Multidrug Resistance Protein (MRP) Attenuates Gemtuzumab Ozogamicin-Induced Cytotoxicity In Acute Myeloid Leukemia Cells

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ABSTRACT

Gemtuzumab ozogamicin (GO) is a novel immunoconjugate therapy for AML. P-glycoprotein (Pgp) confers resistance to GO and is associated with a worse clinical response. To address whether multidrug resistance protein (MRP) affects GO susceptibility we characterized Pgp, MRP1, and MRP2 expression in CD33+ cell lines and CD33+ AML samples and analyzed the effect of the Pgp inhibitor cyclosporine (CSA) and the MRP inhibitor MK-571 on GO-induced cytotoxicity. MRP1 expression, but not MRP2, correlated with MRP activity. MK-571 enhanced GO-induced cytotoxicity in Pgp-negative/MRP-positive NB4 and HL-60 cells. CSA, but not MK-571 alone, restored GO susceptibility in Pgp-positive/MRP positive TF1 cells; however, MK-571 enhanced cytotoxicity in the presence of CSA. All patient samples exhibited MRP activity and 17/23 exhibited Pgp activity. CSA increased GO-induced cytotoxicity in 12 Pgp-positive samples, whereas MK-571 alone was effective in only one case with minimal Pgp activity. In 3 Pgp-positive/MRP-positive samples, MK-571 enhanced GO-induced cytotoxicity in the presence of CSA. Thus, MRP1 may attenuate susceptibility to GO. This effect was comparatively less than Pgp and required inhibition of Pgp to detect in cells that co-expressed both transporters. Because MK-571 and/or CSA failed to affect cytotoxicity in a portion of Pgp-positive/MRP positive AML samples, additional resistance mechanisms are likely important.

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INTRODUCTION

Conventional induction chemotherapy induces complete remissions (CRs) in 50-80% of adults with de novo acute myeloid leukemia (AML). However, a significant proportion of responding patients relapses and ultimately dies from treatment-refractory disease. Thus, novel drugs and treatment strategies are major objectives of research, especially for the elderly who respond poorly to standard therapy regimens and often experience dose-limiting toxicity from intensive chemotherapies.

A humanized anti-CD33 antibody conjugated to a calicheamicin derivative, gemtuzumab ozogamicin (CMA-676, Mylotarg®, Wyeth-Ayerst Laboratories, Philadelphia, PA), was recently approved as a therapeutic option for relapsed AML in elderly patients who are not considered candidates for conventional salvage therapy. Phase II trials demonstrated that two doses of gemtuzumab ozogamicin (GO) given as monotherapy induced remissions in 26% of adults with CD33+, relapsed AML. The mechanisms leading to resistance in the other three-fourths of the patients are so far incompletely understood, however active drug efflux is likely important.

Drug efflux mediated by members of the adenosine triphosphate-(ATP) binding cassette (ABC) superfamily of proteins has been implicated in AML resistance against conventional chemotherapeutic drugs (reviewed in ). The best characterized and most intensively studied is permeability-glycoprotein (Pgp or MDR1 or ABCB1). Pgp is expressed in many normal tissues but is also found on blast cells of 19-75% of de novo AML cases. Lower CR rates and/or decreased overall or disease-free survival have been noted among patients with Pgp-positive AML treated with conventional chemotherapy. Similarly, phase II trials with GO revealed that blast cell Pgp expression correlated with treatment failure. In vitro studies have further demonstrated that Pgp expressing cell lines and patient AML blast cells are resistant to GO, and that Pgp inhibitors can restore drug sensitivity in Pgp expressing cells. Despite this evidence, the Pgp inhibitor cyclosporine (CSA) enhanced in vitro GO-induced cytotoxicity in only one third of Pgp expressing AML blast cell samples, suggesting that other mechanisms play a role in GO resistance.
The multidrug resistance protein 1 (MRP1 or ABCC1), another widely expressed ABC transporter, extrudes conjugated and unconjugated organic anions and modulates the toxicities of chemotherapeutic agents in normal tissues (reviewed in 12). MRP1 is overexpressed (defined as a level surpassing that of normal blood leukocytes) in 7-30% of de novo AML cases (reviewed in 13). Higher frequencies are found in younger patients, in cases of secondary AML, and among more immature CD34+ blast cell subpopulations.8,14,15 Although MRP1 overexpression confers a similar but not identical pattern of drug resistance as Pgp in malignant cells in vitro, its clinical significance in AML is a matter of debate. The majority of studies to date observed no association between MRP1 protein expression and treatment outcome. Nevertheless, isolated reports have identified MRP1 expression or MRP activity alone, or the simultaneous activities of MRP and Pgp, as adverse prognostic factors.16-18 MRP2 (ABCC2) has a more limited normal tissue distribution than MRP1 but transports a similar range of xenobiotics and chemotherapeutic agents.12 MRP2 is also expressed in a significant portion of AML cases, however the role of this transporter in drug resistance of AML is unknown.6,18 There is limited information about a potential role of MRP1 in GO-resistance. In a single study of 27 AML blast cell samples, the in vitro cytotoxic effect of GO did not correlate with the expression of MRP1 protein or MRP activity.11 However, in this particular study, concomitant Pgp activity was detected in most of the samples, thereby potentially confounding interpretation of correlations between MRP expression or function and GO-induced cytotoxicity.

As an immunoconjugate, GO must be internalized and subsequently undergo intracellular processing to release the toxic calicheamicin moiety. Because of this extensive trafficking, surface and intracellular membrane-associated transporter proteins, such as Pgp and MRP1, may have distinct roles in modulating GO susceptibility. The primary objective of the present study was therefore to characterize a potential role of MRP activity in GO-induced cytotoxicity, to correlate this effect with MRP1 and/or MRP2 expression, and to differentiate this effect from that mediated by Pgp.
MATERIALS AND METHODS

**Cell lines:** The two human promyelocytic cell lines, HL-60 and NB4, were maintained in RPMI medium 1640 (GIBCO Invitrogen, Carlsbad, CA) supplemented with 5% heat-inactivated bovine calf serum (BCS, HyClone, Logan, UT). The human erythroblastic TF1 cell line was maintained in RPMI medium 1640 with 25 mM HEPES buffer (GIBCO) supplemented with 10% heat-inactivated BCS, 1 mM MEM sodium pyruvate (GIBCO), and 4 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; Amgen, Thousand Oaks, CA). The anthracycline-resistant subline of HL-60 (HL-60/AR) and the vincristine-resistant HL-60 subline (HL-60/VCR, both kindly provided by Dr. K.N. Bhalla, University of South Florida, Tampa, FL) were grown in RPMI medium 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS, HyClone), 1 mM MEM sodium pyruvate, and 0.1 mM MEM non-essential amino acids (GIBCO). These cell lines were maintained in the presence of selective drugs (1 µM doxorubicin or 1.1 µM vincristine, respectively), which were removed at least 2 days prior to each experiment.

**Patient AML blast cell samples:** Marrow samples were taken from adult patients in untreated first relapse of AML (non-M3 subtypes) who participated in the phase II clinical trials with GO.2-4 Thawed aliquots of frozen samples of density gradient-isolated mononuclear cells containing leukemic blast cells were used for these studies. All patients signed informed consents, and the institutional review boards of the participating institutions approved all protocols.

**Determination of multidrug resistance protein expression:** A multiparameter cytofluorometric immunofluorescence staining method was used to characterize the membrane display of CD33 and the surface or intracellular expression of drug transporter proteins. Monoclonal antibodies included: L4F319 and P67.6 (Becton Dickinson, San Jose, CA) to recognize CD33; 4E3.16 for Pgp (provided by R. Arceci, Johns Hopkins University, Baltimore, MD); MRPm6,8,14,16,20-22 for MRP1; and M2I-4 for MRP2 (both from Kamiya Biomedical Company, Seattle, WA). CD33 expression in cell lines was detected by staining with phycoerythrin (PE)-conjugated P67.6. For detection of the extracellular Pgp epitope, cells were stained with the 4E3.16 antibody followed by a secondary fluorescein
isothiocyanate (FITC)-conjugated antibody as previously described. For detection of the intracellular epitopes of MRP1 and MRP2, cells were first permeabilized (PermeaFix, Ortho Diagnostic Systems, Raritan, NJ) for 30 minutes at room temperature. The cells were then washed twice in ice-cold PBS/2% FBS, and incubated with the primary antibody for 20 minutes on ice, followed by a secondary FITC-conjugated antibody. For a staining negative control, parallel samples were incubated with an irrelevant isotype control antibody at the same protein concentration as the primary monoclonal antibody. To assess CD33 on AML blasts, samples were first incubated with the IgM anti-CD33 antibody L4F3 or the appropriate nonbinding isotype antibody for 20 minutes on ice, followed by staining with a secondary phycoerythrin (PE)-conjugated antibody. To identify nonviable cells, all samples were also stained with propidium iodide (PI, Sigma, St. Louis, MO). At least 10,000 events were acquired and analyzed on a FACScan flow cytometer using the Cellquest software (Becton Dickinson). Data analyses were done on PI− cells (cell line samples) or CD33+/PI− cells (patient AML blast samples) with size and granularity properties consistent with leukemic cells. The staining intensity ratio was calculated by dividing the mean fluorescence intensity of cells stained with a given specific antibody by the mean fluorescence intensity of cells stained with the corresponding nonbinding isotype control antibody. A ratio >1 indicates the presence and a ratio <1 indicates the absence of the assessed antigen, respectively.

**Determination of functional Pgp and MRP activity:** Pgp function was determined by the efflux of the fluorescent dye 3,3′-diethyloxacarbocyanine iodide (DiOC2, Aldrich, St. Louis, MO) in the presence or absence of the Pgp inhibitor CSA (Novartis Pharma AG, Basel, Switzerland), as previously described. The anionic dye 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate (CDCF, Molecular Probes, Eugene, OR) is a substrate for both MRP1 and MRP2 after the lipophilic blocking groups are cleaved by nonspecific esterases. The efflux of CDCF in the presence or absence of the leukotriene D4 receptor antagonist MK-571 (BIOMOL Research Laboratories Inc., Plymouth Meeting, PA) was used as a measure of functional MRP activity. MK 571 is a strong competitive inhibitor of MRP1 but less potent against MRP2. Dose-finding experiments with CDCF/MK-571 revealed that the sensitivity of this drug/inhibitor combination to detect functional MRP activity strongly depended on the dye concentrations. Thus, sensitivity was optimized.
when CDCF was used at 0.05 µM. For dye efflux assays, 6 x 10^5 cells were incubated for 30 minutes at 37°C in the dark in either a 10 ng/mL solution of DiOC2 or a 0.05 µM solution of CDCF in the presence or absence of CSA (2.5 µg/mL) or MK-571 (10 µM), respectively. Cells were then washed three times and resuspended in fresh dye-less medium with or without the inhibitor and incubated for 90 minutes at 37°C to allow time for active efflux. Prior to analysis of dye fluorescence, cells were stained with PI, and additionally, in the case of patient AML blast cell samples, with anti-CD33-PE or the respective isotype control antibody. All samples were analyzed on a FACScan flow cytometer, gating on the PI− cell population (for cell lines) or CD33+/PI− cell population (for patient AML samples). Results are expressed as the ratio of the mean fluorescence intensity in the presence of the inhibitor divided by the mean fluorescence intensity in the absence of the inhibitor, also referred to as the inhibitor-modulating factor.\textsuperscript{15}

**Assays for GO-induced cytotoxicity:** For experiments with cell lines, cells were taken during the log-phase of growth and distributed into 96-well round bottom plates (Falcon®, Becton Dickinson Labware, Franklin Lakes, NJ) in 240 µL culture medium (6.5 x 10^4 cells/well). Various concentrations of GO were added and after 2 hours incubation at 37°C in 5% CO2 and air, the cells were washed and resuspended in 200 µL fresh complete medium without drug. Parallel cultures were incubated with GO in the presence of various concentrations of CSA, MK-571, or combinations of these inhibitors, prior to washing and resuspension in fresh medium containing the inhibitors but no GO. For experiments with patient AML blast cells, 5 x 10^4 cells/well were cultured in 240 µL RPMI medium 1640 with L-glutamine/2% FBS containing either 10 ng/mL GO or 10 ng/mL of the control immunoconjugate hCTM01 (calicheamicin conjugated to an anti-MUC1 antibody; provided by Wyeth-Ayerst)\textsuperscript{4} in the presence or absence of CSA (2.5 µg/mL), MK-571 (20 µM), or combinations thereof. After 2 hours incubation, cells were washed and resuspended in the presence or absence of CSA (1 µg/mL), MK-571 (20 µM), or a combination of CSA and MK 571, in 200 µL Iscoves modified Dulbecco medium (IMDM, GIBCO) with 20% FBS and 100 ng/mL of the following recombinant human cytokines: GM-CSF, stem cell factor, interleukin-3, and granulocyte colony-stimulating factor (all from Amgen) as previously described.\textsuperscript{4} Drug-induced cytotoxicity was determined after 3 days of culture using a cell viability assay and/or an annexin V based apoptosis assay. The 3-day time-course was
chosen based on our preliminary studies and observations by others that apoptotic changes are minimal at 24-48 hours after exposure to GO, but are significantly increased after 72-96 hours. To determine the number of viable cells after exposure to GO, a commercial assay was used that measures the conversion of the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) to a colored formazan product (Promega, Madison, WI). With this method, the absorbance at 490 nm is directly proportional to the number of viable cells. For our assays, 20 µL of the MTS tetrazolium compound was added to 100 µL of cell suspension and incubated for 4 hours at 37°C in 5% CO₂ and air. The absorbance was then determined at 490 nm using a 96 well plate reader (µQuant, Bio-Tek Instruments Inc., Winooski, VT). To assess apoptosis, cells were stained with annexin V (BD PharmMingen, San Diego, CA) according to the manufacturer’s instructions. All samples were also stained with PI and analyzed on the FACScan flow cytometer. Non-permeable apoptotic cells were identified as annexin V⁺/PI⁻, whereas permeable necrotic cells were identified as PI⁺.

**mRNA extraction and quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR).** Total RNA was extracted and purified using a commercial kit and the manufacturer’s instructions (Absolutely RNA™ RT-PCR Miniprep Kit, Stratagene, La Jolla, CA). RNA was then quantitated, aliquoted, and stored at -80°C until further use. Quantitative RT-PCR for Pgp, MRP1, and MRP2 transcripts was performed using the Taqman chemistry and the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Branchburg, NJ). Transporter transcript levels within each sample were normalized for the expression of β2-microglobulin mRNA within that sample. The sequences of each primer set and the respective FAM/BHQ-1 fluorogenic probe (Synthegen, Houston, TX) are shown in table 1. The transporter primer sets were designed to amplify cDNA across one intron-exon boundary, to avoid amplifying genomic DNA. Gel electrophoresis of the RT-PCR product obtained with the ABI Prism 7700 was carried out to confirm amplification of a single product with the expected size. Duplicate RNA samples from cell lines or patient AML blast cells were used as templates with 500 nM of each primer pair and 200 nM of probe in TaqMan® One-Step RT-PCR Master Mix supplemented with Multiscribe™ and RNase Inhibitor Mix (Applied Biosystems), in a total volume of 50 µL. Samples were reverse transcribed for 30 min at 50°C followed by heat-deactivation of
the reverse transcriptase for 5 min at 95°C. The cDNA product was subsequently amplified for 40 cycles, each consisting of 30 s at 95°C, 30 s at 59.5°C, and 30 s at 72°C. Serial dilutions (1:10, from 1 µg to 100 pg) of RNA from the TF1 cell line were used to generate standard curves for each RT-PCR product and to calculate normalized transcript levels. The normalized transcript levels in TF1 cells were used to compare the relative expression levels of Pgp, MRP1, and MRP2 transcripts in all other cell lines and patient AML samples. The TF1 cell line was chosen for this purpose since Pgp, MRP1, and MRP2 are constitutively expressed in these cells. The C_t value (i.e. the cycle number at which emitted fluorescence exceeds the 10 times standard deviation of base-line emissions as measured between cycles 3 and 15) was determined for each transporter and ß2-microglobulin RT-PCR product for each sample. The relative amount of starting mRNA in the reaction sample was then calculated using the Sequence Detection Systems software (version 1.9, Applied Biosystems). For each sample, mRNA levels of each multidrug resistance gene were divided by the ß2-microglobulin mRNA level to obtain a normalized transcript level. To compare the relative transcript levels in the other cell lines and patient samples, the normalized ratios for Pgp, MRP1, or MRP2 (normalized for ß2-microglobulin) in the TF1 RNA standard cells were arbitrarily set at 1.

Statistical analysis. Results are presented as means±SD from several independent experiments with single measurements for data on antibody staining ratios and inhibitor modulating factors, as well as for data on apoptosis/necrosis in patient samples. For data on apoptosis and cell viability in cell lines, results are presented as means±SEM obtained from independent experiments with triplicate or quadruplicate wells. Parametric statistical tests were used throughout. Continuous variables between groups were compared using a two-tailed unpaired Student’s t test or a one-sample t test, as appropriate. In addition, linear correlations (Pearson) were performed. Statistical calculations were done using InStat version 3.05 (GraphPad, San Diego, CA, USA); p<0.05 was considered significant.
RESULTS

MRP and Pgp expression in human CD33^+ cell lines
We first sought to characterize the expression of MRP1, MRP2, and Pgp in the five CD33^+ hematopoietic cell lines: NB4 cells, HL-60 cells, an anthracycline-resistant HL-60 subline (HL-60/AR), a vincristine-resistant HL-60 subline (HL-60/VCR), and TF1 cells. All cell lines stained positively for MRP1 and MRP2 (Table 2). As anticipated, HL-60/AR cells highly overexpressed MRP1 protein. Accordingly, all cell lines showed functional MRP activity, as determined by CDCF efflux and an inhibitor-modulating factor for MK-571 of ≥1.1. The extent of efflux varied considerably with only minimal activity in NB4 cells, intermediate activity in HL-60 and HL-60/VCR cells, and high activity in TF1 and HL-60/AR cells (Table 2). Of note, the inhibitor-modulating factor of HL-60/AR cells likely underestimated the true MRP activity because those cells could not be loaded with CDCF to the same degree as the other cell lines. Whereas the high MRP activity in HL-60/AR cells correlated with the higher surface display of MRP1 protein, the high CDCF efflux activity of TF1 cells did not correlate with MRP1 and MRP2 immunostaining. To investigate this discordance further, Western blots were prepared using cell lysates and methods previously reported. Probing with MRPm6 yielded high background staining that prevented quantitation. Repeat blots were probed with QCRL-1 (Santa Cruz Biotechnology, Santa Cruz, CA), a purified anti-MRP1 antibody that performed similarly to MRPm6 in immunofluorescence assays (data not shown). This revealed relative MRP1 levels (normalized to µg of total protein) of 0.30, 1.00, and 4.52 in NB4, TF1, and HL-60/AR cells, respectively. Real-time RT-PCR assays revealed that HL-60/AR and TF1 cells expressed the highest relative levels of MRP1 mRNA, compared to NB4, HL-60, and HL-60/VCR cells (Table 2). Relative mRNA levels for MRP2 varied less among the individual cell lines (Table 2). Furthermore, within each cell line, the normalized transcript levels of MRP2 were approximately 10-100 fold lower than the normalized levels of MRP1 mRNA (data not shown). Thus, MRP1 expression, reflected by relative mRNA and whole cell protein levels, but not MRP2 expression, appeared to correlate with CDCF efflux and inhibition by MK-571 in these cell lines.
Pgp was detected on the surface of TF1 and HL-60/VCR cells by immunostaining, but not on HL-60, HL-60/AR, or NB4 cells, respectively (Table 2). Consistent with these findings, functional Pgp activity, as assessed by DiOC₂ efflux and inhibition by CSA, was high in TF1 cells and very high in HL-60/VCR cells. Virtually no dye efflux was observed in HL-60 and NB4 cells. Real-time RT-PCR assays similarly detected no measurable levels of Pgp mRNA in NB4 and HL-60 cells. By comparison, minimal DiOC₂ efflux activity and very low, but detectable, relative Pgp transcript levels were found in HL-60/AR cells, whereas 200-fold and 6500-fold higher relative levels of Pgp mRNA were found in TF1 and HL-60/VCR cells, respectively, correlating with the higher protein and activity levels in those cell lines (Table 2).

**GO-toxicity in human CD33⁺ cell lines: effect of inhibition of MRP and/or Pgp**

Based on the phenotypic characterizations for transporter expression, NB4 and HL-60 cells were used as models for Pgp-negative/MRP positive cells, whereas the TF1 cells were used as the model for Pgp-positive/MRP positive cells. Exposure of HL-60 and NB4 cells to GO for 2 hours, at clinically achievable concentrations (1-40 ng/mL), resulted in dose-dependent growth inhibition (Fig 1A) and induction of apoptosis and necrosis (Fig 1B,C). MK-571 enhanced the cytotoxic effect of GO in both cell lines, however the effect on cell number at 72 hours was demonstrable only in HL-60 cells. The lesser effect of MK-571 in NB4 cells is consistent with the lower MRP activity in those cells compared to HL-60 cells. By comparison, the Pgp-positive/MRP positive TF1 cells were resistant to GO at concentrations up to 40 ng/mL (Fig 2). Drug susceptibility was restored by CSA but not by MK 571 alone (up to 10 µM). However, MK-571 further enhanced GO-induced necrotic cell death in the presence of CSA, suggesting that MRP activity contributes to GO resistance in that cell line (Fig 2). HL-60/AR and HL-60/VCR cells were completely resistant to GO up to 50 ng/mL. In contrast to the effects observed in the other cell lines, CSA (up to 2.5 µg/mL) and MK-571 (up to 30 µM), either alone or in combination, failed to enhance GO-cytotoxicity in these drug-selected HL-60 sublines (data not shown), suggesting that either the very high overexpression of Pgp or MRP1 prevented the effects of multidrug reversal agents, or other resistance mechanisms are active in these cells.
**Effect of MK-571 on GO-induced cytotoxicity in patient AML blast cells**

We next addressed whether MK-571 could enhance GO-induced cytotoxicity in blast cells taken from patients with AML in first relapse. A total of 23 samples were analyzed for expression and functional activity of Pgp and MRP, as well as for GO-induced cytotoxicity in the presence or absence of multidrug reversal agents. CDCF efflux was found to a variable degree in all samples, with an inhibitor-modulating factor for MK-571 ranging from 1.10 to 1.41. Of note, the patient blast cell samples did not take up the CDCF dye as well as the cell lines, potentially leading to an underestimation of MRP efflux activity. MRP proteins were detected in all samples; staining fluorescence ratios for MRP1 ranged from 1.02 to 1.23 and for MRP2 ranged from 1.10 to 1.41. Adequate RNA was available for analysis of MRP1 and MRP2 transcripts in 11 samples. The relative levels of MRP1 mRNA, normalized to amounts of β2-microglobulin, ranged from 0.01 to 0.78 compared to the level of 1 arbitrarily established for the TF1 RNA standard. The relative levels of MRP1 mRNA significantly correlated with MRP activity in these samples (r=0.62, p<0.05), whereas the amount of MRP1 protein detected by immunofluorescence did not (r=-0.116, p=0.66). Because of limited sample availability, Western blot assays could not be carried out. MRP2 mRNA was also detectable in all 11 samples. The relative transcript levels, normalized to β2-microglobulin, ranged from 0.04 to 1.39 (compared to the level of 1 arbitrarily established for the TF1 RNA standard). In comparison to MRP1, neither the amount of MRP2 protein detected by immunofluorescence (r=0.168, p=0.51) nor the relative level of MRP2 mRNA (r=0.037, p=0.91) correlated with the MRP activity in these samples. Thus, similar to the observations with the cell lines, MRP1 mRNA expression, but not MRP2, correlated with functional dye efflux associated with MRP activity.

Pgp activity was observed in 17 out of 23 patient samples with DiOC₉ inhibitor-modulating factor values for CSA ranging from 1.02-3.15 (mean: 1.42). In these samples, there was a significant correlation between the staining intensity ratio for Pgp and Pgp activity (r=0.639, p<0.008). For the 13 samples with adequate RNA, a highly significant correlation was also observed between Pgp activity and the relative levels of Pgp mRNA normalized to the amounts of β-2-microglobulin (r=0.95, p<0.0001). Among these samples, the relative normalized mRNA levels ranged from 0.01 to 0.36 compared to the level of 1 arbitrarily.
established for the TF1 RNA standard. These levels were all higher than the relative Pgp mRNA level found in HL-60/AR cells. The level of MRP co-expression among the Pgp-positive samples did not differ from the level in Pgp-negative samples (mean inhibitor-modulating factor values for MK-571: 1.20±0.09 versus 1.23±0.10, p=0.56), suggesting that, at least in this small cohort, Pgp and MRP are independent resistance factors.

CSA enhanced GO-induced cytotoxicity in 12 of the 17 Pgp-positive samples (Fig 3). The percentage of apoptotic cells was increased by the addition of CSA in 5 samples (Fig 3A, patient nos. 5, 7, 12, 16, and 17; range: from +2.1 to +23.0%, mean increase +11.1±7.8%), whereas CSA increased necrotic cells in 12 samples (Fig 3B, patient nos. 1, 2, 5-8, 11-13, and 15-17; range: from +1.5 to +34.6%, mean increase +10.1±9.7%). The ability of CSA to enhance GO-induced cytotoxicity was not strongly associated with the relative level of Pgp functional activity (Fig 3). The mean DiOC2 inhibitor-modulating factor value for CSA-responsive samples was 1.52±0.73 compared to 1.19±0.26 for nonresponsive samples (p=0.35). In contrast to the frequent effects of CSA alone, GO-induced cytotoxicity was increased by addition of MK-571 alone (20 µM) in only one patient sample (Fig 3B, sample no. 1), which co-expressed minimal Pgp activity (CSA inhibitor-modulating factor 1.02) but was responsive to CSA. Because we were concerned that an effect due to MK-571 might be masked by co-expression of Pgp, we studied the effects of combinations of CSA and MK 571 with GO. In 3 of the other 11 Pgp-positive/MRP-positive samples that showed increased GO-cytotoxicity with CSA alone, the addition of MK-571 further enhanced the percentages of apoptotic and/or necrotic cells (Fig 3, sample nos. 2, 5, and 16). Reproducible MK-571 enhancement was seen on repeated assays of responsive samples (data not shown). There were no differences in the MRP activities of the MK-571-responsive samples (mean inhibitor-modulating factor level of 1.26±0.08) compared to the nonresponsive samples (mean inhibitor-modulating factor level of 1.20±0.10; p=0.39). No MK 571 responses were seen among the 6 Pgp-negative AML samples (data not shown).
DISCUSSION

In the present study, the specific MRP-inhibitor MK-571 enhanced GO-induced cytotoxicity in 3 out of 5 MRP-expressing CD33+ hematopoietic cell lines. However, MK-571 alone increased cytotoxicity only in the HL-60 and NB4 cells, which do not concomitantly express Pgp. By comparison, MK-571 enhanced cytotoxicity in the Pgp-positive/MRP-positive TF1 cells only when combined with the Pgp inhibitor CSA. Likewise, GO-induced toxicity was enhanced to a variable degree in a small proportion of blast cell samples from patients with relapsed AML. In only one case was MK-571 alone effective; in 3 cases GO-induced cytotoxicity was enhanced by MK-571 only in combination with CSA. These data indicate that MRP may contribute to GO resistance, but that inhibition of Pgp function may be required to reveal an effect of MRP on GO-susceptibility.

All relapsed AML blast samples assessed in this study showed some level of MRP activity, as indicated by MK-571-sensitive efflux of CDCF. The differences of CDCF efflux between individual samples were rather small. Overall, the inhibitor-modulating factor values for MK 571 were roughly comparable to that observed in NB4 cells and none reached the level found in parental HL-60 cells. These observations support the hypothesis of others that the MRP activity in most clinical AML cases may represent “basal” MRP activity rather than “overexpression” of this transporter protein. 27 Despite this relatively low level of MRP activity, an effect of MK-571 on GO-induced cytotoxicity could be demonstrated in the NB4 and HL-60 cell line as well as in some patient cell samples. Thus, “basal” MRP expression may reduce GO susceptibility, presumably through efflux of the calicheamicin moiety of the conjugate. This finding would be consistent with the notion that even very low levels of MRP can affect the cells’ sensitivity to conventional chemotherapeutic drugs and that baseline MRP expression is sufficient for a protective role against a toxin. 6,12

To date, only one study has addressed a potential role of MRP in GO-resistance. 11 In that study low MRP activity was found in almost all of the 27 AML patient samples studied but neither MRP1 protein expression nor MRP activity correlated with the in vitro cytotoxic effect of GO. The MRP inhibitor probenecid alone also had no effect on GO-induced cytotoxicity. Importantly, most of the samples from that study, similar to our findings, also
expressed Pgp activity.\textsuperscript{11} It is therefore possible that effects of MRP, including the inability
of probenecid alone to increase GO-induced cytotoxicity, were confounded by unblocked
Pgp function.

Our observations concur with recent studies demonstrating that Pgp function mediates GO
resistance in patient blast cell samples and in cell lines.\textsuperscript{4,10,11} Theoretically, Pgp extrudes
the calicheamicin moiety that dissociates from the immunoconjugate after internalization.
Indeed, Pgp was implicated as a drug resistance factor in early studies of calicheamicin
derivatives\textsuperscript{31} and these compounds are similar in size and structure to other known Pgp
substrates.\textsuperscript{32} However, systematic studies to track intracellular calicheamicin are hindered
by the limitations that calicheamicins do not autofluoresce and labeling is difficult. Pgp
activity was detected in 17 out of 23 of our relapsed AML cell samples. However, in many
of the cases, the Pgp activity was relatively low (mean inhibitor-modulating factor level of
1.42). We observed that CSA significantly enhanced \textit{in vitro} GO-induced cytotoxicity in
roughly 70\% of the samples, including some with low Pgp activity. In turn, many of our
results suggest that MRP function plays a comparatively minor role. First, MK-571 alone
enhanced GO-induced cytotoxicity in only 1 sample, which had minimal Pgp activity.
Second, the relative increases in percentages of apoptotic and/or necrotic cells in the
presence of CSA were greater than the increases in the presence of MK-571. And third, an
effect due to MK-571 always required concomitant CSA in cells that co-expressed more
than minimal Pgp activity. We hypothesize that, in those co-expressing cells, unopposed
efflux by Pgp likely prevented intracellular drug accumulation and therefore masked a
minor effect of MK-571 alone. A predominant role of Pgp over MRP is further suggested by
our recent observations that repeated exposure of parental HL-60 cells to incremental
concentrations of GO induces functional Pgp activity without a change in basal MRP
activity (unpublished observations). Together, these findings emphasize the importance of
Pgp in GO-resistance and the rationale for treatment trials combining GO with Pgp
inhibitors.

The functional assays used for these studies were chosen because they are among the
most specific available for assessment of MRP and Pgp. Unfortunately, specific
fluorescent substrates and inhibitors have not yet been developed for unique members of
the MRP family, such as MRP1 and MRP2. CDCF is known to be a substrate for MRP1 and MRP2, but not for Pgp.\textsuperscript{33-35} MK-571 is a competitive inhibitor of transport mediated by MRP1 and, to a lesser degree, MRP2,\textsuperscript{24,36} but does not affect Pgp activity.\textsuperscript{37,38} Our experiments confirmed these findings; MK-571 did not block DiOC\textsubscript{2} efflux in TF1 cells, HL-60/VCR cells, or Pgp-positive blast cell samples. By comparison, Pgp pumps DiOC\textsubscript{2} out of the cell, but the dye is not a substrate for MRP.\textsuperscript{39} Similarly, we found no DiOC\textsubscript{2} efflux in cell lines that express MRP but not Pgp. Thus, these dye/inhibitor combinations served as valid surrogates for MRP and Pgp transporter activities in cells expressing both proteins.

Because MRP1 and MRP2 have both been found in AML blasts,\textsuperscript{6,18} we wanted to understand the potential role of each transporter in GO resistance. The different levels of MRP activity among the CD33\textsuperscript{+} cell lines correlated with whole cell MRP1 protein levels and were partially reflected by cell surface display of MRP1, whereas they were not associated with MRP2 surface immunostaining. Neither MRP1 nor MRP2 immunofluorescence staining correlated with CDCF efflux in the patient AML blast cell samples, likely because of the low levels in this group and the relative insensitivity of the assay. By comparison, a relatively good correlation was found between the functional MRP activity and normalized mRNA levels for MRP1 among both the cell lines and the 11 patient AML blast cell samples tested. MRP2 mRNA levels, on the other hand, showed no correlation with MRP activity in either the CD33\textsuperscript{+} hematopoietic cell lines or the patient AML samples. A similar association between MRP1 mRNA levels and functional MRP dye efflux activity, but no correlation with MRP2 transcript levels, has been observed in AML patient samples by others.\textsuperscript{18} These data suggest that quantitative levels of MRP1 mRNA may be used to assess the relative functional activity of MRP and that MRP2 does not contribute significantly to MRP activity in AML blasts. Although the level of Pgp protein detected by immunofluorescence correlated with Pgp activity in the patient AML blast cell samples an even better correlation was found between the relative level of Pgp mRNA and Pgp activity. This finding could be explained by the recent observation that intracellular, compartmentalized Pgp may be functionally active but not detected by surface immunostaining.\textsuperscript{40} Thus, the real-time quantitative mRNA assays appear to be valid and useful for estimation of relative MRP and Pgp functional activity, especially in cases with low-level expression.
CSA was used in the present study since this drug has been used clinically as a Pgp reversal agent.\textsuperscript{9,41,42} Of note, previous studies have shown that CSA may also weakly inhibit MRP1- and MRP2-mediated transport.\textsuperscript{43-45} Indeed, we observed that CSA partially inhibited CDCF efflux in all cell lines (inhibitor modulating factor levels ranging from 1.13±0.03 for NB4 cells [n=3, p<0.05] to 1.57±0.15 for HL-60/AR cells [n=3, p<0.05]) and in patient AML blast cell samples (inhibitor modulating factor levels ranging from 1.06 to 1.29 [p<0.0001]). Although CSA inhibits MRP function to a much lesser extent than MK-571, it is possible that some of the effect of CSA we observed in \textit{in vitro} cytotoxicity assays was due, at least in part, to inhibition of MRP function. On the other hand, the fact that MK-571 had an additive effect with CSA in enhancing GO-induced cytotoxicity both in some patient samples and the TF1 cell line suggests that CSA does not sufficiently block MRP activity to fully restore GO susceptibility.

Because MK-571 and/or CSA failed to enhance GO-induced cytotoxicity in roughly 30\% of the Pgp-positive/MRP-positive patient cell samples, alternative mechanisms of resistance are likely operative. Hypothetically, a variety of mechanisms could contribute to diminished GO activity: alterations in the process of GO internalization, resulting in decreased GO uptake; differences in intracellular trafficking,\textsuperscript{46} resulting in diminished release of the active calicheamicin moiety; additional drug efflux systems, such as other ABC transporter proteins; and dysregulation of downstream factors that either increase the cells’ capability to repair DNA or prevent cascades leading to cell death. Additional studies will be required to dissect out alternative mechanisms that may be important for GO resistance and may also be relevant for other antibody-based treatments. Knowledge of such mechanisms will facilitate the development of new agents that avoid resistance or clinical interventions that reverse or bypass these barriers.

\textbf{Acknowledgments}

We thank Steven J. Staats for performing Western blot assays, and Deborah E. Banker for helpful discussions.
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12. Leslie EM, Deeley RG, Cole SPC. Toxicological relevance of the multidrug resistance protein 1, MRP1 (ABCC1) and related transporters. Toxicology. 2001;167:3-23.


FIGURE LEGENDS

Figure 1
Effect of MK-571 on GO-induced cytotoxicity in Pgp-negative/MRP-positive cell lines. Exposure of HL-60 and NB4 cells to GO for 2 hours resulted in dose-dependent cytotoxicity, as reflected by decreased viable cell numbers measured by the MTS assay (A), and increased percentages of apoptotic (annexin V+/PI-) and necrotic (PI+) cells (B and C). The MRP inhibitor MK-571 (5 µM) increased GO-induced apoptosis and necrosis in both cell lines at 72 hours and further decreased HL-60 cell numbers compared to controls. Results are shown as mean±SEM from 4-7 individual experiments performed in quadruplicate (A) or triplicate wells (B,C).

Figure 2
Effect of multidrug resistance reversal agents on GO-induced cytotoxicity in Pgp-positive/MRP-positive TF1 cells. Exposure of TF1 cells to GO for 2 hours did not change the frequency of apoptotic (annexin V+/PI-) cells (A) or necrotic (PI+) cells (B) after 72 hours. CSA alone (1.25 µg/mL) enhanced GO-induced apoptosis and cell death, whereas MK-571 (10 µM) alone was ineffective. The combination of CSA and MK-571, however, significantly increased the percentage of necrotic cells compared to the effects of CSA alone. Results are shown as mean±SEM from 3 individual experiments performed in triplicate wells.

Figure 3
Effect of multidrug resistance reversal agents on GO-induced cytotoxicity in Pgp-positive/MRP-positive AML blast cell samples. The 17 patient samples are ordered (1 to 17) from the lowest to the highest DiOC2 inhibitor-modulating factor values for CSA (range: 1.02 to 3.15). The effect of GO alone or GO in combination with the MRP inhibitor MK-571, the Pgp inhibitor CSA, or MK-571+CSA are depicted as the percentage increases in apoptosis (A) or necrosis (B) compared to patient samples treated with the control immunoconjugate CTM01. GO-induced cytotoxicity (apoptosis and/or necrosis) was significantly enhanced by MK-571 alone in sample no. 1, whereas MK-571 further enhanced the effect of CSA on GO-induced cytotoxicity in sample nos. 2, 5, and 16.
(*denotes p-value <0.05). These effects were confirmed in a second independent experiment. CSA alone caused significant enhancement (p-value <0.05) in GO-induced cytotoxicity in sample nos. 1, 2, 5-8, 11-13, and 15-17. Values are shown as mean±SD of triplicate assays.
FIGURE 1

A

HL-60  


NB4  


B

Apoptotic Cells (%)  


C

Necrotic Cells (%)  


GO (ng/mL)  

GO (ng/mL)
FIGURE 2

A

Apoptotic Cells (%)

Control  MK-571
CSA  CSA+MK-571

GO (ng/mL)

B

Necrotic Cells (%)

0 2.5 5.0 7.5 10.0 40
FIGURE 3

A  Apoptotic cells

B  Necrotic cells

% Increase compared to CTM01

Patient sample

% Increase compared to CTM01

Patient sample
<table>
<thead>
<tr>
<th>Target cDNA</th>
<th>Primer/probe combinations</th>
</tr>
</thead>
</table>
| **Pgp**    | Forward 5’-CAGCTCCTGGAGCGGTTCTA  
Revers 5’-CGCTTTATTTCTTTGCCATCAA  
Probe 5’-CCCCTTGGCAGGGAAAGTGCTGC  
PCR product size: 68 bp |
| **MRP1**   | Forward 5’-TCATGGTGCCCGTGTAATG  
Revers 5’-CGATTGTCTTTGCTCTCATGTG  
Probe 5’-ATGGCGATGAAGACCAAGACGTATCAGGT  
PCR product size: 79 bp |
| **MRP2**   | Forward 5’-TGCAGCCTCCATAACCATGA  
Revers 5’-GGACCTCAGATGCCTGCCA  
Probe 5’-TCGAACACTTAGCCGCAGTTCTAGGTCCA  
PCR product size: 88 bp |
| **ß2-microglobulin** | Forward 5’-CTTCCGGCGCCGAGATGTC  
Revers 5’-CTCCAGGCCAGAAAGAGAGAGTAG  
Probe 5’-CCGTCGCTTTAGCTGCTGCTGC  
PCR product size: 70 bp |
Table 2 Cell surface display, functional activity, and relative mRNA levels for Pgp and MRP in human CD33+ hematopoietic cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Pgp</th>
<th>MRP1</th>
<th>MRP2</th>
<th>Pgp</th>
<th>MRP</th>
<th>Relative Normalized mRNA Level&lt;sup&gt;3&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>Cell Surface Staining Intensity Ratio&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Dye Efflux Inhibitor -Modulating Factor&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HL-60</td>
<td>0.96±0.03 (7)</td>
<td>1.17±0.04 (15)</td>
<td>1.30±0.05 (8)</td>
<td>1.01±0.00 (9)</td>
<td>1.85±0.19 (10)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NB4</td>
<td>0.96±0.01 (5)</td>
<td>1.16±0.03 (5)</td>
<td>1.19±0.12 (2)</td>
<td>1.01±0.00 (3)</td>
<td>1.23±0.04 (3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TF1</td>
<td>1.21±0.11 (6)</td>
<td>1.17±0.04 (7)</td>
<td>1.17±0.03 (3)</td>
<td>1.43±0.19 (4)</td>
<td>2.90±0.74 (4)</td>
<td>1</td>
</tr>
<tr>
<td>HL-60/AR</td>
<td>0.95±0.05 (6)</td>
<td>1.43±0.10 (6)</td>
<td>1.23±0.06 (3)</td>
<td>1.02±0.00 (4)</td>
<td>2.39±0.28 (4)</td>
<td>0.006</td>
</tr>
<tr>
<td>HL-60/VCR</td>
<td>1.75±0.08 (4)</td>
<td>1.17±0.03 (5)</td>
<td>1.26±0.03 (2)</td>
<td>4.51±0.23 (3)</td>
<td>1.80±0.23 (4)</td>
<td>38.8</td>
</tr>
</tbody>
</table>

<sup>1</sup> Shown is the mean±SD of the staining intensity ratio (mean fluorescence intensity of cells stained with specific antibodies divided by the mean fluorescence intensity of cells stained with a isotype control antibody).  
<sup>2</sup> Pgp activity was determined by the efflux of the fluorescent dye DiOC<sub>2</sub> with or without the inhibitor CSA. MRP activity was determined by the efflux of CDCF with or without the inhibitor MK-571. Results are expressed as inhibitor modulating factor (ratio of the mean fluorescence intensity in the presence of the inhibitor divided by the mean fluorescence intensity in the absence of the inhibitor). The number of independent experiments is given in parenthesis.  
<sup>3</sup> Relative normalized mRNA levels were determined by real-time RT-PCR, and one representative experiment is shown. Results are expressed as the transcript levels of Pgp, MRP1 or MRP2, divided by the β2-microglobulin transcript level. The normalized ratios for TF1 transcript levels were arbitrarily set at 1.
because that cell line constitutively expresses all three transporters and TF1 RNA was used to generate standard curves for each experiment (see text).
Multidrug resistance protein (MRP) attenuates gemtuzumab ozogamicin-induced cytotoxicity in acute myeloid leukemia cells

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