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ORIGINAL ARTICLE

Multidrug resistance-associated protein 1 expression is under the control of the phosphoinositide 3 kinase/Akt signal transduction network in human acute myelogenous leukemia blasts

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A high incidence of relapses following induction chemotherapy is a major hindrance to patient survival in acute myelogenous leukemia (AML). There is strong evidence that activation of the phosphoinositide 3 kinase (PI3K)/Akt signaling network plays a significant role in rendering AML blasts drug resistant. An important mechanism underlying drug resistance is represented by overexpression of membrane drug transporters such as multidrug resistance-associated protein 1 (MRP1) or 170-kDa P-glycoprotein (P-gp). Here, we present evidence that MRP1, but not P-gp, expression is under the control of the PI3K/Akt axis in AML blasts. We observed a highly significant correlation between levels of phosphorylated Akt and MRP1 expression in AML cells. Furthermore, incubation of AML blasts with wortmannin, a PI3K pharmacological inhibitor, resulted in lower levels of phosphorylated Akt, downregulated MRP1 expression, and decreased Rhodamine 123 extrusion in an *in vitro* functional dye efflux assay. We also demonstrate that wortmannin-dependent PI3K/Akt inhibition upregulated p53 protein levels in most AML cases, and this correlated with diminished MRP1 expression and enhanced phosphorylation of murine double minute 2 (MDM2). Taken together, these data suggest that PI3K/Akt activation may lead to the development of chemoresistance in AML blasts through a mechanism involving a p53-dependent suppression of MRP1 expression.
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Introduction

The phosphoinositide 3 kinase (PI3K)/Akt signaling pathway is a key mediator of cell growth, survival, and apoptosis, and its constitutive activation has been implicated in both the pathogenesis and the progression of a wide variety of neoplasias.[1] Upon activation by different growth factors and cytokines, PI3K generates phosphatidylinositol$^{3-5}$ trisphosphate (PtdIns(3,4,5) P_3), which in turn activates a number of important downstream proteins.[2] The serine (Ser)/threonine (Thr) kinase Akt (also known as protein kinase B or PKB) is a well-characterized downstream target of PI3K, and binding of PtdIns(3,4,5) P_3 to the pleckstrin homology domain of Akt results in its translocation to the proximity of the plasma membrane, where it subsequently undergoes phosphorylation within its catalytic loop at Thr 308, and C-terminally at Ser 473.[2] The former phosphorylation step is effected by phosphoinositide-dependent kinase 1 which is recruited to the vicinity of the plasma membrane by PtdIns 3,4,5 P_3, whereas the latter is effected by a kinase not yet conclusively identified. Activated Akt targets many proteins that are deeply involved in cell growth, survival and apoptosis, including Bad, caspase-9, FoxO transcription factors, glycogen synthase kinase 3β, and Inhibitor kinase B (IκB) kinase (IKK), which then regulates nuclear factor kappa B (NF-κB) function.[3–5]

Acute myelogenous leukemia (AML) is a clonal hematological disease characterized by multiple genetic anomalies resulting in alterations in transcription factor regulation and in signal transduction pathways that lead to impaired cell differentiation, excessive proliferation, and inadequate apoptosis.[6,7] Recent studies showed that PI3K/Akt signaling is frequently activated in AML cell lines and patient blasts and strongly contributes to proliferation, survival, and drug resistance of these cells[8–12] (see Martelli *et al.*[13] for an updated review on this issue). Moreover, constitutive activation of PI3K/Akt is associated with a poorer prognosis of AML.[14]

Drug resistance owing to PI3K/Akt upregulation has been generally interpreted as being the consequence of Akt-dependent blockage of proapoptotic signaling cascades,[10] given that most chemotherapeutic drugs kill cancer cells by inducing apoptosis.[15] However, recent evidence has highlighted that in prostate cancer cell lines, PI3K/Akt activation leads to multidrug resistance-associated protein 1 (MRP1) expression and chemoresistance.[16] MRP1 is a well-characterized member of ATP-binding cassette (ABC) membrane transporters that function as drug efflux pumps. These membrane proteins are responsible for the transport of hundreds of substrates, including hormones, lipids, drugs, and other toxins, across both extracellular and intracellular membranes.[17,18] Several studies have highlighted that overexpression of ABC transporters correlates with *in vitro* and *in vivo* drug resistance of AML cells as well as with poorer prognosis.[19–22] With the above in mind, we decided to investigate whether or not there was a relationship between PI3K/Akt activation and MRP1 expression in AML blasts. Here, we show that in these cells a strong correlation exists between upregulation of the PI3K/Akt survival pathway and MRP1 expression, whereas such a relationship was not found for the

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170-kDa P-glycoprotein (P-gp), the product of the MDR1 gene, which is another well-characterized ABC transporter. We also demonstrate that PI3K/Akt downregulated by means of wortmannin, upregulated p53 in most AML cases, and this correlated with diminished MRP1 levels and enhanced phosphorylation of MDM2. Our results highlight a possible mechanism by which upregulation of the PI3K/Akt pathway leads to drug resistance of AML blasts.

Materials and methods

Antibodies and chemicals

The following antibodies were employed in this study. For Western blot analysis with MRP1, a mouse monoclonal (clone MRPm5) from Chemicon International, Temecula, CA, USA; for flow cytometric analysis with MRP1, a fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal (clone QCRL-3) from BD Biosciences Pharmingen, Milan, Italy. For Western blot analysis with P-gp, a mouse monoclonal (clone F4) from Sigma-Aldrich, St Louis, MO, USA; for flow cytometric analysis with P-gp, a phycoerythrin (PE)-conjugated mouse monoclonal (clone 17F9) from BD Biosciences Pharmingen. For Western blot analysis with Akt, a rabbit polyclonal (catalog number 9272) from Cell Signaling Technology, Beverly, MA, USA. For Western blot analysis with Ser 473 p-Akt, a rabbit polyclonal (catalog 92719) from Cell Signaling Technology). For flow cytometric analysis with Ser 473 p-Akt, either an Alexa Fluor 488-conjugated rabbit polyclonal (catalog 2336) or an Alexa Fluor 647-conjugated rabbit polyclonal (catalog 2337) from Cell Signaling Technology. For Western blot and flow cytometric analysis with p53, a rabbit polyclonal from Cell Signaling Technology (catalog 9282). For Western blot with MDM2, a mouse monoclonal (clone 2A10) from Calbiochem, La Jolla, CA, USA. For Western blot and flow cytometric analysis with Ser 166 p-MDM2, a rabbit polyclonal from Cell Signaling Technology (catalog 3521). PE-conjugated anti-human CD33 (clone P67.6, mouse monoclonal) was from BD Biosciences. Normal rabbit IgG, Alexa Fluor 488-conjugated (catalog number 16–237), was from Upstate, Lake Placid, NY, USA; normal mouse IgG, either FITC- or PE-conjugated and FITC-conjugated anti-rabbit or anti-mouse IgG were from BD Biosciences. For Western blot analysis to β-tubulin, a mouse monoclonal (clone 2-28-33) from Sigma-Aldrich. The MRP1 inhibitor MK571 was from Calbiochem. The Multidrug Resistance Direct Dye Efflux Assay was from Chemicon International, Temecula, CA, USA. Wortmannin was from Sigma.

Cell culture and MRP1 cDNA transfection

NB4 acute human promyelocytic leukemia cells, wild-type (WT) CEM acute human T-lymphoblastic leukemia cells and their multidrug resistant subclone (VBL100),[23] and Jurkat cells were routinely maintained in (RPMI) 1640 supplemented with 10% fetal calf serum (FCS) at an optimal cell density of 3–8×10^5 cells/ml. NB4 cells were transiently transfected with human MRP1 cDNA cloned in pcDNA3.1 (a kind gift form Dr Susan PC Cole, Queen's University, Kingston, Ontario, Canada, USA see ref.[24]). Briefly, cells were transfected with Nucleofactor R using an Amaxa electroporator device set at program T20 (Amaxa, Koln, Germany). Two microgram of cDNA were used for 7×10^6 cells. Saos2 human osteosarcoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS.

Isolation of mononuclear cells from bone marrow and peripheral blood

All samples were obtained at diagnosis before chemotherapy induction, after informed consent of the patients in accordance with institutional guidelines. Peripheral blood mononuclear cells (PBMC) or bone marrow mononuclear cells (BMMC) were isolated by density gradient centrifugation (Ficoll-Paque Plus, 1.077 g/ml, from Amersham Biosciences, Milan, Italy) and were frozen at a concentration of 30–60×10^6cells/ml in 10% dimethylsulfoxide (DMSO), 45% RPMI Rosewell Park Memorial Institute medium 1640, and 45% FCS. The AML cases were defined according to the classification of the French–American–British (FAB) committee. Percentage of blasts in the samples ranged between 75 and 90% and was checked by flow cytometry surface immunostaining, depending on the phenotype of the leukemia (usually CD13, CD33, CD34, alone or in combination). AML blasts (1×10^6/ml) were cultured in methylcellulose medium (Methocult, Stem Cell Technologies, Vancouver, Canada, USA) supplemented with human recombinant growth factors: interleukin (IL)-3 (20 ng/ml), IL-6 (20 ng/ml), and stem cell factor (50 ng/ml) (complete medium). $CD34^+$ cells from normal bone marrow and peripheral blood leukocytes (PBL) were isolated from healthy donors after informed consent using magnetic cell separation (Miltenyii Biotec, Bergisch Gladbach, Germany) or by density gradient centrifugation, respectively.

Flow cytometric analysis of MRP1 and P-gp expression

For detection of MRP1 intracellular epitope, cells were washed with phosphate-buffered saline (PBS, pH 7.4), fixed with Reagent 1 of the Intraprep kit, according to the manufacturer's instructions (Beckman Coulter, Miami, FL, USA), permeabilized with saponin-based Reagent 2, and incubated at 4°C for 12 h with FITC-conjugated anti-MRP1 monoclonal antibody (2 μl/10^5 cells). A double immunostaining procedure combining surface staining with PE-conjugated anti-CD33 (Beckman Coulter) and cytoplasmic staining for MRP1 was also performed. In brief, 5×10^5 AML blast cells were incubated with 10 μl of PE-conjugated anti-CD33 for 20 min at room temperature. Cells were washed with PBS and then fixed and stained for MRP1 as described above. For detection of P-gp cell surface expression, cells were washed with PBS and incubated with 4 μl/10^6cells of PE-conjugated anti-P-gp monoclonal antibody for 1 h at 4°C. After incubation with antibodies, cells were washed with PBS and analyzed on an Epics XL flow cytometer (Beckman Coulter). Appropriately isotype-matched controls (same IgG subclass at the same concentration as the antibody tested) were used as control in all assays. At least 5000 events were analyzed for each sample.

Flow cytometric detection of intracellular Ser 473 p-Akt, p53, and Ser 166 p-MDM2 levels

This was performed essentially as reported previously[25] However, an Alexa Fluor 488-conjugated rabbit polyclonal antibody to Ser 473 p-Akt was employed (5 μl/10^5cells). In some cases, samples were double stained with FITC-conjugated anti-MRP1 and Alexa Fluor 647-conjugated anti-Ser473 p-Akt. They were then washed with PBS and analyzed by flow cytometry, using a dual-laser FC500 flow cytometer (Beckman Coulter), equipped with CXP software. For detection of p53 or Ser 166–MDM2, the primary antibody (3 μl/10^5cells) was revealed by means of an FITC-conjugated anti-rabbit IgG. At least 5000 events were analyzed for each sample.

Multidrug resistance direct dye efflux assay

To assess functional drug efflux, the ability of leukemic blasts to efflux Rhodamine 123 was measured in single-color flow cytometric assay. Rhodamine 123 is a substrate for both P-gp and MRP1.[20] The assay was performed essentially as described in the manufacturer's instructions, with some modifications. Briefly, approximately 5×10^5 AML blasts were centrifuged**,** and the pellet was resuspended in cold Loading Buffer containing Rhodamine 123 and incubated at 4°C for 45 min for measurement of basal dye uptake. Subsequently, cells were washed twice with PBS and incubated for 3 h at 37°C, as it has been reported that MRP1-mediated functional efflux is slower than P-gp-dependent efflux.[20] Then, test tubes were put on ice to stop the reaction, samples were washed twice with ice-cold PBS, and cells resuspended in cold PBS. Cellular fluorescence was analyzed on gated leukemic blasts, selected by forward and side-scatter characteristics, after baseline dye uptake (4°C) and after efflux (37°C), using an Epics XL flow cytometer. The MRP1 inhibitor MK571 was used at 20 μM.[26] At least 5000 events were analyzed for each sample.

Preparations of cell homogenates for Western blot analysis

Cells (at least 2×10^6/sample) were resuspended at $\sim 10^7$/ml in 10 mM Tris–HCl, pH 7.4, 1 mM $MgCl_2$, 1 mM ethylene glycol bis(2-aminoethylether)-N,N,N′,N′,-tetraacetic acid (EGTA), 1% Triton X-100, 250 mM sucrose, 25 mM Na pyrophosphate, supplemented with the COMPLETE Protease Inhibitor Cocktail (Roche Applied Science, Milan, Italy). Samples were incubated at 4°C for 15 min, then centrifuged at 10 000 g for 10 min at 4°C. Protein concentration was assayed using the Protein Assay kit (detergent compatible, from Bio-Rad, Hercules, CA, USA). Lysates were stored at −80°C until Western blot analysis.

Western blot analysis

Protein (40 μg/sample), separated on sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE), was transferred to nitrocellulose membranes. The membranes were saturated in PBS containing 0.1% Tween-20 and 5% w/v nonfat dry milk (blocking buffer) for 60 min at 37°C, then incubated overnight at 4°C with the primary antibodies diluted in PBS containing 0.1% Tween-20 and 5% w/v nonfat dry milk for the monoclonals, or in PBS containing 0.1% Tween-20 and 5% bovine serum albumin for the polyclonals. Dilutions of the antibodies used for Western blotting were as follows: Anti-MRP1: 1:250; anti-P-gp: 1:5000; anti-Akt and anti-Ser 473 p-Akt: 1:1000; anti-p53: 1:500; anti-MDM2: 1:100; anti Ser-166 p-MDM2: 1:500; anti-β-tubulin: 1:10 000. After four washes in PBS containing 0.1% Tween-20, membranes were incubated for 30 min at room temperature with the appropriate peroxidase-conjugated secondary antibodies (Cell Signaling Technology), diluted 1:2000 in PBS, 0.1% Tween-20, and washed as described above. Protein bands were visualized by the enhanced chemiluminescence (ECL) method (Phototope-HRP Western blot detection kit, from Cell Signaling Technology). Blots shown are representative of three different experiments.

Statistical analysis

The Pearson rank correlation and the two-tailed Students *t*-test were used to describe correlations between variables and evaluate their significance. Analyses were performed using the Statistical Package for the Social Sciences (SPSS) software, version 11 · 0.1. A $P < 0.05$ was considered to be significant.

Results

Specificity of the antibodies

As in some cases of AML, the number of blasts available for analytical purposes represents a limiting factor, we decided to evaluate MRP1, P-gp, and Ser 473 p-Akt levels in AML patient primary cells by flow cytometry. Indeed, this approach allows the use of less cells when compared with Western blot and is rapidly becoming the preferred technique for the analysis of drug resistance-linked proteins[27] and phosphorylated proteins[28–30] in AML. Therefore, a very critical issue concerned the specificity of the antibodies used in our study. For this reason, a series of control experiments were first performed to validate the antibodies employed in this investigation. NB4 human leukemia cells were transiently transfected with a human MRP1 cDNA and the MRP1 protein expression was analyzed 24 h after transfection by both flow cytometry and Western blot (Figure 1a). Although control (untransfected) cells were practically negative by both techniques, flow cytometric analysis with an FITC-conjugated antibody revealed that approximately 70% of the cells expressed MRP1. Western blot with a monoclonal antibody to human MRP1 supported this conclusion, by showing that transfected cells expressed a predominant band migrating at approximately 190-kDa.

To assess specificity of the antibody employed to detect P-gp, we took advantage of CEM cells that are P-gp negative, and of their drug-resistant variant, VBL100 cells, which overexpress P-gp.[23] As shown in Figure 1b, flow cytometric analysis demonstrated that WT CEM cells stained negatively, whereas VBL100 stained positively for P-gp. Once again, Western blot confirmed these findings.

To control for Ser 473 p-Akt, we took advantage of HL60 cells, which have low or undetectable levels of this phosphorylated Akt form,[25] and of VBL100 cells, which, on the contrary, have elevated levels, because they lack PTEN lipid phosphatase.[23,31] As illustrated in Figure 1c, flow cytometric analysis of samples stained with an Alexa Fluor 488-conjugated antibody to Ser 473 p-Akt showed HL60 cells to be negative, whereas VBL100 cells were positive. Western blot analysis corroborated these findings. The specificity of the Alexa Fluor 647-conjugated antibody to Ser 473 p-Akt was also tested, employing Jurkat cells which, like CEM and VBL-100 cells, lack PTEN and consequently have elevated levels of Ser 473 p-Akt.[31] When treated with wortmannin (300 nM for 24 h), a selective PI3K pharmacological inhibitor, Jurkat cells displayed a much lower level of Ser 473 p-Akt, as revealed by flow cytometric analysis of samples stained with the Alexa Fluor 647-conjugated antibody to the phosphorylated Akt form (Figure 1d). Once again, Western blot analysis confirmed these results. Overall, these experiments validated the antibodies we used for cytofluorimetric investigations aimed at evaluating the levels of MRP1, P-gp, and Ser 473 p-Akt.

Patients

BMMC and PBMC fractions from 32 patients diagnosed with AML were examined in this study. Median age was 55.3 (range: 21–85 years). For patient demographics and disease characteristics, refer to Table 1.

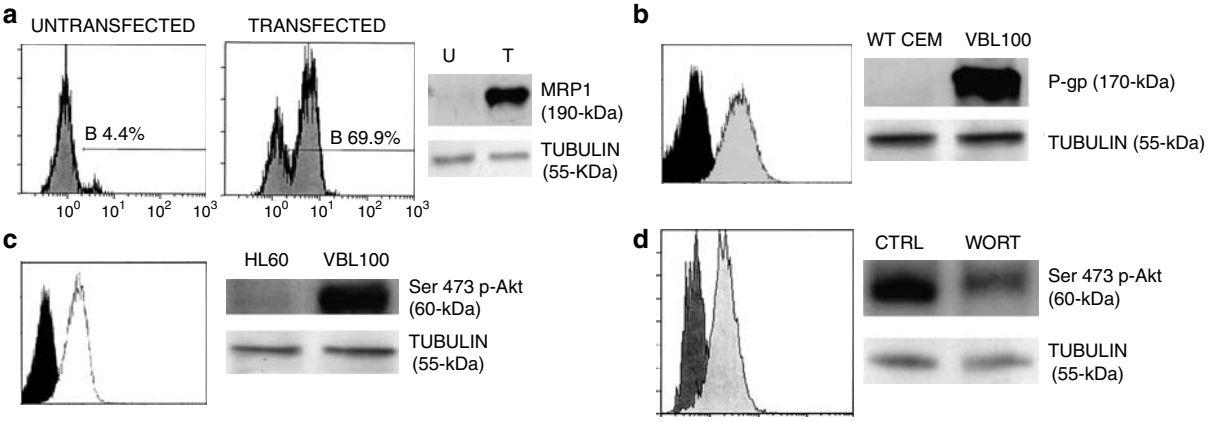

Figure 1 Specificity of the antibodies. (**a**) NB4 cells were transiently transfected with MRP1 cDNA and then analyzed by both flow cytometry and Western blot with antibodies recognizing MRP1. For Western blot analysis: U, untransfected cells; T, transfected cells. (**b**) WT CEM cells and their VBL100 drug-resistant variant were analyzed by flow cytometry and Western blot for the expression of P-gp. For flow cytometry analysis, dark-shaded histogram represents WT CEM, whereas light-shaded histogram indicates VBL100 cells. (**c**) HL60 and VBL100 cells were analyzed for the levels of Ser 473 p-Akt using both flow cytometry (dark-shaded histogram: HL60; light-shaded histogram: VBL100) and Western blot. For flow cytometrical analysis, an Alexa Fluor 488-conjugated antibody to Ser 473 p-Akt was employed. (**d**) Jurkat T-lymphoblastoid leukemia cells were analyzed for Ser 473 p-Akt levels using both flow cyotmetry with an Alexa Fluor 647-conjugated antibody (dark-shaded histogram: wortmannin-treated cells; light-shaded histogram: control cells) and Western blot. For the blot: CTRL, control cells; WORT, wortmannin-treated cells.

Table 1 Biological characteristics of AML patients

Case	*Age*	*Sample*	*Fab classification*	*Karyotype*
1	55	BMMC	M4	Inv16
2	65	BMMC	M0	Normal
3	38	PBMC	M1	Normal
4	68	PBMC	M2	Inv9, dup1p
5	74	BMMC	M1	Normal
6	64	PBMC	M5	NA
7	57	PBMC	M5	Normal
8	48	PBMC	M1	Normal
9	39	PBMC	M0	Normal
10	85	PBMC	M4	46xy, inv16
11	21	PBMC	M2	Normal
12	65	PBMC	M0	NA
13	84	PBMC	M2	Normal
14	61	PBMC	M1	NA
15	57	PBMC	M1	NA
16	45	PBMC	M3	t(15;17)
17	39	PBMC	M3	t(15;17)
18	45	PBMC	M1	Complex
19	69	BMMC	M0	Normal
20	82	BMMC	M0	+11
21	35	BMMC	M3	t(15;17)
22	66	PBMC	M4	46xy, inv(3) (q21q26)
23	58	PBMC	M4	46xy, inv(3) (q21q26)
24	58	PBMC	M1	46xx, inv(3) (q21q26)
25	63	PBMC	M5	46xx, t(4,11) (q21q23)
26	47	PBMC	M1	46xx, inv(3) (q21q26)
27	31	PBMC	M5	Complex
28	45	BMMC	M5	Normal
29	86	PBMC	M0	+8
30	41	PBMC	M4	46xy, inv16
31	35	BMMC	M2	t(8;21)
32	61	PBMC	M2	t(8;21)

Abbreviations: AML, acute myelogenous leukemia; NA, not available.

Analysis of MRP1 and P-gp expression in AML patient blasts

In these patients, expression of MRP1 and P-gp was investigated by means of flow cytometry. Samples were considered positive for MRP1 when staining intensity was higher than that of normal mature leukocytes from healthy donors,[32] whereas for P-gp expression, the threshold intensity was represented by $CD34^+$ cells from normal bone marrow (data not shown). The results for this analysis are presented in Table 2. Overall, MRP1 expression was detected in 9/32 patients (28%), whereas P-gp was positive in 11/32 patients (34%). Representative histograms, showing positivity for either MRP1 or P-gp, are illustrated in Figure 2a. As a control, an irrelevant antibody, either FITC- or PE-conjugated mouse IgG, did not stain the samples. Double immunostaining, employing an antibody to CD33, was also performed, to ensure that the population that was positive for MRP1 corresponded to AML blasts, as shown in Figure 2b.

Relationship between MRP1/P-gp expression and Ser 473 p-Akt positivity

We then investigated by flow cytometry the activation state of the PI3K/Akt pathway, by analyzing the levels of Ser 473 p-Akt. To this end, we employed an Alexa Fluor 488-conjugated antibody to Ser 473 p-Akt. The results are presented in Table 2 and in Figure 3a. Overall, 19/32 of patients were positive for Ser 473 p-Akt (59%), in agreement with other studies.[14] Of the nine patients expressing MRP1, eight (89%, $P<0.05$) were also positive for Ser 473 p-Akt. In contrast, of the 11 patients expressing P-gp, only three were positive for activated Akt (27%, $P>0.05$) (Table 2). We also performed double immunolabeling using FITC-conjugated antibody to MRP1 and Alexa Fluor 647-conjugated antibody to Ser 473 p-Akt, to verify that cells positive for MRP1 were also positive for the phosphorylated Akt form. Two examples of double staining are illustrated in Figure 3b.

Down regulation of Ser 473 p-Akt with wortmannin results in reduced expression of MRP-1

To establish a functional correlation between activation of the PI3K/Akt pathway and expression of MRP1, AML blasts were incubated for 24 h in the presence of either DMSO (solvent carrier) or wortmannin, an irreversible pharmacological inhibitor selective for PI3K. Samples were then stained for both Ser

Table 2 Expression of MRP1, P-gp, and levels of p-Akt and p-Erk 1/2 in AML patients, as analyzed by flow cytometry.

Case	*MRP1*	*P-gp*	*p-Akt*	*p-Erk 1/2*	*MRP1 expression inhibited by wort*	*Dye efflux inhibited by wort*
1	–	–	+	n/a	n/a	n/a
2	–	+	–	n/a	n/a	n/a
3	–	–	–	n/a	n/a	n/a
4	–	+	–	n/a	n/a	no
5	–	+	+	n/a	n/a	n/a
6	–	+	–	n/a	n/a	no
7	–	+	+	n/a	n/a	n/a
8	–	–	–	n/a	n/a	n/a
9	+	–	–	+	no	no
10	–	–	+	n/a	n/a	n/a
11	–	–	+	n/a	n/a	n/a
12	–	+	–	n/a	n/a	n/a
13	–	–	+	n/a	n/a	n/a
14	+	–	+	–	Yes	Yes
15	+	–	+	+	Yes	Yes
16	–	–	–	n/a	n/a	Yes
17	–	–	–	n/a	n/a	n/a
18	+	–	+	–	Yes	Yes
19	–	+	–	n/a	n/a	no
20	–	+	–	n/a	n/a	n/a
21	–	–	+	n/a	n/a	n/a
22	–	–	+	n/a	n/a	n/a
23	+	–	+	–	Yes	Yes
24	+	–	+	+	Yes	Yes
25	–	+	-	n/a	n/a	no
26	+	–	+	+	Yes	Yes
27	+	–	+	–	Yes	Yes
28	–	–	+	n/a	n/a	n/a
29	–	+	–	n/a	n/a	no
30	–	-	+	n/a	n/a	n/a
31	+	–	+	+	no	no
32	–	+	+	n/a	n/a	no

Abbreviations: AML, acute myelogenous leukemia; MRP1, multidrug resistance-associated protein 1; n/a, not assayed; P-gp, P-glycoprotein; wort, wortmannin.

473 p-Akt and MRP1, and analyzed by flow cytometry (Figure 3c). From this set of experiments, it became evident that, when compared with DMSO-incubated samples, wortmannin (300 nM) effectively down regulated Ser 473 p-Akt levels of AML blasts in seven out of eight patients ($P<0.05$) and this was accompanied by a reduction of MRP1 reactivity (Table 2). In contrast, P-gp expression was not down regulated by wortmannin treatment (data not shown).

To rule out that the effect of wortmannin was not specific or owing to toxicity, we investigated Erk 1/2 and p-Erk 1/2 levels in AML blasts. Indeed, it is well known that the Erk 1/2 pathway is up regulated in most cases of AML.[33] Controls were represented by Jurkat cells treated with 80 nM 4α-Phorbol 12–myristate 13-acetate (PMA), a powerful activator of the Erk 1/2 pathway (see Figure 4a). Five out of nine patients, who were positive for MRP1, also had activated Erk 1/2 (Table 2). However, in none of the samples did wortmannin treatment result in down regulated levels of either Erk 1/2 or p-Erk 1/2, as exemplified by case 15 (see Figure 4b). Instead, we reproducibly observed an increase in p-Erk 1/2 levels in all cases treated with wortmannin (Figure 4b). This might depend on the fact that Akt exerts an inhibitory effect on Raf-1 through direct phosphorylation. Hence, inhibition of the PI3K/Akt by wortmannin could remove this inhibition resulting in up regulation of p-Erk 1/2 levels.[34] Moreover, the viability of blasts treated with wortmaninn was analyzed by Trypan blue staining which that demonstrated no significant changes with respect to untreated cells. Indeed, about 80–85% of AML blasts were viable after exposure to the drug (Figure 4c).

Consequences of wortmannin treatment on drug efflux of AML blasts

To determine whether or not exposure to wortmannin had consequences on MRP1 activity in AML blasts, a functional dye efflux assay was performed. To analyze the activity of MRP1, Rhodamine 123 was used, which is a substrate for both P-gp and MRP1.[20] Control cells were pre-incubated for 24 h with solvent carrier (DMSO) or wortmannin. Cells were then loaded with the fluorescent dye at 4°C to measure basal uptake and further incubated for 3 h at 37°C to measure dye efflux. Previous results have shown that a longer efflux period better reflects the activity of MRP1.[20] It is evident from Figure 5 that, in the samples pre-incubated with wortmannin, the dye drug efflux at 37°C was strongly reduced when compared with samples exposed to DMSO only. The basal uptake was not affected by wortmannin treatment (data not presented). Overall, the results obtained with wortmannin were similar to those obtained when control cells (i.e., exposed to DMSO only) were exposed to the MRP1-selective inhibitor MK571 (Figure 5). Wortmannin down regulated dye efflux in seven out of nine patient samples ($P<0.05$), and it also diminished the levels of MRP1 in these samples (Table 2). In contrast, wortmannin did not have any effects on P-gp-mediated dye efflux (Figure 5 and Table 2).

Exposure to wortmannin resulted in p53 up regulation in AML blasts

The transcriptional regulation of MRP1 is only partially understood.[17] However, it has been shown that WT p53 represses

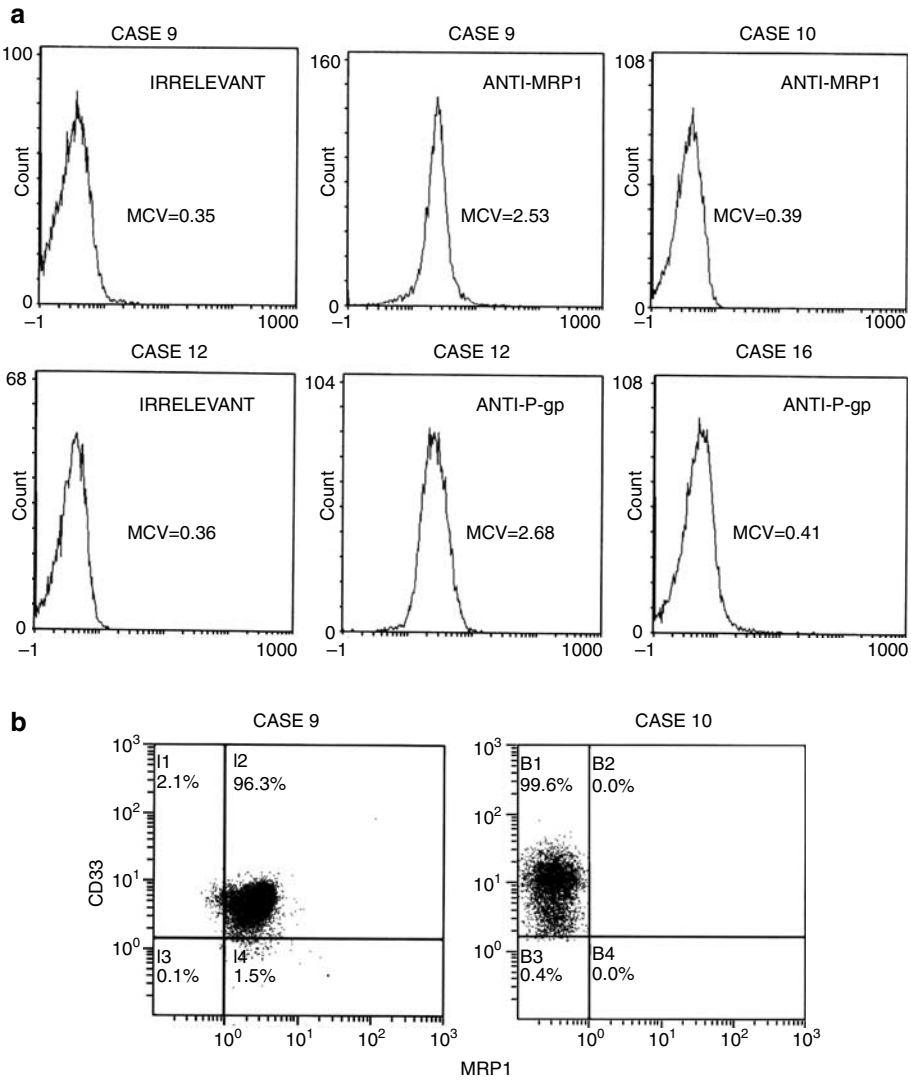

Figure 2 Flow cytometric histograms demonstrating detection of MRP1 or P-gp in AML blasts. (**a**) Samples were single stained using an FITC-conjugated antibody to MRP1 or a PE-conjugated antibody to P-gp. As controls, irrelevant antibodies (either FITC- or PE-conjugated mouse IgG) were employed. MCV: mean channel value. (**b**) Double immunolabeling employing a PE-conjugated anti-CD33 antibody and an FITC-conjugated anti-MRP1 antibody.

transcription of the human MRP1 promoter[35] and loss of p53 expression is correlated with increased MRP1 expression in colorectal cancer.[36] The PI3K/Akt signaling pathway could control p53 expression through interactions with MDM2. Indeed, Akt-mediated MDM2 phosphorylation at Ser 166 increases its interaction with p300, providing a platform to allow the assembly of the protein complex necessary for MDM2-mediated ubiquitination and degradation of p53.[37] Consistently, a PI3K/Akt-dependent down regulation of p53 activity has been reported in some AML cases[10] We, therefore, sought to determine whether or not PI3K/Akt inhibition with wortmannin correlated with increased p53 expression and lowered MDM2 phosphorylation levels in AML blasts. For p53 expression, the negative control was represented by Saos2 human osteosarcoma cells, which are p53 null, whereas the positive control was represented by WT CEM cells, which are known to express p53. Flow cytometry and Western blot analysis demonstrated specificity of the antibody to p53 (Figure 6a). In six out of the seven patients (85%, $P<0.05$) in which wortmannin down regulated MRP1 expression, there was a concomitant increase in the levels of 53 expression (see Table 3). Four cases are illustrated in Figure 6a. The control for MDM2 expression was Jurkat cells, either untreated or treated with wortmannin. As shown in Figure 6b, both flow cytometry and Western blot analysis revealed a decrease in the amount of Ser 166 p-MDM2 in Jurkat cells. Western blot analysis demonstrated that wortmannin treatment did not result in a decrease in the levels of MDM2 protein. Wortmannin effectively reduced the levels of phosphorylated MDM2 in all patient cases in which there was a concomitant increase in p53 expression ($P<0.05$) (Figure 6b and Table 3).

Discussion

Conventional induction chemotherapy with intensive regimens including doxorubicin, idarubicin, etoposide, and mitoxantrone induces complete remission in 65–75% of adults with *de novo* acute AML. However, despite the intensive treatments, a significant proportion of responding patients have relapses and

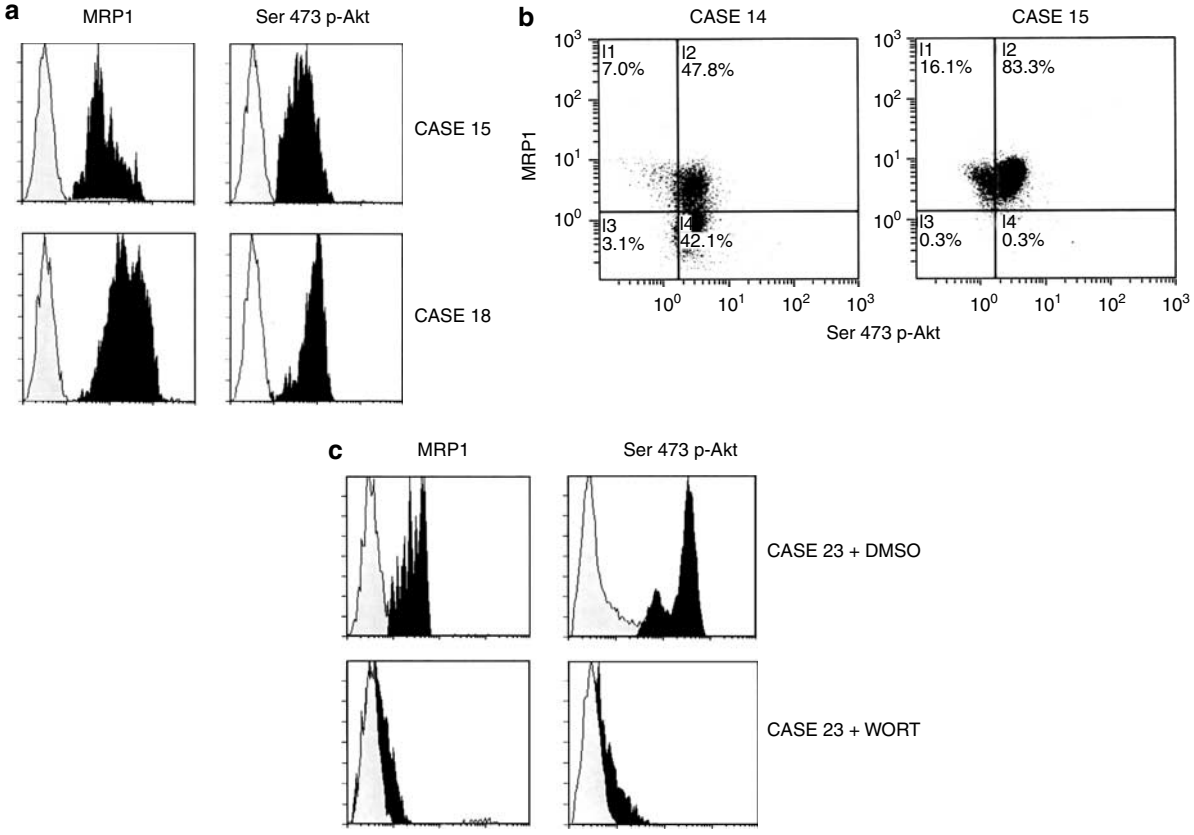

Figure 3 Flow cytometric histograms showing the relationship between MRP1 expression and activation of the PI3K/Akt pathway in AML blasts. (**a**) Samples were either stained with FITC-conjugated anti-MRP1 antibody or Alexa Fluor 488-conjugated anti-Ser 473 antibody (dark-shaded histograms). Irrelevant antibodies (FITC-conjugated mouse IgG or Alexa Fluor 488-conjugated rabbit IgG) were employed as control (light-shaded histograms). (**b**) Double labeling analysis for FITC-conjugated anti-MRP1 antibody and Alexa Fluor 647-conjugated anti-Ser 473 p-Akt antibody. (**c**) Samples were incubated for 24 h in complete medium in the presence of 300 nM wortmannin or an equivalent concentration of DMSO solvent carrier. They were then stained for either MRP1 or Ser 473 p-Akt (dark-shaded histograms). Appropriate irrelevant antibodies, as described above, were employed (light-shaded histograms).

ultimately die of therapy-refractory disease, so that disease-free survival at 5 years ranges from 25 to 35%.[38] Therefore, AML remains a clinical challenge with poor long-term survival, and the identification of aberrantly activated signaling pathways, which contribute to drug resistance, could provide an opportunity to improve the standard therapeutic treatments. The ABC family of drug transporters confer resistance to multiple chemotherapeutic agents when overexpressed in human tumors, including AML. The best-characterized ABC family member is 170-kDa P-gp, the product of the MDR1 gene. However, it has become clear that additional ABC transporters could be involved in drug resistance of AML blasts. MRP1 is encoded by the MRP1 gene located on chromosome 16p13.[39] More recently, additional members, MRP2–MRP9, have been identified; however,their role in multidrug resistance awaits elucidation.[40]

The PI3K/Akt signal transduction pathway plays a key role in controlling neoplastic cell growth, survival, motility and invasion.[41] There is now increasing evidence for a PI3K/Akt-mediated role in the regulation of AML cell cycle, survival, and drug resistance.[13,42] In this study, we showed that, in AML blasts, the expression of MRP1, but not of P-gp, is under the control of the PI3K/Akt axis. As far as we know, this is the first report showing a relationship between MRP1 expression and activation of the PI3K/Akt axis in primary tumor cells. Several observations support this conclusion. There was a statistically significant correlation ($P<0.05$) between the levels of Ser 473 p-Akt and the expression of MRP1 in AML primary cells, whereas this was not true for P-gp. Moreover, treatment of AML blasts with wortmannin, a selective PI3K pharmacological inhibitor, resulted in lower levels of MRP1 expression and in a decreased capability of these cells to extrude Rhodamine 123 in an *in vitro* functional dye efflux assay. This reduced dye extrusion was similar to that obtained with MK571, a chemical that selectively inhibits MRP1 function. In our study, we have analyzed 32 patients for MRP1 and P-gp expression. We found MRP1 expression in 9/32 patients (28%), whereas P-gp was positive in 11/32 patients (34%). These values are consistent with much larger studies demonstrating that MRP1 is overexpressed (defined as a level surpassing that of healthy blood leukocytes) in 7–30% of patients with *de novo* AML,[43] whereas P-gp positivity, exceeding the positivity of normal bone marrow $CD34^+$ cells, is found in 25–40% of patients.[19] The expression of P-gp has been reported to be linked with increasing age of the patients[44] reaching 71% in a group with median age 68 years. Interestingly, although we have investigated a much smaller cohort of patients, the median age of patients with P-gp expression was 68.5 years, whereas the median age of patients with MRP1 expression was 48. Indeed, MRP1 expression is known to be more frequent in patients who are younger.[44] We did not find patients positive for both P-gp and MRP-1. However, according to a study performed on a much larger

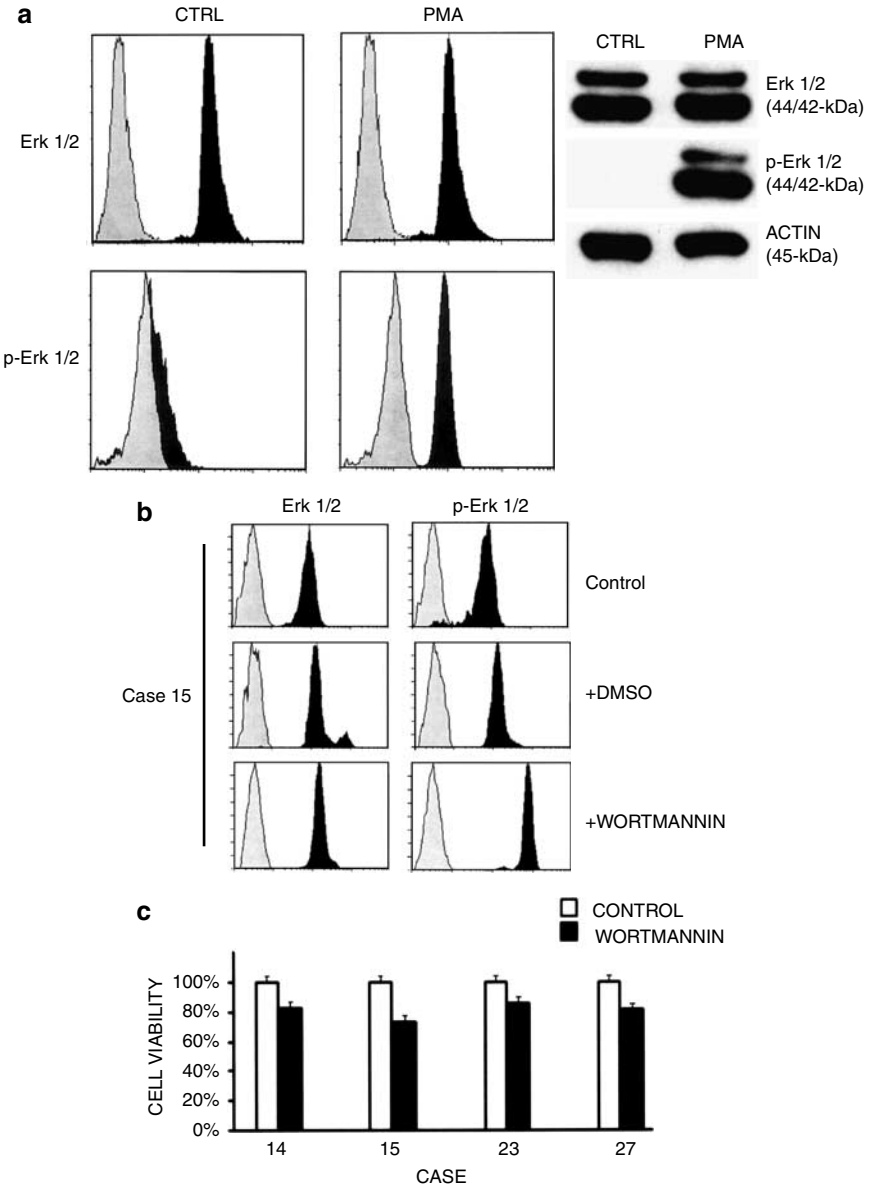

Figure 4 Wortmannin does not affect the levels of either Erk 1/2 or p-Erk 1/2, or viability of AML blasts. (**a**) Permeabilized Jurkat T cells (either untreated, CTRL, or treated for 30 min with 80 nM 4α-phorbol 12-myristate 13-acetate (PMA)) were stained with anti-Erk 1/2 (catalog 9102) or anti-Thr 202/Tyr 204 p-Erk 1/2 antibody (catalog 9106), both from Cell Signaling Technology. The primary antibodies (3 μl/10^5 cells) were revealed by a FITC-conjugated anti-rabbit (Erk 1/2) or anti-mouse (p-Erk 1/2) antibody, then analyzed by flow cytometry. Light-shaded histograms represent samples stained with the secondary antibody only, whereas dark-shaded histograms represent samples reacted with both the primary and the secondary antibody. Western blot analysis of the same samples is shown on the right. Both antibodies to Erk 1/2 were diluted 1:1000. Antibody to β-actin (1:1000, catalog 4967 from Cell Signaling Technology) demonstrated equal loading. Bands were revealed by the ECL technique. (**b**) AML blasts were stained as above immediately after thawing (untreated) or incubated for 24 h in complete medium with either 300 nM wortmannin or an equivalent concentration of DMSO solvent carrier. Light-shaded histograms represent samples stained with the secondary antibody only, whereas dark-shaded histograms represent samples reacted with both the primary and the secondary antibody. (**c**) For evaluation of cell viability, cells were stained with 0.2% Trypan blue in PBS. A total of 300 cells were counted under a microscope for each sample. Results are the mean of three different experiments $\pm$ s.d.

cohort of patients, this condition was found in only 15% of the cases whereas 32% were positive for P-gp and 27% for MRP-1.[19] Thus, it might be that the number of patients we have investigated is too low to detect doubly positive cases.

It is worth reminding here that inhibition of the PI3K/Akt pathway enhances *in vitro* sensitivity of AML blasts to etoposide,[45] which is a well-established MRP1 substrate.[46] This has been interpreted mainly as a consequence of downregulated NF-κB activity, but it could not be ruled out that it was owing to decreased drug efflux by MRP1.

In advanced prostate cancer cells, MRP1 expression has been reported to be under the control of the PI3K/Akt axis at the mRNA and protein level.[16] In this experimental model, silencing of MRP1 expression by siRNA rendered cultured prostate cancer cells sensitive to doxorubicin, a typical MRP1 substrate. Nevertheless, a recent report has shown that LY294002, a

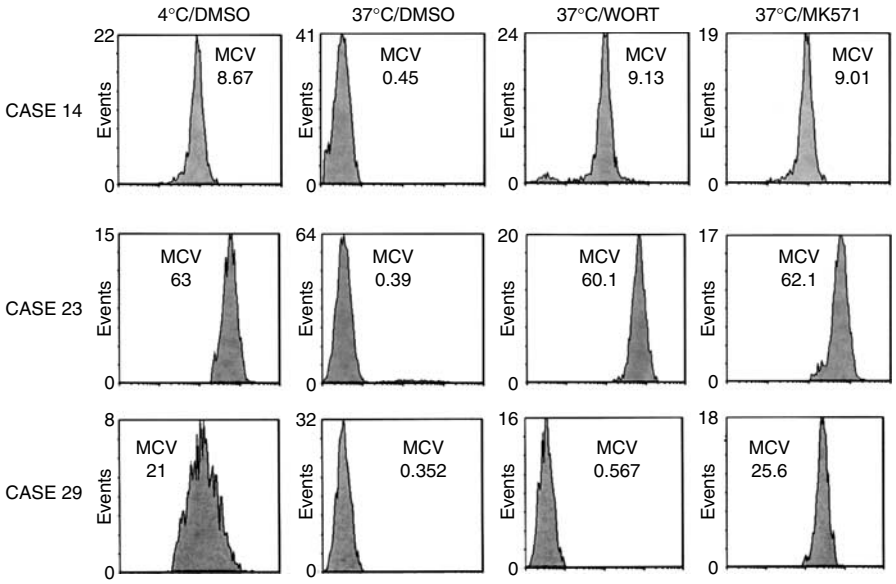

Figure 5 Flow cytometric histograms showing that wortmannin down regulates MRP1-dependent Rhodamine 123 dye efflux in AML blasts. AML blasts were incubated for 24 h in complete medium with either 300 nM wortmannin or an equivalent concentration of DMSO solvent carrier. They were then incubated at 4°C for 45 min to measure Rhodamine 123 dye basal uptake. Basal uptake in wortmannin-treated sample was equal to that of DMSO-incubated sample (not shown). Incubation at 37°C was for 3 h, at the end of which intracellular fluorescence was measured again by flow cytometry. In selected experiments, the MRP1 selective inhibitor MK571 (20 μM) was present during the 37°C incubation. In contrast, wortmannin did not affect P-gp-dependent dye efflux. MCV: mean channel value.

Table 3 Effects of wortmannin on MRP1 and p53 expression, and on phosphorylation of MDM2 at Ser 166 in AML blasts

Case	*MRP1 expression down regulated by wort*	*p53 levels up regulated by wort*	*Ser 166 p-MDM2 levels down regulated by wort*
14	Yes	Yes	Yes
15	Yes	Yes	Yes
18	Yes	Yes	Yes
23	Yes	Yes	Yes
24	Yes	Yes	Yes
26	Yes	no	no
27	Yes	Yes	Yes

Abbreviations: AML, acute myelogenous leukemia; MDM2, murine double minute 2; MRP1, multidrug resistance-associated protein 1; wort, wortmannin.

PI3K pharmacological inhibitor, blocked drug export in a competitive manner from drug-resistant HT29RDB colon carcinoma cells overexpressing MRP1.[47] LY294002 increased the intracellular doxorubicin concentration threefold without any effect on MRP1 expression level or intracellular distribution and was as effective as the MRP1 inhibitor MK571. Competitive drug export blockage was not achieved with wortmannin. For these reasons, we used wortmannin instead of LY294002 to down regulate PI3K/Akt signaling, even if wortmannin is not entirely specific for PI3K. However, it should be reminded here that LY294002 also has off-target activity, because it inhibits casein kinase 2.[48] Information concerning transcriptional regulation of ABC transporters is quite limited. In this work, we focused on a possible p53-dependent mechanism of MRP1 regulation, because it has been demonstrated that WT p53 represses transcription of MRP1 promoter, whereas mutated p53 enhances it.[49] A similar phenomenon has been described in p53-null Saos-2 cells overexpressing gain-of-function mutated p53.[50] It should be pointed out, however, that to date there is no report of any p53-binding motifs located within the MRP1 promoter region.[49] Therefore, one has to hypothesize that p53-mediated repression of MRP1 occurs through an indirect mechanism rather than an immediate interaction with the MRP1 promoter. Nevertheless, we have found that, when MRP1 activity was inhibited by wortmannin treatment, there was a concomitant increase in the amount of p53 as well as a decrease in the levels of Ser 166 p-MDM2. Dominant negative p53 mutations are rare in AML; however, inactivation of WT p53 frequently occurs through interaction with MDM2.[51] While MDM2 overexpression is controversial in AML, it could not be ruled out that in AML blasts, p53 inactivation was also related to a PI3K/Akt-linked increase in MDM2 phosphorylation, as suggested by our findings. In this connection, it is worth remembering that recent work has established that nutlin-3, a small molecule antagonistic of MDM2, synergistically enhanced cytotoxicity of doxorubicin (a well-established MRP1 substrate) in AML blasts.[52] Such an increase in drug sensitivity might also be related to p53-dependent MRP1 down regulation. It has also been reported that specificity protein 1 (Sp1) transcription factor is a strong activator of MRP1 expression.[53] Sp1 activity is PI3K-dependent,[54] and Sp-1 is known to be expressed, albeit to low levels, in some AML cell lines, including THP-1 and HL60.[55] Therefore, future investigations should also aim at establishing the relevance of Sp-1 in controlling MRP1 expression in AML cells.

At clinically relevant concentrations of doxorubicin, drug resistance was related to MRP1 overexpression, but not to P-gp expression, in human myeloid leukemia cell lines;[56] however, MRP1 clinical relevance in AML patients is still a matter of debate, as some studies have reported a negative impact on clinical outcome, whereas other investigations could not confirm this hypothesis.[57] A possible explanation to these

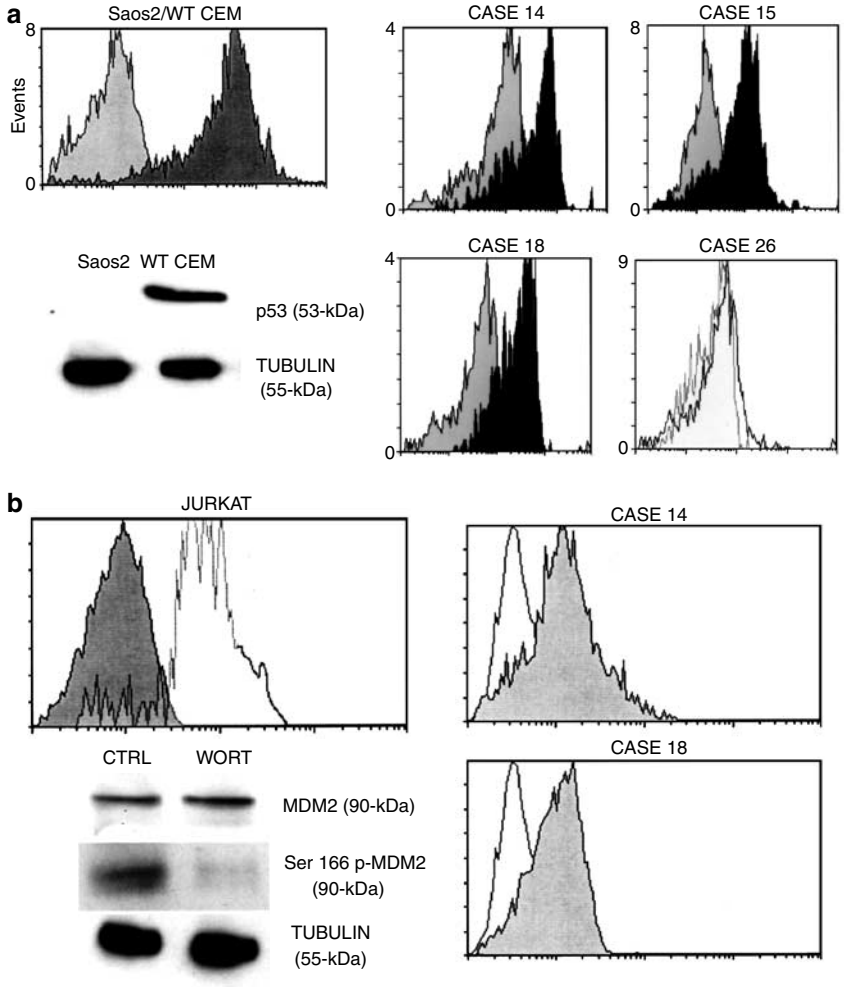

Figure 6 Wortmannin treatment results in p53 up regulation and in reduced levels of Ser 166 p-MDM2. (**a**) Saos2 human osteosarcoma cells (light-shaded histogram) and WT CEM cells (dark-shaded histogram) were fixed, permeabilized, and incubated with the anti-p53 antibody, followed by incubation with the FITC-conjugated anti-rabbit IgG. They were then analyzed by flow cytometry. Western blot analysis confirmed the absence of p53 in Saos2 cells. For patient analysis, samples were incubated for 24 h in complete medium in the presence of 300 nM wortmannin (dark-shaded histograms) or an equal concentration of DMSO solvent carrier (light-shaded histograms). They were immunostained for p53 and analyzed by flow cytometry as detailed above. In the case of patient 26, who did not respond to wortmannin, the blank-shaded histogram represents wortmannin-exposed cells. (**b**) Untreated (control) Jurkat cells (light gray-shaded histogram) or wortmannin-exposed Jurkat cells (dark gray-shaded histogram) were stained for intracellular Ser 166 p-MDM2 as described above, using an FITC-conjugated anti-rabbit IgG, and then analyzed by flow cytometry. Western blot analysis demonstrated a marked decrease in the levels of Ser 166 p-MDM2 in wortmannin (WORT)-treated Jurkat cells as compared with control (CTRL) cells. Note that the levels of MDM2 protein did not decrease. For patient analysis, samples were incubated for 24 h in the presence of 300 nM wortmannin (blank-shaded histograms) or DMSO solvent carrier (gray-shaded histograms). They were analyzed by flow cytometry as described above.

conflicting findings is that multiple mechanisms contribute to drug resistance of AML cells.

In conclusion, we have identified a possible signaling mechanism that controls MRP1 expression in AML blasts. Even though the role of MRP1 in determining drug resistance in AML cells is still controversial, a better knowledge of the molecular pathways that regulate its expression could be of great benefit for the outcome of this disease.

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