Activity and Expression of the Multidrug Resistance Proteins MRP1 and MRP2 in Acute Myeloid Leukemia Cells, Tumor Cell Lines, and Normal Hematopoietic CD34+ Peripheral Blood Cells

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INTRODUCTION

Development of cross-resistance to structurally and functionally unrelated drugs, MDR, is a major cause of treatment failure in myeloid leukemia. Several mechanisms of MDR are known (1), but the best-studied mechanism is the overexpression of the MDR1 gene product P-gp, a Mr 170,000 protein that acts as an ATP-dependent membrane efflux pump, increasing transport of drugs out of the cell (2–5). Inhibitors of P-gp, including cyclosporin A and its derivative PSC833, have been shown to interfere with P-gp function and can reverse MDR in vitro (6). P-gp expression is a poor prognostic factor in AML and is frequently associated with the expression of the CD34 antigen on AML blast cells (7).

Another membrane transporter, the multidrug resistance-associated protein 1 (MRP1), has been identified and characterized as a Mr 190,000 protein that is encoded by the MRP1 gene, which is located on chromosome 16p13 (8, 9). MRP1, like P-gp, belongs to the ATP-binding cassette (ABC) superfamily of membrane transport proteins. It is shown that MRP1 is identical to a GSH-S conjugate carrier described in many cells, because the endogenous leukotriene C4 and other GSH-S conjugates are transported by MRP1 (10, 11). Recently, it is shown that ATP-dependent uptake of the unmodified drug vincristine by membrane vesicles derived from MRP1-transfected HeLa cells is dependent on the presence of GSH (12). It is possible that GSH interacts directly with MRP1 and that this interaction is necessary for transport. In addition, GSH depletion increases the accumulation of DNR and Rh123 in several MRP1-positive cell lines (13). A specific inhibitor of MRP-mediated transport is MK-571, a leukotriene D4 receptor antagonist (11, 14).

Thus far, limited data are available regarding the functional role of MRP1 in clinical drug resistance in AML (15). However, MK-571 has been shown to increase cytotoxicity of anticancer drugs in a MRP1-positive myeloid leukemic cell line (16).

Different compounds are available to study P-gp and MRP-mediated efflux (17–19). Rh123 acts as a substrate for P-gp but may also be transported by MRP1 in a GSH-dependent manner, similar to VCR (12, 20, 21). CFDA is a nonfluorescent compound, which permeates the plasma membrane, and upon cleavage of the ester bonds by intracellular esterases, it is transformed into the fluorescent anion CF (22), which might be a substrate for MRP but not for P-gp.

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The abbreviations used are: MDR, multidrug resistance; P-gp, P-glycoprotein; AML, acute myeloid leukemia; MRP, multidrug resistance protein; GSH, glutathione; VCR, vincristine; DNR, daunorubicin; DOX, doxorubicin; CF, carboxyfluorescein; CFDA, CF diacetate; FAB, French-American-British; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; VP-16, etoposide; Rh123, rhodamine 123.
Recently, a homologue of MRP1, the canalicular multispecific organic anion transporter (cMOAT), called MRP2, was cloned (23). The locus of the human MRP2 gene was demonstrated on chromosome 10q24. Expression of MRP2 was enhanced in cisplatin- and doxorubicin-resistant human cancer cell lines. The expression level of MRP2 correlated with the cisplatin but not the doxorubicin resistance of the cell lines (23, 24).

In the present study, we analyzed MRP1 activity in human AML cells by evaluating the effects of MK-571 on CF efflux. The effects in several tumor cell lines were compared with cancer cell lines. The expression level of MRP2 was determined by trypan blue exclusion, and in all cases treated, and washed with RPMI 1640 medium. Viability of the AML cell lines (23, 24).

MATERIALS AND METHODS

Cell Lines. The in vitro DOX-selected, MRP1-overexpressing, P-gp-negative subline GLC4/ADR (25), derived from the human small cell lung cancer cell line GLC4, was maintained in the presence of 1.2 μM doxorubicin (Farmitalia Carlo Erba, Brussels, Belgium). The doxorubicin-resistant human ovarian carcinoma cell line 2780AD, derived in vitro from the A2780 cell line, overexpresses P-gp and expresses some MRP and was maintained in the presence of 2 μM doxorubicin (26).

These cell lines were cultured in RPMI 1640 (BioWhittaker, Brussels, Belgium) supplemented with FCS (Hyclone, Logan, UT).

S1(MRP) is a P-gp-negative subline of the non-small cell lung carcinoma cell line SW-1573/S1, which is also P-gp negative (27). This cell line (kindly provided by Dr P. Borst, Dutch Cancer Institute, Amsterdam, the Netherlands) is obtained after transfection of SW-1573/S1 cells with an expression vector containing MRP1 cDNA and a neoR gene (pRo/RSV-MRP), followed by selection with geneticin (200 μg/ml). S1 and S1(MRP) cell lines were cultured in DMEM (BioWhittaker) supplemented with 10% FCS.

Patients. After informed consent, peripheral blood or bone marrow aspirates from 15 de novo AML patients at presentation were collected. Patients were classified according to the FAB classification (28). Leukemic blasts from bone marrow and blood were enriched by Ficoll-Isopaque (Nycomed, Oslo, Norway) density gradient centrifugation. Normal hematopoietic CD34+ cells were isolated from a peripheral blood stem cell collection using the Isolox system, which is based on incubation with Dynabeads and subsequently chymopapain for releasing the target cells from the beads (29). 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 rethawed, centrifuged in newborn calf serum (Life Technologies, Breda, the Netherlands), DNase treated, and washed with RPMI 1640 medium. Viability of the cells was determined by trypan blue exclusion, and in all cases was over 90%. For cell phenotyping, 10⁶ cells were incubated with 10 μl of FITC- or phycoerythrin-labeled mouse monoclonal antibodies to CD34, CD33, CD3, or IgG isotype-matched control (Becton Dickinson, Mountain View, CA) for 30 min at 4°C, washed with RPMI 1640 medium, and analyzed with a FACStar flow cytometer (Becton Dickinson Medical Systems, Sharon, MA).

To study cytogenetics, bone marrow was cultured for 24 and 48 h in RPMI 1640 supplemented with 15% FCS. The cultures were harvested and chromosome preparations were made according to standard cytogenetic techniques. The chromosomes were G-banded using trypsin or pancratin, and karyotypes were described according to the International System for Human Cytogenetic Nomenclature guidelines for cancer cytogenetics (30).

T lymphocytes were isolated by 2-aminoethylisothiourea bromide-treated sheep RBC (SRBC) rosetting. The SRBCs were lysed with 155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA, and the remaining cell preparations contained more than 97% T lymphocytes, as assessed by flow cytometry after staining with an anti-CD3 monoclonal antibody (Becton Dickinson). After isolation, T lymphocytes were cultured overnight at 37°C in RPMI 1640 (BioWhittaker) containing 10% FCS (Hyclone).

Flow Cytometric Detection of Functional Drug Efflux. The ability of tumor cell lines, normal hematopoietic CD34+ cells, and leukemic blasts to extrude Rh123 (Sigma Chemical Co., Bornem, Belgium) or CF in the absence or presence of the P-gp inhibitor PSC833 (provided by Sandoz, Basel, Switzerland) and the leukotriene C4 receptor antagonist and MRP inhibitor MK-571 (Ref. 31; kindly provided by Dr. Ford-Hutchinson, Merck Sharp, Kirkland, PQ, Canada) was measured in a flow cytometric assay. Cells (1 × 10⁶) were incubated for 20 min at 37°C, 5% CO₂, in 200 ng/ml Rh123 or 0.1 μM CFDA (Sigma) with or without inhibitor (2 or 5 μg/ml PSC833, 20 or 50 μg/ml MK-571) in RPMI 1640. Cells were pelleted and divided into two aliquots. One aliquot was placed on ice for measurement of baseline Rh123 and CFDA uptake. The second part was resuspended in drug-free medium with or without inhibitor and incubated for 60 min at 37°C, 5% CO₂ to give the opportunity for efflux or efflux-blocking to occur. Efflux was stopped by pelleting the cells and adding ice-cold medium.

Fluorescence of Rh123 and CF was analyzed with a FACStar flow cytometer that was equipped with an argon laser. The blast population was gated by forward and side scatter characteristics. The Rh123 and CF fluorescence of 10,000 events was logarithmically measured at a laser excitation wavelength of 488 nm through a 530 nm band-pass filter. The logarithmically measured fluorescence was converted into linear values and expressed as relative fluorescence units using the Winlist 2.0 program (Verity Software House, Inc., Topsham, ME). The effectiveness of efflux-blocking by the inhibitors, called efflux-blocking factor, was defined as the ratio between median relative fluorescence units in inhibitor-blocked versus unblocked cells (19, 32) after 60 min of efflux.

Drug Sensitivity Assay. The microculture tetrazolium test (MTT) (33) was used for determination of drug sensitivities of the cell lines GLC4 and GLC4/ADR in the presence or absence of inhibitors. GLC4 cells (3.7 × 10⁴ cells/well) or GLC4/ADR cells (10⁵ cells/well) were seeded in quadruplicate into 96-well microtiter plates and incubated for 72 h with or without the drugs vincristine VCR (1.0 ng/ml for the GLC4 cell line and 5.0 ng/ml for the GLC4/ADR cell line), DOX (40 nm and 4.0 μM), VP-16 (0.2 and 20 nm), and DNR (0.1 and 6 μg/ml), in combination with or without the inhibitors MK-571 (50 μM) and PSC833 (1 μg/ml). Higher concentrations of MK-571 and PSC833 resulted in cytotoxic effects. After a 72-h
culture period, cells were processed as described before (33). The survival of cells that were not incubated with drug or inhibitor was expressed as 100%. Assays were performed at least three times.

**Preparation of Cell Membrane Fractions and MRP Western Blot Analysis.** Cells (5 × 10^6) were washed twice in ice-cold PBS (0.14 M NaCl, 2.7 mM KCl, 6.4 mM Na₂HPO₄, and 1.5 mM KH₂PO₄; pH 7.4), centrifuged again, and permeabilized by 40-fold dilution in a hypotonic buffer (1 mM Tris/HCl, pH 7) in the presence of 400 units of recombinant *Serratia* marcescens nuclease (benzonase, grade II, protease-free; Merck, Darmstadt, Germany). The cell lysate was centrifuged at 4°C for 30 min. The resulting pellets were resuspended in TS buffer. Protein content was measured by the Lowry method (34). Equal amounts of proteins were size fractionated in a SDS-7.5% polyacrylamide gel. MRP1 was detected with the monoclonal antibody MRP1 (kindly provided by Dr. R. J. Schepers, Free University Hospital, Amsterdam, the Netherlands; Ref. 35). Enhanced chemiluminescence (Amersham International, Buckinghamshire, United Kingdom) was used for detection of the antibodies. Densitometric scanning was performed with an Image Master VDS (Pharmacia, Woerden, the Netherlands), and optical density (OD) was expressed as OD/mm² using the program Diversity One 1D (PDI, New York, NY).

**RNA Extraction and RT-PCR Analysis for MRP1 and MRP2.** Total cellular RNA was isolated from 10–30 × 10^6 AML blasts or 5 × 10^6 cell line cells using 1 ml of RNAzol B (Campro Scientific, Veenendaal, the Netherlands) and A2780 and 2780AD, 20 μg/ml. The concentrations used of PSC833 are: GLC4, GLC4/ADR, S1, and S1(MRP), 2 μg/ml; and A2780 and 2780AD, 5 μg/ml.

**Table 1** Efflux studies in tumor cell lines

<table>
<thead>
<tr>
<th></th>
<th>CF efflux-blocking factor</th>
<th>Rh123 efflux-blocking factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MK-571 PSC833</td>
<td>MK-571 PSC833</td>
</tr>
<tr>
<td>GLC4</td>
<td>11.5 (2.7) 3.2 (1.0)</td>
<td>0.8 (0.2) 0.7 (0.2)</td>
</tr>
<tr>
<td>GLC4/ADR</td>
<td>45.9 (5.8) 2.9 (1.5)</td>
<td>1.0 (0.0) 0.7 (0.2)</td>
</tr>
<tr>
<td>A2780</td>
<td>12.7 (4.0) 2.9 (0.7)</td>
<td>1.2 (0.1) 1.2 (0.2)</td>
</tr>
<tr>
<td>2780AD</td>
<td>15.3 (2.2) 2.7 (0.4)</td>
<td>1.9 (0.5) 93.0 (10.8)</td>
</tr>
<tr>
<td>S1</td>
<td>2.8 (0.4) 1.6 (0.4)</td>
<td>1.3 (0.5) 0.7 (0.2)</td>
</tr>
<tr>
<td>S1(MRP)</td>
<td>14.4 (3.2) 2.5 (0.6)</td>
<td>1.8 (0.2) 1.0 (0.2)</td>
</tr>
</tbody>
</table>

The results represent the mean (SD) of at least three independent experiments. The concentrations used of MK-571 are: GLC4 and GLC4/ADR, 50 μM; and S1, S1(MRP), A2780, and 2780AD, 20 μM. The concentrations used of PSC833 are: GLC4, GLC4/ADR, S1, and S1(MRP), 2 μg/ml; and A2780 and 2780AD, 5 μg/ml.

**Fig. 1** Fluorescence histograms showing CF content and autofluorescence control (white) after 60 min of efflux with (black) or without (gray) 50 μM MK-571 in GLC4 (efflux-blocking factor of 11.5 ± 2.7; A) and GLC4/ADR (efflux-blocking factor of 45.9 ± 5.8; B) cell lines.
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sequences of the MRP1 primers are as follows: sense, 5'-AATGCGCCAAGACTAGGAAG-3'; and antisense, 5'-ACCGAGGATGTGAACAG-3'. For MRP2, amplified product consists of 1067 bp. Primer pairs corresponding to bases 961–981 (sense) and 2007–2027 (antisense) are chosen. MRP2 primer sequences are: sense, 5'-CTGGTGATGAAGGGCCTGT-3'; and antisense, 5'-CTGCCATAATGTCCAGGTT-3'. GAPDH primer sequences are the following: sense, 5'-CCATCACCATCTCCACCAACG-3'; and antisense, 5'-CCTGCTCCACACCTTCTTG-3'. Primer pairs correspond to bases 242–262 (sense) and 797–817 (antisense), and from these primers a 576-bp product is obtained.

Statistical Analysis. The paired Student’s t test was used to calculate significances, and correlations were calculated using the Pearson bivariate correlation test. Ps < 0.05 were considered significant.

RESULTS

Inhibitory Effects of MK-571 on CF Efflux in Cell Lines. To test whether CF was effluxed by MRP and whether the inhibitor MK-571 could specifically block this efflux pump, we studied the effects of MK-571 on several cell lines with varying degrees of MRP1 expression. The GLC4/ADR cell line

Fig. 2 Effects of 1 μg/ml PSC833 (white) and 50 μM MK-571 (gray) on cell survival of GLC4 (A) and GLC4/ADR (B) cell lines incubated with the drugs VCR, DOX, VP-16 (VP16), and DNR (■).

A

B

- Controlled
- MK-571
- PSC833

Survival (%)

0 20 40 60 80 100

VCR (1 ng/ml) DOX (40 nM) VP16 (0.2 nM) DNR (0.1 μg/ml)

Survival (%)

0 20 40 60 80 100

VCR (5 ng/ml) DOX (4 μM) VP16 (10 nM) DNR (6 μg/ml)
shows overexpression of MRP1 compared with the GLC4 cell line (25). As demonstrated in Fig. 1 and Table 1, for GLC4/ADR a CF efflux blocking factor of 45.9 ± 5.8 (mean ± SD, n = 3) was observed in the presence of 50μM MK-571, the maximum nontoxic dose for GLC4 and GLC4/ADR, whereas a limited effect of PSC833 (5μg/ml) was found (2.9 ± 1.5, n = 3). The GLC4 cell line showed a CF efflux blocking factor for MK-571 of 11.5 ± 2.7 (n = 3).

To further examine the substrate specificity of MK-571, the P-gp-overexpressing, MRPI-expressing 2780AD cell line and its P-gp-negative, MRPI-expressing counterpart A2780 (26) were loaded with Rh123 and CFDA in the absence and presence of MK-571 and PSC833 (Table 1). The P-gp-specific inhibitor PSC833 inhibited Rh123 efflux with a factor 93.0 ± 10.8 (n = 4), whereas MK-571 showed a efflux blocking factor of 1.9 ± 0.5 (n = 4). In the A2780 cells, neither PSC833 nor MK-571 inhibited Rh123 efflux (1.2 ± 0.2 and 1.2 ± 0.1, respectively; n = 3). CF efflux, however, was inhibited by MK-571 in both MRPI-expressing cell lines. In the 2780 AD cell line, MK-571 inhibited CF efflux with a factor 15.3 ± 2.2 (n = 4), whereas PSC833 showed an efflux blocking factor of 2.7 ± 0.4 (n = 4). In the A2780 cell line, MK-571 showed a efflux blocking factor of CF from 12.7 ± 4.0 (n = 4), and PSC833 inhibited CF efflux with a factor 2.9 ± 0.7 (n = 4).

Substrate specificity was further underscored by studying the effects of MK-571 and PSC833 in the S1 and in the MRPI-transfected S1(MRP) cell lines. In the S1 cell line, a CF efflux blocking factor of 2.8 ± 0.4 (n = 3, Table 1) was observed in the presence of MK-571. In S1(MRP), MK-571 showed a CF efflux blocking factor of 14.4 ± 3.2 (n = 3). These results demonstrate that CF is specifically effluxed by MRPI, and that efflux is inhibited by the MRPI inhibitor MK-571.

**Drug Sensitivity Assay.** Next it was analyzed whether MK-571 and PSC833 enhance the cytotoxic effects of VCR, DOX, VP-16, and DNR in the cell lines GLC4 and GLC4/ADR (Fig. 2). The cell lines were exposed to the different drugs in combination with or without MK-571 and PSC833.

<table>
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<tr>
<th>Patient no.</th>
<th>FAB class</th>
<th>Karyotype</th>
<th>Efflux-blocking CF</th>
<th>Efflux-blocking Rh123</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>MK*</td>
<td>PSC</td>
</tr>
<tr>
<td>1</td>
<td>M4</td>
<td>t(8;21),t(2;5),−7</td>
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<tr>
<td>2b</td>
<td>M1</td>
<td>+19</td>
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<tr>
<td>3</td>
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<td>+8</td>
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<tr>
<td>4</td>
<td>M1</td>
<td>del(7q),del(9p)</td>
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<td>1.5</td>
</tr>
<tr>
<td>5</td>
<td>M4Eo</td>
<td>inv(16)</td>
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<td>1.5</td>
</tr>
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<td>6</td>
<td>M1</td>
<td>−Y</td>
<td>4.0</td>
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<tr>
<td>7</td>
<td>M4Eo</td>
<td>inv(16)+,+8,+22</td>
<td>1.9</td>
<td>1.3</td>
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<tr>
<td>8b</td>
<td>M4Eo</td>
<td>inv(16)+,+8</td>
<td>2.4</td>
<td>1.4</td>
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<td>9</td>
<td>M2</td>
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<tr>
<td>10</td>
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<tr>
<td>14</td>
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</tr>
<tr>
<td>15</td>
<td>M4</td>
<td>dic(7;8)</td>
<td>3.4</td>
<td>1.7</td>
</tr>
</tbody>
</table>

*a MK, MK-571; PSC, PSC833; ND, not done.

*b In these cases, bone marrow is used; in other cases, peripheral blood is used.

**Table 2** AML patient results

In the GLC4 cell line, PSC833 did not enhance the cytotoxic effects of any of the drugs. MK-571 showed no significant effect on DOX and DNR cytotoxicity but augmented the cytotoxic effect of VCR (survival: 87% ± 10 versus 27% ± 5; P < 0.05) and of VP-16 (99% ± 6 versus 72% ± 4, P < 0.05; Fig. 2A).

In the GLC4/ADR cell line, PSC833 (1μg/ml) did not enhance the cytotoxic effects of VCR, DOX, and VP-16; DNR cytotoxicity, however, was enhanced by PSC833 (86% ± 5 versus 66% ± 3, P < 0.05; Fig. 2B). MK-571 augmented the cytotoxicity of VCR (85% ± 7 versus 39% ± 6, P < 0.05), DOX (82% ± 8 versus 37% ± 5, P < 0.05), VP-16 (94% ± 5 versus 66% ± 4, P < 0.05), and DNR (86% ± 5 versus 42% ± 3, P < 0.05).

**Effects of MK-571 on CF Efflux in CD34+ Cells.** To obtain data about P-gp- and MRP-mediated efflux in normal hematopoietic stem cells, CD34+ sorted cells were loaded with Rh123 and CFDA, in combination with efflux-blockers PSC833 and MK-571. MK-571 caused a retention of CF with a blocking factor of 11.5 ± 2.0 (n = 3). In the GLC4 cell line, PSC833 did not enhance the cytotoxic effects of VCR, DOX, and MK-571. MK-571 also influenced the Rh123 efflux (efflux blocking factor, 13.5 ± 4.9; n = 3) in CD34+ cells. However, Rh123 efflux appears to be not specifically mediated by P-gp, because MK-571 also influenced the Rh123 efflux (efflux blocking factor, 13.5 ± 4.9; n = 3). Therefore, it seems that P-gp as well as MRP is present and functional in these normal hematopoietic stem cells and can be blocked by the specific inhibitors PSC833 and MK-571.

Because contaminating T lymphocytes could possibly influence the efflux-blocking effects, the CD34+ sorted cells were analyzed for CD34 and CD3 antigen expression. CD34 positivity was observed in >99%, whereas 0.8% of the cells was also positive for CD3.

To exclude that the contaminating T cells have an effect on the CF efflux, we determined Rh123 and CF efflux blocking factors in purified T lymphocytes (>97% CD3 positive). CF...
efflux-blocking factors of MK-571 (20 μM) and PSC833 (2 μg/ml) were 1.7 ± 0.4 (n = 5) and 1.4 ± 0.3 (n = 5), respectively, whereas Rh123 efflux was blocked with a factor 8.6 ± 2.3 (n = 5) by MK-571 and with a factor 3.7 ± 1.9 (n = 5) by PSC833. We conclude that, although P-gp is functional, MRP activity seems to be very low in purified T lymphocytes.

**MRP1 and MRP2 mRNA and MRP1 Protein Expression.** To gain more insight in MRP expression and activity in the malignant counterpart of CD34+ cells, we studied MRP1 and MRP2 at the mRNA level and MRP1 at the protein level, and we measured the efflux capacity in the absence and presence of PSC833 and MK-571 in 15 AML patients. According to the FAB classifications, the patients included one M0, five M1, two M2, two M4, three M4Eo, one M5, and one M5A. Cytogenetic analysis demonstrated 11 patients with karyotypes with clonal chromosomal abnormalities consistent with the diagnosis of myeloid leukemia and four patients with a normal karyotype. A summary of all of the results is shown in Table 2.

MRP1 and MRP2 mRNA expression was analyzed by RT-PCR. MRP1 expression levels are shown, together with expression levels of the housekeeping gene GAPDH. MRP1 mRNA levels were shown in 10 of 11 patients with varying degrees of expression (Fig. 3). The expression of MRP1 protein as detected by Western blotting is shown in Fig. 4. One patient (no. 13) showed some MRP1 mRNA expression, although no MRP1 protein was detected.

The expression of MRP2 mRNA was also studied in the AML samples and compared with expression in GLC4 and GLC4/ADR cell lines. MRP2 mRNA expression in GLC4/ADR was very low, whereas the patient samples showed variable levels (Fig. 5). Patients with a high expression of MRP1 mRNA also showed expression of MRP2 mRNA, whereas patients with a low expression of MRP1 mRNA did not have detectable MRP2 levels.

**MRP and P-gp Activity in AML.** To test functional activity of P-gp and MRP in AML blasts, a flow cytometric assay was performed using Rh123, CFDA, and the inhibitors PSC833 and MK-571. To check the effect of freezing and thawing on the efflux capacity of AML blasts, efflux and efflux-blocking studies were performed before and after freezing, but no difference was found between fresh and rethawed samples (data not shown).

A great variability in CF efflux-blocking factors of MK-571 was observed (Fig. 6 and Table 2). In three cases, efflux-blocking factor was ≤2; in three cases, the blocking factor varied between 2 and 3, and nine cases showed an efflux-blocking factor >3. The patients with inv(16) showed low efflux-blocking factors between 1.9 and 2.4. A correlation was observed between MRP1 protein expression and CF efflux-blocking factor of the MRP-specific inhibitor MK-571 (P = 0.013, r = 0.39; Fig. 7), although the correlation was dependent on the patients showing the highest MRP1 expressions. PSC833 did not affect the CF efflux (CF efflux blocking factors vary between 1.3 and 2.0; Fig. 6) but was efficient in inhibiting Rh123 efflux.

No correlation was found between MRP1 protein expression and CF efflux-blocking factor caused by PSC833 (P = 0.654). However, just like normal hematopoietic CD34+ cells, MK-571 inhibited Rh123 efflux more efficiently than PSC833 (Fig. 6). No correlation existed between efflux-blocking factor of MK-571 on CF and Rh123 efflux (P = 0.807). In addition, no correlation was observed between MRP1 protein expression and Rh123 efflux-blocking factor of MK-571 (P = 0.637). An overview of all AML patient results is given in Table 2. Fluorescence histograms of CF and Rh123 efflux blocking in AML blasts of three representative patients are shown in Fig. 8. CD33 and CD34 expressions are shown in Table 2. A correlation exists between CD33 expression and Rh123 efflux-blocking factor of PSC833 (P = 0.01).

**DISCUSSION**

Both P-gp and MRP1 overexpression appear to be of potential importance in drug resistance in AML (36–41), and overexpression of MRP1 might precede the overexpression of
Fig. 5 Expression of MRP2 and GAPDH mRNA in AML patient samples and in GLC4 and GLC4/ADR cell lines. The patient numbers correspond with the numbers in Table 2. patient nr, patient no.

Fig. 6 Efflux-blocking factors in AML patients of MK-571 (20 \( \mu \)M) and PSC833 (2 \( \mu \)g/ml) on CF and Rh123. O, AML patients with inv(16).

Fig. 7 Correlation between MRPI protein levels determined by Western blotting and CF efflux-blocking factors of MK-571 in AML patient samples. AML patient sample numbers are shown. O, patients with inv(16).

P-gp in human myeloid leukemia cells (42). Many fluorescent compounds are available to detect activity of P-gp-mediated MDR. However, to detect MRPI-mediated MDR, only a few fluorescent compounds are described (13, 19, 20). The interpretation of cellular drug levels is hampered by subcellular compartmentalization. Intracellular MRPI-positive vesicles can accumulate drugs, thereby lowering effective drug levels in the cell but not changing overall cellular drug levels. This difficulty can be overcome by using MRPI inhibitors, which interfere with the pump function. The compound CF, which is specifically effluxed by MRPI and can be inhibited by a leukotriene D4 receptor antagonist, provides the possibility to study MRP-
mediated MDR in a flow cytometric assay. To examine activity and compound specificity of P-gp and MRP, Rh123 and CFDA were used in combination with the P-gp inhibitor PSC833 and the MRP inhibitor MK-571 in several tumor cell lines, which include a MRP1-transfected cell line, and in blasts from 15 AML patients. CF appeared to be a specific substrate for MRP1, and CF efflux can be specifically inhibited by the MRP1 inhibitor MK-571. Rh123 on the contrary was found to be specific in nonhematopoietic cells but not in hematopoietic cells for studying P-gp-mediated export, and it can be inhibited by MK-571 in hematopoietic cells. Thus, it is possible to discriminate between P-gp- and MRP-mediated efflux of Rh123 by using the specific inhibitors PSC833 and MK-571.

MRP1 efflux blocking by MK-571 seems to be promising, but to examine the clinical significance of MK-571, it should be studied in a larger number of patient samples. Almost all AML patient samples showed efflux blocking of Rh123 by MK-571, whereas the cell lines 2780AD, GLC4, and GLC4/ADR, which express MRP1 in varying degrees, did not show efflux blocking of Rh123 by MK-571. This difference is not elucidated but might be related to the studied cell type, hematopoietic versus nonhematopoietic cells. Alternatively, the difference might reflect the presence of an additional MRP1-related efflux pump. This possibility cannot be excluded at the moment because different MRP homologues have been identified (24, 43), which may have similar activity as the MRP1 gene product. One of these homologues, MRP2, which in this study is described for the first time in AML patient samples, is present in these AML cell lines, in view of the results of the RT-PCR analysis. However, it seems unlikely that MRP2 accounts for the Rh123 efflux in the AML patient samples, because Rh123 efflux is inhibited by MK-571 in all patient samples, whereas only a few samples showed detectable MRP2 mRNA expressions.

When we used the combination of the two inhibitors MK-571 and PSC833 in the Rh123 efflux assay, a higher efflux-blocking factor was observed than when both inhibitors were used separately (data not shown). This observation suggests that PSC833 and MK-571 inhibit different pumps, or that the pump is not maximally blocked when PSC833 and MK-571 are used separately in the flow cytometric assay. However, because the used concentrations of MK-571 and PSC833 were the maximum nontoxic doses, the last possibility cannot be excluded.

All patients with inv(16) show low CF efflux-blocking factors of MK-571, reflecting the dysfunction of the MRP1 pump as result of the chromosomal inversion. Whether this determines the relatively good prognosis is not yet clear, but these results suggest that MRP1 deletion may have a key role in determining outcome in AML patients with inv(16).

In conclusion, the present study shows that the analysis of CF accumulation in combination with the MRP1 inhibitor MK-571, although it does not discriminate between different MRPs, is a specific test for detecting MRP1-mediated resistance. In addition, it shows that MRP2 mRNA is present in AML patient samples.

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