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5-Aza-2'-deoxycytidine Sensitizes Busulfan-resistant Myeloid Leukemia Cells By Regulating Expression of Genes Involved in Cell Cycle Checkpoint and Apoptosis

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Abstract

Busulfan (Bu) is a DNA-alkylating drug used in myeloablative pretransplant conditioning therapy for patients with myeloid leukemia (ML). A major obstacle to successful treatment is cellular Bu-resistance. To investigate the possible contribution of DNA hypermethylation to Bu-resistance, we examined the cytotoxic activity of combined 5-aza-2'-deoxycytidine (DAC) and Bu. Exposure of Bu-resistant B5/Bu250⁶ ML cells to 0.5 μ M DAC resulted in G2-arrest and apoptosis. The observed G2-arrest was associated with hypomethylation and subsequent expression of epigenetically controlled genes including *p16^{INK4A}*, activation of the p53 pathway, and phosphorylation of CDC2. The DAC-mediated apoptosis was partly due to hypomethylation and up-regulation of *XAF1*, which resulted in down-regulation of the anti-apoptotic proteins XIAP, cIAP1 and cIAP2. The pro-apoptotic PUMA and BNIP3 proteins were up-regulated while pro-survival STAT3 and c-MYC were suppressed. Combination of 0.05 μ M DAC and 5 μ g/ml Bu resulted in synergistic cytotoxicity, which was associated with PARP1 cleavage and activation of caspases 3 and 8, suggesting induction of an apoptotic response. P53 inhibition in B5/Bu250⁶ cells using pifithrin- α alleviated these effects, suggesting a role for p53 therein; this observation was supported by the relative resistance of p53-null K562 cells to [DAC+Bu] combinations and by the effects of an anti-p53 shRNA on the OCI-AML3 cell line. We conclude that the synergistic effects of [DAC+Bu] are p53-dependent and involve cell-cycle arrest, apoptosis induction and down-regulation of pro-survival genes. Our results suggest that, depending on tumor p53 status, incorporation of DAC might synergistically improve the cytoreductive efficacy of Bu-based pre-transplant regimen in patients with ML.

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Keywords

busulfan; 5-aza-2'-deoxycytidine; myeloid leukemia; drug resistance; DNA methylation; cell cycle signaling; apoptosis

INTRODUCTION

Acquired resistance to anticancer agents is mediated by both genetic and epigenetic mechanisms [1]. Mutations in the DNA sequences, including amplification of drug-resistance genes, are known to play a major role in the development of cellular drug resistance [2,3]. Epigenetic changes, including DNA hypermethylation and histone modifications, may play equally significant roles in resistance development through transcriptional suppression of genes involved in pro-drug activation, cell cycle checkpoint control, apoptosis, and other cellular processes [4,5]. DNA methyltransferases (DNMTs), histone deacetylases (HDACs) and histone methyltransferase have been identified to be responsible for these epigenetic modifications. Inhibition of these enzymes has been shown to reverse gene silencing by chromatin remodelling [5,6]. One such inhibitor, 5-aza-2'-deoxycytidine (DAC) or decitabine, is known to inhibit DNMTs which results in DNA hypomethylation and expression of silenced genes including those involved in apoptosis [7,8]. Facilitation of apoptosis clearly has a therapeutic potential in various malignancies, and the activity of DAC has been investigated in patients with hematologic diseases [9]. Based on the favorable results of these studies, DAC received regulatory approval for the treatment of myelodysplastic syndromes (MDS) and chronic myelomonocytic leukemia (CMML) in the United States in 2006.

The combination of DAC with other chemotherapeutic agents has been shown to result in additive or synergistic biological activities and possible reversal of clinical drug resistance [10–13]. Such studies have hitherto been performed with nucleoside analogs and/or DNA-intercalating agents that are commonly used in antileukemic therapy. There are, however, no data to support the combination of DAC and DNA-alkylating drugs. DNA-alkylating agent-based leukemia treatment is typically followed by hematopoietic stem cell transplantation as an alternative salvage modality for advanced leukemia, and it affords an option for long-term disease control in patients not responding to conventional chemotherapy. Whether the addition of DAC to an alkylating agent-based pretransplant regimen will alter DNA methylation status and translate into a significantly improved long-term disease control is unknown.

We hypothesized that DAC can sensitize alkylating agent-resistant cells to DNA alkylators. Considering Bu as a prototypic bifunctional alkylating agent used in high-dose pretransplant conditioning therapy, we utilized a previously established Bu-resistant cell line B5/Bu250⁶ [14] to examine the effects of DAC on myeloid leukemia (ML) cells with acquired Bu-resistance. We demonstrate in the present report that exposure of B5/Bu250⁶ cells to DAC synergistically increases their sensitivity to Bu in a p53-dependent manner, and we describe possible molecular mechanisms for the synergistic effects of Bu and DAC on Bu-resistant ML cells.

MATERIALS AND METHODS

Cells and drugs

The Bu-resistant B5/Bu250⁶ cells used in this study were established from the human KBM7/B5 cell line as described previously [14]. B5/Bu250⁶ and K562 CML cell lines [15] were cultured in Iscove's Modified Dulbecco's Medium (IMDM; Sigma-Aldrich, St. Louis, MO) supplemented with 20% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) at 37°C in a humidified atmosphere of 5% CO₂ in air. The OCI-AML3, OCI-AML3/empty vector and OCI-

AML3/p53 shRNA cell lines [16] were grown in RPMI 1640 (Mediatech, Herndon, VA) supplemented with 10% FBS. Busulfan (Sigma-Aldrich) and 5-aza-2'-deoxycytidine (DAC; Sigma-Aldrich) were both freshly dissolved in dimethyl sulfoxide (DMSO) immediately prior to cellular drug exposure(s). The final concentration of DMSO in all experiments did not exceed 0.08% (by volume), a level that does not induce differentiation of these cell lines (data not shown).

Cytotoxicity assay and graphical analysis

Cell suspensions were aliquoted (50 μ l of 4×10^5 cells/ml) into 96-well plates in the presence of drug(s) or solvent alone, incubated as above at 37°C for 4 d, and analyzed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [17]. Graphical analyses including calculations of IC₅₀ values (the concentration of drug required for 50% growth inhibition) were done using Prism 5 (GraphPad Software, San Diego, CA). The combination effects of Bu and DAC were estimated based on the combination index (CI) values [18] calculated using the CalcuSyn software (Biosoft, Ferguson, MO). This program was developed based on the median-effect method: CI < 1 indicates synergy, CI $\approx$ 1 is additive, and CI > 1 suggests antagonism.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Actively growing B5/Bu250⁶ cells (2.5×10^5 cells/ml) were exposed to the indicated concentrations of the drug(s) at 37°C for 4 d. Cells were analyzed for apoptosis using the Apo-Direct™ TUNEL assay kit (Millipore, Billerica, MA) according to the procedure provided by the manufacturer. TUNEL-positive cells were determined by analysis of at least 15,000 cells using a FACS Calibur instrument (BD Biosciences, San Jose, CA).

Flow-cytometric analysis of cell cycle phase distribution

Cells in logarithmic growth phase (1×10^6 cells/ml) were incubated with the indicated concentrations of the drug(s) at 37°C. Following incubation for 48 h, the cells were centrifuged, resuspended in 70% ethanol in phosphate-buffered saline (PBS), and fixed at -20°C overnight. Fixed cells were pelleted at $3,000 \times g$ at room temperature, washed with PBS, and treated with 0.25 ml of 500 U/ml RNase A in PBS containing 1.12% sodium citrate at 37°C for 30 min. After addition of 0.25 ml propidium iodide (PI, 50 μ g/ml) solution, the cells were kept in subdued light for at least 1 h prior to analysis by flow cytometry. The cellular DNA content of at least 10,000 cells was analyzed using a BD FACS Calibur instrument and the proportion of cells in the different phases of the cell cycle was determined using the CellQuest™ software (Becton Dickinson, Franklin Lakes, NJ).

Real-time PCR

Total RNA was extracted using TRIzol™ Reagent (Invitrogen). The high capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) was used to synthesize cDNA. Real-time PCR amplification was performed using a SYBR Green-based assay with the 7500 Real Time PCR System (Applied Biosystems). A dissociation step was included in the amplification, and only primers with a single dissociation peak were included in the final analysis. The quantification of gene expression was carried out by comparative C_T methodology (i.e., threshold cycle number above which the increase in fluorescence was logarithmic); the *GAPDH* gene was used as an internal control. Primers used for all PCR analyses are listed in Table 1.

Methylation-specific PCR analysis

Genomic DNA was isolated from the cell lines using a Wizard Genomic DNA Purification kit (Promega, Madison, WI). Bisulfite modification of the genomic DNA and its purification was

performed using a MethylDetector kit (Active Motif, Carlsbad, CA). Methylation-specific PCR (MSP) was performed using 30 ng bisulfite-modified DNA, 0.5 μ M of each primer (Table 1), 1 mM each of dATP, dCTP, dGTP and dTTP, 5 mM MgSO₄, and *Taq* DNA polymerase with ThermoPol II buffer (New England Biolabs, Ipswich, MA). The amplification method included initial heating at 95°C for 3 min, followed by 30 cycles of 95°C for 30 s, 57°C or 65°C for 30 s depending on the primers (see Table 1), and 72°C for 33 s, followed by extension at 72°C for 10 min. MSP products were visualized on a 2.5% agarose gel and DNA band intensities were analyzed using Quant One software (Bio-Rad, Hercules, CA).

Western blot analysis

Cells were collected by centrifugation, washed with PBS and lysed with cell lysis buffer (Cell Signaling Technology, Danvers, MA) as recommended by the manufacturer. Total protein concentrations in cell lysates were determined using a BCA Protein Assay kit (Thermo Fisher Scientific, Rockford, IL). Western blot analysis was done by separating protein extracts on polyacrylamide-SDS gels and blotting onto nitrocellulose membranes (Bio-Rad). Immunoblot analysis by chemiluminescence was done using the SuperSignal West Pico/Dura Substrate (Thermo Fisher, Waltham, MA) or the Immobilon Western Chemiluminescent HRP Substrate (Millipore, Bedford, MA). All antibodies, their sources and other relevant information are listed in Table 2. The β -actin protein was used as an internal control.

RESULTS

Bu and DAC combinations have synergistic effects on the survival of B5/Bu250⁶ cells

Recent phenotypic characterizations of Bu-resistant B5/Bu250⁶ cells demonstrated decreased expression of several genes known to be epigenetically regulated [14]. We, therefore, sought evidence that exposure of B5/Bu250⁶ cells to DAC might alter gene expression and sensitize these cells to Bu. Cells were exposed to various concentrations of Bu or DAC alone, or in combination, and their survival was determined using the MTT assay. B5/Bu250⁶ cells have calculated IC₅₀ values of 25 μ g/ml Bu and 0.17 μ M DAC when continuously incubated with the individual drug for 4 d (data not shown). Approximately 86% cell survival was observed using 5 μ g/ml Bu alone and 83% cell survival using 0.04 μ M DAC alone (Figure 1A). A more efficient inhibition of cell survival (down to 57% of control) was observed when the two drugs were combined at the same concentrations (Figure 1A). When values for cell survival (logarithmic scale) at three different Bu concentrations were plotted against DAC concentration (linear scale), the slopes of the three lines increased with Bu concentration, suggesting possible synergistic effects of Bu and DAC. The possibility of a synergistic cytotoxicity of combined Bu and DAC was further indicated by analysis using the median-effect method [18]. The combination indexes (CIs) at fractional effects of 0.3–0.7 (i.e., 30%–70% cytotoxicity) were significantly lower than 1.0, suggesting strong synergism in these concentration intervals (Figure 1B).

Analysis of the isobologram for the different [Bu+DAC] combinations that resulted in 50% cell survival (Figure 1C) shows that [6 μ g/ml Bu + 0.07 μ M DAC] would be as cytotoxic for the Bu-resistant B5/Bu250⁶ cells (Figure 1C) as 6 μ g/ml Bu alone was for the Bu-sensitive parental B5 cells (i.e., its IC₅₀ value, data not shown). These results further illustrate that DAC can sensitize Bu-resistant cells to Bu.

The observed synergistic effects of DAC and Bu using the MTT assay are further validated by the results obtained using the TUNEL assay, a method for detecting DNA fragmentation as a result of apoptotic responses [19]. Approximately 1.8% of the cells were positive for the TUNEL assay in the absence (solvent control) or presence of 0.02 μ M DAC (Figure 1D). Exposure of B5/Bu250⁶ cells to 15 μ g/ml Bu for 4 d resulted in 18.3% apoptotic cells and the

combination of 0.02 μ M DAC and 15 μ g/ml Bu increased the level of TUNEL-positive cells to 30.8% (Figure 1D), which strongly indicates synergistic cytotoxicity.

DAC induces G2-arrest, cell death and DNA hypomethylation of B5/Bu250⁶ cells

The observed synergism between Bu and DAC prompted us to determine the molecular mechanisms underlying these cytotoxic effects. We recently reported that the resistance of B5/Bu250⁶ cells to Bu alone is partly due to their ability to evade drug-induced G2-arrest and apoptosis [14]. Others have attributed the cytotoxic activity of DAC to its ability to arrest cells in the G1 and G2-M phases of cell cycle [20]. We therefore examined how DAC alone would affect the phenotype of B5/Bu250⁶ cells. Exposure of cultures to 0.5 μ M DAC for 48 h and analysis by flow cytometry showed an ~5-fold increase in cells in G2 phase and an ~5-fold increase in the sub-G1 fraction, which likely reflects the induction of apoptosis (Figure 2A). The accumulation of cells in G2 phase is further manifested by a 10-fold increase in G2/G1 and G2/S values (bottom of Figure 2A). These results suggest that DAC exposure results in alterations in cell cycle checkpoint regulation and in the death of B5/Bu250⁶ cells presumably by apoptosis.

To determine whether DAC affects gene expression in B5/Bu250⁶ cells, we initially examined its effect on the methylation of selected genes known to be epigenetically regulated. Using the methylation-specific PCR (MSP) methodology [21] we identified the *p16^{INK4A}* and *XAF1* genes to be hypermethylated in B5/Bu250⁶ cells (Figure 2B). Exposure to DAC for 48 h resulted in a dose-dependent reversal of the hypermethylation of the *p16^{INK4A}* and *XAF1* gene promoters as suggested by an increasing ratio of unmethylated to methylated (U/M) DNA (Figure 2B). These changes in the methylation status of the *p16^{INK4A}* and *XAF1* promoters correlated with a dramatic increase in their transcription level as measured by real-time PCR (Figure 2B, right hand panel). Exposure of B5/Bu250⁶ cells to 0.5 μ M DAC for 48 h resulted in increased expression of *p16^{INK4A}* and *XAF1* by more than 500- and ~70-fold, respectively, compared with the control cells exposed to solvent alone. Thus exposure of B5/Bu250⁶ cells to DAC results in hypomethylation and transcriptional activation of the *p16^{INK4A}* and *XAF1* genes which encode the p16^{INK4A} cyclin-dependent kinase (CDK) inhibitor and the pro-apoptotic XAF1 (XIAP Associated Factor 1) protein, respectively.

To further understand how DAC causes hypomethylation of the *p16^{INK4A}* and *XAF1* genes we examined the status of DNA methyl transferase (DNMT) isozymes which are known to catalyze DNA methylation. Using real-time PCR analysis, the constitutive expression of *DNMT1*, *DNMT2*, *DNMT3A* and *DNMT3B* was found to be 1.5, 1.9, 0.9 and 23-fold higher in Bu-resistant B5/Bu250⁶ cells relative to Bu-sensitive parental B5 cells (data not shown). Because DNMT1 and DNMT3B are believed to maintain ~95% of the DNA methylation in human cancer cells [22], we determined how the protein levels of these two isozymes are affected by DAC. B5/Bu250⁶ cells were exposed to various concentrations of the drug, and protein extracts were analyzed by immunoblotting. Figure 2C shows a dose-dependent disappearance of the two enzymes in the presence of DAC, which is consistent with studies using other cell lines [23,24]. The observed higher expression of *DNMT3B* in B5/Bu250⁶ versus B5 cells (23-fold) combined with the previous report that the DNMT3B enzyme functions principally as a *de novo* DNA methyltransferase [25] suggest that DNMT3B could play a significant role in epigenetic regulation of the phenotypic expression of Bu-resistance in B5/Bu250⁶ cells.

DAC alters the expression of several proteins involved in cell cycle regulation, apoptosis and survival

As noted above, DAC treatment invokes a G2-arrest in B5/Bu250⁶ cells (Figure 2A). To identify the cell cycle regulatory proteins involved in DAC-mediated checkpoint control, we

examined the expression and phosphorylation status of several key proteins involved in this response. Exposure of B5/Bu250⁶ cells to 0.5 μ M DAC for 48 h resulted in a dramatic phosphorylation of the variant H2A histone, H2AX, to its activated form γ -H2AX (Figure 3A), suggesting a DNA-damage response that could trigger checkpoint signaling. Downstream in this signaling cascade is the phosphorylation of the tumor suppressor protein, p53, and its subsequent induction (by transcriptional transactivation) of the CDK inhibitor protein p21^{WAF1/CIP1} (Figure 3A), an observation consistent with previous studies using this drug in other tumor cell lines [7, 8]. The up-regulation of p21^{WAF1/CIP1} in the presence of DAC is likely related to the observed phosphorylation of p53 at Ser15 as previously reported [26, 27] and discussed further below. Although p21^{WAF1/CIP1} is known to be capable of triggering G1-arrest, it also causes G2-arrest by interacting with CDK1/CDC2 [28, 29]. The DAC-induced increase in p21^{WAF1/CIP1} correlates with the observed accumulation of P-CDC2 (Figure 3A), a known hallmark of G2-M checkpoint activation [30]. Another gene encoding a CDK inhibitor that was induced by DAC was *p16^{INK4A}* (Figure 2B). The DAC-mediated hypomethylation and transcription of the *p16^{INK4A}* gene correlates closely with the dramatic increase in p16^{INK4A} protein expression shown in Figure 3A. As will be discussed below, p16^{INK4A} is best known as an activator of the G1 checkpoint, although some studies also indicate a role for p16^{INK4A} at the G2-M checkpoint.

Prolonged arrest of cells in G2 phase may result in apoptosis or mitotic catastrophe and subsequent cell death. Indeed, protracted exposure (48 h) of B5/Bu250⁶ cells to 0.5 μ M DAC increased the proportion of cells with sub-G1 DNA content (Figure 2A), which is a strong indication of cells undergoing apoptosis. Immunoblot analysis was used to identify anti- and pro-apoptotic proteins affected by DAC (Figure 3B). As shown in Figure 2B, hypomethylation and transcription of the *XAF1* gene were induced by DAC, and Figure 3B shows the concomitant up-regulation of XAF1 protein expression. The constitutive low expression of XAF1 (Figure 3B, lane 1) correlated with the low abundance of the unmethylated form of the *XAF1* gene in untreated B5/Bu250⁶ cells (Figure 2B, lane 2). XAF1 protein is known to antagonize the pro-survival activities of the inhibitor of apoptosis (IAP) family of proteins, such as XIAP, cIAP1 and cIAP2 [31, 32]. Indeed, the increased level of expression of XAF1 protein correlated with decreased levels of its target proteins XIAP, cIAP1 and cIAP2 (Figure 3B). The down-regulation of these pro-survival proteins by DAC was accompanied by up-regulation of pro-apoptotic proteins such as PUMA (p53-upregulated modulator of apoptosis) and BNIP3 (BCL2/adenovirus E1B 19kDa interacting protein 3) (Figure 3B). The up-regulation of PUMA might be attributed to p53 trans-activation [33] and the increase in BNIP3 expression could be due to DNA hypomethylation of its promoter [34].

In addition to proteins involved in apoptosis, we examined the effects of DAC on other pro-survival factors. Because the STAT3 (signal transducer and activator of transcription 3) protein is up-regulated in B5/Bu250⁶ cells [14], we examined the effects of DAC exposure on the expression of STAT3 and its target genes. Figure 3B showed a DAC-dose-dependent decrease in expression of STAT3 protein; a less marked effect was observed at 0.1 μ M DAC, but an almost 50% decrease in STAT3 expression was consistently observed at 0.5 μ M DAC after a 48-h exposure. This down-regulation of STAT3 is consistent with a dramatic decrease in the expression of c-MYC (Figure 3B), a known downstream target gene of STAT3 [35]. Overall, the down-regulation of anti-apoptotic and pro-survival proteins and up-regulation of pro-apoptotic proteins is associated with and presumably contributes to the cytotoxic activity of DAC towards B5/Bu250⁶ cells.

The synergistic effects of DAC and Bu in B5/Bu250⁶ cells include exacerbation of G2-arrest and apoptosis

The observed G2-arrest and apoptosis described above were obtained at relatively high concentrations of DAC. We decided to combine DAC and Bu at concentrations much lower than IC₅₀ values and determine their cytotoxicity on Bu-resistant B5/Bu250⁶ cells. Exposure of cells to 0.05 μ M DAC for 48 h resulted in an ~2-fold increase in G2/G1 ratio without a substantial change in the sub-G1 fraction; exposure to 5 μ g/ml Bu resulted in an ~4-fold increase in G2/G1 ratio and small increase in the sub-G1 fraction (Figure 4A). Exposure to the combination of 0.05 μ M DAC and 5 μ g/ml Bu dramatically increased the G2/G1 ratio and sub-G1 fraction by ~7-fold and ~3-fold, respectively (Figure 4A). This marked increase of cells in the sub-G1 fraction was also associated with activation of apoptosis as indicated by cleavage of PARP1 (poly-ADP ribose polymerase-1) and activation (by cleavage) of two key apoptotic proteins, caspases 3 and 8 (Figure 4B), but not of caspase 2 (data not shown). A less dramatic increase in cleavage of PARP1, caspase 3 and caspase 8 was observed when B5/Bu250⁶ cells were exposed to either 0.05 μ M DAC alone or 5 μ g/ml Bu alone. Thus, combination of the two drugs resulted in an increased activation of the apoptotic pathway (Figure 4B). These results suggest that the cytotoxic activity of low concentrations of DAC and Bu can be increased by combining the two drugs.

The [DAC+Bu]-mediated synergistic effects on myeloid leukemia cells are p53-dependent

As shown in Figure 3, DAC alone induces the phosphorylation of p53 and enhances its transcriptional transactivation function as suggested by the associated up-regulation of p21^{WAF1/CIP1} and PUMA. Recognizing that p53 plays a critical role in checkpoint signaling, we examined the effects of combined [DAC+Bu] on the expression and phosphorylation of p53. Figure 5A shows that whereas 0.05 μ M DAC alone or 5 μ g/ml Bu alone induced p53 phosphorylation at Ser15 (Figure 5A, lanes 2 and 4), their combination (lane 5) had an even more dramatic effect, suggesting that the activation of p53 might play a role in the cytotoxic activity of combined [DAC+Bu]. We therefore sought to examine the dependence of [DAC+Bu] cytotoxicity on p53. We used pifithrin- α to inhibit the transactivation function of p53 in B5/Bu250⁶ cells and compared the results to those seen in the p53-null K562 CML cell line. Cells pre-exposed to pifithrin- α prior to treatment with the [DAC+Bu] combination were analyzed by flow cytometry and immunostaining. We used a slightly higher DAC concentration (0.2 μ M) in these experiments to elicit more pronounced effects. Exposure of B5/Bu250⁶ cells to 0.2 μ M DAC and 5 μ g/ml Bu resulted in a considerable increase of cells in the sub-G1 compartment (39%, versus 15% in control cells), a response that was inhibited in a dose-dependent manner by exposing the cells to pifithrin- α prior to [DAC+Bu] treatment (Figure 5B). The sub-G1 fraction was reduced from 39% to 12% in the presence of 20 μ M pifithrin- α . The [DAC+Bu]-mediated G2-arrest, as measured by the G2/G1 or G2/S ratio, was alleviated from 0.46 to 0.38 or 0.88 to 0.63, respectively, in B5/Bu250⁶ cells pre-exposed to pifithrin- α (bottom of Figure 5B). This observation is consistent with the previously-reported requirement for p53 and p21^{WAF1/CIP1} to sustain G2-arrest in human cells [28]. In contrast, the p53-null K562 cells showed resistance to the [DAC+Bu] combination as suggested by the lack of increase in sub-G1 fraction, but interestingly a marked G2-arrest was observed in these cells (Figure 5B). Pre-exposure of K562 cells to 20 μ M pifithrin- α prior to [DAC+Bu] treatment did not substantially change the proportion of cells in sub-G1 but further enhanced the G2-arrest (G2/G1 and G2/S ratios increasing from 0.45 to 0.58 and 0.41 to 0.70, respectively; Figure 5B).

The results from flow cytometry were consistent with immunostaining studies. Exposure of B5/Bu250⁶ cells to 0.2 μ M DAC and 5 μ g/ml Bu dramatically increased the level of p21^{WAF1/CIP1} protein, and pre-exposure of cells to 20 μ M pifithrin- α inhibited this up-regulation (Figure 5C, left hand panel). This abrogation of p21^{WAF1/CIP1} induction implies

that pifithrin- α is an effective inhibitor of p53 transcriptional transactivation in this cell line under these conditions. Surprisingly, the observed [DAC+Bu]-mediated phosphorylation of histone H2AX in B5/Bu250⁶ cells (Figure 5C) was also abrogated by pifithrin- α . Although exposure of K562 cells to [DAC+Bu] combination also induced the phosphorylation of histone H2AX, this response was not blocked by pifithrin- α (Figure 5C). In addition to blocking p21^{WAF1/CIP1} induction in B5/Bu250⁶ cells, the p53 inhibitor pifithrin- α also blocked the [DAC+Bu]-mediated cleavage of PARP1, caspase 9 and caspase 8 (Figure 5C). These collective results, including the decrease in sub-G1 fraction shown in Figure 5B, suggest that the [DAC+Bu]-mediated apoptosis of B5/Bu250⁶ cells is largely p53-dependent. This conclusion is further supported by the resistance of p53-null K562 cells to apoptosis when treated with [DAC+Bu] as shown by lack of increase in sub-G1 fraction (Figure 5B, right hand panel) and minimal cleavage of PARP1, caspase 8 and caspase 9 (Figure 5C, right hand panel).

The relevance of p53 and p21^{WAF1/CIP1} in the maintenance of G2-arrest, as mentioned above, is further suggested by the observed p53-dependent changes in the level of phosphorylation of CDK1/CDC2. Pre-exposure of p53-positive B5/Bu250⁶ cells to pifithrin- α reduced the level of [DAC+Bu]-induced P-CDK1/CDC2 (Figure 5C), which coincides with the observed reduction in G2/G1 and G2/S values (Figure 5B). This result was not observed in the p53-null K562 cells; interestingly, a slight upregulation of P-CDK1/CDC2 was observed when these cells were exposed to pifithrin- α (Figure 5C) with a concomitant increase in G2/G1 and G2/S ratios (Figure 5B).

To further define the relevance of p53 in [Bu+DAC]-mediated cytotoxicity, we used a previously established clone of OCI-AML3 cells where p53 was down-regulated using an shRNA construct [16]. Initial analysis showed that the parental cell line OCI-AML3 was more resistant to [Bu+DAC] combinations compared with B5/Bu250⁶ cells (data not shown). Exposure of OCI-AML3 and OCI-AML3/empty vector cells to combined 30 μ g/ml Bu and 4 μ M DAC for 48 h resulted in G2-arrest and significant cell death as indicated by 40–50% cells in sub-G1; in contrast, OCI-AML3/p53 shRNA cells showed greater resistance to [Bu+DAC] exposure (Figure 5D). This resistance may be attributed to a lack of p53 expression and activation of its downstream signaling pathways as suggested by Western blot analysis. Figure 5E shows expression of p53 in the parental and empty vector cell lines but not in OCI-AML3/p53 shRNA cells. The expressed p53 was phosphorylated at Ser15 when cells were exposed to [Bu+DAC] and resulted in p21^{WAF1/CIP1} expression and PARP1 cleavage. Together, the results from cell cycle and Western blot analyses underscore the importance of p53-dependent pathways in mediating the cellular effects of [Bu+DAC] combinations in the ML background.

DISCUSSION

Based on our cellular model for acquired Bu resistance in ML cells, it is apparent from these findings that DAC can be effectively used to sensitize these Bu-resistant cells to Bu *in vitro*. The combination of DAC and Bu over a broad range of concentrations displayed a synergistic increase in cytotoxicity compared to the individual drugs as evaluated by the median-effect method. Our results suggest that this synergism can be attributed to the combined efficacies of these two drugs in inducing cell cycle arrest and apoptosis when administered at low concentrations.

Since we had already established that Bu alone causes G2-arrest and induces the expression of pro-apoptotic genes [14], we first evaluated the mechanism of cytotoxicity of DAC alone in Bu-resistant B5/Bu250⁶ cells. Like Bu, DAC induces both G2-arrest and apoptosis in these cells. The ability of DAC to induce these two cellular responses might be attributed to its inhibition of DNA methylation and activation of cell cycle checkpoint signaling, similarly to previous reports for DNA repair responses [36,37]. These two activities – DNA

hypomethylation and cell cycle checkpoint regulation – may not be entirely independent of each other given that the expression of some genes involved in cell cycle regulation is epigenetically controlled. However, the incorporation of DAC into DNA strands may elicit responses independent of its DNA-hypomethylating activity. Whether DNA hypomethylation or cell cycle checkpoint regulation prevails in a given situation in this cell type most likely depends on factors such as the DAC concentration relative to exposure time.

DAC induces DNA hypomethylation by inhibiting DNA methyltransferases. We observed that B5/Bu250⁶ cells express ~1.5-fold and ~23-fold higher levels of DNMT1 and DNMT3B, respectively, compared with the Bu-sensitive parental B5 cells. This elevated expression of DNMT1 and DNMT3B could be a contributory factor to the Bu-resistance of B5/Bu250⁶ cells. Exposure of these cells to 0.5 μ M DAC for 48 h resulted in decreased levels of both DNMT1 and DNMT3B (Figure 1C), presumably mediated by enhanced protein degradation, which is believed to be initiated by covalent trapping of the enzymes to DNA-incorporated DAC [23]. This trapping/degradation of DNMTs is expected to result in a reversal of DNA hypermethylation and re-expression of silenced genes. Among the epigenetically regulated pro-apoptotic genes that we examined in this context (data not shown), *XAF1* showed a dramatic change in expression in the presence of DAC (Figure 2B). Analysis of the methylation status of the *XAF1* promoter by MSP showed an approximately 4-fold increase in the ratio of unmethylated to methylated sequences when B5/Bu250⁶ cells were exposed to 0.5 μ M DAC (Figure 2B).

XAF1 is a pro-apoptotic protein which binds to and antagonizes the anti-apoptotic activities of several members of the IAP family of proteins, including XIAP, cIAP1 and cIAP2; its binding to XIAP reverses the anti-caspase activity of XIAP [31] and its interaction with cIAP1 and cIAP2 releases pro-apoptotic Smac/DIABLO from their complexes [32]. Our results are consistent with these previous reports. Exposure of B5/Bu250⁶ cells to DAC resulted in up-regulation of XAF1 protein and down-regulation of all three of these IAP proteins, i.e., XIAP, cIAP1 and cIAP2 (Figure 3B).

DNA hypomethylation could also play a role in the DAC-mediated G2-arrest of B5/Bu250⁶ cells. Examination of the expression of various cell cycle-regulatory proteins in our cellular model suggests that DAC controls the cell cycle at various signaling checkpoints. It caused DNA hypomethylation of the *p16^{INK4A}* gene and up-regulation of the p16^{INK4A} protein (Figure 2B), an inhibitor of CDKs which are critical regulators of cell cycle progression. DAC stimulated the phosphorylation of p53, which was most likely responsible for the up-regulation of another CDK inhibitor, p21^{WAF1/CIP1} (Figure 3A). Although both the p16^{INK4A} and p21^{WAF1/CIP1} proteins are known to arrest cells in G1 phase [38], they have been shown to contribute to the arrest of cells in G2-M phase as well [28, 39–41].

Although DAC alone can perturb cell cycle progression, the combination of DAC and Bu at lower concentrations resulted in a more marked G2-arrest of Bu-resistant B5/Bu250⁶ cells (Figure 4A). We reported previously that Bu causes G2 cell cycle arrest through the CHK2-CDC25-CDC2 pathway [14]. The [DAC+Bu]-mediated activation of p53 and up-regulation of p21^{WAF1/CIP1}, which eventually inhibits CDC2 [28, 29], may also enhance/maintain G2-arrest [40, 41]. Together these responses may elicit stronger signals for the activation of cell cycle checkpoints.

The combination of DAC and Bu also elicited a more efficient activation of the apoptotic response. The observed phosphorylation of histone H2AX, cleavage of PARP1 and activation of caspases 3 and 8 are all consistent with DNA-damage responses, which might be caused by the DNA crosslinking activity of Bu [42] and the incorporation of DAC into DNA strands as a nucleoside analogue [27]. How inhibition of p53 activity by pifithrin- α alleviates the [DAC

+Bu]-induced phosphorylation of H2AX in B5/Bu250⁶ cells remains to be defined, although the possibility of off-target effects cannot be discounted. Another possibility is that H2AX phosphorylation is triggered by the attempt of cells to repair such DNA modifications, which probably results in DNA nicks and single-stranded DNA. It is well known that such repair events are often p53-dependent [43].

Although further studies are required to dissect the cascade of events and identify specific caspases involved in [DAC+Bu]-mediated apoptosis, our study using B5/Bu250⁶ cells suggests a major involvement of caspases 3, 8 and 9, but not of caspase 2, in this apoptotic response. Caspase 3 is a known executioner caspase, but recent reports have shown its role upstream of the mitochondria [44,45] and may therefore be classified as an initiator caspase.

The cytotoxic activity of combined [DAC+Bu] in ML cells clearly depends on p53 status. The p53-null K562 cell line exhibited greater resistance to these drugs than the B5/Bu250⁶ cell line as shown by cell cycle analysis (Figure 5B) and cytotoxicity/MTT assay (data not shown). Inhibition of p53 function and associated abrogation of transactivation of gene expression in p53-wild type B5/Bu250⁶ cells with pifithrin- α alleviated the [DAC+Bu]-mediated G2-arrest and apoptosis (Figure 5B) with a concomitant decrease in P-CDC2 and inhibition of PARP1, caspase 8 and caspase 9 cleavage (Figure 5C). Similar results were obtained when p53 was knocked down with shRNA in OCI-AML3 cells (Figure 5D and 5E). As a guardian of chromosome integrity, it is not unreasonable to imagine that p53 would mediate the effects caused by incorporation of DAC as a nucleoside analogue into DNA strands or by DNA crosslinks caused by Bu. These results suggest that analysis of the p53 status of the leukemia cell population may have predictive value when evaluating the potential benefit of combining DAC with an alkylating agent-based pretransplant conditioning regimen.

The inclusion of DAC in a Bu-based therapy for ML might not only result in a synergistic cytoreduction but might also offer additional safety. Our observation that combined 70 nM DAC + 6 μ g/ml Bu exerts the same degree of cytotoxicity to Bu-resistant cells as 6 μ g/ml Bu alone does to Bu-sensitive cells suggests that the proposed strategy might ultimately offer advantages for treating Bu-resistant ML by overcoming resistance and reducing toxic Bu exposure, and for treating Bu-sensitive ML by reducing the amount of Bu exposure required for therapeutic benefit. We should note that the major side effect of DAC therapy in leukemia patients is severe and prolonged myelosuppression [46]; the major side effects of Bu include irreversible myelosuppression, hepatic veno-occlusive disease and neurotoxicity [47].

In conclusion, DAC and Bu have partly unique and partly common effects on cell cycle progression and cell survival. When combined, their cytotoxic effects become synergistic against Bu-resistant ML cells. Our findings provide a mechanistic explanation for the observed synergistic cytotoxicity. Based on these findings, we propose that inclusion of DAC in Bu-based pre-transplant conditioning therapy may increase the cytoreductive efficacy against myeloid leukemic cell clones with acquired cross-resistance to bifunctional alkylating agents.

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Figure 1.

Cytotoxic effects of Bu and DAC, alone or in combination, on B5/Bu250⁶ cells. (A) Cells were incubated for 4 d with the indicated concentrations of Bu or DAC alone, or in combination, and cell survival was determined by the MTT assay. Values for cell survival (%) on the Y-axis are in logarithmic scale and were calculated relative to control cells exposed to solvent alone. (B) Results from the MTT assay were analyzed for synergism by calculating the combination index (CI) values using the CalcuSyn software. The values in panels A and B are the average ($\pm$ SE) of five independent experiments. (C) Equipotent combinations of various concentrations of Bu and DAC that result in 50% cell survival. (D) The synergistic cytotoxicity of [DAC+Bu] after 4 d was further evaluated by determining the proportion of cells with fragmented DNA using the TUNEL assay.

Figure 2.

Effects of DAC on cell cycle phase distribution, DNA methylation and DNA methyltransferase levels. (A) Representative DNA histograms for B5/Bu250⁶ cells following exposure to solvent or 0.5 μ M DAC for 48 h prior to staining with propidium iodide and flow cytometry. These experiments were repeated at least 3 times with very similar results. (B) Left hand panel: Typical genomic DNA methylation profiles from cells exposed to different concentrations of DAC for 48 h and analyzed by MSP using primers for methylated (M) and unmethylated (U) DNA. The numbers at the bottom of each picture refer to the ratio of unmethylated to methylated DNA (U/M). These experiments were repeated at least 3 times with very similar results. Right hand panel: mRNA levels of the *p16^{INK4A}* and *XAF1* genes as measured by real-time PCR. The data represent the average ($\pm$ SE) of three independent experiments. (C) Protein expression of DNMT1 and DNMT3B was determined by immunoblot analysis using extracts from cells exposed to various concentrations of DAC for 48 h. These experiments were repeated at least twice, and a typical result is shown.

Figure 3.

DAC affects the expression of key cell cycle regulatory (A) and survival (B) proteins. Cells were exposed to solvent or the indicated concentrations of DAC for 48 h and cell extracts were analyzed for protein expression by Western blot. Representative results of two experiments are shown.

Figure 4. Synergistic effects of Bu and DAC on Bu-resistant B5/Bu250⁶ cells. (A) Typical cell cycle histograms from flow cytometric analysis of cells exposed to Bu or DAC, alone or combined. (B) Representative Western blot showing the more efficient activation of the apoptotic response by [Bu+DAC] combinations than by single drug exposures. These experiments were repeated at least twice with similar results. [Casp = caspase]

Figure 5.

Effect of inhibition of p53 using pifithrin- α (PF α) on cellular responses to Bu and/or DAC. (A) B5/Bu250⁶ cells were exposed to the indicated concentrations of DAC and Bu, alone or combined, for 48 h prior to Western blot analysis to determine the phosphorylation status of p53. (B) Cells were or were not pre-exposed to pifithrin- α for 24 h then treated with [DAC +Bu] for 48 h prior to flow-cytometric analysis or (C) Western blot analysis. (D) OCI-AML3 cells were exposed to DMSO solvent or to the indicated concentrations of Bu and DAC for 48 h and analyzed by flow cytometry and (E) Western blot. All panels are representative: in each case the experiments were repeated at least twice with similar results.

Table 1

Primers for PCR analysis

Real-time PCR			
Gene	Forward	Reverse	Annealing Temp (°C)
<i>GAPDH</i>	CAACAGCCTCAAGATCATCAGC	TCCTAGACGGCAGGTCAGGTC	60
<i>DNMT1</i>	CCGGCCCCGGTTCTT	GGACCATGGAGCGCTTGA	60
<i>DNMT2</i>	AGCCCCTGGTCAGGTACTGA	CATTGCATATTTTGTGGATGTACAGA	60
<i>DNMT3A</i>	AGAGAGCGGCTGGTGTACGA	CTCCCACAGGAGATGCAGATG	60
<i>DNMT3B</i>	AAGCTCGCGACTCTCCAAGAG	ATCGCCTGTCAAGTCCTGTGT	60
<i>p16</i>	CCCAACGCACCGAATAGTTAC	CGCTGCCCATCATCATGAC	60
<i>XAF1</i>	CTCGGTGTGCAGGAAGTGTAAA	CAGGAACCGCAGGCAGTAA	60
MSP			
<i>p16</i> (Methylated)	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	65
<i>p16</i> (Unmethylated)	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	65
<i>XAF1</i> (Methylated)	TTTGTAAGAAACGAAATTTAATCGA	CCTACCCCTTAAACCCACGAT	57
<i>XAF1</i> (Unmethylated)	TTTGTAAGAAATGAAATTTAATTGA	CTCCTACCCCTTAAACCCACAAT	57

Table 2

List of primary antibodies, their sources and dilutions

Antigen	Source/Cat. #	Clone type*	Dilution**
BNIP3	Cell Signaling/3769	pAb	2000
Caspase 8	Cell Signaling/9746	mAb	1500
Caspase 9	Cell Signaling/9502	pAb	1500
CDC2	Cell Signaling/9112	pAb	1500
cIAP1	Cell Signaling/4952	pAb	1500
cIAP2	Cell Signaling/7944	pAb	500
Cleaved Caspase 3	Cell Signaling/9661	pAb	1500
c-MYC	Cell Signaling/9402	pAb	1500
DNMT1	Santa Cruz Biotech/10222	pAb (goat)	500
DNMT3B	Santa Cruz Biotech/10236	pAb (goat)	500
MAPKAPK2	Cell Signaling/3042	pAb	2500
p16	BD Pharmingen/554079	mAb	1000
p21	Upstate Biotech/05-345	mAb	1000
p53	Santa Cruz Biotech/1315	pAb	1000
p38	Cell Signaling/9212	pAb	2500
PARP1	Santa Cruz Biotech/8007	mAb	1000
P-CDC2 (Tyr15)	Cell Signaling/9111	pAb	1500
P-MAPKAPK2(Thr334)	Cell Signaling/3041	pAb	2500
P-p38(Thr180/Tyr182)	Cell Signaling/9211	pAb	2000
P-p53(Ser15)	Cell Signaling/9284	pAb	2000
PUMA	Cell Signaling/4976	pAb	2000
STAT3	Cell Signaling/9132	pAb	1000
XAF1	Santa Cruz Biotech/19193	pAb (goat)	500
XIAP	BD Transduction Lab/610717	mAb	1500
β-actin	Sigma/A5316	mAb	10000
γ-H2AX	Upstate Biotech/05-636	mAb	1000

* pAb: polyclonal antibody; used anti-rabbit IgG (or anti-goat as indicated) for secondary antibody from Bio-Rad Lab

mAb: monoclonal antibody; used anti-mouse IgG for secondary antibody from Bio-Rad Lab

** Fold dilution in PBS with 0.05% Tween 20