

Decitabine Induces Delayed Reactive Oxygen Species (ROS) Accumulation in Leukemia Cells and Induces the Expression of ROS Generating Enzymes

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Abstract

Purpose: Azanucleoside DNA methyltransferase (DNMT) inhibitors are currently approved by the U.S. Food and Drug Administration for treatment of myelodysplastic syndrome. The relative contributions of DNMT inhibition and other off-target effects to their clinical efficacy remain unclear. Data correlating DNA methylation reversal and clinical response have been conflicting. Consequently, it is necessary to investigate so-called off-target effects and their impact on cell survival and differentiation.

Experimental Design: Flow cytometry was used for cell cycle, apoptosis, and reactive oxygen species (ROS) accumulation analysis. Gene expression analysis was performed using real-time PCR. DNA methylation was detected by methylation-specific PCR. Mitochondrial membrane potential was analyzed using JC-1 dye staining. Western blotting was used for quantitative protein expression analysis.

Results: 5-Aza-2'-deoxycytidine (DAC) induced cell-cycle arrest and apoptosis in leukemia cells. p53 expression was dispensable for DAC-induced apoptosis. DAC induced delayed ROS accumulation in leukemia cells but not in solid tumor cells and p53 expression was dispensable for ROS increase. ROS increase was deoxycytidine kinase dependent, indicating that incorporation of DAC into nuclear DNA is required for ROS generation. ROS accumulation by DAC was caspase-independent and mediated the dissipation of the mitochondrial membrane potential. Concordantly, ROS scavengers diminished DAC-induced apoptosis. DAC induced the expression of different NADPH oxidase isoforms and upregulated Nox4 protein expression in an ATM-dependent manner, indicating the involvement of DNA damage signaling in Nox4 upregulation.

Conclusion: These data highlight the importance of mechanisms other than DNA cytosine demethylation in modulating gene expression and suggest investigating the relevance of ROS accumulation to the clinical activity of DAC. *Clin Cancer Res*; 20(5); 1249–58. ©2014 AACR.

Introduction

The 5-azacytosine nucleosides 5-azacytidine and 5-aza-2'-deoxycytidine (decitabine, DAC) are approved by the U.S. Food and Drug Administration (FDA) for the treatment of myelodysplastic syndrome (MDS); however, the mechanism underpinning their clinical activity remains uncertain.

Understanding the mechanism of their clinical activity is crucial for the development of more effective and safer analogues. The pleiotropic biologic effects of these compounds in addition to their indirect DNA methyltransferase (DNMT) inhibition complicate understanding their mode of clinical activity. Recent data has called into question the relationship between DNA methylation reversal and clinical activity of DNMT inhibitors (1, 2), increasing interest in the study of the impact of these compounds on apoptosis and gene expression through mechanisms other than DNMT inhibition (3–7).

Generation of reactive oxygen species (ROS) is either mitochondrial or enzyme dependent and affects several vital cellular functions, including differentiation and intracellular signaling (8). The NADPH oxidase (Nox) enzyme complex produces superoxide anion from oxygen using NADPH as an electron donor (9). The NADPH oxidase activity was first identified in phagocytes and then in nonphagocytic cells (10). Nox family proteins are the catalytic subunits of the NADPH oxidase enzyme complex and there are currently 7 identified isoforms of Nox proteins (Nox1, Nox2, Nox3,

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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doi: 10.1158/1078-0432.CCR-13-1453

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Translational Relevance

Although DNA methyltransferase (DNMT) inhibitors such as 5-azacytidine and 5-aza-2'-deoxycytidine (DAC) are currently approved by the U.S. Food and Drug Administration for the treatment of myelodysplastic syndrome, the relative contributions of DNMT inhibition and other off-target effects to their clinical efficacy remain unclear. We observed that these drugs increase reactive oxygen species (ROS) accumulation in leukemia cell lines and patient with acute myelogenous leukemia (AML) samples when used in clinically relevant doses. We investigated the mechanism of ROS accumulation in leukemia cells after DAC treatment and observed gene expression changes in ROS generating enzymes. The differential response of patient with AML samples to ROS accumulation suggests that the response of AML samples to DAC treatment is not always accompanied by increase in ROS accumulation. Our data suggest that it is important to study the relevance of ROS accumulation to the clinical activity of these drugs in future studies.

Nox4, Nox5, Duox1, and Duox2) (11). Nox isoforms are widely distributed in different tissues and also in cancer cells (12, 13). *Nox4*, *Duox1*, and *Duox2* have a putative CpG island around their promoter region and the effect of DNMT inhibitors on the expression of these genes is unknown. Detoxification of ROS by superoxide dismutases (SOD) convert 2 superoxide molecules into oxygen and 1 hydrogen peroxide (H_2O_2) molecule. In turn, H_2O_2 is detoxified by glutathione peroxidase (GPx) and/or catalase enzymes. Interestingly, all these antioxidant genes (*SOD1*, *SOD2*, *GPx1*, and *catalase*) have CpG islands around their promoters and their expression can be silenced by DNA methylation.

In this study, we investigated DNMT-independent effects of clinically relevant concentrations of DAC. Leukemia cells exhibited differential sensitivity to DAC-induced apoptosis and p53 expression was dispensable for apoptosis induction by DAC. DAC induced delayed and sustained ROS accumulation in leukemia cells. ROS increase was caspase- and p53-independent but deoxycytidine kinase (DCK) dependent. ROS scavengers diminished DAC-induced apoptosis. ROS-generating enzymes with putative CpG islands (*Nox4*, *Duox1*, and *Duox2*) were expressed basally in leukemia cells, suggesting absence of gene silencing by DNA methylation. DAC induced the expression of these genes and upregulated the protein expression of Nox4. However, the specific DNMT1 enzyme inhibitor RG108 neither upregulated Nox4 protein expression nor induced ROS accumulation. Inhibition of ATM abrogated DAC-induced Nox4 upregulation supporting a role for DNA damage signaling in Nox4 expression. These data highlight the significance of the DNMT-independent effects of DAC and should encourage further investigation of the effect of ROS increase on gene expression and intracellular signaling both *in vitro* and *in vivo*.

Materials and Methods

Cell culture and patient samples

ML-1, KG-1a, HL-60, CEM, DCK deficient (CEM/DCK^{-/-}), kindly provided by Professor Beverly Mitchell, Stanford School of Medicine), BV-173 leukemia cell lines, DLD-1 colon cancer cells, and MCF-7 breast cancer cells (provided by Professor Ben Ho Park, Johns Hopkins University) were grown in RPMI 1640 (Invitrogen) supplemented with 10% fetal calf serum. Wild-type and isogenic HCT116 colon cancer cells (DNMT1^{-/-}, DNMT3b^{-/-}, and the double knockout cells, provided by Professor B. Vogelstein, Johns Hopkins University) were cultured in McCoy's 5A (Mediatech, Inc.) supplemented with 10% FBS (Sigma). MCF-10 breast epithelial cells (provided by Professor B.H. Park, Johns Hopkins University) were grown in complete media (DMEM:Ham's F-12 medium supplemented with 5% serum, 10 μ g/mL insulin, 20 ng/mL EGF, 0.5 μ g/mL hydrocortisone, and 100 ng/mL cholera toxin). All cultures were incubated in a humidified atmosphere containing 5% CO₂ at 37°C. Peripheral blood from 5 patients with acute myelogenous leukemia (AML) was provided by the Specimen Accessioning Core (SAC) at the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins University. Mononuclear cells were isolated using Ficoll-Hypaque and were cultured in RPMI 1640 supplemented with 10% FBS.

Chemicals

DAC was purchased from Sigma and dissolved in PBS to a concentration of 1 mmol/L stock solution. KU55933 (ATM inhibitor), pifithrin- α , a cell permeable inhibitor of p53 transactivation, Bcl-2 inhibitor (YC137), RG108, nutlin-3 racemic (MDM2 antagonist), and Z-VAD-FMK (pan caspase inhibitor) were purchased from EMD Biosciences, Inc. *N*-acetyl-L-cysteine (NAC) and DL-buthionine-sulfoximine (BSO) were from Sigma. JC-1 and dihydroethidium (DHE) dyes were from Molecular Probes. Primers were ordered from IDT.

ROS detection and DNMT1 knockdown

ROS were measured by using the fluorescent dye DHE. For suspension cells, 1×10^6 cells were incubated with 2.5 μ mol/L DHE (in RPMI) for 25 minutes in the dark at 37°C. Cells were washed one time with PBS and then red fluorescence was detected by flow cytometry. For adherent cells, the medium was aspirated and replaced with a new medium supplemented with DHE (2.5 μ mol/L). Cells were incubated under the same condition as above then collected for analysis by flow cytometry. The average of the mean fluorescence intensity for at least 3 replicates was calculated. DNMT1 knockdown was performed using siRNA as described previously (14).

Real-time quantitative PCR

RNA was extracted using RNA extraction columns (Qiagen). RNA was further treated with DNase enzyme to remove DNA contamination (Turbo DNA-Free Kit; Applied Biosystems). cDNA synthesis was performed using cDNA

Reverse Transcription Kit (Applied Biosystems). Real-time PCR was performed using KAPA SYBR Fast Master Mix using a cycling protocol that starts with heating at 95°C for 3 minutes followed by 40 cycles of denaturation (95°C for 3 seconds), annealing (60°C for 20 seconds), and extension (72°C for 10 seconds). Supplementary Table S1 shows the primers and the reference sequence (RefSeq) accession number for each gene.

Protein extraction and immunoblotting

Cells were lysed in radioimmunoprecipitation assay buffer containing an EDTA-free protease inhibitor cocktail, at 4°C for 30 minutes. Lysates were collected by centrifugation at 14,000 rpm for 10 minutes. Protein concentration was determined by a Bicinchoninic Acid Assay Kit (Pierce). Proteins were separated by 8% SDS-PAGE and immunoblotted using antibodies for Nox4 (rabbit polyclonal; Novus Biologicals) and mouse β -actin monoclonal antibody (Oncogene Research Products). The immunoreactive proteins were detected using enhanced chemiluminescence Western blotting system (GE Healthcare).

Methylation-specific PCR

EZ DNA Methylation Kit (ZymoResearch) was used for the bisulfite treatment of DNA as per the manufacturer's instructions. Methylation-specific PCR (MSP) was performed in 25 μ L reaction volume using 3 μ L of the bisulfite-treated DNA. Primers for *E-cadherin* was used as described previously (1). The annealing temperature was 58°C and PCR amplification was done for 35 cycles. The PCR product was resolved on a 6% nondenaturing polyacrylamide gel and poststained with ethidium bromide.

Flow cytometric analyses of cell cycle and apoptosis

For cell-cycle analysis, cells were synchronized by overnight serum starvation and DAC was added to the cells growing in regular media for 48 or 72 hours. A total of 1×10^6 cells were washed once with $1 \times$ PBS, fixed with 70% alcohol at 4°C for at least 30 minutes, and incubated with propidium iodide solution (50 μ g/mL) containing RNase (10 units/mL) at 37°C for 30 minutes. DNA fluorescence was measured using a Becton Dickinson FACScan flow cytometer and analyzed by CellQuest software (BD Biosciences). Apoptosis was measured using the Annexin V-FITC Detection Kit (BD Pharmingen) as per the manufacturer's instructions.

Mitochondrial membrane potential measurement

Dissipation of the mitochondrial membrane potential (MMP) is a hall mark for apoptosis. The cationic dye JC-1 stains the mitochondria of healthy cells red and apoptotic cells green. JC-1 (5 mg/ml) stock solution was diluted $500 \times$ in RPMI and vortexed. 1×10^6 cells were resuspended in JC-1/RPMI medium and incubated for 15 minutes in the dark at 37°C. Cells were washed 2 times in $1 \times$ PBS and then analyzed for red and green fluorescence by flow cytometry.

Statistical analysis

Data represent the mean \pm SD for 3 to 4 replicates. Student *t* test was used to detect significant differences and $P < 0.05$ was considered statistically significant.

Results

Leukemia cells exhibit differential sensitivity to DAC-induced apoptosis

DAC induces DNA damage in leukemia cells and solid tumors (15–18). DAC induced G_1 or G_2 -M cell-cycle arrest, depending on cell type, following DNA damage. DAC induced G_2 -M arrest in all leukemia cell lines but with variable kinetics (Supplementary Table S2). In BV-173 cells, G_2 -M arrest was observed after 24 hours (Fig. 1A). DAC induced G_2 -M arrest in other leukemia cells after 48 hours with HL-60, showing a slight but still significant G_2 -M arrest (Supplementary Table S2 and Fig. 1A). Various cell lines exhibited a range of sensitivity to DAC-induced apoptosis despite the common G_2 -M arrest induced in leukemia cells. BV-173 cells were highly sensitive to DAC-induced apoptosis (Fig. 1B). Low doses of DAC (250 nmol/L) induced 48% and 54% apoptosis after 48 and 72 hours, respectively (100 nmol/L induced 46% apoptosis after 72 hours, data not shown). Other leukemia cells, including ML-1, KG-1a, and HL-60, were relatively resistant to DAC-induced apoptosis (Fig. 1B): high doses (1,000 nmol/L) of DAC induced 19%, 20%, and 11% apoptosis after 72 hours of DAC exposure, respectively. These results indicate that G_2 -M arrest is a common response to DAC treatment in leukemia cells; however, leukemia cells exhibit differential sensitivity to DAC-induced apoptosis.

p53 transactivation or accumulation is dispensable for DAC-induced apoptosis

p53 mutations are a major cause of resistance to chemotherapeutic agents. Previous reports demonstrated conflicting data regarding the requirement for p53 expression in DAC-induced apoptosis (5, 19, 20). In this study, HL-60 cells (p53 null) were the most resistant to DAC-induced apoptosis (Fig. 1C) whereas BV-173 cells (p53 wild type) were the most sensitive, suggesting that p53 expression may be required for apoptosis induction by DAC. However, pretreatment of p53 wild-type leukemia cells with pifithrin- α , a p53 transactivation inhibitor, did not significantly decrease DAC-induced apoptosis in wild-type p53 BV-173 cells (Fig. 2A) and ML-1 cells (data not shown). However, pifithrin- α significantly decreased cytarabine (ara-C)-induced apoptosis in both cell lines (Fig. 2A). Furthermore, induction of p53 accumulation by pretreatment of p53 wild-type leukemia cells with the MDM-2 inhibitor, nutlin 3, did not significantly increase DAC-induced apoptosis in BV-173 cells (Fig. 2B) and ML-1 cells (data not shown), whereas ara-C-induced apoptosis was significantly increased by nutlin-3 pretreatment (Fig. 2B). Collectively, these results indicate that p53 expression is dispensable for DAC-induced apoptosis in leukemia cells. Because HL-60 was highly resistant to DAC-induced apoptosis, factors

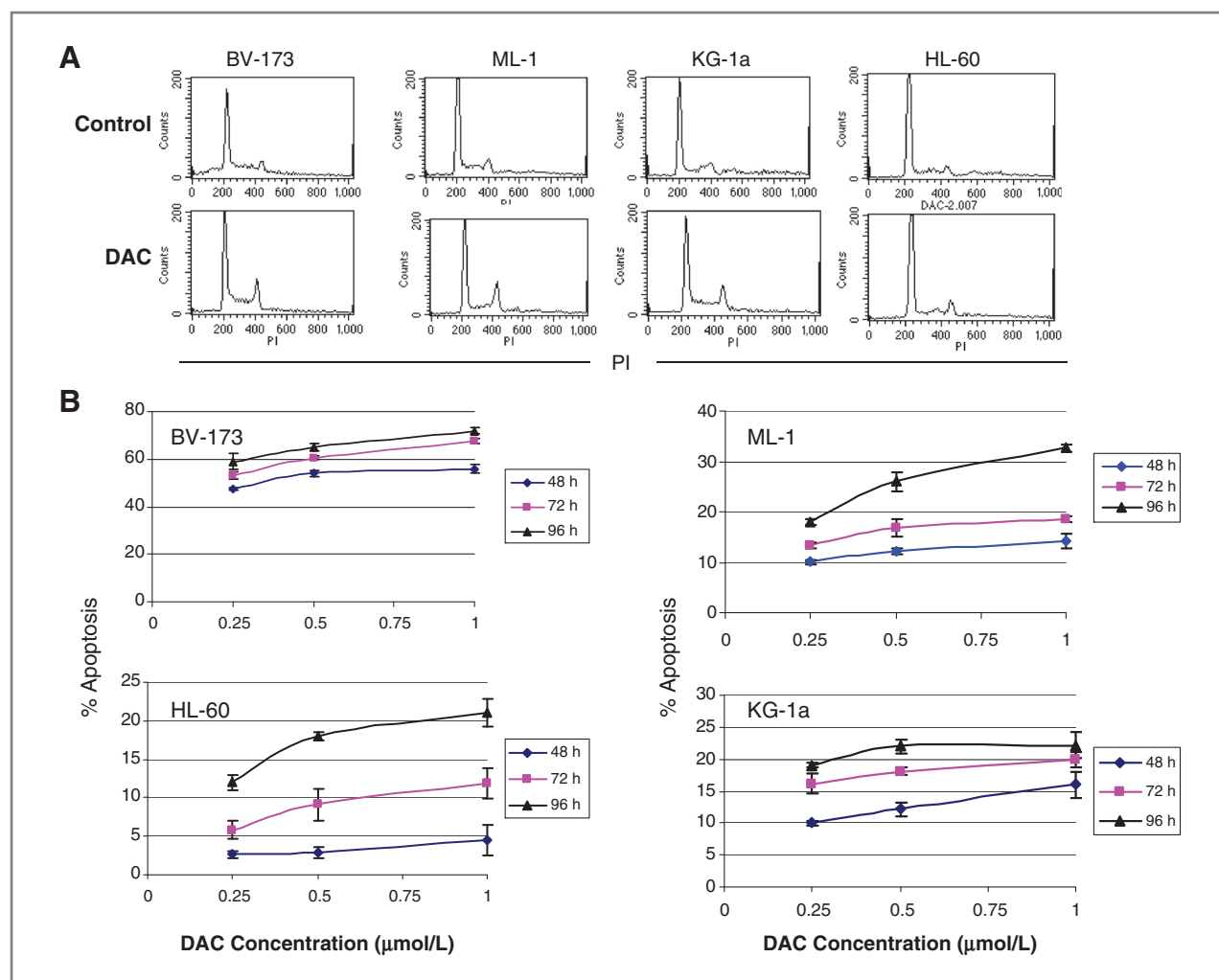


Figure 1. Impact of DAC on cell cycle and apoptosis in leukemia cells. A, representative figures for cell-cycle analysis after treatment with DAC (250 nmol/L) for 24 hours (BV-173) or 48 hours (other cells) after staining with propidium iodide (PI) as described in Materials and Methods. B, leukemia cells were treated with a single application of different concentrations of DAC (0.25, 0.5, and 1 $\mu\text{mol/L}$) for varying intervals (48, 72, and 96 hours) and apoptosis determined as described in Materials and Methods. Data represent the mean value of 3 replicates \pm SD.

other than p53 expression must contribute to such resistance. Pretreatment of HL-60 with a specific inhibitor (YC137) for the antiapoptotic protein Bcl-2 sensitized HL-60 to DAC-induced apoptosis in a dose-dependent fashion (Fig. 2C), further supporting that DAC can induce apoptosis in p53 null cells.

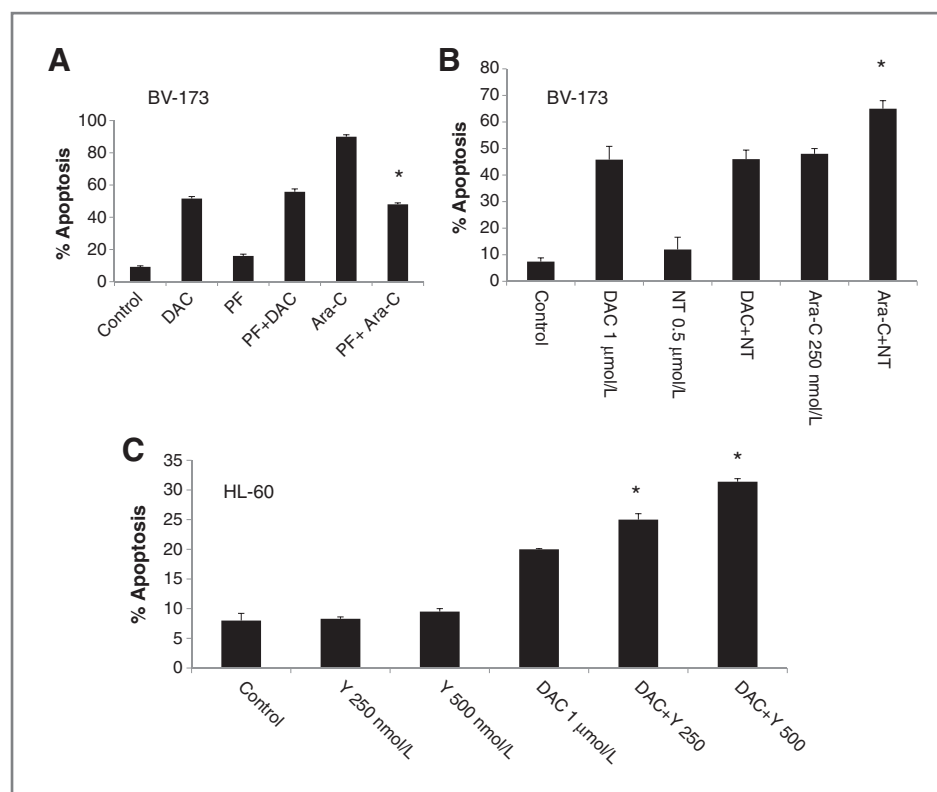
DAC induces delayed and sustained ROS increase in leukemia cells

Histone deacetylase (HDAC) inhibitors induce ROS generation in leukemia cells (21) and the selectivity of these compounds has been attributed to the higher basal oxidative stress of neoplastic cells compared with normal cells (22). We therefore investigated DAC-induced ROS in different leukemia cell lines using the ROS detection dye DHE. No significant increase in ROS was detected at early time points after DAC treatment (1, 3, 6, and 12 hours) in BV173, HL-60, ML-1, and CEM cells (data not shown). Further

incubation with single treatment of low doses of DAC for 24, 48 and 72 hours resulted in a significant increase in ROS in BV-173 cells after 24 hours and in other cells after 48 and 72 hours. Representative figures for 48-hour treatment with 250 and 500 nmol/L DAC are shown in Fig. 3A and even lower dose of DAC (100 nmol/L) also induced ROS accumulation. Collectively, these data indicate that low doses of DAC induce ROS accumulation in leukemia cells. Because DNMT inhibition by DAC is observed in both leukemia and adherent cells, it was compelling to test ROS accumulation in adherent cells. Surprisingly, DAC treatment did not increase ROS in colon cancer (HCT116, DLD-1) or breast cancer cell lines (MCF-7, MCF-10), at doses as high as 5 $\mu\text{mol/L}$ and time points ranging from 12 to 72 hours. Figure 3A shows representative figures for DLD-1 and HCT-116 cells after 72-hour treatment with 5 $\mu\text{mol/L}$ DAC.

To investigate the role of DNMT inhibition in ROS increase, we compared ROS increase in leukemia cells after

Figure 2. Impact of p53 and Bcl-2 activity on DAC-induced apoptosis in leukemia cells. **A**, BV-173 cells were pretreated with pifithrin- α (PF, 20 $\mu\text{mol/L}$) for 24 hours then treated with either DAC (250 nmol/L) or ara-c (500 nmol/L) for 48 hours followed by apoptosis analysis. **B**, BV-173 cells were pretreated with nutlin-3 (NT, 0.5 $\mu\text{mol/L}$) for 1 hour then treated with either DAC (250 nmol/L) or ara-c (250 nmol/L) for 48 hours. **C**, HL-60 cells were pretreated with the Bcl-2 inhibitor YC137 (Y250 is 250 nmol/L and Y500 is 500 nmol/L) for 1 hour followed by DAC (1 $\mu\text{mol/L}$) treatment for 72 hours and apoptosis analysis. Data represent the mean value of 3 replicates \pm SD. * indicates significant difference from the corresponding control at $P < 0.05$.



treatment with DAC, 5-azacytidine, and the non-nucleoside, direct DNMT1 inhibitor RG108. Low doses of DAC (100 nmol/L) increased ROS in leukemia cells after 48 hours, whereas RG108 (10 $\mu\text{mol/L}$) did not (Fig. 3B). 5-Azacytidine increased ROS significantly (500 nmol/L) after 48 hours; the low concentration (100 nmol/L) led to a smaller increase, which was not statistically significant based on mean fluorescence intensity (Fig. 3B). RG108 (10 $\mu\text{mol/L}$ for 12, 24, 48, and 72 hours) also failed to increase ROS among ML-1, CEM, and HL-60 cells (data not shown). However, RG108 (10 $\mu\text{mol/L}$) reversed *E-cadherin* methylation in ML-1 cells, indicating effective DNMT1 inhibition by RG108 (Fig. 3C). Moreover, silencing DNMT1 expression by siRNA in ML-1 cells did not induce ROS accumulation (data not shown), further indicating that DNMT1 inhibition is not inducing ROS accumulation. Collectively, these data suggest that DNMT1 inhibition by RG108 or by genetic approach is not sufficient for inducing ROS accumulation in leukemia cells. Also, it indicates that DAC is more potent on a molar basis than 5-azacytidine in inducing ROS accumulation in leukemia cells, similar to their relative molar activities in DNMT inhibition.

The effect of DAC treatment on ROS accumulation in patient with primary AML samples would provide a better clinical model than cell lines. DAC induced ROS accumulation in 3 (P1, P4, and P5) of 5 patient samples after 72-hour treatment (Fig. 3D), indicating that the response of patient samples to DAC-induced ROS accumulation is variable.

DAC-induced ROS increase is caspase independent and mediates MMP dissipation

Both apoptosis and caspase activation can generate ROS. Therefore, it was essential to investigate whether the detected increase in ROS is a cause or a consequence of DAC-induced caspase activation and apoptosis in leukemia cells. Pretreatment of ML-1 and BV-173 cells with the pan-caspase inhibitor Z-VAD-FMK did not significantly decrease DAC-induced ROS increase (Fig. 4A), indicating that the observed increase in ROS is not a consequence of caspase activation. However, pretreatment with Z-VAD-FMK significantly decreased DAC-induced apoptosis, indicating that DAC-induced apoptosis is caspase dependent and that Z-VAD-FMK efficiently inhibits different caspase enzymes under these experimental conditions (Fig. 4B).

To further investigate the role of ROS increase in DAC-induced apoptosis, the effect of the ROS scavenger NAC on DAC-induced dissipation of MMP was monitored, because MMP dissipation is a critical event in apoptosis and leads to release of different apoptogenic mitochondrial proteins in the cytoplasm (23). NAC significantly reduced DAC-induced MMP dissipation in a dose-dependent manner (Fig. 5A), indicating that the increase in ROS is required for the loss of MMP. Moreover, NAC pretreatment of ML-1 and BV-173 cells significantly reduced DAC-induced apoptosis (Fig. 5B) whereas glutathione depletion by BSO significantly increased DAC-induced apoptosis in the 2 cell lines (Fig. 5C). It is worth mentioning that NAC also inhibited 5AC-induced apoptosis in BV-173 and ML-1 cells

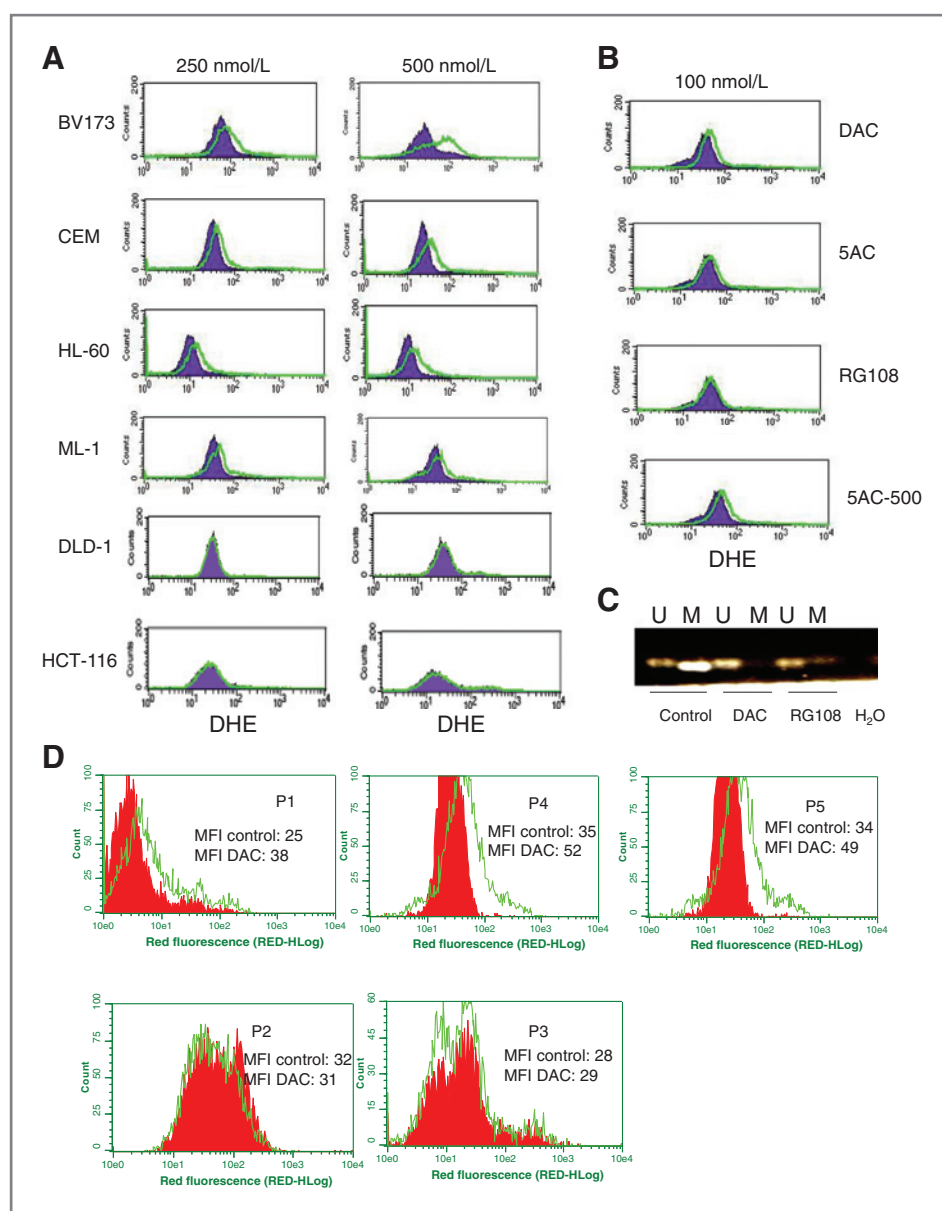


Figure 3. Impact of DAC on generation of ROS. A, representative figures of 4 replicates for leukemia and colon cancer cell lines treated with DAC (250 and 500 nmol/L) for 48 hours followed by DHE staining and flow cytometry. B, CEM cells were treated with DAC (100 nmol/L), 5-azacytidine (5AC, 100 or 500 nmol/L), and RG108 (10 μ mol/L) for 48 hours and ROS detected by DHE staining. Representative data for 6 replicates are shown. C, ML-1 cells were treated with DAC (500 nmol/L) and RG108 (10 μ mol/L) for 72 hours. Analysis of methylation reversal of *E-cadherin* was performed using MSP. U indicates unmethylated lane and M indicates methylated lane. D, peripheral blood from 5 AML patients were treated with 500 nmol/L DAC for 72 hours and then stained with DHE for ROS detection. The figures are representatives of 3 replicates for each sample. The red filled line represents the control cells and the green line represents DAC-treated cells. P# indicates the sample number. Samples P1, P4, and P5 showed increase in ROS accumulation, whereas P2 and P3 did not. MFI indicates mean fluorescence intensity.

(data not shown). Collectively, these data indicate that DAC-induced ROS mediates MMP dissipation and apoptotic induction in leukemia cells.

DAC-induced ROS accumulation requires DCK activity

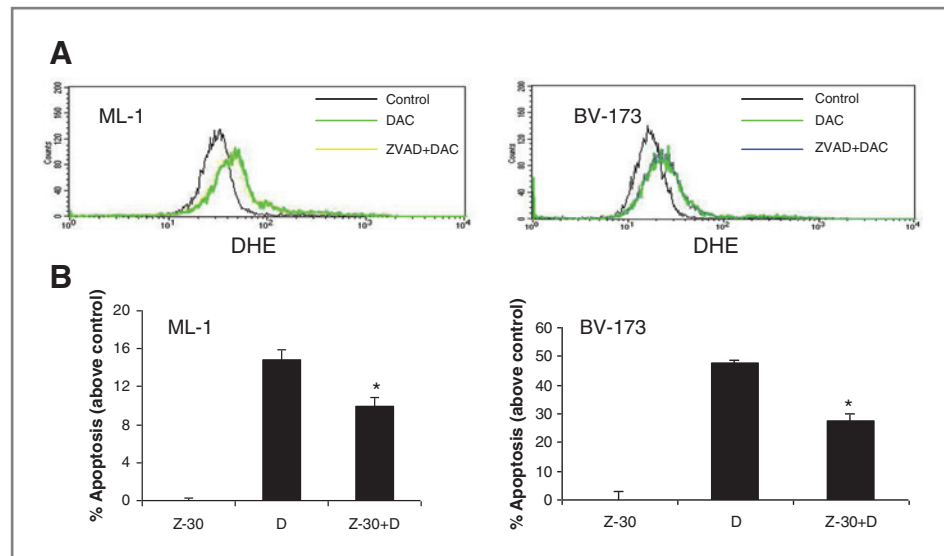
The DNMT inhibitory activity of DAC requires its phosphorylation by DCK and nuclear DNA incorporation. However, DAC can still be phosphorylated to a small extent by the mitochondrial thymidine kinase in absence of DCK and incorporate into mitochondrial DNA (24). Mitochondrial DNA incorporation of nucleoside analogues induces cytotoxicity with consequent ROS generation (25). DAC treatment induced increased ROS in wild-type CEM cells but not in the DCK-deficient CEM cells (CEM/DCK^{-/-}; Fig. 5D), indicating that DCK activity is essential for DAC-induced

increase in ROS. Also CEM/DCK^{-/-} cells were highly resistant to apoptosis induction by DAC compared with CEM cells (Supplementary Fig. S1), indicating that DCK activity is required for apoptosis induction by DAC.

DAC modulates the expression of ROS-generating enzymes and antioxidants

Analysis of the promoter region of the 7 isoforms of the superoxide anion-generating enzymes Nox demonstrated the presence of CpG islands in 3 isoforms (Nox4, Duox1, and Duox2). The criteria for defining a CpG island were CG content $\geq 50\%$, a ratio of ≥ 0.6 for observed to expected CGs, and a minimal island length of 200 bp. As shown in Supplementary Table S3, the 3 isoforms were expressed in all leukemia cells (except for Nox4 in CEM and CEM/DCK^{-/-} cells), suggesting absence of epigenetic silencing.

Figure 4. Impact of caspase inhibition on DAC-induced ROS increase. **A**, ML-1 and BV-173 cells were pretreated with the caspase inhibitor Z-VAD-FMK (30 μ mol/L, Z-30) for 2 hours then treated with DAC (D, 250 nmol/L) for 48 hours. Note that there is almost complete overlap between DAC and Z-VAD + DAC in the 2 cell lines. Half of the cells were analyzed for ROS detection by DHE staining and the other half used for apoptosis analysis. **B**, ML-1 and BV-173 cells from **A** were analyzed for apoptosis as described in Materials and Methods. Data represent the mean value of 3 replicates \pm SD. *, indicates significant difference from the corresponding control at $P < 0.05$.



DAC treatment (250 nmol/L) for 24 hours induced the expression of at least 2 genes of the 3 isoforms in each cell line. 5AC treatment induced comparable induction of Nox4, Duox1, and Duox2 in the same cell lines (Supplementary Table S4).

Analysis of the promoter region of different antioxidant enzymes involved in superoxide anion detoxification revealed the presence of promoter CpG islands in all the enzymes except for SOD3 (Supplementary Table S3). Similar to the Nox enzymes, all of the antioxidant enzymes were expressed at the basal level indicating absence of baseline gene silencing. The effect of DAC on the expression of antioxidant enzymes was heterogeneous with minor repression in HL-60, CEM, and CEM/DCK^{-/-} and minor induction in ML-1 cells. To gain insight into further events downstream of induction of NOX4 gene expression, we analyzed the protein expression of Nox4 in 2 leukemia cell lines that showed Nox4 induction following DAC treatment. DAC induced Nox4 protein upregulation in leukemia cells after 48 and 72 hours (Fig. 6A). Pretreatment of ML-1 cells with the specific ATM inhibitor KU55933 diminished DAC-induced Nox4 upregulation (Fig. 6B), indicating that Nox4 upregulation is dependent on DNA damage signaling. Unfortunately, the contribution of ATM to the increase in ROS could not be determined because the specific ATM inhibitor by itself increased ROS levels (data not shown).

Discussion

The successful development of low-dose schedules of 5-azacytosine nucleosides for the treatment of MDS paralleled the emergence of epigenetic science. Despite their well-documented ability to form irreversible adducts with and thus deplete cellular DNMT when incorporated into DNA, it remains unclear whether the clinical activity of these compounds derives from reversal of DNA methylation (1, 2). In this study, we investigated the effects of clinically relevant

low doses of DAC on leukemia cells. DAC induced G₂-M cell-cycle arrest and apoptosis in leukemia cells and increased the level of ROS in a delayed but sustained manner. The increase in ROS level was caspase independent and required DCK activity for nuclear DNA incorporation. The specific DNMT1 inhibitor RG108 did not induce ROS accumulation. DAC induced the expression of ROS-generating enzymes by a methylation reversal independent mechanism and induced Nox4 protein upregulation, which required ATM.

The activities of 5-azacytosine nucleosides vary with dose. At higher doses, they act as DNA synthesis inhibitors and cytotoxic agents (26). In low doses, their cytotoxicity is minimal and their DNA methylation reversal and gene re-expression is dominant. The current suggests that DAC activity also varies with the cell type. DAC can elicit significant apoptosis in leukemia cells (BV-173) at low doses after a single exposure or treatment. The reason for the exquisite sensitivity of BV-173 to low doses of DAC is not yet clear; however, it seems p53 independent. Previous studies of the role of p53 expression in azacytosine nucleoside-induced apoptosis in different tumors reported conflicting results (5, 6, 14, 16–18, 26). Although p53 expression was essential for apoptosis in lung and colon cancer cells (5, 27), it was dispensable in leukemia cells (6, 19). In addition, p53 was shown to have antiapoptotic effect in mouse embryonic fibroblasts after DAC exposure by promoting cell-cycle arrest and DNA repair (20). In concordance with the findings of others in leukemia cells, our results demonstrate a dispensable role of p53 in DAC-induced apoptosis. This observation might affect leukemias and MDS with mutated p53 (resistant-to-chemotherapeutic agents), if cytotoxicity is the mechanism of elimination of the tumor clone.

The role of oxidative stress in regulating gene expression and apoptosis is well established (28). However, low levels of oxidative stress can also mediate cell survival by upregulating antiapoptotic Bcl-2 proteins and other intracellular

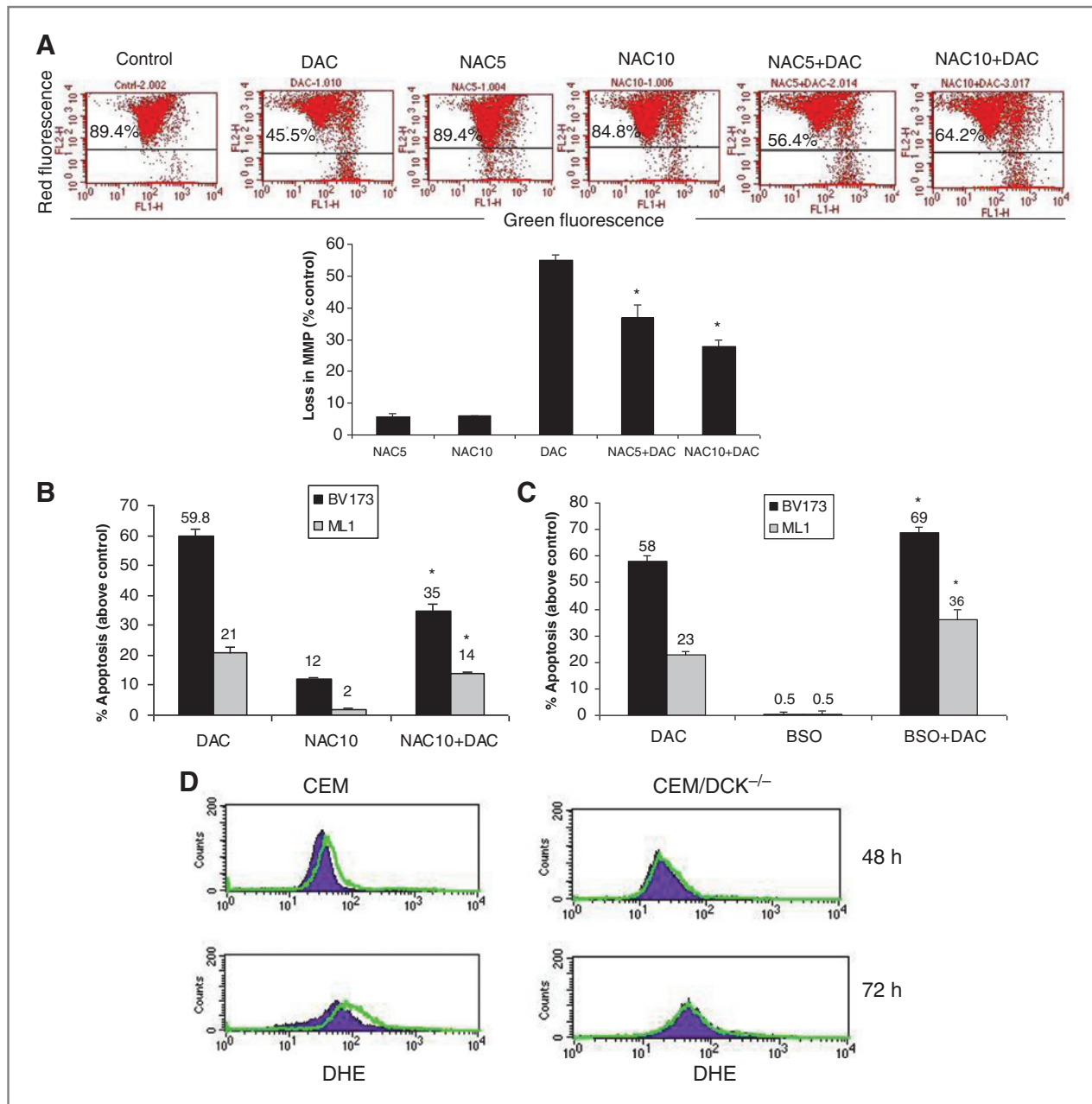


Figure 5. Impact of ROS increase on MMP and DAC-induced apoptosis in leukemia cells. **A**, BV-173 cells were pretreated with 5 mmol/L NAC (NAC5) and 10 mmol/L NAC (NAC10) for 1 hour then treated with DAC (250 nmol/L) for 72 hours. Cells were stained with JC-1 dye and MMP was detected as described in Materials and Methods. Figures are representative for 3 replicates. Values in the top quadrant represent the percentage of cells with intact MMP. The bar graph represents the mean value of 3 replicates \pm SD of A. * indicates significant difference from the corresponding control. **B**, BV-173 and ML-1 cells were pretreated with 10 nmol/L NAC (NAC10) for 1 hour then treated with DAC (500 nmol/L) for 72 hours followed by apoptosis analysis. **C**, BV-173 and ML-1 cells were pretreated with 0.5 and 0.1 mmol/L BSO for 2 hours, respectively, and then treated with DAC (500 nmol/L) for 72 hours. Data represent the mean value of 3 replicates \pm SD. **D**, CEM and CEM/DCK^{-/-} cells were treated with 250 nmol/L DAC for 48 and 72 hours and stained with DHE for ROS detection by flow cytometry. * indicates significant difference from the corresponding control.

signaling mechanisms (29–31). ROS were considered previously as metabolic byproducts whose degradation is ensured by various antioxidant enzymes. Currently, ROS are seen as intra- and extracellular messengers whose production is controlled by the Nox/Duox family of enzymes. The Nox family of enzymes serves as a major source of

intracellular ROS that has important signaling roles. For instance, NIH3T3 fibroblasts overexpressing Nox4 were found to develop signs of cellular senescence (32). Moreover, ROS generation may promote cellular differentiation (33), proliferation (34), or apoptosis (35) depending on the cellular context. Recent findings also support ROS

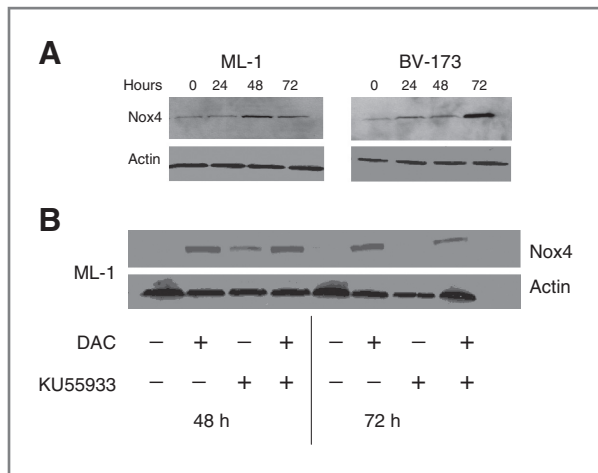


Figure 6. Impact of DAC on Nox4 protein expression. A, BV-173 and ML-1 cells were treated with 500 nmol/L DAC for 24, 48, and 72 hours then Nox4 protein expression was monitored by Western blotting. Actin was used as a loading control. B, ML-1 cells were pretreated with ATM inhibitor (KU55933, 10 μ mol/L) for 1 hour and then treated with 500 nmol/L DAC for 48 and 72 hours and Nox4 expression was monitored by Western blotting. Actin was used as a loading control.

detoxification as a promoter of tumorigenesis (36). These reports highlight the diverse role of ROS in modulating vital cellular functions. In this study, we demonstrate caspase-independent increase in ROS; however, inhibition of ROS accumulation diminished DAC-induced apoptosis. Although ROS generation is usually an early event after drug treatment, the increase in ROS following DAC treatment was delayed. This may indicate that the ROS accumulation is a consequence of DAC incorporation into DNA and consequent DNA damage signaling. Consistent with this hypothesis, DCK-deficient CEM cells that do not incorporate DAC into DNA did not show any increase in ROS or significant apoptosis after DAC treatment (Fig. 5D and Supplementary Fig. S1). Moreover, the non-nucleoside direct DNMT1 inhibitor RG108 or DNMT1 gene silencing did not induce any increase in ROS. Thus, ROS induction seems to derive from incorporation of the nucleoside analogues into DNA, but not through DNMT inhibition *per se*. Furthermore, ROS was induced in both p53 null (HL-60) and wild-type (ML-1, BV-173) cells, indicating that induction of ROS by 5-azacytosines does not require p53 expression, in contrast to other chemotherapeutic agents (37, 38). The differential response of patient with AML samples to DAC-induced ROS accumulation is intriguing. It is not clear why some samples demonstrate ROS accumulation and by what mechanism. The correlation of ROS accumulation to the clinical activity of DAC, if any, is also unknown and requires further investigation.

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Nox4 and p21^{WAF1} upregulation are examples of proteins upregulated by DAC in a methylation reversal-independent manner despite presence of a putative CpG island around their promoter region (15). Indeed, the number of induced genes with a putative CpG island after DAC treatment is almost equal to the number of induced genes that do not possess a CpG island (4), highlighting the significance of the DNMT-independent effects of DAC on genes, which are not silenced by DNA methylation. Although ROS accumulation-mediated apoptosis after DAC treatment in all tested leukemia cells, apoptosis induction among these cell lines was highly variable. This can be explained by the various downstream targets of ROS and by the differential expression of the proapoptotic and antiapoptotic proteins in each cell line.

A variety of clinical trials aim to develop combinations of DNMT inhibitors with HDAC inhibitors based on the synergy of these 2 classes of drugs in effecting the re-expression of methylated genes (1, 39–41). Such combinations synergistically induce expression of p21 in a p53-dependent and DNMT-independent fashion (15). Induction of ROS in leukemia cells by 5-azacytosines provides another rationale for combining these classes of drugs because such combinations synergize ROS generation and accumulation (42). Successful dissection of molecular mechanisms underpinning the clinical activity of these putative epigenetic modifiers will lead to more effective applications of these drugs, alone and in combination, as well as to the development of more specific clinical reagents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: T.E. Fandy, M. Thakar, S.D. Gore
Development of methodology: T.E. Fandy, S.D. Gore
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T.E. Fandy, M. Thakar, S.D. Gore
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T.E. Fandy, P. Rhoden, S.D. Gore
Writing, review, and/or revision of the manuscript: T.E. Fandy, M. Thakar, L. Suarez, S.D. Gore
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Jiemjit, M. Thakar, P. Rhoden, S.D. Gore
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Acknowledgments

This work was supported by NCI grants K24 CA111717 and R01 CA125635 (to S.D. Gore) and Faculty startup funds from Albany College of Pharmacy (to T.E. Fandy).

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Received May 30, 2013; revised December 5, 2013; accepted December 20, 2013; published OnlineFirst January 14, 2014.

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