Decitabine induces delayed reactive oxygen species (ROS) accumulation in leukemia cells and induces the expression of ROS generating enzymes

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Running Title: DAC induces ROS accumulation

Key Words: decitabine, apoptosis, DNA methyltransferase inhibitors, ROS, RG108

Conflict of interest: the authors have no conflicts to disclose

Acknowledgements: Supported by NCI Grants K24 CA111717 and R01 CA125635 to SDG and Faculty startup funds from Albany College of Pharmacy to TEF.

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Translational relevance

Although DNA methyltransferase (DNMT) inhibitors like 5-azacytidine and 5-aza-2’-deoxycytidine are currently FDA-approved for the treatment of myelodysplastic syndrome (MDS), 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 AML patient 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 AML patient 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.
Abstract

Purpose. Azanucleoside DNA methyltransferase (DNMT) inhibitors are currently FDA-approved for treatment of myelodysplastic syndrome (MDS). 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 RealTime 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.
Introduction

The 5-azacytosine nucleosides 5-azacytidine and 5-aza-2'-deoxycytidine (decitabine, DAC) are FDA-approved 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 biological effects of these compounds in addition to their indirect DNA methyltransferase (DNMT) inhibitoin 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 non-phagocytic cells (10). Nox family proteins are the catalytic subunits of the NADPH oxidase enzyme complex and there are currently seven identified isoforms of Nox proteins (Nox1, Nox2, Nox3, 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 (SODs)
convert two superoxide molecules into oxygen and one hydrogen peroxide (H$_2$O$_2$) molecule. In turn, H$_2$O$_2$ 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. On the other hand, 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, Carlsbad, USA) supplemented with 10% fetal calf serum. Wild type (WT) and isogenic HCT116 colon cancer cells (DNMT1\(^{-/-}\), DNMT3b\(^{-/-}\) and the double knock out (DKO) cells, provided by Professor Bert Vogelstein, Johns Hopkins University) were cultured in McCoy's 5A (Mediatech, Inc, Herndon, VA) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO). MCF-10 breast epithelial cells (Provided by Professor Ben Ho Park, Johns Hopkins University) were grown in complete media (DMEM/Ham's F-12 medium supplemented with 5% serum, 10 μg/ml insulin, 20 ng/ml epidermal growth factor, 0.5 μg/ml hydrocortisone, and 100 ng/ml cholera toxin). All cultures were incubated in a humidified atmosphere containing 5% CO\(_2\) at 37°C. Peripheral blood from 5 AML patients was provided by the Specimen Accessioning Core (SAC) at the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins. Mononuclear cells were isolated using Ficoll-Hypaque and were cultured in RPMI-1640 supplemented with 10% fetal bovine serum.

Chemicals

DAC was purchased from Sigma and dissolved in PBS to a concentration of 1mM stock solution. KU55933 (ATM inhibitor), Pifithrin-\(\alpha\), 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. (San Diego, CA). N-acetyl-L-cysteine (NAC) and DL-buthionine-sulfoximine (BSO) were from Sigma. JC-1 and dihydroethidium (DHE) dyes were from Molecular Probes (Eugene, OR). Primers were ordered from IDT (Coralville, IA).

**ROS detection and DNMT1 knockdown**

ROS were measured by using the fluorescent dye DHE. For suspension cells, 1 X 10^6 cells were incubated with 2.5 μM DHE (in RPMI) for 25 min in the dark at 37°C. Cells were washed once 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 μM). 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).

**RealTime quantitative PCR**

RNA was extracted using RNA extraction columns (Qiagen, CA). RNA was further treated with DNase enzyme to remove DNA contamination (Turbo DNA-free kit, Applied Biosystems, CA). cDNA synthesis was performed using cDNA reverse transcription kit (Applied Biosystems). RealTime 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 RIPA lysis buffer containing an EDTA-free protease inhibitor cocktail, at 4°C for 30 min. Lysates were collected by centrifugation at 14,000 rpm for 10 min. Protein concentration was determined by a bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL). Proteins were separated by 8 % SDS-PAGE and immunoblotted using antibodies for Nox4 (rabbit polyclonal, Novus biologicals, Littleton, CO) and mouse β-actin monoclonal antibody (Oncogene Research Products, San Diego, CA). The immunoreactive proteins were detected using ECL western blotting system (GE Healthcare, Piscataway, NJ).

**Methylation specific PCR (MSP)**

EZ DNA methylation kit (ZymoResearch, Orange, CA) was used for the bisulfite treatment of DNA as per the manufacturer’s instructions. MSP was performed in 25 μl reaction volume using 3 μl 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% non-denaturing polyacrylamide gel and post stained 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 h. 1 x 10^6 cells were washed once with 1X PBS, fixed with 70% alcohol at 4 °C for at least 30 min and incubated with propidium iodide solution (50 μg/mL) containing RNase (10 units/ml) at 37 °C for 30 min. DNA fluorescence was measured using a Becton Dickinson FACScan flow cytometer and analyzed by CellQuest software (BD Biosciences, San Jose, CA). Apoptosis was measured using the Annexin V-FITC detection kit (BD Pharmingen, San Diego, CA) as per the manufacturer’s instructions.

Mitochondrial membrane potential measurement

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

Statistical Analysis

Data represent the mean ± standard deviation (SD) for 3-4 replicates. Student's 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 G1 or G2/M cell cycle arrest, depending on cell type, following DNA damage. DAC induced G2/M arrest in all leukemia cell lines but with variable kinetics (supplementary table S2). In BV-173 cells, G2/M arrest was observed after 24 h (Fig 1a). DAC induced G2/M arrest in other leukemia cells after 48 h with HL-60 showing a slight but still significant G2/M arrest (supplementary table S2 and Fig 1a). Various cell lines exhibited a range of sensitivity to DAC-induced apoptosis despite the common G2/M arrest induced in leukemia cells. BV-173 cells were highly sensitive to DAC-induced apoptosis (Fig 1b). Low doses of DAC (250nM) induced 48% and 54% apoptosis after 48 and 72 h, respectively (100 nM induced 46% apoptosis after 72 h, 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 (1000 nM) of DAC induced 19%, 20% and 11% apoptosis after 72 h of DAC exposure, respectively. These results indicate that G2/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 1b) while 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 (PF), 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). On the other hand, PF 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), while 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. Since HL-60 was highly
resistant to DAC-induced apoptosis, factors 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 to 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 12h) 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 h, resulted in a significant increase in ROS in BV-173 cells after 24 h and in other cells after 48 and 72 h. Representative figures for 48 h treatment with 250 nM and 500 nM DAC are shown in Fig. 3a and even lower dose of DAC (100 nM) also induced ROS accumulation (see below). Collectively, these data indicate that low doses of DAC induce ROS accumulation in leukemia cells. Since 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 µM and time points ranging from 12 – 72 hours. Figure 3a shows representative figures for DLD-1 and HCT-116 cells after 72 h treatment with 5 µM DAC.

To investigate the role of DNMT inhibition in ROS increase, we compared ROS increase in leukemia cells after treatment with DAC, 5-azacytidine and the non-nucleoside, direct DNMT1 inhibitor RG108. Low doses of DAC (100 nM) increased ROS in leukemia cells after 48h, while RG108 (10 µM) did not (Fig 3b). 5-azacytidine increased ROS significantly (500 nM) after 48h; the low concentration (100 nM) led to a smaller increase which was not statistically significant based on mean fluorescence intensity (Fig 3b). RG108 (10 µM for 12, 24, 48, and 72 h) also failed to increase ROS among ML-1, CEM, and HL-60 cells (data not shown). On the other hand, RG108 (10 µM) 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 primary AML patient samples would provide a better clinical model than cell lines. DAC induced ROS accumulation in 3 (P1, P4 and P5) out of 5 patient samples after 72 h 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, since 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) while glutathione depletion by BSO significantly increased DAC-induced apoptosis in the two cell lines (Fig 5c). It is worth mentioning that NAC also inhibited 5AC induced apoptosis in BV-173 and ML-1 cells (data not shown). Collectively, these data indicate that DAC-induced ROS mediates MMP dissipation and apoptosis induction in leukemia cells.

**DAC-induced ROS accumulation requires deoxycytidine kinase (DCK) activity**

The DNA methyltransferase 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<sup>−/−</sup>) (Fig 5d), indicating that DCK activity is essential for DAC-induced
increase in ROS. Also CEM/DCK\textsuperscript{-/-} cells were highly resistant to apoptosis induction by DAC compared to CEM cells (supplementary figure 1), 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 ≥ 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 three isoforms were expressed in all leukemia cells (except for Nox4 in CEM and CEM/DCK\textsuperscript{-/-} cells) suggesting absence of epigenetic silencing. DAC treatment (250 nM) for 24 h induced the expression of at least two genes of the three 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 3). 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\textsuperscript{-/-} 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 two leukemia...
cell lines that showed Nox4 induction following DAC treatment. DAC induced Nox4 protein upregulation in leukemia cells after 48 and 72 h (Figure 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 nucelosides 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 appears 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). While p53 expression was essential for apoptosis in lung and colon cancer cells (5, 27), it was dispensable in leukemia cells (6, 19). Additionally, 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 impact 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 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
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 which do not incorporate DAC into DNA did not show any increase
in ROS or significant apoptosis after DAC treatment (fig 5d and supplementary fig1).
Moreover, the non-nucleoside direct DNMT1 inhibitor RG108 or DNMT1 gene silencing
did not induce any increase in ROS. Thus ROS induction appears 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-azanucleosides does not require
p53 expression, in contrast to other chemotherapeutic agents (37, 38). The differential
response of AML patient 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.

Nox4 and p21\textsuperscript{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 two 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-azanucleosides provides another rationale for combining these classes of drugs since 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.
References


**Figure legends**

**Figure 1. Impact of DAC on cell cycle and apoptosis in leukemia cells.**

1a. Representative figures for cell cycle analysis after treatment with DAC (250 nM) for 24 h (BV-173) or 48h (other cells) after staining with propidium iodide (PI) as described under methods. 1b. Leukemia cells were treated with a single application of different concentrations of DAC (0.25, 0.5 and 1 μM) for varying intervals (48, 72 and 96h) and apoptosis determined as described under Methods. Data represent the mean value of 3 replicates ± SD.

**Figure 2. Impact of p53 and Bcl-2 activity on DAC-induced apoptosis in leukemia cells.**

2a. BV-173 cells were pretreated with pifithrin-α (PF, 20 μM) for 24h then treated with either DAC (250 nM) or ara-c (500 nM) for 48h followed by apoptosis analysis. 2b. BV-173 cells were pretreated with nutlin-3 (NT, 0.5 μM) for 1h then treated with either DAC (250 nM) or ara-c (250 nM) for 48h. 2c. HL-60 cells were pretreated with the Bcl-2 inhibitor YC137 (Y250 is 250 nM and Y500 is 500 nM) for 1h followed by DAC (1μM) treatment for 72h and apoptosis analysis. Data represent the mean value of 3 replicates ± SD. * indicates significant difference from the corresponding control at P<0.05.
Figure 3. Impact of DAC on generation of ROS 3a. Representative figures of 4 replicates for leukemia and colon cancer cell lines treated with DAC (250 and 500nM) for 48h followed by dihydroethidium (DHE) staining and flow cytometry. 3b. CEM cells were treated with DAC (100 nM), 5-azacytidine (5AC, 100 nM or 500 nM), and RG108 (10 μM) for 48h and ROS detected by DHE staining. Representative data for 6 replicates are shown. 3c. ML-1 cells were treated with DAC (500 nM) and RG108 (10 μM) for 72 h. Analysis of methylation reversal of E-cadherin was performed using MSP. U indicates unmethylated lane and M indicates methylated lane. 3d. Peripheral blood from 5 AML patients were treated with 500 nM DAC for 72 h 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, while P2 and P3 did not. MFI indicates mean fluorescence intensity.

Figure 4. Impact of caspase inhibition on DAC-induced ROS increase. 4a. ML-1 and BV-173 cells were pretreated with the caspase inhibitor Z-VAD-FMK (30 μM, Z-30) for 2h then treated with DAC (D, 250 nM) for 48h. 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. 4b. ML-1 and BV-173 cells from 4a were analyzed for apoptosis as described under methods. Data represent the mean value of 3 replicates ± SD.
Figure 5. Impact of ROS increase on mitochondrial membrane potential and DAC-induced apoptosis in leukemia cells. 5a. BV-173 cells were pretreated with 5 mM NAC (NAC5) and 10 mM NAC (NAC10) for 1h then treated with DAC (250 nM) for 72h. Cells were stained with JC-1 dye and MMP was detected as described under methods. Figures are representative for 3 replicates. Values in the upper quadrant represent the percentage of cells with intact MMP. The bar graph represents the mean value of 3 replicates ± SD of figure 5a. * indicates significant difference from the corresponding control. 5b. BV-173 and ML-1 cells were pretreated with 10nM NAC (NAC10) for 1h then treated with DAC (500 nM) for 72h followed by apoptosis analysis. 5c. BV-173 and ML-1 cells were pretreated with 0.5 mM and 0.1 mM BSO for 2h, respectively, and then treated with DAC (500 nM) for 72h. Data represent the mean value of 3 replicates ± SD. 5d. CEM and CEM/DCK<sup>−/−</sup> cells were treated with 250 nM DAC for 48 and 72h and stained with DHE for ROS detection by flow cytometry. * indicates significant difference from the corresponding control.

Figure 6. Impact of DAC on Nox4 protein expression. 6a. BV-173 and ML-1 cells were treated with 500 nM DAC for 24, 48 and 72 h then Nox4 protein expression was monitored by western blotting. Actin was used as a loading control. 6b. ML-1 cells were pretreated with ATM inhibitor (KU55933, 10 μM) for 1h and then treated with 500 nM DAC for 48 and 72h and Nox4 expression was monitored by western blotting. Actin was used as a loading control.
Figure 1

1a. Control

1b. DAC

% Apoptosis

DAC Concentration (μM)

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Research.


Published OnlineFirst on January 14, 2014; DOI: 10.1158/1078-0432.CCR-13-1453
Figure 2

2a. % Apoptosis for BV-173

- Control
- DAC
- PF
- PF+DAC
- Ara-C
- PF+ Ara-C

2b. % Apoptosis for BV-173

- Control
- DAC 1μM
- NT 0.5μM
- DAC+NT
- Ara-C 250 nM
- Ara-C+NT

2c. % Apoptosis for HL-60

- Control
- Y 250nM
- Y 500nM
- DAC 1μM
- DAC+Y 250
- DAC+Y 500
3a.

<table>
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<td>CEM</td>
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<td><img src="image4" alt="Graph" /></td>
</tr>
<tr>
<td>HL-60</td>
<td><img src="image5" alt="Graph" /></td>
<td><img src="image6" alt="Graph" /></td>
</tr>
<tr>
<td>ML-1</td>
<td><img src="image7" alt="Graph" /></td>
<td><img src="image8" alt="Graph" /></td>
</tr>
<tr>
<td>DLD-1</td>
<td><img src="image9" alt="Graph" /></td>
<td><img src="image10" alt="Graph" /></td>
</tr>
<tr>
<td>HCT-116</td>
<td><img src="image11" alt="Graph" /></td>
<td><img src="image12" alt="Graph" /></td>
</tr>
</tbody>
</table>

3b.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Graphs</th>
</tr>
</thead>
<tbody>
<tr>
<td>100nM DAC</td>
<td><img src="image13" alt="Graph" /></td>
</tr>
<tr>
<td>5AC</td>
<td><img src="image14" alt="Graph" /></td>
</tr>
<tr>
<td>RG108</td>
<td><img src="image15" alt="Graph" /></td>
</tr>
<tr>
<td>5AC-500</td>
<td><img src="image16" alt="Graph" /></td>
</tr>
</tbody>
</table>

3c.

- U M U M U M
- Control DAC RG108 H₂O
3d.

P1
MFI control: 25
MFI DAC: 38

P4
MFI control: 35
MFI DAC: 52

P5
MFI control: 34
MFI DAC: 49

P2
MFI control: 32
MFI DAC: 31

P3
MFI control: 28
MFI DAC: 29
Figure 4

4a.

ML-1

Control DAC ZVAD+DAC

DHE

BV-173

Control DAC ZVAD+DAC

DHE

4b.

% Apoptosis (above control)

ML-1

Z-30 D Z-30+D

% Apoptosis (above control)

BV-173

Z-30 D Z-30+D

* indicates significant difference compared to control.
Figure 5

5a. Red fluorescence

Control

DAC

NAC5

NAC10

NAC5+DAC

NAC10+DAC

89.4% 45.5% 89.4% 84.8% 56.4% 64.2%

Green fluorescence

5b. Green fluorescence

Loss in MMP (% control)

NAC5

NAC10

DAC

NAC5+DAC

NAC10+DAC

5c. Green fluorescence

% Apoptosis (above control)

DAC

NAC10

NAC10+DAC

BV173

ML1

59.8

21

12

2

35

14

58

23

36

% Apoptosis (above control)

DAC

BSO

BSO+DAC

0.5

0.5

*
Figure 5

5d. CEM

48h

DHE

Counts

0 10 100 200

Counts

0 10 100 200

DHE

CEM/DCK−/−

72h

Counts

0 10 100 200

Counts

0 10 100 200

48h
Figure 6

a.

- -
+ +
- -
+ +

48h

72h

ML-1

DAC

KU55933

b.

Actin

Nox4

Hours

0 24 48 72

0 24 48 72

ML-1

BV-173
Clinical Cancer Research

Decitabine induces delayed reactive oxygen species (ROS) accumulation in leukemia cells and induces the expression of ROS generating enzymes

Tamer Fandy, Anchalee Jiemjit, Manjusha Thakar, et al.

Clin Cancer Res  Published OnlineFirst January 14, 2014.

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