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The histone deacetylase inhibitor SAHA sensitizes acute myeloid leukemia cells to a combination of nucleoside analogs and the DNA-alkylating agent busulfan

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Abstract

Fludarabine (Flu), clofarabine (Clo) and busulfan (Bu) are used in allogeneic hematopoietic stem cell transplant (allo-HSCT). We reported that combining [Flu + Clo + Bu] had a synergistic cytotoxicity in AML cells. We hypothesized that combining [Flu + Clo + Bu] with the histone deacetylase inhibitor SAHA will further enhance cytotoxicity. We exposed the acute myeloid leukemia (AML) cell lines KBM3/Bu250⁶ and OCI-AML3 to Flu, Clo, Bu and SAHA alone and in various combinations. [Flu + Clo + Bu + SAHA] resulted in synergistic cytotoxicity, which can be attributed to (1) activated DNA-damage response and cell cycle checkpoint activation through the ATM–CHK2–P53 (or P73) pathway or ATM–CHK2–cdc25–cdc2 pathway, (2) histone modifications and (3) activated apoptosis pathway. The [Flu + Clo + Bu + SAHA] combination causes mitochondrial outer membrane permeabilization, leakage of cytochrome c and Smac/Diablo into the cytosol with caspase activation, and release of apoptosis-inducing factor (AIF) into the nucleus resulting in nuclear fragmentation and cell death. These results provide a mechanistic basis for using SAHA in future clinical trials with double nucleoside analog-busulfan combinations in pretransplant conditioning therapy.

Keywords

DNA alkylator; nucleoside analog; SAHA; AML; drug cytotoxicity

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Introduction

It has become evident that dose-intensity is of importance for outcome after allogeneic stem cell transplant in patients with myeloid malignancies [1]. However, there are concerns about serious toxicity after myeloablative pre-transplant conditioning therapy based on either one alkylating agent and total body irradiation or 2–3 DNA-alkylating agents. These concerns led to the design of the myeloablative reduced-toxicity regimens based on nucleoside analogs and intravenous busulfan (IV Bu) [1,2]. Such combinations resulted in a high-safety conditioning platform for patients with various hematologic malignancies. These variant regimens contributed to greatly increased safety of the allogeneic stem cell transplant procedure [1–7], but relapsing disease remains a major problem, especially when transplanting patients with active leukemia.

The recent introduction of clofarabine (Clo), a second-generation nucleoside analog with greatly enhanced anti-leukemic activity, held promise of improved clinical activity without added toxicity [8–11]. We explored the supplementation of Clo with fludarabine (Flu) and Bu in myeloid leukemia cell lines, and, based on the observed synergistic cytotoxicity of [Flu + Clo + Bu] *in vitro* as compared with either [Flu + Bu] or [Clo + Bu] [12], we introduced the double nucleoside analog–IV Bu concept in a clinical trial, which demonstrated its efficacy yet retained safety in patients undergoing hematopoietic stem cell transplant (HSCT) for high-risk acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) [13]. Although the results promise to be significantly improved when compared with the Flu–IV Bu regimen, a sizeable fraction of patients still suffered progressive leukemia after HSCT. These findings underscore the importance of further improving leukemia control achieved with the [Flu + Clo + Bu] regimen. Our mechanistic studies suggest that further synergistic augmentation of cytotoxicity may be achieved through the judicious use of histone deacetylase (HDAC) inhibitors, which can induce proliferation arrest, terminal differentiation and death of transformed cells [14–18].

Suberoylanilide hydroxamic acid (SAHA) is the first member of the HDAC inhibitor family that received Food and Drug Administration approval for treatment of patients with cutaneous T-cell lymphoma [19,20]. This drug induces mitochondrial apoptosis through the Bcl-2 protein family. It is also clinically appealing in that it is quite selective in causing cancer cell death at doses that cause little or no death of normal cells, i.e. negligible clinical adverse effects [20–23] by inducing DNA damage, which normal, but not transformed, cells can repair [24]. We hypothesized that SAHA in combination with two nucleoside analogs and Bu would invoke synergistic cytotoxicity in leukemia cells of myeloid origin.

Nucleoside analogs are known to inhibit DNA synthesis and repair, and induce histone modifications, while SAHA induces histone acetylation. Such histone modifications result in chromatin remodeling and facilitate the access of Bu to genomic DNA for alkylation. Our data show that the [Flu + Clo + Bu + SAHA] combination induces cytotoxicity in the KBM3/Bu250⁶ and OCI-AML3 AML cell lines via activation of DNA-damage response, chromatin remodeling and mitochondrial control of apoptosis. In addition to providing a mechanistic explanation for the observed synergistic cytotoxicity, we suggest that the results offer a rationale for modifying the nucleoside analog–Bu conditioning platform for patients with active, chemotherapy-refractory leukemia.

Materials and methods

Reagents

Flu, Bu and pifithrin- α (Sigma-Aldrich, St. Louis, MO) were dissolved in dimethyl sulfoxide (DMSO). SAHA (Cayman Chemical Co., Ann Arbor, MI) was dissolved in ethanol. Clo (Genzyme Corporation, Cambridge, MA) was dissolved in phosphate-buffered saline (PBS).

Cell culture

The Bu-resistant AML cell line KBM3/Bu250⁶ used in this study was established from KBM3 cells as described previously [25,26]. HL60, KBM3, KBM3/Bu250⁶ and OCI-AML3 were grown in RPMI 1640 medium (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Inc., Lawrenceville, GA). Kasumi-1 was grown in RPMI 1640 medium supplemented with 20% FBS and KG-1 was grown in Iscove's modified Dulbecco's medium (Mediatech). All growth media were supplemented with 100 U/mL of penicillin and 100 μ g/mL of streptomycin. All cells were maintained in a humidified incubator at 37°C in an atmosphere of 5% CO₂ in air. The cytogenetic characteristics of the cell lines used in this study are listed in Table I [27–29].

Cytotoxicity assay

All cells (5×10^5 cells/mL) were exposed to Flu, Clo, Bu and SAHA alone, or in combinations, for 96 h. Cells were analyzed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [30]. All cytotoxicity data are the average $\pm$ SEM of at least three independent experiments.

Preparation of cytosolic, mitochondrial and nuclear protein fractions

Control and drug-treated cells were collected and washed with ice-cold PBS, resuspended in buffer A (10 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid [HEPES] [pH 7.6], 10 mM KCl, 100 μ M ethylene diamine tetraacetic acid [EDTA], 100 μ M ethylene glycol tetraacetic acid [EGTA], 1 mM dithiothreitol [DTT], 500 μ M phenylmethylsulfonyl fluoride [PMSF] with proteinase inhibitors) and incubated on ice for 30 min. Cell disruption was completed by passing the cells 10 times through a needle (27½ gauge) on ice. Cell homogenates were centrifuged at $800 \times g$ for 5 min at 4°C to separate nuclei from the mitochondrial and cytosolic fractions. The nuclear pellet was washed with ice-cold buffer A and resuspended in lysis buffer B (20 mM HEPES [pH 7.6], 400 mM NaCl, 100 μ M EDTA, 100 μ M EGTA, 1 mM DTT, 500 μ M PMSF with proteinase inhibitors). The mitochondrial and cytosolic fractions were further subjected to 8 min of centrifugation at $12\,500 \times g$ at 4°C to pellet mitochondria and the supernatant was saved as the cytosolic fraction. The pelleted mitochondria were washed with ice-cold buffer A, and then resuspended in ice-cold lysis buffer C (10 mM HEPES [pH 7.6], 300 mM KCl, 1 mM EDTA, 0.5% Triton X-100, 5 % glycerol, 1 mM DTT with proteinase inhibitors). Particle-free cytosolic, mitochondrial and nuclear lysates were obtained by centrifugation of the three fractions at $15\,000 \times g$ for 20 min at 4°C.

Western blot analysis

Cells were incubated with the drug(s) at 37°C for 48 h, centrifuged, washed with ice-cold PBS and lysed with cell lysis buffer (Cell Signaling Technology, Danvers, MA). Protein concentrations were determined using a BCA (bicinchoninic acid) Protein Assay kit (Thermo Fisher Scientific, Rockford, IL). Western blot analysis was done by separating protein extracts on polyacrylamide–sodium dodecyl sulfate (SDS) gels, transferring onto nitrocellulose membranes (Bio-Rad, Hercules, CA) and probing with primary antibodies. β -ACTIN, COX IV and nucleoporin p62 were used as internal controls for cytosolic, mitochondrial and nuclear extracts, respectively. Signals were detected using Immobilon Western Chemiluminescent HRP (horseradish peroxidase) Substrate (Millipore, Bedford, MA).

Analysis of mitochondrial transmembrane potential

Changes in the mitochondrial transmembrane potential (Ψ_m) were measured using a JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbo cyanine iodide) mitochondrial membrane potential detection kit (Cayman Chemical Co., MI). KBM3/Bu250⁶ and OCI-AML3 cells were exposed to 1 μ M valinomycin for 4 h prior to incubation with the JC-1 reagent as positive controls. Cells were incubated with the mitochondrial membrane potential-sensitive fluorescent dye JC-1 for 20 min at 37°C, and then immediately analyzed with a flow cytometer for signal recording using the 530 nm (FL-1 channel, green) and 585 nm (FL-2 channel, red) band-pass filters simultaneously. Healthy cells with functional mitochondria and high Ψ_m exhibit red fluorescence while apoptotic or dying cells with collapsed mitochondria or low Ψ_m show green fluorescence. Each determination was based on the ratio of red to green mean fluorescence intensities measured in arbitrary units from at least 10 000 cells per sample [31].

Patient cell samples

Mononuclear cell samples from peripheral blood of patients with AML were collected and purified using lymphocyte separation medium (Mediatech). The purified cells were incubated in suspension in RPMI 1640 supplemented with 10% FBS, 100 IU/mL penicillin and 100 μ g/mL streptomycin with the indicated drugs. Cells were analyzed after 48 h of incubation. All studies were performed according to a protocol approved by the Institutional Review Board of the University of Texas M. D. Anderson Cancer Center.

Statistical analyses

All experiments were conducted at least three times, and results are expressed as mean $\pm$ SEM. *p*-Values (Student *t*-test) less than 0.05 are considered statistically significant.

Results

Combinations of Flu, Clo, Bu and SAHA inhibit proliferation of AML cell lines

We first examined the effects of drug combinations on the proliferation of KBM3/Bu250⁶ and OCI-AML3 cells by exposing them to the individual drugs, or in various combinations, for 96 h and analyzing them by MTT assay. As shown in Figure 1(A), exposure of KBM3/

Bu250⁶ cells to Flu, Clo, Bu or SAHA alone, or to two-drug combinations [Clo + Bu], [Flu + Bu] and [Flu + Clo], did not significantly affect cell proliferation relative to control at the concentrations used. The combination of two nucleoside analogs with Bu resulted in ~20% inhibition of cell proliferation. Addition of 0.7 μ M SAHA to the [Flu + Clo + Bu] mixture further enhanced the inhibition of proliferation to 60%. Similar results were obtained using another AML cell line; exposure of OCI-AML3 cells to Flu, Clo or Bu alone, or to two-drug combinations [Clo + Bu], [Flu + Bu] and [Flu + Clo], did not significantly affect cell proliferation when compared with the control [Figure 1(B)]. Exposure of OCI-AML3 cells to SAHA alone or [Flu + Clo + Bu] inhibited the cell proliferation by approximately 30%, and their combination inhibited cell proliferation by 80% [Figure 1(B)]. These results suggest that [Flu + Clo + Bu + SAHA] had a greater synergistic cytotoxicity in AML cells when compared with using single drugs or other drug combinations.

Drug combinations activate DNA-damage signaling pathway and trigger apoptosis

To better understand the mechanism of drug-induced cytotoxicity, we analyzed the changes in levels and modifications of proteins associated with DNA-damage response. We focused our study on OCI-AML3 cells that harbor wild-type P53, since about 95% of AMLs carry wild-type P53 [32,33]. To test whether the combination of Flu, Clo, Bu and SAHA would activate the DNA-damage response through the ATM (ataxia telangiectasia mutated protein) pathway, OCI-AML3 cells were exposed to various drug combinations and analyzed for modifications in relevant proteins. As shown in Figure 2(A), exposure to single drug or two-drug combinations did not greatly alter ATM phosphorylation, whereas the combination of [Flu + Clo + Bu] or [Flu + Clo + Bu + SAHA] dramatically increased ATM phosphorylation at Ser1981.

To further confirm activation of the ATM pathway, we analyzed the phosphorylation of histone 2AX, an earlier event regulated by ATM activation and an indicator of DNA-damage response [34]. Cells exposed to SAHA alone demonstrated increased phosphorylation of histone 2AX and a combination of SAHA with [Flu + Clo + Bu] further enhanced this phosphorylation [Figure 2(A)]. We also analyzed the phosphorylation of two additional ATM substrates, CHK2 and P53. OCI-AML3 cells exposed to SAHA alone or to the [Flu + Clo + Bu] combination showed a marginal increase in the phosphorylation of CHK2 at Ser19, whereas exposure to [Flu + Clo + Bu + SAHA] enhanced the phosphorylation of CHK2 [Figure 2(A)]. Cells exposed to Bu, [Clo + Bu], [Flu + Bu], [Flu + Clo + Bu] or [Flu + Clo + Bu + SAHA] all showed P53 phosphorylation at Ser15. Taken all together, these results strongly suggest activation of the ATM pathway in OCI-AML3 cells exposed to [Flu + Clo + Bu + SAHA].

The observed extensive activation of the ATM-CHK2 pathway [Figure 2(A)] is strongly suggestive of a sequential cellular response to high levels of complex genomic injury that ultimately may induce apoptosis. Exposure of cells to single agents (except SAHA) or two- or three-drug combinations did not result in poly-ADP ribose polymerase 1 (PARP1) and caspase cleavage [Figure 2(B)]. However, exposure to [Flu + Clo + Bu + SAHA] resulted in cleavage of PARP1, caspase 8, caspase 9 and caspase 3, and, therefore, suggest activation of apoptosis.

Drug combinations increase histone modifications

Histone modifications contribute to chromatin relaxation, which may render the genomic DNA more accessible to the effects of chemotherapeutic agents. To test how these drug combinations would affect histones in AML cells, OCI-AML3 cells were exposed to various drug combinations. As shown in Figure 3, OCI-AML3 cells that were exposed to Flu, Clo or Bu, or to any two-drug combinations, did not show any significant changes in the level of histone acetylation or methylation, whereas exposure to SAHA alone or [Flu + Clo + Bu] greatly increased acetylation of histone 4 at Lys 5 and histone 3 at Lys 4 (Figure 3). Methylation of histone 3 at Lys 27 and Lys 4 was also observed when OCI-AML3 cells were exposed to SAHA. The addition of SAHA to [Flu + Clo + Bu] further increased these histone modifications. The results suggest induction of chromatin remodeling through histone modifications when OCI-AML3 cells were exposed to [Flu + Clo + Bu + SAHA].

Drug combinations trigger mitochondrial outer membrane permeabilization

Although activation of DNA-damage response would explain the observed drug-induced apoptosis, we wanted to determine whether exposure of AML cells to [Flu + Clo + Bu + SAHA] would also decrease the mitochondrial transmembrane potential (Ψ_m), a known contributing factor to apoptosis [35,36]. KBM3/Bu250⁶ and OCI-AML3 cells were exposed to [Flu + Clo + Bu] or [Flu + Clo + Bu + SAHA] for 48 h. [Flu + Clo + Bu] exposure decreased the mitochondrial membrane potential in both cell lines by approximately 40%, and [Flu + Clo + Bu + SAHA] decreased it by 80% (Figure 4). This suggests that addition of SAHA to [Flu + Clo + Bu] contributes to apoptosis through activation of the intrinsic mitochondrial pathway.

Drug combinations induce release of death factors from the mitochondria to the cytosol and/or the nucleus

To further test whether Flu, Clo, Bu and SAHA alone, or in combinations, contributed to apoptosis through activation of the intrinsic mitochondrial pathway, we exposed OCI-AML3 cells to drugs for 48 h; the cytosolic, mitochondrial and nuclear fractions were then isolated and protein levels of the Bcl-2 family members were determined. The pro-apoptotic proteins Bax and cleaved Bid increased in the mitochondria when cells were exposed to [Flu + Clo + Bu + SAHA] (Figure 5, lane FCBS). Phosphorylated and pan P53 increased in the mitochondria when cells were treated with [Flu + Clo + Bu] or [Flu + Clo + Bu + SAHA], consistent with previous reports that a fraction of stress-induced wild-type p53 rapidly translocate to mitochondria and initiate a first wave of mitochondrial outer membrane permeabilization [37–39]. Finally, the pro-survival protein MCL-1 was cleaved and yielded a pro-apoptotic 28 kDa protein, and the level of the pro-survival protein Bcl-xL decreased.

The observed changes in the levels of these proteins may have resulted in an alteration of the outer mitochondrial membrane, and led to release of death factors (AIF, Smac/Diablo and cytochrome c) that normally reside in the mitochondria. The released death factors would in turn trigger other downstream events in the apoptotic cascade. To test whether the drug combinations would indeed cause death factor-release from mitochondria to the cytosol and/or the nuclei, we examined the levels of these proteins in cytosol, mitochondria and nuclei of OCI-AML3 cells. As shown in Figure 5, the level of AIF decreased in the

mitochondria and dramatically increased in the nuclei when cells were exposed to Clo alone and more significantly when the combination of [Flu + Clo + Bu + SAHA] was used. When cells were exposed to Flu alone, [Flu + Clo + Bu] or [Flu + Clo + Bu + SAHA], the level of Smac/Diablo decreased in the mitochondria with a concomitant increase in the cytoplasm. Finally, when cells were exposed to Flu alone, [Flu + Clo + Bu] and [Flu + Clo + Bu + SAHA], the level of cytochrome c increased in the cytoplasmic fraction compared with the control cells, suggesting leakage of cytochrome c from the mitochondria to the cytosol. Overall, these results demonstrate that exposure of AML cells to [Flu + Clo + Bu + SAHA] decreases the mitochondrial transmembrane potential (Figure 4), causes release of death factors to the cytoplasm and/or nucleus (Figure 5) and activates the cell death pathways [Figure 2(B)].

Importance of P53 and P73 activity for [Flu + Clo + Bu + SAHA]-induced cytotoxicity

The P53 tumor suppressor protein plays a major role in cellular responses to DNA damage and other genomic aberrations. Activation of p53 can lead to cell cycle arrest, DNA repair and apoptosis [40]. To examine whether drug-induced apoptosis is P53-dependent, we used a previously established clone of OCI-AML3 cells where P53 was down-regulated using a shRNA GFP construct [41]. The OCI/vector and OCI/P53 shRNA cell lines were analyzed by Western blot analysis using antibodies against P53 and GFP [Figure 6(A)] and confirmed the shRNA-mediated down-regulation of P53. The cell lines were then exposed to SAHA alone, [Flu + Clo + Bu] and [Flu + Clo + Bu + SAHA], and the resulting cytotoxicity was determined by the MTT assay. As shown in Figure 6(B), 40% and 60% inhibition of cell proliferation was observed when OCI/vector cells were exposed to SAHA alone or [Flu + Clo + Bu + SAHA], respectively, whereas only marginal inhibition of cell proliferation was recorded when OCI/P53 shRNA cells were exposed to these drugs. The results were confirmed by the observed partial inhibition of drug-induced cleavage of PARP1 and phosphorylation of H2AX in the P53 shRNA clone, further supporting the notion that the observed synergistic effects of [Flu + Clo + Bu + SAHA] are P53-dependent [Figure 6(C)].

Loss of P53 function has been associated with carcinogenesis and drug resistance [40,42]. However, HL60, KBM3, KBM3/Bu250⁶ and KG-1 cell lines, which possess mutant P53, remain sensitive to [Flu + Clo + Bu + SAHA] when examined with the MTT assay [Figure 6(D)]. P73, a member of the P53 gene family, can replace P53 function in response to DNA damage in P53-deficient or P53-mutated cancers [43–45]. The P73 variant P73 α inhibits drug-induced apoptosis, while P73 β promotes apoptosis [46]. To test whether the utilized drug combination would induce changes in P73 levels, we exposed the AML cell lines HL60, KBM3, KBM3/Bu250⁶ and KG-1 cells, all of which have truncated or mutated P53, and Kasumi-1 and OCI-AML3 cells, which harbor wild-type P53, to [Flu + Clo + Bu + SAHA] at approximately 10% inhibitory concentrations (IC₁₀) of the respective drug for 48 h. Nuclear extracts were prepared, and the level of P73 α and P73 β proteins and the phosphorylation status of P53 were examined by Western blot. As shown in Figure 6(E), [Flu + Clo + Bu + SAHA] decreased the level of P73 α in HL60, KBM3 and KBM3/Bu250⁶ cells with a concomitant increase in P73 β . In contrast, P73 α and P73 β were not changed with the same drug exposure in KG-1, Kasumi-1 and OCI-AML3 cells. Finally, the [Flu +

Clo + Bu + SAHA] combination induced phosphorylation of P53 at Ser15 in OCI-AML3 cells but not in Kasumi-1 cells [Figure 6(E), lower panel].

P21^{Cip1} is the target gene for the transcription factors P53 and P73. To test whether the increased P73 β or phosphorylated P53 would increase P21^{Cip1} expression, the same six AML cell lines were exposed to [Flu + Clo + Bu + SAHA] and total proteins were isolated. As shown in Figure 6(F), the P21^{Cip1} protein level increased in HL60, KBM3, KBM3/Bu250⁶ and OCI-AML3 cells but not in KG-1 and Kasumi-1 cells. Taken together, these results suggest that [Flu + Clo + Bu + SAHA]-induced cytotoxicity was mediated through activated P73 in HL60, KBM3 and KBM3/Bu250⁶ cells, and through activated P53 in OCI-AML3 cells, but independent of both P53 and P73 in KG-1 and Kasumi-1 cells.

Since drug-induced cytotoxicity may also be attributed to activation of the ATM–CHK2–cdc25 pathway, we examined the cdc25A level in the six AML cell lines exposed to [Flu + Clo + Bu + SAHA]. The cdc25A protein level decreased in all of the cell lines exposed to [Flu + Clo + Bu + SAHA] when compared with the control cells [Figure 6(F)], whereas p-cdc2 (Thr15), a substrate protein for cdc25A phosphatase activity, increased, suggesting that [Flu + Clo + Bu + SAHA]-induced cytotoxicity was partly mediated through activation of the ATM–CHK2–cdc25 pathway in all of the cell lines.

In summary, the [Flu + Clo + Bu + SAHA]-induced cytotoxicity can be attributed to activation of DNA-damage and cell cycle checkpoint through the CHK2–cdc25A pathway in these cell lines. In addition, the observed effects of these drugs can be partly attributed to activation of the P53–P21^{Cip1} pathway in OCI-AML3 cells, and P73–P21^{Cip1} pathway in HL60, KBM3 and KBM3/Bu250⁶ cells.

[Flu +Clo +Bu +SAHA] combination has similar synergistic effects in patient-derived AML cells

To assess the potential clinical implications of the observed synergism of [Flu + Clo + Bu + SAHA] in AML cell lines, we isolated mononuclear cells from blood samples of patients with AML. Cells were exposed to SAHA, [Flu + Clo + Bu] or [Flu + Clo + Bu + SAHA]. Immunoblot analysis showed increased phosphorylation of P53 in cells exposed to [Flu + Clo + Bu] or [Flu + Clo + Bu + SAHA] (Figure 7). The phosphorylation of histone 2AX increased in cells exposed to [SAHA] or [Flu + Clo + Bu], and was further enhanced in cells exposed to [Flu + Clo + Bu + SAHA], suggesting robust activation of the DNA-damage response pathway. The elevated baseline level of γ -H2AX in patient 2 and patient 3 could be due to other drugs the patients were taking at the time of blood sampling. The synergistic activity of [Flu + Clo + Bu + SAHA] in these patient samples is further supported by an increased cleavage of PARP1, a hallmark of apoptosis (Figure 7).

Discussion

Our earlier studies indicated that the DNA alkylating agent Bu, combined with the nucleoside analogs Flu and Clo and HDAC inhibitor SAHA, exerts synergistic cytotoxicity in lymphoma cell lines [47]. The present study shows a similar synergism in AML cells. These effects are achieved through (1) activation of DNA-damage response, (2) activation of

the cell cycle checkpoint pathway through ATM–CHK2–P53, ATM–CHK2–P73 and/or ATM–CHK2–cdc25 and (3) chromatin remodeling through altered methylation- and acetylation-status of histones. Furthermore, activation of caspases 3/8/9 and pro-apoptotic proteins (tBID and BAX), as well as down-regulation of members of the Bcl-2 family (MCL-1 and Bcl-xL), which subsequently decreases mitochondrial membrane potential and induces death factor (AIF, Smac/Diablo and cytochrome c) release from the mitochondria to the cytosol and/or the nucleus, all contribute to the observed cytotoxic effects.

Clo and Flu may affect mitochondria by directly disrupting membrane integrity, leading to release of cytochrome c and AIF or by an indirect mechanism [48]. In CLL cells, these nucleoside analogs have been shown to cause DNA damage, induce P53 phosphorylation and consequently mediate the translocation of BAX from the cytosol to the mitochondria, leading to release of cytochrome c and activation of the intrinsic apoptotic machinery [49–52]. Flu also decreases the level of the anti-apoptotic protein MCL-1 in CLL cells [53]. Our results are consistent with the aforementioned findings when AML cells were exposed to the combination of [Flu + Clo + Bu] or [Flu + Clo + Bu + SAHA]. However, the described molecular readouts were not observed in our study when drugs were used individually. We used IC₅–IC₂₀ concentrations of the individual drugs to allow the detection of synergism when the various agents were combined, but these low concentrations may explain the observed differences between the present observations and previous reports when comparing the corresponding single-agent effects.

SAHA may induce cell cycle arrest, terminal differentiation and apoptosis in leukemia [20,54,55]. Cell death occurs through the intrinsic apoptotic pathway, which involves the release of mitochondrial membrane proteins, such as cytochrome c, and activation of caspases [55,56]. Mechanistically this may explain how SAHA synergistically enhances the [Flu + Clo + Bu]-induced cytotoxicity.

Histone modifications including acetylation, methylation, ubiquitination and phosphorylation are known to regulate gene expressions. H3K27 tri-methylation is a characteristic of Polycomb group target genes and is associated with transcriptional repression [57]. Less methylation at H3K27 is often correlated with up-regulation of oncogenes and repression of tumor suppressor genes in lymphoid neoplasms, prostate cancer and myeloid transformation [58–60]. Our study showed that SAHA alone or in combination with [Flu + Clo + Bu] induced H3K27 trimethylation (Figure 3), suggesting that such histone modification may have repressed expression of oncogenes and up-regulated tumor suppressor genes in AML cells.

P53 may cause apoptosis through a transcription-dependent or -independent pathway. Wild-type P53 rapidly translocates to the mitochondria in response to apoptosis-inducing stress signals [37–39]. Our study shows that the level of p-P53 increased in the mitochondria when OCI-AML3 cells were treated with [Flu + Clo + Bu] or [Flu + Clo + Bu + SAHA]. Although SAHA alone did not increase p-P53 in the mitochondria, it increased the level of p-P53 in the nucleus. The observed cytotoxicity of [Flu + Clo + Bu + SAHA] is P53-dependent in cells harboring wild-type P53 (OCI-AML3). The observed cytotoxicity of [Flu + Clo + Bu + SAHA] is P73-dependent in HL60, KBM3 and KBM3/Bu250⁶, all of which have mutated

P53. P73 is a member of the P53 gene family [61], which, in contrast to P53 [62], rarely is mutated or lost in cancer cells [44]. P73 can regulate P53 target genes in P53-deficient cancers and replace P53 function(s) in response to DNA damage [32,33,63]. Furthermore, drug-induced cytotoxicity may also be dependent on the cdc25A pathway, independent of the P53 family-members, as suggested by our findings in KG-1 cells and Kasumi-1 cells. Overall, the results suggest that addition of SAHA may further improve chemotherapeutic effects of [Flu + Clo + Bu] in tumor cells harboring wild-type or mutated P53.

In summary, combinations of an HDAC inhibitor with nucleoside analogs and the DNA alkylating agent Bu are synergistically cytotoxic in AML cell lines and patient-derived cells. We further suggest that our results can be used to mechanistically justify the incorporation of (an) HDAC inhibitor(s) in a modified [Flu + Clo + Bu] pre-transplant conditioning regimen for patients with AML.

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Figure 1. Anti-proliferative effects of Flu, Clo, Bu and SAHA in AML cell lines. KBM3/Bu250⁶ (A) and OCI-AML3 (B) cells were exposed to drugs alone, or in combination, for 96 h and analyzed by MTT assay. Results are expressed as the mean $\pm$ SEM of three independent experiments. Flu or F, fludarabine; Clo or C, clofarabine; Bu or B, busulfan; SAHA or S, suberoyl-anilide hydroxamic acid.

Figure 2.

Drug combinations activate the DNA-damage response and induce apoptosis. OCI-AML3 cells were exposed to Flu, Clo, Bu and SAHA alone, or in combination, for 48 h and changes in level of proteins and phosphorylation of protein involved in ATM pathway (A) and apoptosis (B) were analyzed by Western blot. Flu or F, fludarabine; Clo or C, clofarabine; Bu or B, busulfan; SAHA or S, suberoyl-anilide hydroxamic acid.

Figure 3.

Drug exposures cause modifications of histones. OCI-AML3 cells were exposed to Flu, Clo, Bu and SAHA alone, or in combination, for 48 h and histone modifications were analyzed by Western blot. Flu or F, fludarabine; Clo or C, clofarabine; Bu or B, busulfan; SAHA or S, suberoyl-anilide hydroxamic acid.

Figure 4.

Changes in mitochondrial membrane potential in KBM3/250⁶ (A) and OCI-AML3 (B) cells exposed to F (0.5 μ M), C (0.01 μ M), B (6.1 μ M) $\pm$ S (0.7 μ M) for 48 h. Results are expressed as percentage of change of calculated ratio of red/green fluorescence relative to control. Results are expressed as the mean $\pm$ SEM of three independent experiments. § p < 0.05 relative to control. † p < 0.05 relative to FCB treated group. F, fludarabine; C, clofarabine; B, busulfan; S, suberoyl-anilide hydroxamic acid.

Figure 5.

Drug combinations change the level of proteins related to mitochondrial control of apoptosis. OCI-AML3 cells were exposed to Flu, Clo, Bu or SAHA alone, or in combination, for 48 h and cytoplasmic, mitochondrial and nuclear proteins were isolated as described in "Materials and methods." Protein changes were analyzed by Western blot. β -ACTIN, COX IV and nucleoporin p62 were used as loading controls for the cytoplasmic (Cyto), mitochondrial (Mito) and nuclear (Nuc) fractions, respectively. Flu or F, fludarabine; Clo or C, clofarabine; Bu or B, busulfan; SAHA or S, suberoyl-anilide hydroxamic acid.

Figure 6.

Dependence of [Flu + Clo + Bu + SAHA] cytotoxicity on P53 family or cdc25–cdc2 pathway. OCI-AML3 cells with empty vector or with P53 shRNA were exposed to drugs and analyzed by Western blot (A, C) and MTT assay (B). HL60, KBM3, KBM3/Bu250⁶ and KG-1 cell lines with mutant P53, and Kasumi-1 and OCI-AML3 cell lines with wild-type P53 were treated with solvent or four-drug combination for 48 h and analyzed by MTT assay (D) and Western blot (E, F). The lower panel in (E) shows results for one-third of the nuclear extracts from Kasumi-1 cells used on top panel. FCB, fludarabine + clofarabine + busulfan; FCBS, FCB + SAHA.

Figure 7.

Analysis of mononuclear cells from the peripheral blood of three patients with AML. Cells were isolated, exposed to the indicated concentrations of SAHA, FCB or both for 48 h and analyzed using Western blot for levels of P53, γ H2AX and PARP1. Patient (Pt) number is indicated. FCB, fludarabine + clofarabine + busulfan; FCBS, FCB + SAHA. 1 $\times$ = 0.7 μ M SAHA, 0.5 μ M Flu, 0.01 μ M Clo and 6.1 μ M Bu.

Table I

Cell lines used in this study*.

Cell line	Disease	Cytogenetic karyotype	Unique gene alteration	P53 status
HL-60	AML M2	Flat-moded hypotetraploid, hypodiploid sideline, 1.5% polyploidy; 82(78-88)<4n>XX, -X, -X, -8, -8, -16, -17, -17, +18, +22, +2mar, ins(l;8)(p?31;q24hsr)×2, der(5)t(5;17)(qll;qll) ×2, add(6)(q27)×2, der(9)del(9)(pl3)t(9;14)(q?22;q?22)×2, der(14)t(9;14)(q?22;q?22)×2 der(16)t(16;17)(q22;q22)×1-2, add(18)(q21); sideline with-2, -5, -15, del(11)(q23.1q23.2)	Amplification of <i>MYC</i> , <i>NEU</i> , <i>NRAS</i> , <i>P15INK4B</i> GG/DG, <i>P16INK4A</i> GG/DG	Mutant
Kasumi-1	AML M2	45 < 2n>X, -Y, -9, -13, -16, +3mar, t(8;21)(q22;q22), der(9)t(9;?) (p22;?), der(15)t(?9;15)(?qll;?pll)	<i>AML1-ETO</i> fusion gene	Wild type
KG-1	AML	Hypodiploid, 4.5% polyploidy; 45(42-47)<2n>X/XY, -4, +8, +8, -12, -17, -20, +2mar, der(5;17)(ql0;ql0) del(5)(q?llq?13), dup(7)(ql2q33), del(7)(q22q35), i(8q)×2, der(8)t(6;8)(pll;q22), der(8)t(8;12)(pll;q13), der(11)t(1;11)(ql3-21;pll-pl3), der(16)t (?12;16) (?pl3;q13/21)	<i>NRAS</i> mutation <i>P15INK4B</i> methylation <i>P16INK4A</i> methylation <i>RB1</i> rearrangement	Mutant
KBM3	AML M4	48(41-45), -4, -12, +13, del(12)ins(12q+?), del(5)(pl2-ter), del(9)(pll-ter), del(16)t(16q+?), del(7)ins(7p+HSR)		Mutant
OCI-AML3	AML		<i>NPM</i> mutation	Wild type

AML, acute myeloid leukemia.

* From (except OCI-AML3) reference [27], OCI-AML3 from references [28,29].