AML blasts (33–36) but not in ALL blasts (6, 8, 37). Apparently, the connection between transporters and maturation stage is differently regulated in ALL than normal hematopoietic cells and AML blasts.

A mutation in the ABCG2 gene can alter substrate specificity in drug-resistant cell lines (19). Expression of the mutated form, with threonine or glycine, can confer resistance to a broader spectrum of drugs, including mitoxantrone, Rh123, and doxorubicin. The three patient samples, in which ABCG2 was suggested to transport Rh123, did not show the mutation. This finding underscores the limited role of this mutation in the ABCG2 gene in clinical samples.

Although no gold standard exists for the detection of ABCG2 functional activity, Minderman et al. (38) showed that flow cytometry using mitoxantrone in combination with
ABCG2 inhibitor FTC can be used to detect functional activity. Evidently, other inhibitors could have been used, such as the FTC-analogue Ko143, but we have no reason to expect them to be more suitable than FTC. Moreover, FTC is used most often in functional studies, and the application of a standardized detection method is recommended (39).

Mitoxantrone is a substrate for several other transporters, including P-gp and MRP. Therefore, the functional activity of these transporters was investigated as well as their role in mitoxantrone transport. The contribution to mitoxantrone transport of the individual transporters is reflected in Table 3. ABCG2-mediated mitoxantrone transport in B-lineage ALL is comparable with that of P-gp and MRP. In T-lineage ALL, the mitoxantrone transport by ABCG2 is negligible compared with P-gp- and MRP-mediated mitoxantrone transport.

Remarkable was the effect of PSC 833 on mitoxantrone accumulation in patients who did not show P-gp activity, as assessed with the Rh123 efflux assay. This could possibly be explained by PSC 833 interfering with another transporter than P-gp, which is also capable of transporting mitoxantrone. In general, the lack of specificity of P-gp and ABCG2 inhibitors could explain the inconclusive data, because new transporters are still being identified that may be inhibited by P-gp and/or ABCG2 inhibitors. Also the occurrence of polymorphisms in the ABCG2 or P-gp encoding genes, altering the substrate specificity, may be of influence.

In patients with functionally active P-gp, FTC showed an increase in mitoxantrone accumulation when P-gp was inhibited. This suggests that even in patients in which mitoxantrone is transported by P-gp, ABCG2 contributes to mitoxantrone resistance.

The ability of MRP to transport mitoxantrone is not very clear. We observed an effect of MK-571 in mitoxantrone transport in most of the ALL cases. The MRP-overexpressing cell line GLC4/ADR also showed an increase in mitoxantrone accumulation when MK-571 was added. However, there are studies suggesting that mitoxantrone is not a substrate of MRP (18, 40). The conflicting reports could also be the result of a functional polymorphism that has not been discovered yet.

When P-gp and MRP were inhibited, FTC did not cause an overall increase in mitoxantrone accumulation in B- and T-lineage ALL. In B-lineage ALL, an effect of FTC could be observed in half of the patients, and the increase in mitoxantrone accumulation correlated with the ABCG2 protein expression. In patient samples in which PSC 833 and MK-571 showed a significant effect on mitoxantrone accumulation, the addition of FTC did not cause an extra increase. Consequently, in these samples the effect of FTC is most likely masked by the effect of PSC 833 and MK-571. The effect of inhibitors was expressed as a percentage, and consequently, if two inhibitors have a large effect, the effect of the addition of a third inhibitor is likely to be underestimated.

The distribution of the functional activity of transporters within patients was determined by calculating the correlation between the effect of FTC and P-gp and/or MRP functional activity. No correlation was found between the three transporters in B- and T-lineage ALL. Surprisingly, the effect of FTC on mitoxantrone accumulation correlated with the effect of only PSC 833 or MK-571 on mitoxantrone accumulation. Cross-resistance of inhibitors could account for this phenomenon, although this has not been described in studies using cell lines overexpressing only one of the transporters (12, 21). In cell lines overexpressing P-gp or MRP, we and others showed that FTC did not reverse resistance (12, 21). These observations in cell lines imply that FTC acts as a specific ABCG2 inhibitor. The specificity of PSC833 was described in the same study (21) in which PSC833 showed little effect on the ABCG2-overexpressing cell line. However, in contrast to cell lines overexpressing specific transporters, clinical samples could express other (unknown) transporters, which can also be inhibited by FTC or PSC383. The discrepancies in our data could be explained by the presence of these transporters, which are capable of effluxing mitoxantrone as well.

In summary, this study shows that ABCG2 is expressed and functional in B-lineage ALL and at a lower level in T-lineage ALL. The next step will be to determine the clinical impact of this transporter in a larger prospective trial. Because ABCG2 is mainly functionally active in B-lineage ALL, the trial will only be useful in this ALL subtype. Hopefully, it will elucidate whether these patients with ALL could benefit from treatment adaptations based on this knowledge.

References


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