

Decitabine, a DNA Methyltransferase Inhibitor, Reduces P-Glycoprotein mRNA and Protein Expressions and Increases Drug Sensitivity in Drug-resistant MOLT4 and Jurkat Cell Lines

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Abstract. Multidrug resistance (MDR) is a major clinical obstacle in the treatment of several cancers including hematological malignancies and solid tumors. The ATP-binding cassette transporter B1 (ABCB1) gene and its product, P-glycoprotein (P-gp), is one molecule that is involved in drug resistance. Here we report on the effect of decitabine (5-aza-2'-deoxycytidine), an inhibitor of DNA methyltransferase, on ABCB1 mRNA and P-gp expressions in drug-resistant MOLT4 and Jurkat cells. We found that decitabine treatment reduced ABCB1 mRNA and P-gp expressions in MOLT4/daunorubicin-resistant and Jurkat/doxorubicin-resistant cells. The decrease in the expression of ABCB1 mRNA and P-gp was accompanied by increased sensitivity to anticancer drugs in both drug-resistant cell lines. Our data suggest that DNA methylation is one of the mechanisms underlying ABCB1/P-gp overexpression in drug-resistant hematopoietic cell lines. The modulation of ABCB1/P-gp by DNA methylation inhibitors may be an effective strategy to overcome P-gp-related drug resistance.

Multidrug resistance (MDR) is a major clinical obstacle in the treatment of several types of cancer including hematological malignancies and solid tumors. The ATP-binding cassette transporter B1 (ABCB1) gene or its product, P-glycoprotein (P-gp), is one molecule involved in drug resistance (1, 2). DNA methylation is an important epigenetic mechanism that controls gene expression (3). Decitabine (5-aza-2'-deoxycytidine) is an inhibitor of DNA methyltransferase, which methylates the 5-position of cytosine in CpG dinucleotides.

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There have been many studies on the effect of DNA de-methylation agents on cancer cells and drug-resistant cells (4-6). However, in order to explore the possibility of epigenetic modifiers in clinical settings, more research is required to characterize the association of the effect of DNA de-methylation agents with the drug-resistant features of several types of cancer cell. In the current study, we investigated the effect of decitabine treatment on the drug sensitivity of two drug-resistant hematopoietic cell lines, MOLT4/resistant to daunorubicin (DNR) and Jurkat/resistant to doxorubicin (DOX), established in our laboratory by culturing cells with stepwise concentrations of daunorubicine and doxorubicine, respectively (7). To elucidate the involvement of DNA methylation, the effects of decitabine treatment on ABCB1/P-gp expression were also assessed.

Materials and Methods

Reagents. Decitabine (5-aza-2'-deoxycytidine), doxorubicin hydrochloride (DOX), and daunorubicin hydrochloride (DNR) were purchased from Sigma-Aldrich (Tokyo, Japan). A mouse monoclonal antibody against human P-gp (17F9), conjugated with R-phycoerythrin (R-PE) and a R-PE-conjugated mouse IgG2b isotype control monoclonal antibody were obtained from PharMingen (San Diego, CA, USA). All other reagents were of the best available grade.

Cell culture and drug-resistant hematopoietic cells. The human acute lymphoblastic leukemia cell line MOLT-4 and the T-cell leukemia cell line Jurkat were obtained from DS pharma biomedical (Tokyo, Japan) and maintained in an RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100,000 IU/l penicillin, and 100 mg/l streptomycin. The DNR-resistant MOLT4 cell subline (MOLT4/DNR) was established in our laboratory (7). For the development of drug-resistant Jurkat sublines, Jurkat cells were exposed to DOX for three months. During the selection of drug-resistant Jurkat cells, the concentration of DOX was gradually increased (7). Accordingly, resistant sublines designated as Jurkat/DOX were developed. Investigations of the characteristics of the cell line were performed on cells grown in drug-free medium for at least 10 days. Before the analysis of ABCB1 mRNA, P-gp

expression, and drug sensitivity, cells were treated with 5 μ M decitabine for four days.

RNA extraction and real-time reverse transcription-PCR. RNA extraction was performed by using a QuickPrep Micro mRNA purification kit (GE Healthcare, Tokyo, Japan) according to the manufacturer's instructions. cDNA was constructed by reverse transcription. The PCR was performed using real-time SYBR green technology and analyzed by an ABI 7700 sequence detector (Life Technologies, Tokyo, Japan). Primer pairs for *ABCB1* and β -actin (*ACTB*) were designed to span an intron sequence and to amplify 128-bp and 148-bp products, respectively. The primer sequences were as follow: *ABCB1* 5'-GGCCTAATGCCGAACACATT-3' and 5'-CAGCGTCTGGCCCTTCTTC-3', *ACTB* 5'-CCCAGGCACCAGGTAGTGAT-3' and 5'-TGCCAGATTTTCTCCATGTGC-3'. The quantity of the *ABCB1* and *ACTB* transcript in each sample was determined from the calibration curve. To compare the expression levels of *ABCB1* mRNA among the cell lines, the ratio of *ABCB1* transcript to *ACTB* transcript was calculated. Each sample was analyzed in triplicate and all PCR products were subjected to 2% agarose gel electrophoresis to ensure that no non-specific amplicon was obtained. To further confirm their sequences, the resultant PCR products were applied to the sequencing analysis.

Western blotting. Parental and drug-resistant cells were harvested and lysed with a lysis buffer (Cellytic-M; Sigma-Aldrich, Tokyo, Japan). Protein concentration was determined by a Bradford protein assay. The samples were diluted with a lysis buffer containing β -mercaptoethanol. Equal amounts of protein were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Immobilong-P; Millipore, Tokyo, Japan). Proteins were detected by immunoblotting followed by ECL chemiluminescence detection (GE Biosciences, Tokyo, Japan). Chemiluminescence signals were detected by a Luminoimage Analyzer LAS-1500 (Fujifilm, Tokyo, Japan).

Flow cytometry. Aliquots of 1×10^6 cells were washed twice in ice-cold PBS and resuspended in 50 μ l of binding buffer, and then incubated with either 20 μ l R-PE-conjugated mouse monoclonal antibody 17F9 against human P-gp, or 20 μ l R-PE-conjugated mouse IgG2b isotype control monoclonal antibody for 30 min at 4°C. After incubation and two further washes in ice-cold PBS, the cells were resuspended in an ice-cold binding buffer and kept on ice in the dark until analysis. P-gp expression was determined using a FACSCalibur analyzer (Becton Dickinson, Tokyo, Japan).

Drug sensitivity test. Cells were washed and suspended in the medium at a density of 5×10^5 cells/ml. Cell suspensions were placed in each well of a 96-well flat-bottom plate (Iwaki, Chiba, Japan). The cells were incubated for 72 h at 37°C in an atmosphere comprising of 5% CO₂/95% air. After the incubation period, the cell viability was measured by either the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay or by the amount of radiation produced by [³H]-thymidine incorporated into the cells. Dose-response curves were plotted and the concentrations that gave 50% inhibition of cell growth (IC₅₀) were calculated.

Statistical analysis. Statistical analysis was performed by using the GraphPad Prism3.0 software (GraphPad, CA, USA). $p < 0.05$ was regarded as statistically significant.

Results

ABCB1/P-gp expression and drug sensitivity in drug-resistant MOLT4 and Jurkat cells. We have previously reported that drug-resistant MOLT4/DNR cells highly express *ABCB1* mRNA and P-gp and that this transporter is instrumental in acquiring drug resistance (7) (Figure 1A and C). In the current study, we have newly-established another drug resistant hematopoietic cell line, Jurkat/DOX, through the continuous exposure of Jurkat cells to a low concentration of DOX treatment. Through the characterization of Jurkat/DOX, we found that *ABCB1* mRNA and P-gp were also highly expressed in cells of this drug-resistant cell line (Figure 1B and D). We confirmed that both MOLT4/DNR and Jurkat/DOX cells have drug-resistant phenotypes of at least 10-fold, as compared to their parental cells (Table I).

Decitabine reduces ABCB1 mRNA expression in drug-resistant cells. Next, we investigated the effect of decitabine treatment on the expression of *ABCB1* mRNA in MOLT4/DNR and Jurkat/DOX cell lines. The drug-resistant (MOLT4/DNR, Jurkat/DOX) cells were treated with 5 μ M decitabine for four days, and then the amount of *ABCB1* mRNA was measured by real-time RT-PCR. The results showed that decitabine treatment significantly reduced the *ABCB1* mRNA expression in both drug-resistant cell lines (Figure 2).

Decitabine reduces P-gp expression in drug-resistant cells. We also investigated the effect of decitabine treatment on P-gp expression in MOLT4/DNR and Jurkat/DOX cell lines by FACS analysis. The drug-resistant MOLT4/DNR and Jurkat/DOX cells were treated with 5 μ M decitabine for four days, and then the P-gp expression on the cell surface was measured by FACS. The results showed that decitabine treatment reduced the mean fluorescence intensity for P-gp detection by 69% and 77% in drug-resistant MOLT4/DNR and Jurkat/DOX cells, respectively (Figure 3A and B).

Decitabine restores drug sensitivity in the drug-resistant MOLT4/DNR and Jurkat/DOX cell lines. As we observed significant decreases in *ABCB1* mRNA and P-gp expressions in MOLT4/DNR and Jurkat/DOX cells by decitabine treatment, we next investigated the effect of decitabine treatment on drug sensitivity of both drug-resistant cell lines. We observed that 5 μ M of decitabine treatment significantly reduced the IC₅₀ value for daunorubicin sensitivity in MOLT4/DNR cells (Figure 4A). In the MOLT4/DNR cells, the IC₅₀ for daunorubicin in the absence of decitabine was 392×10^{-8} M, whereas the IC₅₀ for daunorubicin on pre-treatment of cells with 5 μ M decitabine for four days was 90×10^{-8} M, for which the resistant factor (fold change) was

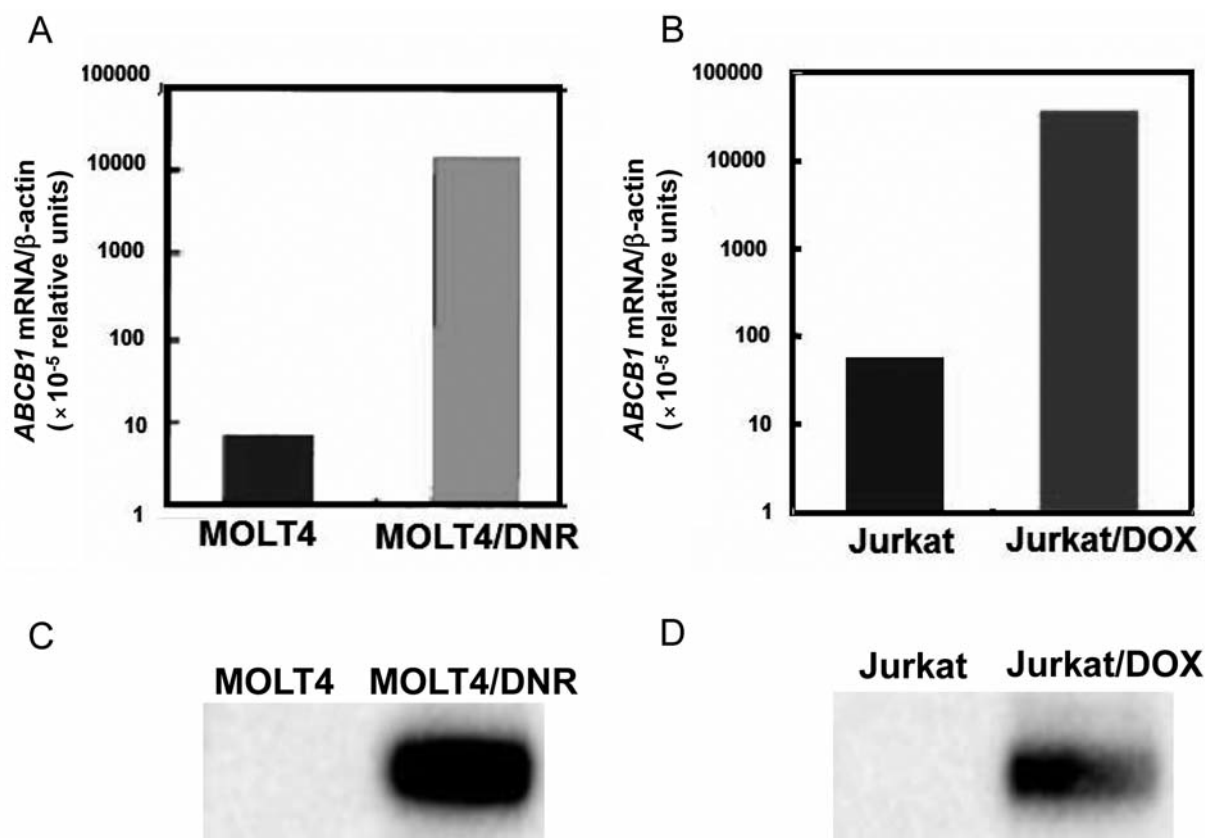


Figure 1. *ABCB1* mRNA expressions in the parental MOLT4 and drug-resistant MOLT4/DNR (A), as well as parental Jurkat and drug-resistant Jurkat/DOX (B) cell lines. The expression for *ABCB1* mRNA was determined using real-time RT-PCR. Western blot analysis for the detection of P-glycoprotein (P-gp) in MOLT4 and MOLT4/DNR (C) as well as Jurkat and Jurkat/DOX cells (D). DNR: daunorubicin, DOX: doxorubicin.

calculated as 3.08. On the other hand, the resistant factor in the absence of decitabine treatment in MOLT4/DNR cells was calculated as 11.9 (Table II). The chemosensitizing effect for doxorubicin was also observed in Jurkat/DOX cells (Figure 4B), in which the resistant factor was markedly reduced from 51.8 to 3.7 by pre-treatment with 5 μ M decitabine for four days.

Discussion

In the current study, we observed that decitabine treatment significantly reduced *ABCB1* mRNA and P-gp expressions in MOLT4/DNR and Jurkat/DOX, two drug-resistant hematopoietic cell lines. In our preliminary experiment, we observed that 5 μ M of decitabine treatment for four days did not affect cell viability, neither of the parental (MOLT4, Jurkat) nor of the drug-resistant (MOLT4/DNR, Jurkat/DOX) cell lines (data not shown). As such, we found that 5 μ M decitabine treatment significantly restored sensitivity to the growth-inhibitory effect of chemotherapeutic agents in the

Table I. Fifty percent inhibitory concentration (IC_{50}) values for anticancer drugs in parental (MOLT4, Jurkat) and drug-resistant (MOLT4/DNR, Jurkat/DOX) cell lines determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. DNR: daunorubicin, DOX: doxorubicin.

	IC_{50} ($\times 10^{-8}$ M)		
	MOLT4	MOLT4/DNR	RF (-fold)
Daunorubicin	2.9	37.2	12.8
	Jurkat	Jurkat/DOX	RF (-fold)
Doxorubicin	18.9	215	11.4

drug-resistant cell lines. Our observation suggests that DNA hypermethylation is associated with *ABCB1*/P-gp overexpressions in drug-resistant MOLT4/DNR and Jurkat/DOX cells.

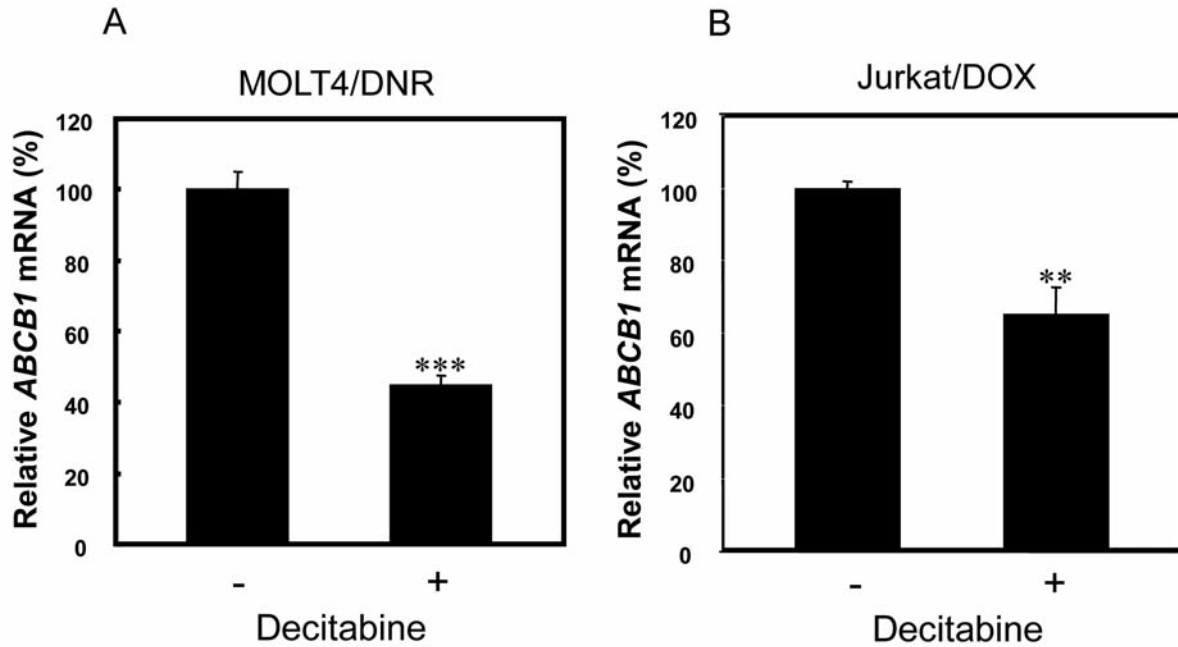
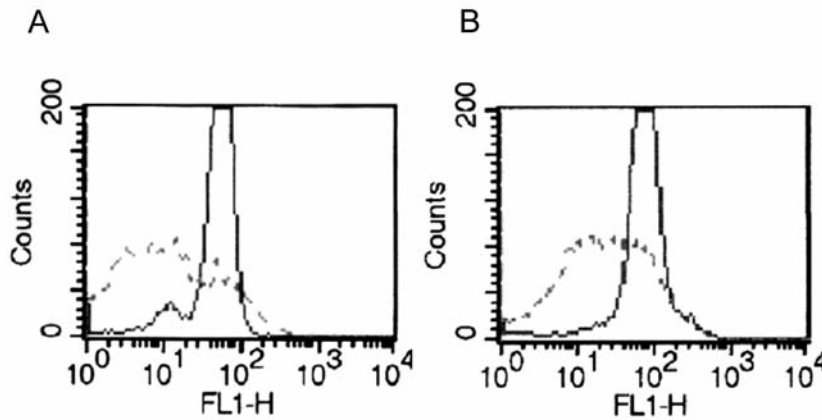


Figure 2. Effect of decitabine treatment on ABCB1 mRNA expressions in drug-resistant MOLT4/DNR (A) and Jurkat/DOX (B) cells determined by real-time RT-PCR. The cells were treated with 5 μM decitabine (+) for four days before the assay. ***p*<0.01 and ****p*<0.001. Compared to cells without treatment (-).



Cell line	Δ	D value
MOLT4/DNR	69	0.58
Jurkat/DOX	77	0.64

$$\Delta = \text{Relative decrease in MFI} = (\text{MFI with decitabine} - \text{MFI without decitabine}) / \text{MFI without decitabine} \times 100\%$$

Figure 3. Effect of decitabine treatment on P-glycoprotein (P-gp) expressions in drug-resistant MOLT4/DNR (A) and Jurkat/DOX (B) cells, determined by flow cytometry. The cells were treated with 5 μM decitabine for four days before the assay. The solid line shows the control, whereas the dotted line shows the fluorescent signal detecting P-gp expression in the cells treated with 5 μM decitabine for four days. The effect of decitabine on the relative decrease in the mean fluorescence intensity (MFI) and KS statistical D values were calculated in both drug-resistant cell lines.

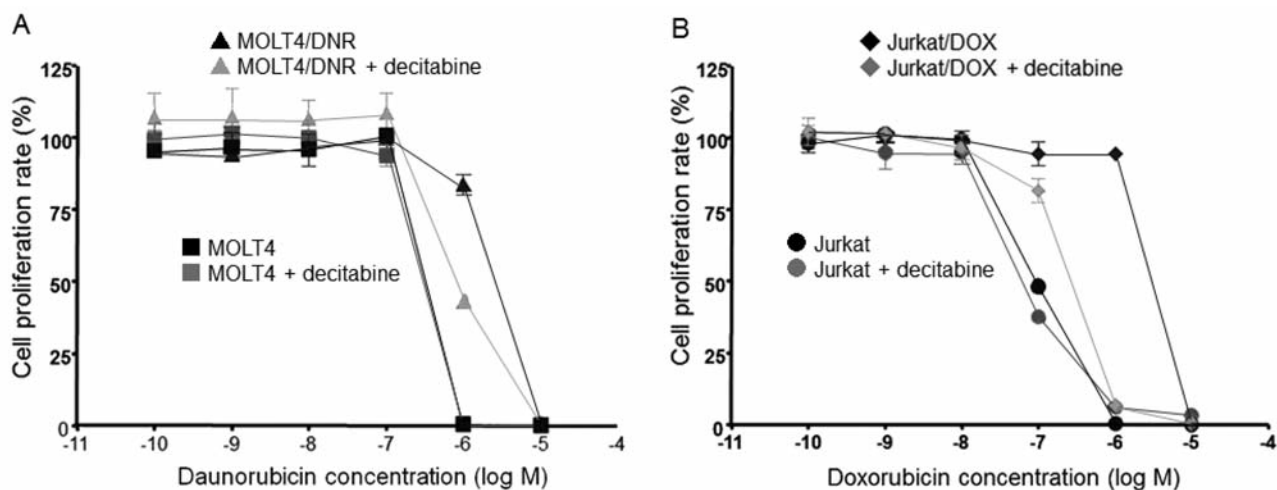


Figure 4. Dose-response curves for chemotherapeutic drug effect on cell proliferation of parental and drug resistant sublines. A: Sensitivity to daunorubicin (DNR) in MOLT4 and MOLT4/DNR cells in the presence and absence of decitabine treatment. B: Sensitivity to doxorubicin (DOX) in Jurkat and Jurkat/DOX cells in the presence and absence of decitabine treatment. Cells were treated with or without 5 μ M decitabine for four days before the sensitivity test.

Studies have shown that DNA hypermethylation in genes such as tumor suppressor genes is associated with cancer development (8-11). In addition, it has been reported that epigenetic changes also underlie drug resistance to several chemotherapeutic agents in cancer cells (12-14). It is generally understood that DNA hypermethylation suppresses gene expression, whereas DNA de-methylation facilitates gene expression. However, our experimental data demonstrating that decitabine reduced *ABCB1* mRNA expression, seem to be discordant with this notion. To date, there have been contrary observations regarding the relationship between epigenetic modifiers and drug resistance in cancer cells. Kusaba *et al.* reported that the treatment of T-cell leukemia CEM cell line with decitabine altered the methylation status of the *ABCB1* promoter and resulted in an increased resistance to epirubicin and reduced daunorubicin accumulation, both of which were reversible by verapamil, a P-gp inhibitor (15). On the other hand, Ando *et al.* showed that drug-resistance of acute myeloid leukemia K562/ADR cells is accompanied by an overexpression of P-gp, and this phenomenon is related to a change in the methylation status of the *ABCB1* promoter (16, 17). However, they also reported that decitabine or 5-azacytidine treatment significantly reversed the MDR phenotype of K562/ADR cells (16). In addition, Eferth *et al.* reported that sensitivity to cisplatin, a non P-gp substrate, was increased by 5-azacytidine treatment in K562/ADR cells (17), suggesting that non-P-gp-mediated mechanisms in K562/ADR cells also contributed to drug resistance. They reported that hypermethylation in the suppressor region of the *ABCB1* gene promoter contributes to the decrease in the *ABCB1* gene expression by decitabine treatment. Although

Table II. Effect of decitabine treatment on sensitivity to chemotherapeutic agents (daunorubicin and doxorubicin) in parental and drug-resistant cell lines. The resistance factor (RF) was calculated by the formula: IC_{50} for drug-resistant cells/ IC_{50} for parental cells.

	IC_{50} ($\times 10^{-8}$ M)			RF
	Decitabine	MOLT4	MOLT4/DNR	
Daunorubicin	-	32.9	392	11.9
	+	29.2	90	3.08
Doxorubicin	Decitabine	Jurkat	Jurkat/DOX	RF
	-	9.56	495	51.8
	+	6.87	25.4	3.70

our data suggest that *ABCB1* hypermethylation contributes to drug resistance in the two hematopoietic cell lines studied, further research is needed to clarify the methylation status in the genes, including *ABCB1*, and transcription factors that regulate *ABCB1* gene expression.

Clinical trials have been carried out to explore the efficacy and safety of decitabine in hematological malignancies, such as myelodysplastic syndrome and acute myeloid leukemia (18-20). Low-dose decitabine treatment has provided promising results. Recently, Scandura *et al.* reported that epigenetic priming, namely pre-treatment with decitabine, before intensive chemotherapy can be safely delivered in an attempt to improve response rates (21). Our data may underlie such clinical usefulness of decitabine treatment for hematological malignancies.

In conclusion, our study showed that decitabine treatment reduced *ABCB1* mRNA and P-gp expressions in drug-resistant MOLT4/DNR and Jurkat/DOX cells, suggesting that DNA methylation underlies the overexpression of *ABCB1*/P-gp in these cells. Decitabine treatment also significantly increased sensitivity to anticancer drugs in MOLT4/DNR and Jurkat/DOX cells. Our results suggest that the use of a DNA methylation inhibitor, such as decitabine, is an effective way to overcome anticancer drug resistance by reducing P-gp expression.

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