Published in final edited form as:

Exp Hematol. 2015 June ; 43(6): 448–461.e2. doi:10.1016/j.exphem.2015.02.001.

Comparison of the cytotoxicity of cladribine and clofarabine when combined with fludarabine and busulfan in AML cells: enhancement of cytotoxicity with epigenetic modulators

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Abstract

Clofarabine (Clo), fludarabine (Flu) and busulfan (Bu) combinations are efficacious in hematopoietic stem cell transplantation (HSCT) for myeloid leukemia. We now determined if the more affordable drug cladribine (Clad) can provide a viable alternative to Clo, with or without panobinostat (Pano) and 5-aza-2'-deoxycytidine (DAC). Both [Clad+Flu+Bu] and [Clo+Flu+Bu] combinations showed synergistic cytotoxicity in KBM3/Bu250⁶, HL60 and OCI-AML3 cell lines. Cell exposure to these drug combinations resulted in 60%-80% inhibition of proliferation, activation of the ATM pathway, increase in histone modifications, decrease in HDAC3, HDAC4, HDAC5 and SirT7 proteins, decrease in mitochondrial membrane potential, activation of apoptosis and stress signaling pathways, and down-regulation of the AKT pathway. These drug combinations activated DNA-damage response and apoptosis in primary cell samples from AML patients. At lower concentrations of Clad/Clo, Flu and Bu, inclusion of Pano and DAC enhanced cell killing, increased histone modifications and DNA demethylation, and increased the level of P16/INK4a, P15/INK4b and P21/Waf1/Cip1 proteins. The observed DNA demethylating activity of Clad and Clo may complement DAC activity, increase demethylation of the gene promoters for the *SFRP1*, *DKK3* and *WIF1*, and cause degradation of β -catenin in cells exposed to [Clad/Clo+Flu+Bu+DAC+Pano]. The overlapping activities of [Clad/Clo+Flu+Bu], Pano and DAC in DNA-damage formation and repair, histone modifications, DNA demethylation and apoptosis may underlie their synergism. Our results provide a basis for supplanting Clo with Clad and for including epigenetic modifiers in the pre-HSCT conditioning regimen for myeloid leukemia patients in regions where economic factors might restrict the use of Clad.

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The authors declare no financial conflict(s) of interest.

Introduction

An important factor that contributes to the long-term success of hematopoietic stem cell transplantation (HSCT) is the efficacy of the pre-transplant conditioning regimen, which alleviates tumor burden and suppresses host immunoreactivity to secure engraftment. Combinations of nucleoside analogs (NAs) and DNA-alkylating agents are rapidly becoming part of standard conditioning therapy for acute myeloid leukemia/myelodysplastic syndrome (AML/MDS) patients undergoing HSCT. The efficacy of combinations of these drugs is exemplified by the synergistic cytotoxicity of the adenosine analogs clofarabine (Clo) and fludarabine (Flu) with the DNA-alkylating agent busulfan (Bu). In AML cell lines and primary patient cell samples it has been demonstrated that their combination induces complex DNA damage, chromatin remodeling and activation of the ATM pathway, leading to massive cell-cycle checkpoint activation and apoptosis [Valdez et al., 2011]. A combination of Clo with myeloablative doses of Bu was well-tolerated and showed significant antitumor activity in refractory AML patients [Magenau et al., 2011; Farag et al., 2011].

Despite the efficacy of Clo when combined with Flu and Bu, its more widespread use is hampered by its excessive cost, especially in countries outside the United States. Clofarabine is a second generation analog of 2'-deoxyadenosine and is considered more efficacious than its prototype predecessor, cladribine (Clad), when used as a single agent in patients with myeloid leukemia. We hypothesized that Clad may be combined with Flu and Bu to obtain synergistic cytotoxicity at least comparable with a [Clo+Flu+Bu] combination.

Cladribine is readily available. It is FDA-approved for treatment of hairy cell leukemia. It shares some common mechanisms of action with other nucleoside analogs yet it has its own unique clinical activity. In addition to its nucleoside analog activities, Clad alters the epigenomic status of tumor cells by acting as a hypomethylating agent through inhibition of S-adenosylhomocysteine hydrolase which leads to a deficiency in S-adenosylmethionine, a methyl donor for DNA methylation reactions catalyzed by DNA methyltransferases [Warzocha et al., 1997; Wyczzechowska and Fabianowska-Majewska, 2003]. The ability of Clad to inhibit adenosine deaminase also contributes to its unique properties [Warzocha et al., 1997].

As a single agent, Clad has a low response rate in AML but it has shown significant activity when used in combination with other drugs such as daunorubicin+cytarabine (DA). Thus, patients receiving [Clad+DA] induction therapy for refractory or relapsed AML showed a significantly better overall response rate and improved survival compared with patients receiving either the DA by itself or the [Flu+DA] variant regimen [Holowiecki et al., 2012]. A combination of Clad and Bu with or without low-dose total-body irradiation (TBI) as reduced intensity conditioning for allogeneic-HSCT also provided a satisfactory outcome in older patients with high-risk myeloid malignancies [Hamaki et al., 2004; Markova et al., 2007]. In light of these clinical studies, we hypothesized that there would be an improved efficacy of a [Clad+Flu+Bu] combination compared with [Flu+Bu] and it would be comparable with the efficacy of [Clo+Flu+Bu] if used as pretransplant conditioning for

HSCT in patients with advanced hematologic malignancies, moreover with greatly reduced cost.

To evaluate the possibility of using [Clad+Flu+Bu] in AML patients, we used AML cell line models and primary patient cell samples to study their synergistic cytotoxicity and mechanisms of action. Their combination showed strong synergistic effects, which correlated with activation of a DNA-damage response (through ATM) and apoptosis. We also report here that epigenetic modifications using the histone deacetylase (HDAC) inhibitor panobinostat (Pano) and the DNA demethylating agent 5-aza-2'-deoxycytidine (DAC) further enhance the cytotoxicity, at lower concentrations, of [Clad/Clo+Flu+Bu].

Materials and Methods

Cell lines and drugs

KBM3/Bu250⁶ is a Bu-resistant AML cell line established in our laboratory [Valdez et al., 2008]. The OCI-AML3 AML cell line was kindly provided by Dr. Michael Andreeff's laboratory (University of Texas MD Anderson Cancer Center, Houston, TX), and the HL60 AML cell line was obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in RPMI-1640 medium (Mediatech, Manassas, VA) supplemented with 10% heat-inactivated fetal bovine serum (FBS: Sigma-Aldrich, St. Louis, MO) and 100 IU/mL penicillin and 100 µg/mL streptomycin (Mediatech) at 37°C in a humidified atmosphere of 5% CO₂ in air. Cladribine, Flu, Bu and DAC were obtained from Sigma-Aldrich. Clofarabine (Clo, Clolar™; 1 mg/mL solution) and Pano (10 mM solution in DMSO) were purchased from Genzyme Oncology (Cambridge, MA) and Selleck Chemicals LLC (Houston, TX), respectively. Clad and Flu were dissolved in DMSO and stored at -20°C; Bu was prepared fresh in DMSO immediately prior to each experiment.

Cytotoxicity and apoptosis assays

The cytotoxicity of drugs was determined as previously described using the 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [Valdez et al., 2010]. Cell proliferation was determined relative to the control cells exposed to solvent alone. Graphical analyses including calculations of IC₁₀–IC₂₀ values (the concentration of drug required for 10–20% growth inhibition) were done using Prism 5 (GraphPad Software, San Diego, CA). Apoptosis was determined by flow-cytometric measurements of phosphatidylserine externalization with Annexin-V-FLUOS (Roche Diagnostics, Indianapolis, IN) and 7-aminoactinomycin D (BD Biosciences, San Jose, CA) using a Muse Cell Analyzer (EMD Millipore, Billerica, MA). Drug combination effects were estimated based on the combination index (CI) values [Chou and Talalay, 1984] calculated using the CalcuSyn software (Biosoft, Ferguson, MO).

Western blot analysis

Cells were exposed to drugs for 48 hours, collected by centrifugation, washed with cold PBS, lysed and analyzed for protein levels as previously described [Valdez et al., 2010]. Western blot analyses were done as described [Valdez et al., 2010] using antibodies listed in Table 1 (Supplemental Materials).

Analysis of mitochondrial membrane potential

Cells were exposed to drugs for 48 hours and aliquoted (0.5 mL) into 5-ml tubes. Changes in the mitochondrial membrane potential (MMP) were determined using an MMP detection kit (Cayman Chemical Co., Ann Arbor, MI) that included an MMP-sensitive fluorescent dye JC-1 reagent (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide). Diluted JC-1 reagent (1:10 with cell growth medium, 40 µL) was added to cell aliquots and incubated at 37°C for 20 min, and the samples were immediately analyzed by flow cytometry as described by the manufacturer. Valinomycin (1 µM) was added to a positive control tube containing untreated cells and incubated at 37°C, 5% CO₂ for 1 hour prior to addition of the JC-1 reagent.

Isolation of cytosolic fraction

Cells were exposed to drugs for 48 hours, collected by centrifugation, washed with ice-cold PBS, resuspended in buffer A (10 mM HEPES (pH 7.6), 10 mM KCl, 100 µM EDTA, 100 µM EGTA, 1 mM DTT, 500 µM phenylmethanesulfonyl fluoride and proteinase inhibitor cocktail) and incubated on ice for 30 min. Cells were lysed by passing the cells 10 × through a fine needle (27½ gauge). Cell lysates were centrifuged at 800 x g for 5 min at 4°C to separate the nuclei. The supernatant was further centrifuged at 12,500 x g at 4°C for 8 min to pellet mitochondria and the resulting supernatant was further centrifuged at 15,000 x g for 20 min at 4°C to pellet residual cellular debris. The final supernatant (cytosolic fraction) was analyzed by Western blotting.

Real-time PCR

Real-time PCR analysis was performed to determine the extent of DNA demethylation and level of gene expression. For demethylation analysis, genomic DNA was isolated from cells 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). The modified genomic DNA (12.5 –50 ng) was used in the methylation-specific PCR which included 1 x iTaq™ Fast SYBR Green Supermix with ROX (BIO-RAD, Hercules, CA) and 0.5 µM primers (Table 2 under Supplemental Materials). The amplification method included initial heating at 95 °C for 2 min, followed by 35 cycles of 95 °C for 3 sec and the indicated annealing temperature (Table 2 under Supplemental Materials) for 32 sec using the 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). Relative demethylation was determined using comparative C_T methodology (i.e., threshold cycle number above which the increase in fluorescence was logarithmic) and C_T calculations ($C_T = (C_{T,U} - C_{T,M})_{\text{drug X}} - (C_{T,U} - C_{T,M})_{\text{Control}}$ where U and M refer to unmethylated and methylated DNA, respectively (see Table 2 under Supplemental Materials), in cells exposed to drug combination X or solvent alone (Control)). Fold-change in the level of unmethylated DNA was calculated using the 2^{-C_T} method [Livak and Schmittgen, 2001].

For gene expression analysis, total RNA was extracted and purified using the RNeasy Mini Kit (QIAGEN, Valencia, CA). The high capacity cDNA Archive kit (Applied Biosystems) was used to synthesize cDNA. Real-time PCR amplification was performed as above using the primers listed in Table 2 (Supplemental Materials). The quantification of gene

expression was carried out by comparative C_T methodology using the *GAPDH* gene as an internal control. Fold-change in the level of expression was calculated using the 2^{-C_T} method, where $C_T = (C_{T,target} - C_{T,GAPDH})_{drug\ X} - (C_{T,target} - C_{T,GAPDH})_{Control}$.

Patient cell samples

Peripheral blood samples from patients with AML were collected after obtaining written informed consent. Mononuclear cells were purified using lymphocyte separation medium (Mediatech) and incubated in suspension with the indicated drugs in RPMI 1640 medium supplemented with 10% FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin. After 48 hours of incubation, cells were centrifuged and analyzed by Western blotting. All studies were performed according to a protocol approved by the Institutional Review Board of the University of Texas MD Anderson Cancer Center, in accordance with the Declaration of Helsinki.

Statistical analysis

Results are presented as the mean $\pm$ standard deviation of at least three independent experiments and statistical analysis was performed using a Student's paired t-test with a two-tailed distribution.

Results

Cladribine provides synergistic cytotoxicity with busulfan and fludarabine in AML cell lines

We previously showed synergistic cytotoxicity of Clo, Flu and Bu in AML cell lines and patient cell samples [Valdez et al., 2011]. Their efficacy as a part of the conditioning regimen prior to HSCT has been confirmed in AML, MDS and chronic myeloid leukemia (CML) patients [Andersson et al., 2011]. To determine if Clad would provide a similar synergism with Flu and Bu *in vitro*, we compared the cytotoxic activity of [Clad+Flu+Bu] and [Clo+Flu+Bu] combinations in AML cell lines. Exposure of KBM3/Bu250⁶ cells to 100 µM Bu, 0.6 µM Flu, 20 nM Clad or 10 nM Clo (equivalent to their IC₁₀–IC₂₀ values) showed negligible effects on cell proliferation and apoptosis (Fig. 1A). Exposure to [Flu+Bu] combination resulted in ~18% inhibition of cell proliferation but little apoptosis, as measured by Annexin V assay. Growth inhibition was significantly increased to ~77% when Clad was added, suggesting strong synergism of the three drugs. Addition of Clo to [Flu+Bu] inhibited cell proliferation by ~67% (Fig. 1A), consistent with our previous report [Valdez et al., 2011]. Apoptosis increased to ~23% for both the Clad/Clo triple-drug combinations, versus ~4% in the control samples.

Similar results were obtained when other AML cell lines, HL60 and OCI-AML3, were exposed to the same drug combinations. Exposure of HL60 cells to 40 µM Bu, 1.3 µM Flu, 40 nM Clad, 13 nM Clo or [Flu+Bu] combination resulted in less than 10% inhibition of cell proliferation; addition of Clad or Clo to [Flu+Bu] resulted in ~58% and ~60% growth inhibition, respectively (Fig. 1B). Apoptosis increased from ~3% in the control samples to ~19% for the Clad/Clo triple-drug combinations. OCI-AML3 cells were as sensitive as the KBM3/Bu250⁶ cells to [Clad+Flu+Bu] and [Clo+Flu+Bu]. While the individual drugs or

[Flu+Bu] combination did not significantly inhibit the proliferation of OCI-AML3 cells, [Clad+Flu+Bu] and [Clo+Flu+Bu] combinations inhibited cell proliferation by ~80% and ~66%, respectively (Fig. 1C). Apoptosis increased to ~63% and ~58% in the presence of [Clad+Flu+Bu] and [Clo+Flu+Bu], respectively, versus ~3% in the control samples. The results obtained using the three cell lines therefore suggest that the observed synergism was not cell line-specific. Moreover, the *in vitro* efficacy of [Clad+Flu+Bu] did not obviously depend on *P53*-status; KBM3/Bu250⁶ and HL60 cells are *P53*-negative and OCI-AML3 harbors wild-type *P53*.

To validate quantitatively the observed synergism, KBM3/Bu250⁶ cells were exposed to various combinations of the drugs ([Flu+Bu], [Clad/Clo+Flu+Bu]) at a constant ratio but at increasing concentrations for 48 hours and the relative cell proliferation was determined. Based on the observed effects/inhibition of proliferation, combination indexes (CI) were calculated for different drug combinations as described under Materials and Methods. Flu and Bu showed synergism (i.e., CI < 1.0) at high drug concentrations. Addition of Clad or Clo to [Flu+Bu] combination showed CI values of 0.2 and 0.3, respectively, at 50% fraction affected (Fa = 0.5), suggesting strong drug synergism (Fig. 1D).

[Clad/Clo+Flu+Bu] combination activates the ATM and apoptosis pathways

Exposure of KBM3/Bu250⁶ cells to [Clo+Flu+Bu] was previously shown to strongly activate the ATM and apoptosis pathways [Valdez et al., 2011]. We sought to determine if these pathways are also activated in cells exposed to [Clad+Flu+Bu]. Phosphorylation of ATM at Ser 1981 greatly increased in the presence of [Clad+Flu+Bu] or [Clo+Flu+Bu] (Fig. 2A). To further examine activation of the ATM kinase activity, we determined the phosphorylation of several of its substrates including the histone 2 AX, CHK2, KAP1 and SMC1 proteins. [Flu+Bu] increased the phosphorylation of histone 2 AX (γ -H2AX) relative to the control or to each individual drug, and this effect further increased in the presence of Clad or Clo (Fig. 2A). Phosphorylations of CHK2 at Ser432 and SMC1 at Ser957 both increased with exposure to triple-drug combinations. The level of P-KAP1 (Ser824) increased in the presence of Clo alone, [Flu+Bu], [Clad+Flu+Bu] and [Clo+Flu+Bu], but not after Flu alone or Clad alone (Fig. 2A). The observed phosphorylation of KAP1 at Ser824 in the presence of Clo alone, but not Clad, suggests inherent differences in their damage-signaling responses that presumably contribute to their cytotoxic activity. Overall, the results suggest activation of the ATM-mediated DNA-damage response pathway when Clad or Clo is combined with Flu and Bu.

Since cell proliferation was markedly inhibited in the presence of [Clad+Flu+Bu] or [Clo+Flu+Bu] (Fig. 1A) and the DNA-damage response was clearly activated (Fig. 2A), we investigated if activation of programmed cell death or apoptosis was a contributory factor in the response to these agents. Cleavages of PARP1 and caspase 3 are commonly-used indicators of apoptosis [Bouchard et al., 2003; Cohen, 1997]. Fig. 2B shows that exposure of KBM3/Bu250⁶ cells to either [Clad+Flu+Bu] or [Clo+Flu+Bu] resulted in a marked increase in the cleavage of both PARP1 and caspase 3, suggesting activation of apoptosis. This observation was further supported by a decrease in the level of pro-survival proteins XIAP and c-MYC in cells exposed to triple-drug combinations (Fig. 2B). Similar activation of

apoptosis was observed in the other AML cell lines. Cleavage of PARP1 and caspase 3 proteins increased in HL60 and OCI-AML3 cells exposed to [Clad+Flu+Bu] or to [Clo+Flu+Bu], and the levels of XIAP and c-MYC proteins significantly decreased (Figs. 2C and 2D). Moreover, phosphorylation of histone 2AX (γ -H2AX) increased when HL60 and OCI-AML3 cells were exposed to the triple-drug combinations, consistent with activation of the ATM pathway and DNA-damage response. These results suggest activation of the ATM and apoptosis pathways and DNA-damage response in all three AML cell lines, KBM3/Bu250⁶, HL60 and OCI-AML3, when exposed to [Clad+Flu+Bu] or [Clo+Flu+Bu].

To further define the involvement of caspases in the drug-mediated cell death, OCI-AML3 cells were pre-exposed to the pan-caspase inhibitor Z-VAD-FMK for 1 hour prior to a 48-hour triple-drug exposure. Subsequent Western blot analysis showed inhibition of cleavage of PARP1, caspase 3 and caspase 8, and of phosphorylation of histone 2 AX in Z-VAD-FMK -treated cells (Fig. 2E). Drug-mediated inhibition of cell proliferation was also alleviated when cells were pre-exposed to 30 μ M Z-VAD-FMK (data not shown). The results suggest a major role of caspases in the cytotoxicity of [Clad/Clo+Flu+Bu] in OCI-AML3 cells.

[Clad/Clo+Flu+Bu] combination decreases mitochondrial membrane potential and causes leakage of pro-apoptotic proteins

The role of mitochondria in the induction of apoptosis is well established [Petit et al., 1996]. It is known that leakage of mitochondrial pro-apoptotic proteins into the cytoplasm activates the caspase-mediated cascade of events leading to DNA fragmentation and cell death (Green and Reed, 1999). To confirm if this mechanism contributes to [Clad+Flu+Bu]-mediated cytotoxicity in AML cells, we examined the decrease in the MMP using the JC-1 assay as described under Methods. The compound JC-1 forms aggregates in the mitochondria and dissociates to a corresponding monomeric form in the cytoplasm. The associated colorimetric changes provide an efficient assay using flow cytometry-based evaluation. KBM3/Bu250⁶ cells were exposed to drugs, individually or in various combinations, and mixed with JC-1 reagent. Flow cytometric analysis indicated the relative abundance of JC-1 monomer and aggregate forms using 1 μ M valinomycin as a positive control. Whereas cells exposed to Bu, Flu, Clad, Clo or [Flu+Bu] exhibited mostly JC-1 aggregates (~84% or greater), exposure to [Clad+Flu+Bu] or [Clo+Flu+Bu] resulted in JC-1 dissociation to ~70% and ~58% monomer, respectively (Fig. 3A), suggesting leakage of JC-1 from the mitochondria into the cytoplasm, possibly due to decreased MMP.

To confirm mitochondrial leakage, we examined the change in the level of cytochrome *c* in the cytoplasm. Exposure of KBM3/Bu250⁶ cells to [Flu+Bu] did not significantly change the level of cytochrome *c* in the cytoplasm; however, addition of Clad or Clo to this combination increased the level of this protein in the cytoplasm (Fig. 3B), suggesting possible leakage. Cytochrome *c* is known to form a complex with Apaf-1 and caspase 9 resulting in caspase activation [Gross et al., 1999]. A key cytoplasmic protein involved in triggering the release of cytochrome *c* is BID. Upon exposure of cells to cytotoxic mediators, BID is cleaved and the carboxyl terminal p15 (tBID) translocates to the mitochondrial outer membrane and causes release of cytochrome *c* [Luo et al., 1998].

Exposure of KBM3/Bu250⁶ cells to [Clad+Flu+Bu] or [Clo+Flu+Bu] increased the level of tBID in the cytoplasm (Fig. 3B), representing a possible contributory factor in the observed decrease in MMP (Fig. 3A) and release of cytochrome *c* (Fig. 3B).

A pro-apoptotic protein normally localized to the mitochondria and released in response to apoptotic stimuli is the Apoptosis-inducing factor, AIF [Susin et al., 1999]. Exposure of KBM3/Bu250⁶ cells to [Clad+Flu+Bu] or [Clo+Flu+Bu] resulted in an increased level of AIF in the cytoplasm (Fig. 3B), suggesting its release from the mitochondria.

An anti-apoptotic protein that interacts with and antagonizes pro-apoptotic proteins is MCL-1 [Sato et al., 1994]. Its level is regulated post-translationally by cleavage near its amino-terminus [Yang et al., 1995]. We, therefore, examined the extent of cleavage of MCL-1 in the presence of [Clad+Flu+Bu] or [Clo+Flu+Bu]. Fig. 3B shows an increase in the level of cleaved MCL-1 in the presence of [Flu+Bu], which was further increased when either Clad or Clo was added into this combination.

These collective results suggest that the cytotoxicity of the [Clad+Flu+Bu] and [Clo+Flu+Bu] combinations may be partly attributed to a decrease in MMP which causes the release of pro-apoptotic factors from the mitochondria to the cytoplasm that triggers activation of caspases. This mechanism is further enhanced by decreasing the levels of anti-apoptotic proteins such as MCL-1, XIAP, and c-MYC.

[Clad/Clo+Flu+Bu] combination increases post-translational modifications of histones

The synergistic cytotoxicity of nucleoside analogs and DNA-alkylating agents may be partly explained by the NA-mediated changes in chromatin structure, which results in greater exposure of genomic DNA to alkylating agents, provided that the drugs are added in optimum sequence; the target cell population has to be exposed to the NA(s) prior to the DNA-alkylating agent-exposure [Yamauchi et al., 2001; Valdez and Andersson, 2010; Valdez et al., 2011]. Histone modifications are known to effect chromatin remodeling to facilitate transcription, DNA replication and repair of damaged DNA [Price and D'Andrea, 2013; Swygert and Peterson, 2014]. We therefore examined relative changes in the status of acetylated and methylated histones 3 and 4, as well as possible changes in the level of enzymes involved in these post-translational modifications. Both acetylation and tri-methylation of histone 3 at Lys 4 markedly increased in cells exposed to [Clad+Flu+Bu] or [Clo+Flu+Bu], while acetylations of histone 3 at Lys 9 and Lys 18 and histone 4 at Lys 5 did not significantly change, suggesting lysine site-specificity (Fig. 4A). The levels of histone deacetylases HDAC3, HDAC4, HDAC5 and SirT7 correspondingly decreased in the presence of triple-drug combinations, suggesting a correlation with increased levels of histone 3 acetylation (Figs. 4A and 4B). The levels of HDAC1 and HDAC2 proteins did not significantly change in KBM3/Bu250⁶ cells exposed to [Clad+Flu+Bu] or [Clo+Flu+Bu], also suggesting specificity for this effect. Similar results were obtained in HL60 and OCI-AML3 cells (Figs. 4C and 4D).

[Clad/Clo+Flu+Bu] combination activates the stress signaling pathway

Combination of nucleoside analogs and the synthetic alkyl-lysophospholipid anticancer drug edelfosine decreases the phosphorylation of AKT and activates the stress signaling pathway through phosphorylation of SAPK/JNK in multiple myeloma cells [Valdez et al., 2014]. We therefore examined if the combinations of [Clad/Clo+Flu+Bu] would have similar effects in AML cells. Exposure of KBM3/Bu250⁶ cells to [Clad+Flu+Bu] or [Clo+Flu+Bu] resulted in decreased levels of p-AKT (Ser473), suggesting that the AKT-mediated survival pathway was compromised (Fig. 5). On the other hand, phosphorylation of SAPK/JNK at Thr183/Tyr185 was increased in the presence of the triple-drug combinations. This finding correlates with an increase in the level of phosphorylation of ATF-2 (a known substrate for the SAPK/JNK kinase) at Thr 71 (Fig. 5). Taken together, our results suggest down-regulation of the pro-survival AKT pathway and up-regulation of the stress signaling pathway by the triple-drug combinations.

[Clad/Clo+Flu+Bu] combination activates apoptosis in primary cell samples from AML patients

To confirm a potential clinical relevance of the observed synergism of [Clad/Clo+Flu+Bu] in AML cell lines, we exposed mononuclear cells isolated from AML patients to these drugs, individually or in the combinations investigated above. The demographic characteristics of the patients from whom samples were isolated are shown in Table 3 (Supplemental Materials). We then examined the changes in cleavage of PARP1 and caspase 3, known indicators of apoptosis activation, as well as the level of γ -H2AX, an indicator of the DNA-damage response. In all four patient samples examined, the cleavage of PARP1 and caspase 3, as well as phosphorylation of histone 2AX (γ -H2AX), increased in the presence of [Clad+Flu+Bu] or [Clo+Flu+Bu] (Fig. 6), suggesting activation of a DNA-damage response and programmed cell death, analogous to that observed in the cell lines.

A histone deacetylase inhibitor and a DNA demethylating agent enhance the cytotoxicity of [Clad/Clo+Flu+Bu] in AML cell lines

If epigenetic changes are involved in the efficacy of the respective [Clad/Clo+Flu+Bu] combination, as suggested by increased acetylation and methylation of histone 3 (Fig. 4), we hypothesized that increasing the level of acetylation of histones and decreasing the methylation of genomic DNA at the same time would further enhance their cytotoxicity. To this effect, we analyzed the OCI-AML3 cell line, which has wild-type/functional p53. Cells were exposed to lower concentrations of Clad, Clo, Flu and Bu than those used in the previous experiments (Figs. 1, 2 and 4) in the absence or presence of panobinostat (Pano), a pan-HDAC inhibitor, and/or 5-aza-2'-deoxycytidine (DAC), a DNA hypomethylating agent. Exposure of OCI-AML3 cells to [1.5 μ M Flu+32 μ M Bu] in the absence or presence of 7 nM Clad or 13 nM Clo had only a modest effect on cell proliferation (~ 5% inhibition); 35 nM DAC and 10 nM Pano had a similar lack of effect (Fig. 7A). Addition of DAC to [Clad +Flu+Bu] or [Clo+Flu+Bu] did not change the cell proliferation profile, but addition of Pano to either three-drug combination decreased cell proliferation to 58% – 63%. Addition of both DAC and Pano to [Clad+Flu+Bu] or [Clo+Flu+Bu] further decreased cell proliferation

to ~42% and ~51%, respectively. These results show that the cytotoxicity of [Clad/Clo+Flu+Bu] may be further enhanced by addition of epigenetic agents such as DAC and Pano.

To determine if the inhibition of cell proliferation seen in Fig. 7A correlates with activation of apoptosis, we examined the cleavage of PARP1 and caspase 3, and the level of γ -H2AX, in OCI-AML3 cells exposed to drugs, individually or in various combinations. [Clad/Clo+Flu+Bu+DAC] exposure resulted in negligible to weak cleavage of PARP1 and caspase 3 and a moderate increase in the phosphorylation of histone 2AX (Fig. 7B). On the other hand, [Clad/Clo+Flu+Bu+Pano] exposure resulted in a marked increase in these molecular readouts; inclusion of DAC in these four-drug combinations further moderately increased the level of these protein modifications (Fig. 7B). These results correlate with the inhibition of cell proliferation shown in Fig. 7A.

We next determined possible mechanisms by which Pano and DAC enhance the cytotoxicity of [Clad/Clo+Flu+Bu]. The activity of Pano was examined by determining its effects on the acetylation status of histone 3. While 10 nM Pano exposure by itself resulted in negligible increase in acetylation of histone 3 at Lys 4, Lys 9 and Lys 27, the combinations of [Clad/Clo+Flu+Bu+Pano] and [Clad/Clo+Flu+Bu+DAC+Pano] dramatically increased histone 3 acetylations (Fig. 7B). These results correlate with a decrease in histone deacetylases HDAC3, HDAC4, HDAC5 and SirT7 (Fig. 7B).

Phosphorylation of HDACs is known to facilitate their interactions with 14-3-3 proteins, which results in HDAC translocation to the cytoplasm and inhibition of their ability to deacetylate histones in the nucleus [Grozinger and Schreiber, 2000]. We, therefore, examined the phosphorylation status of HDACs in cells exposed to drugs. Fig. 7B shows increased phosphorylations of HDAC5 and HDAC7 at Ser259 and Ser155, respectively, in cells exposed to [Clad/Clo+Flu+Bu+Pano] and [Clad/Clo+Flu+Bu+Pano+DAC]. Taken together, the increase in the levels of acetylated histone 3 at Lys 4, Lys 9 and Lys 27 correlates with decreased levels of HDAC3, HDAC4 and HDAC5 and increased phosphorylation of HDAC5 and HDAC7.

Histone deacetylases are known to functionally interact with histone demethylases [Hayakawa and Nakayama, 2011]. With the observed changes in histone acetylations, we determined if the level of methylated histones would also change. A corresponding increase in the levels of dimethylated histone 3 at Lys 4 and Lys 9 was observed in cells exposed to [Clad/Clo+Flu+Bu+Pano] and [Clad/Clo+Flu+Bu+DAC+Pano] and the levels of histone demethylases JMJD1B, JMJD2A and JMJD2B were correspondingly decreased while the level of the histone methyltransferase SET8 was increased (Fig. 7C).

DAC is known to inhibit DNA methylation by triggering the degradation of DNA methyltransferases [Santi et al., 1984]. Examination of the level of DNA methyltransferase 1 (DNMT1) in cells exposed to DAC, alone or in combination with other drugs, showed a decrease in its protein level. DAC (35 nM) decreased DNMT1 by ~50%; addition of DAC to [Clad/Clo+Flu+Bu] further decreased its level and [Clad/Clo+Flu+Bu+DAC+Pano] almost eliminated the expression of DNMT1 (Fig. 8A). The observed down-regulation of DNMT1 was expected to increase the expression of genes that are known to be epigenetically

silenced due to hypermethylation of their promoters. In fact, increased expression of cell-cycle related proteins P16/INK4a, P15/INK4b and P21/Waf1/Cip1, and pro-apoptotic BNIP3, was observed in cells exposed to [Clad/Clo+Flu+Bu+DAC+Pano], correlating with the decreased expression of DNMT1 (Fig. 8A). The observed increase in the levels of these proteins in cells exposed to [Clad/Clo+Flu+Bu+Pano], in spite of the absence of DAC, suggests an involvement of Panobinostat-mediated mechanisms in this effect. Panobinostat is also known to alter the expression of cell cycle-related proteins [Xie et al., 2013]. The known cyclin-dependent kinase inhibitors P15/INK4b, P16/INK4a and P21/Waf1/Cip1 are involved in cell-cycle regulation and may be involved in the observed inhibition of cell proliferation, shown in Fig. 7A.

To ascertain whether the observed drug-mediated increase in the level of P16/INK4a protein (Fig. 8A) did indeed involve DNA demethylation, we examined changes in the methylation status of the *P16/INK4a* gene promoter. Methylation-specific PCR analysis showed 2.5- and 4-fold increases in the level of unmethylated promoter DNA in the presence of [Clad+Flu+Bu+DAC+Pano] and [Clo+Flu+Bu+DAC+Pano], respectively (Fig. 8B, left panel). These changes in methylation status correlated with a significant increase in the expression of *P16/INK4a* mRNA as measured by RT-PCR analysis (Fig. 8B, right panel).

The observed efficacy of Clad/Clo and DAC, in the presence of [Flu+Bu+Pano], may be partly attributed to the previously reported DNA demethylating activities of Clad [Spurgeon et al., 2009] and Clo [Zhang et al., 2009]; it is possible that their combinations would have synergistic DNA demethylation effects. Therefore, we were prompted to examine the DNA-demethylating activities of these two adenosine analogs. Exposure of OCI-AML3 cells to increasing concentrations of the nucleoside analogs showed a corresponding decrease in the level of DNMT1 and a dose-dependent increase in the level of P15/INK4b, P16/INK4a and P21/Waf1/Cip1 proteins (Fig. 8C). At equimolar concentration (40 nM), Clad exposure resulted in a greater than 50% decrease in the level of DNMT1 protein and increased the levels of P15/INK4b, P16/INK4a and P21/Waf1/Cip1 while Clo exposure showed negligible effects (Fig. 8C, left panel). Equitoxic concentrations of these adenosine analogs (50 nM Clad, 80 nM Clo) exerted similar effects on these molecular responses (decreased levels of DNMT1, increased levels of P15/INK4b, P16/INK4a and P21/Waf1/Cip1). These results are consistent with the observed dose-dependent increase in the DNA demethylation of the *P16/INK4A* gene promoter and its expression as determined by real-time PCR (Fig. 8C, right panels). Overall, these results suggest that both Clad and Clo possess DNA demethylating activities, similar to DAC, in OCI-AML3 cells.

Effects of [Clad/Clo+Flu+Bu+DAC+Pano] combinations on the Wnt/ β -catenin pathway

The Wnt/ β -catenin survival pathway is constitutively activated in some hematological malignancies [Lento et al., 2013]. It is partly regulated by its antagonists whose expression depends on the methylation status of their gene promoters [Varol et al., 2014]. We, therefore, examined whether the Wnt/ β -catenin pathway is inhibited in OCI-AML3 cells exposed to [Clad/Clo+Flu+Bu+Pano $\pm$ DAC]. Fig. 9A shows a dramatic decrease in the level of β -catenin protein in cells exposed to the triple-drug combination plus Pano, with or without DAC. This result correlates with a decrease in the expression of the β -catenin-target

genes including *c-MYC*, *MET* and *SURVIVIN* [Kawano and Kypka, 2003] as shown by a decrease in their protein levels in cells exposed to [Clad/Clo+Flu+Bu+DAC+Pano] (Fig. 9A). Concomitantly, we observed an increase in the level of SFRP1 protein (Fig. 9A); SFRP1 is a known antagonist of the Wnt/ β -catenin pathway which causes β -catenin degradation [Arend et al., 2013].

SFRP1 gene expression is known to be regulated by the methylation status of its promoter; demethylation increases its expression [Arend et al., 2013]. To determine if the [Clad/Clo+Flu+Bu+DAC+Pano] combinations decreased the methylation of *SFRP1* and other genes that encode Wnt/ β -catenin antagonists (*DKK3* and *WIF1*), methylation-specific PCR analysis was performed. Fig. 9B shows significant drug-mediated increases in the demethylated status of the promoters of the *SFRP1*, *DKK3* and *WIF1* genes with a corresponding increase in their expression (Fig. 9C; $P < 0.05$). These results suggest that the cytotoxicity of the [Clad/Clo+Flu+Bu+DAC+Pano] combinations is partly due to inhibition of the pro-survival Wnt/ β -catenin signal transduction pathway through epigenetic up-regulation of its antagonists.

Discussion

The safety and efficacy of a double-nucleoside-analog/DNA-alkylating agent combination [Clo+Flu+Bu] as a pretransplant conditioning regimen in patients with myeloid leukemia has been established [Andersson et al., 2011]. Our present study provides *in vitro* evidence, using both AML cell lines and primary patient-derived cell samples, that Clad may be used as a practical and cost-effective alternative to Clo in such a double NA/DNA-alkylating agent-based combination. A [Clad+Flu+Bu] combination may, in terms of synergistic cytotoxicity, be clinically comparable with [Clo+Flu+Bu] but superior in terms of the significantly reduced cost. The molecular mechanisms for the anti-neoplastic activity of [Clad+Flu+Bu] include activation of the DNA-damage response through the ATM pathway, apoptosis, histone modifications, and up-regulation of the stress signaling pathway. Pro-survival and anti-apoptotic pathways are also compromised, as exemplified by a decrease in the level of *c-MYC* and decreased phosphorylation of AKT. The complex interactions of the various pathways affected by the [Clad+Flu+Bu] combination are illustrated in Fig. 10.

The synergistic cytotoxicity of [Clad+Flu+Bu] may be partly explained by the ability of the nucleoside analogs Clad and Flu to induce chromatin remodeling through histone modifications. Such unraveling of chromatin more effectively exposes the genomic DNA to Bu-mediated alkylation, which results in DNA crosslinking that stalls DNA replication. Attempts to restore the integrity of damaged DNA would be impaired due to NA-mediated inhibition of DNA repair [Yamauchi et al., 2001] and a resulting increase in DNA strand breaks would activate the DNA-damage response, resulting in cell death (Fig. 10). If, as we propose, histone modifications enhance Bu-mediated alkylation, then a further increase in histone acetylations (which should further relax the chromatin) should augment cell death. In fact, addition of an HDAC inhibitor, panobinostat, to [Clad+Flu+Bu] further potentiated the cytotoxicity of the combined NAs and DNA-alkylating agent (Fig. 7). This activity may also depend on changes in global gene expression involving up-regulation of tumor suppressor genes and/or abrogation of cell-cycle checkpoints, ultimately resulting in

apoptosis. Xie et al. (2013) reported that panobinostat mediated the down-regulation of the transcription factor E2F, resulting in suppression of cell cycle-related proteins, which is consistent with our observations.

As an HDAC inhibitor, panobinostat may inhibit DNA repair, cause DNA damage and increase reactive oxygen species (ROS) production [Robert and Rassool, 2012]. These processes decrease MMP and result in leakage of pro-apoptotic factors into the cytoplasm (Fig. 10). Although these HDAC inhibitor-mediated effects may in themselves not be as pronounced as those elicited by [Clad/Clo+Flu+Bu], the combination of the HDAC inhibitor with [Clad/Clo+Flu+Bu] may still synergistically exacerbate mitochondrial damage and commit cells to apoptosis.

DAC is another drug with a different mechanism of action but with pharmacological effects that partly overlap with [Clad/Clo+Flu+Bu]. It is a DNA demethylating agent which inhibits DNA methyltransferases. It causes DNA damage by trapping DNMTs on DNA and, like other nucleoside analogs, it incorporates into growing DNA strands during replication [Covey et al., 1986]. DAC increases ROS production and decreases MMP [Fandy et al., 2014], resulting in mitochondria-mediated apoptosis [Ruiz-Magana et al., 2012]. It also regulates the expression of genes involved in cell-cycle checkpoint control and apoptosis in AML cells [Valdez et al., 2010]. All of these DAC-mediated effects could be enhanced by combining DAC with [Clad/Clo+Flu+Bu+Pano]. In fact, increased down-regulation of DNMT1 and up-regulation of cell-cycle checkpoint proteins were observed in cells exposed to [Clad/Clo+Flu+Bu+DAC+Pano] when compared with DAC alone (Fig. 8). The up-regulation of pro-apoptotic BNIP3 and down-regulation of pro-survival SURVIVIN (Figs. 8 and 9) in cells exposed to [Clad/Clo+Flu+Bu+Pano+DAC] may also contribute to the observed increase in cytotoxicity. Another likely contributing factor to the observed synergism of [Clad/Clo+Flu+Bu+Pano+DAC] is the demethylation-mediated up-regulation of Wnt/ β -catenin antagonists. Such events would result in β -catenin degradation and down-regulation of its pro-survival target genes (Fig. 9A). This mechanism is consistent with the observed increase in the unmethylated forms of the *SFRP1*, *DKK3* and *WIF1* Wnt/ β -catenin antagonist genes in cells exposed to this drug combination, which correlates with their increased expression (Figs. 9B and 9C).

These overlapping pharmacological effects on various signal transduction pathways caused by Clad/Clo, Flu, Bu, DAC and Pano, as illustrated in Fig. 10, may largely explain their synergistic cytotoxicity in OCI-AML3 cells and potentially in other AML cell lines and primary cell populations. The cell injuries caused by one drug are probably exacerbated by the presence of the other drug(s), and a massive increase in these injuries commits cells to apoptosis. Activation of the stress signaling pathway (Fig. 5) and inhibition of the pro-survival AKT and Wnt/ β -catenin signal transduction pathways appear to contribute further to the observed drug synergism.

Although Clo appears to be more potent than Clad as a single agent in KBM3/Bu250⁶ and HL60 cell lines, Clad seems to provide enhanced cytotoxicity compared with Clo when combined with [Flu+Bu] in some primary patient cell samples. Using equimolar concentrations of Clad and Clo, stronger activation of the DNA-damage response, as

indicated by phosphorylation of histone 2AX, and increased activation of apoptosis, as indicated by cleavage of Caspase 3, was observed with Clad-containing combinations (patients 1, 3 and 4 in Fig. 6). Moreover, Clad has more potent demethylating activity than Clo in OCI-AML3 cells (Fig. 8C). Whether cells expressing wild-type P53, such as OCI-AML3 cells, are more sensitive to Clad than cells harboring mutant P53 remains to be determined.

It should be noted that our *in vitro* studies involved simultaneous exposure of cells to various drugs. Sequential drug exposure may further reflect its clinical significance; the *in vitro* results, however, will have their own limitation as far as optimal timing of drug exposures in relation to their individual pharmacokinetics is concerned and may not necessarily be extrapolated to clinical settings. Nevertheless, our study provides a strong basis for considering Clad as a potential alternative to Clo to reduce cost and for enhancing efficacy with the addition of epigenetic modifiers in the pre-transplant conditioning regimen in future clinical trials in patients with myeloid leukemia. In a more general setting, the described drug combinations may be used to enhance conventional salvage regimens for patients with chemotherapy refractory acute leukemia.

Acknowledgment

This work was supported in part by a grant from the National Institutes of Health (CCSG Core CA16672), and the Stephen L. and Lavinia Boyd Fund for Leukemia Research, and by funds donated by grateful patients.

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Highlights

- Cladribine, fludarabine and busulfan are synergistic in AML cell lines and patient-derived leukemic cell samples.
- The less expensive cladribine is as efficacious as clofarabine when combined with fludarabine and busulfan in AML cells.
- Panobinostat and DAC enhance the efficacy of Clad/Clo+Flu+Bu in AML cells.
- DNA-damage response, apoptosis and Wnt/ β -catenin down-regulation are involved.

Figure 1.

Synergistic cytotoxicity of nucleoside analogs and the DNA-alkylating agent busulfan in AML cell lines. KBM3/Bu250⁶ (A), HL60 (B) and OCI-AML3 (C) cells were continuously exposed to drugs alone, or in various combinations (i.e., increasing drug concentrations but maintaining a constant ratio) for 48 hours and analyzed by the MTT assay for cell proliferation and by the Annexin V (Ann V) assay for early cell death. (D) KBM3/Bu250⁶ cells were exposed to increasing concentrations of the drugs (constant ratio) for 48 h and inhibition of proliferation (“fraction affected”) was determined. Synergism of cytotoxicity of the indicated drugs was determined by calculating combination indexes (CI) according to the method of Chou and Talalay (1984). The results are the average of at least three independent experiments. Bu, busulfan; Flu, fludarabine; Clad, cladribine; Clo, clofarabine

(A) KBM3/Bu250⁶ – ATM activation **(B) KBM3/Bu250⁶ - Apoptosis** **(C) HL60 - Apoptosis** **(D) OCI-AML3 - Apoptosis** **(E) OCI-AML3 – Caspase inhibition** **Figure 2.**

Activation of the ATM and apoptosis pathways in AML cells continuously exposed to drugs alone, or in various combinations, for 48 hours. Cell lysates from the indicated cells were analyzed by Western blot for changes in the level of expression, cleavage or phosphorylation of proteins involved in these pathways. Bu, busulfan; Flu, fludarabine; Clad, cladribine; Clo, clofarabine

(A) Decrease in mitochondrial membrane potential

(B) Protein translocation to cytoplasm

Figure 3.

Drug-induced effects on mitochondrial membrane potential. KBM3/Bu250⁶ cells were exposed to drugs for 48 hours and analyzed using (A) the JC-1 assay and flow cytometry, and (B) Western blot of the cytosolic fractions for levels/cleavage of proteins involved in apoptosis. The nature of the middle band recognized by the anti-MCL-1 antibody is unknown. The results are the average (A) or representatives (B) of at least three independent experiments. Bu, busulfan; Flu, fludarabine; Clad, cladribine; Clo, clofarabine

(A) KBM3/Bu250⁶ – Histone modifications **(B) KBM3/Bu250⁶ – Histone deacetylases** **(C) HL60 – Histone modifications and HDACs** **(D) OCI-AML3 – Histone modifications and HDACs** **Figure 4.**

Drug-mediated changes in the status of histone modifications and levels of histone modifying enzymes. Cells were continuously exposed to drugs alone, or in different combinations, for 48 hours and total cell fractions were analyzed by Western blot. Bu, busulfan; Flu, fludarabine; Clad, cladribine; Clo, clofarabine

Figure 5.

Inhibition of the AKT pro-survival pathway and activation of the JNK-ATF2 stress signaling pathway by [Clad+Flu+Bu] and [Clo+Flu+Bu]. KBM3/Bu250⁶ cells were continuously exposed to drugs for 48 hours and total cell lysates were analyzed by Western blot. Bu, busulfan; Flu, fludarabine; Clad, cladribine; Clo, clofarabine

Figure 6.

Analysis of mononuclear cells from peripheral blood of AML patients. Cells were isolated, exposed to the indicated concentrations of the drugs for 48 hours, and analyzed for cleavage of PARP1 and caspase 3 and phosphorylation of histone 2AX using Western blot. Bu, busulfan; Flu, fludarabine; Clad, cladribine; Clo, clofarabine

Figure 7. Effects of epigenetic modifying agents on the cytotoxicity of [Clad/Clo+Flu+Bu] and on histone modifications and modifying enzymes. OCI-AML3 cells were exposed to low concentrations of Clad, Clo, Flu and Bu alone, or in combinations, in the absence or presence of DAC and Pano. After 48 hours of drug exposure, cell proliferation (A) was determined by the MTT assay (average of four independent experiments) and total cell lysates were analyzed by Western blot for their levels and/or post-translational modification of various key proteins (B and C). Bu, busulfan; Flu, fludarabine; Clad, cladribine; Clo, clofarabine; DAC, 5-aza-2'-deoxycytidine; Pano, panobinostat

(A) DNMT1 down-regulation **(B)** **(C) Clad and Clo as demethylating agents** **Figure 8.**

Effects of [Clad/Clo+Flu+Bu+DAC+Pano] combinations on the expression of DNMT1 and of known methylation-regulated genes. (A) OCI-AML3 cells were exposed to drugs for 48 hours and analyzed by Western blot. (B) Real-time PCR was used to determine the methylation status of the *P16* gene promoter and expression of the *P16* gene. (C) To determine if Clad and Clo themselves have DNA demethylating activities, OCI-AML3 cells were exposed to different concentrations of Clad or Clo for 48 hours and analyzed by Western blot (left panel) and real-time PCR (right panels). Bu, busulfan; Flu, fludarabine; Clad, cladribine; Clo, clofarabine; DAC, 5-aza-2'-deoxycytidine; Pano, panobinostat

Figure 9. [Clad/Clo+Flu+Bu+DAC+Pano] combination inhibits the Wnt/ β -catenin pathway via up-regulation of its antagonists. OCI-AML3 cells were exposed to drugs for 48 hours and analyzed by Western blot (A) and real-time PCR (B and C). The two asterisks indicate a statistically significant difference ($P < 0.05$) when individual treatments in each group were compared with the corresponding controls. Bu, busulfan; Flu, fludarabine; Clad, cladribine; Clo, clofarabine; DAC, 5-aza-2'-deoxycytidine; Pano, panobinostat

Figure 10.

Proposed mechanisms underlying the synergistic cytotoxicity of [Clad/Clo+Flu+Bu+Pano +DAC] in myeloid leukemia cells. Arrows represent activation and barred lines represent inhibition. Bolder lines suggest stronger effects than thinner lines. The model is described under “Discussion”.

Table 1

List of primary antibodies, their sources and dilutions

Antigen	Source/Cat. #	Clone type*	Dilution**
AcH3K4	Active Motif/39381	pAb	2500
AcH4K5	Active Motif/39170	pAb	3000
AcH3K9	Active Motif/39138	pAb	2500
AcH3K18	Active Motif/39694	pAb	3000
AcH3K27	Active Motif/39134	pAb	3000
AIF	Cell Signaling/5318	mAb - R	2500
AKT	Santa Cruz Biotech/8312	pAb	1000
ATF2	Cell Signaling/9226	mAb - R	2500
ATM	Santa Cruz Biotech/23921	mAb	750
BID	Cell Signaling/2002	pAb	2500
BNIP3	Cell Signaling/3769	pAb	2000
Caspase 8	Cell Signaling/9746	mAb	2000
CHK2	Cell Signaling/2662	pAb	2500
Cleaved Caspase 3	Cell Signaling/9661	pAb	2500
c-MYC	Cell Signaling/9402	pAb	2000
Cytochrome c	BD Biosciences/556433	mAb	2000
DNMT1	Santa Cruz Biotech/10222	pAb - G	1000
HDAC1	Cell Signaling/5356	mAb	2500
HDAC2	Cell Signaling/5113	mAb	3000
HDAC3	Cell Signaling/3949	mAb	2500
HDAC4	Cell Signaling/5392	mAb	2500
HDAC5	Cell Signaling/2082	pAb	2500
JMJD1B	Cell Signaling/2621	pAb	3000
JMJD2A	Cell Signaling/5328	pAb	1500
JMJD2B	Cell Signaling/8639	pAb	2500
KAP1	Bethyl Laboratories A300-274A	pAb	3000
MCL-1	Santa Cruz Biotech/819	pAb	1000
MET	Cell Signaling/8198	mAb	2000
PARP1	Santa Cruz Biotech/8007	mAb	1000
P-AKT (Ser473)	Cell Signaling/4060	mAb - R	2500
P-ATF2 (Thr71)	Cell Signaling/5112	mAb - R	2500
P-ATM (Ser1981)	Rockland/200-301-400	mAb	2000
P-CHK2 (Ser432)	Cell Signaling/2667	pAb	2500
P-HDAC4/P-HDAC5/P-HDAC7	Cell Signaling/3443	mAb - R	2000
P-KAP1 (Ser824)	Bethyl Laboratories A300-767A	pAb	1500
P-SAPK/JNK (Thr183/Tyr185)	Cell Signaling/4668	pAb	2000

Antigen	Source/Cat. #	Clone type*	Dilution**
P-SMC1 (Ser957)	Novus Biolog/NB100-205	pAb	2000
P15/INK4b	Santa Cruz Biotech/612	pAb	700
P16/INK4a	BD Biosciences/554079	mAb	800
P21/Waf1/Cip1	Abcam/7960	pAb	1000
SAPK/JNK	Cell Signaling/9258	pAb	2500
SET8	Cell Signaling/2996	pAb	2000
SFRP1	Cell Signaling/3534	mAb	1500
SirT7	Cell Signaling/5360	pAb	2500
SMC1	Cell Signaling/4802	pAb	2500
SURVIVIN	Cell Signaling/2802	pAb	1000
XIAP	Cell Signaling/2045	mAb	2000
p-ACTIN	Sigma/A5316	mAb	6000
p-CATENIN	Cell Signaling/8480	mAb	2000
Y-H2AX	EMD Millipore/05-636	mAb	3000
2MeH3K4	Active Motif/39679	mAb	2500
3MeH3K4	Active Motif/39159	pAb	3000
2MeH3K9	Active Motif/39683	mAb	3000

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

** Fold dilution in PBS with 0.05% Tween 20

Table 2

Primers for real-time PCR analysis

Gene	Forward	Reverse	Annealing Temp (°C)
A)MSP			
<i>P16</i> (methylated)	GGGGGAGACCCAACCTGG	CCCTCCTCTTTCTTCCTC	60
<i>P16</i> (unmethylated)	GGGGGAGATTTAATTGG	CCCTCCTCTTTCTTCCTC	60
<i>SFRP1</i> (methylated)	TGTAGTTTTTCGGAGTTAGTGTGCGC	CCTACGATCGAAAACGACGCGAACG	63
<i>SFRP1</i> (unmethylated)	GTTTTGTAGTTTTTGGAGTTAGTGTGTGT	CTCAACCTACAATCAAAAACAACACAAACA	63
<i>DKK3</i> (methylated)	CGGTTTTTTTTTCGTTTTTCGGG	CAAACCGCTACATCTCCGCT	54
<i>DKK3</i> (unmethylated)	TTTTGGTTTTTTTTTGTTTTTGGG	CCAAACCACTACATCTCCACT	54
<i>WIF1</i> (methylated)	GGGCGTTTTATTGGGCGTAT	AAACCAACAATCAACGAAAC	58
<i>WIF1</i> (unmethylated)	GGGTGTTTTATTGGGTGTAT	AAACCAACAATCAACAAAAC	58
B) Gene expression			
<i>GAPDH</i>	CAACAGCCTCAAGATCATCAGC	TCCTAGACGGCAGGTCAGGTC	55
<i>P16</i>	CCCAACGCACCGAATAGTTAC	CGCTGCCCATCATCATGAC	55
<i>SFRP1</i>	GCTGGAGCACGAGAC	TGGCAGTTCTTGTGAGCA	55
<i>DKK3</i>	TGGGAGGGGAAGAGATTTAGA	GCACACACCTGGGGAAATAA	55
<i>WIF1</i>	CCAGGGAGACCTCTGTTCAA	TTGGGTTCATGGCAGGTT	55

Table 3

Characteristics of leukemia patients whose cell samples were used in the study

Patient #	Source	Age	Gender	Diagnosis	Blast (%)	Monocyte (%)	Cytogenetics	Medications at the time of sampling
1	PB	47	F	Refractory AML	96	2	<i>JAK2</i> mutation	Decitabine, cytarabine and hydroxyurea
				with CNS involvement			NM_004972.3:c.3323A>G p.N1108S	
							<i>KIT</i> mutation	
							NM_000222.2:c.1621A>C p.M541L	
							<i>MPL</i> mutation	
							NM_005373.2:c.340G>A p.V114M	
							<i>NOTCH1</i> mutation	
							NM_017617.3:c.6853G>A p.V2285I	
2	PB	55	M	Acute biphenotypic leukemia	81	1	<i>BCR-ABL1</i> fusion t(9;22)(q34;q11.2)	Ciprofloxacin and valacyclovir
				with CNS involvement				
							<i>ABL1</i> mutation	Allopurinol and hydroxyurea
							NM_007313:c.944c>T p.Thr315Ile	
3	PB	30	F	Diploid AML	97	1	<i>NRAS</i> mutation	Fludarabine, cytarabine and hydroxyurea
							NM_002524.4:c.182A>G p.Q61R	
4	PB	73	F	Refractory AML	78	5	<i>CEBPA</i> mutations	
							NM_004364.2:c.230del p.Phe77fs c.630_644del p.Ile210fs	

PB: Peripheral Blood