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Synergistic cytotoxicity of the DNA alkylating agent busulfan, nucleoside analogs and SAHA in lymphoma cell lines

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Abstract

Hematopoietic stem cell transplantation (HSCT) is a promising treatment for lymphomas. Its success depends on effective pre-transplant conditioning regimens. We previously reported on the efficacy of DNA alkylating agent-nucleoside analog (NA) combinations for conditioning in AML. We hypothesized that a similar combinatory approach can be used for lymphomas. A combination of busulfan (Bu) with two NAs – clofarabine (Clo), fludarabine (Flu) or gemcitabine (Gem) – resulted in synergistic cytotoxicity in lymphoma cell lines. We demonstrated that the [2 NAs+Bu] combination activates a DNA damage response through the ATM-CHK2 and ATM-CHK1 pathways, leading to cell cycle checkpoint activation and apoptosis. Histone modifications and KAP1 phosphorylation are indicative of chromatin relaxation mediated by the nucleoside analogs which sequentially increase Bu alkylation. Addition of suberoylanilide hydroxamic acid (SAHA) enhanced chromatin relaxation through increased histone acetylation and further augmented the cytotoxicity of [2 NAs+Bu]. Our results provide a preclinical basis for a clinical trial on using [2 NAs+Bu±SAHA] combinations as conditioning therapy for chemotherapy-refractory lymphoma patients undergoing HSCT.

Keywords

DNA alkylating agent; busulfan; clofarabine; gemcitabine; SAHA; lymphoma

Introduction

Malignant lymphomas are hematologic clonal disorders that originate from B-cells (~90% of cases), T-cells (~10%) and occasionally natural killer (NK) cells [1]. These diseases are heterogeneous [2], and their intricate pathogenesis and molecular defects make treatment challenging. Although promising therapies have been reported for specific subtypes of lymphoma, no single drug or drug combination is universally effective. However, the development and inclusion of rituximab, a monoclonal antibody that targets the B-cell CD20 antigen, to combination chemotherapy regimens such as CHOP (cyclophosphamide + doxorubicin + vincristine + prednisone) has improved the prognosis of patients with B-cell lymphomas [3, 4].

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Conflict of interest

No conflict of interest.

A significant fraction of patients still suffer recurrent or progressive disease, and in this setting hematopoietic stem cell transplantation (HSCT) is increasingly recognized as an important therapeutic alternative [5–7]. It is, therefore, important to identify efficacious regimens for pre-transplant conditioning therapy.

We recently combined the nucleoside analog (NA) fludarabine (Flu) with the DNA alkylating agent busulfan (Bu) as part of conditioning therapy for acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) patients undergoing HSCT (8–10). Its success has been further improved with the introduction of a later generation nucleoside analog, clofarabine (Clo) [11, 12]. In analogy with our studies in AML, lymphoma patients in need of HSCT may benefit from a critical appraisal of how currently available knowledge of cytotoxic mechanisms can be optimally incorporated into the conditioning therapy. In human myeloid leukemia cell lines we showed that a sequence-specific combination of two nucleoside analogs [Flu + Clo] followed by Bu yielded even higher synergistic cytotoxicity than combining either NA with Bu [13]. In a subsequent clinical trial we tested the utility of the two NAs with Bu in pre-transplant conditioning therapy for patients with refractory AML or MDS. The results demonstrated further improved leukemia control with retained patient safety when compared with historical controls [9–11].

Extrapolating from our preclinical and clinical HSCT studies on the combinations of nucleoside analogs and DNA alkylating agents, and our recent findings on their synergistic cytotoxicity in AML cells [11, 13], we then hypothesized that a combination of [2 NAs+Bu] would synergistically inhibit lymphoma cell proliferation. Nucleoside analogs are known to inhibit DNA synthesis and induce histone modifications which cause chromatin relaxation [14, 15]. Such histone modifications resulting in chromatin relaxation may facilitate access of Bu to genomic DNA for alkylation [13]. Based on this premise, augmented histone modifications could be expected to further enhance Bu-mediated DNA alkylation. Indeed, we report here synergistic cytotoxicity in lymphoma cells when Bu was combined with nucleoside analogs and these effects could be further enhanced by the addition of a histone deacetylase (HDAC) inhibitor, SAHA (suberoylanilide hydroxamic acid).

Materials and Methods

Cell Culture

Lymphoma cell lines Daudi, J45.01 and U937 were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were grown in RPMI 1640 medium (Mediatech, Manassas, VA) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Inc., Lawrenceville, GA) and 100 U/ml penicillin and 100 µg/ml streptomycin (Mediatech) at 37°C in a humidified atmosphere of 5% CO₂.

Reagents

Busulfan (Sigma-Aldrich, St. Louis, MO) and fludarabine (Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO), SAHA (Cayman Chemical Co., Ann Arbor, MI) was dissolved in ethanol, gemcitabine (Eli Lilly, Indianapolis, IN) was dissolved in phosphate-buffered saline (PBS), and clofarabine (Clolar[®]) was obtained from Genzyme Oncology, Cambridge, MA (1 mg/ml) and diluted in RPMI 1640 medium. The final concentration of DMSO and ethanol in all experiments did not exceed 0.08% by volume.

Cytotoxicity assay

Cell suspensions were aliquoted (100 µl of 2×10^5 cells/ml) into 96-well plates in the presence of drug(s) or solvent alone and continuously incubated at 37°C for 48 or 96 hr. The cells were analyzed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

(MTT) assay [16]. The advantage of using a 96-hr drug exposure in this assay is that the cells are allowed to proliferate for several cell cycle times before staining with MTT. Graphical analyses including calculations of IC₂₀ values (the concentration of drug required for 20% growth inhibition) were done using Prism 5 (GraphPad Software, San Diego, CA). All cytotoxicity data are the average $\pm$ standard deviation of at least 3 independent experiments.

Flow-cytometric analysis of sub-G1 DNA content

Cells in logarithmic growth phase (5×10^5 cells/ml) were continuously incubated with the indicated concentrations of the drug(s) at 37°C for 48 hr. The cells were centrifuged, resuspended in 70% ethanol in 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 hr prior to analysis by flow cytometry. The cellular DNA content of at least 10,000 cells was analyzed using a BD FACS Calibur instrument (BD Biosciences, San Jose, CA) and the proportion of cells in the different phases of the cell cycle was determined using the CellQuestTM software (Becton Dickinson, Franklin Lakes, NJ). Histograms were analyzed for proportion of cells with sub-G1 DNA content using ModFit LT (Verity Software House, Topsham, ME). All flow cytometry experiments were done at least 3 times.

Western blot analysis

Cells were incubated with the indicated concentrations of the drug(s) at 37°C for 48 hr and collected by centrifugation. Cells were washed with PBS and lysed with cell lysis buffer (Cell Signaling Technology, Danvers, MA) as recommended by the manufacturer. Total protein concentrations in the 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-SDSgels and blotting onto nitrocellulose membranes (Bio-Rad, Hercules, CA). Immunoblot analyses by chemiluminescence were done using the Immobilon Western Chemiluminescent HRP Substrate (Millipore, Bedford, MA). All Western blots were done at least 2 times, and representative data are shown. All antibodies, their sources and other relevant information are listed in Table 1. The β -actin protein was used as an internal control.

Results

Combinations of nucleoside analogs and Bu are cytotoxic towards lymphoma cell lines

Three lymphoma cell lines with different cell origins were continuously exposed to drugs, singly or in combination, for 96 hr and analyzed for cytotoxicity by the MTT assay. We used drug concentrations close to their pre-determined IC₁₀-IC₂₀ values (data not shown). Of the three cell lines analyzed, U937 (histiocytic lymphoma) exhibited the highest sensitivity to [2 NAs+Bu]. Exposure of this cell line to 100 μ M Bu, 0.020 μ M Clo, 1 μ M Flu or 0.04 μ M Gem resulted in 0% – 20% inhibition of proliferation (Figure 1A). Combination of two nucleoside analogs ([Clo+Gem], [Gem+Flu] or [Clo+Flu]) resulted in 70% – 80% growth inhibition, and addition of Bu to any of these combinations almost totally abrogated U937-cell proliferation (Figure 1A).

A similar cytotoxicity study was performed using Daudi, a B-cell lymphoblastic cell line. Exposure of Daudi cells to 20 μ M Bu, 0.075 μ M Clo, 20 μ M Flu or 0.004 μ M Gem for 96 hr resulted in only modest effects on cell proliferation (Figure 1B) whereas exposure to combination of two nucleoside analogs ([Clo+Gem], [Gem+Flu] or [Clo+Flu]) resulted in

60% – 80% inhibition of growth. Addition of Bu to these combinations slightly increased the growth inhibition to 75% – 84% (Figure 1B).

A lymphoma cell line with T-cell origin (J45.01) exhibited less sensitivity to these drug combinations. Exposure of J45.01 cells to 40 μ M Bu, 0.040 μ M Clo, 1 μ M Flu or 0.020 μ M Gem resulted in negligible inhibition of proliferation, and exposure to combination of two nucleoside analogs resulted in a maximum inhibition of only 17% (Figure 1C). Addition of Bu to the two-nucleoside analog combinations increased growth inhibition to 45% with [Gem+Clo+Bu], 26% with [Gem+Flu+Bu] and 58% with [Clo+Flu+Bu].

These results suggest differences in the efficacy of Bu, Clo, Flu and Gem in various lymphoma cell lines. Synergistic cytotoxicities are observed for certain drug combinations.

Drug combinations activate the DNA-damage response signaling network

To better understand the mechanism of cytotoxicity, cells exposed to various drug combinations were analyzed for changes in protein levels and modifications associated with the DNA-damage response (DDR) network. We focused our study on Daudi cells since about 90% of lymphomas are derived from B-cells [1]. We examined the effects of each drug combination on the activation of the ATM (Ataxia Telangiectasia Mutated)-CHK2 pathway, which we previously showed to be activated in AML cells exposed to [Clo+Flu+Bu] [13]. Exposure of Daudi cells to various drug combinations resulted in the autophosphorylation of ATM at Ser1981 (a marker for ATM activation) with the highest signal being obtained in cells exposed to [Gem+Flu+Bu] (Figure 2). This drug-mediated phosphorylation of ATM correlates with the phosphorylation of its known kinase substrates including H2AX, CHK2, p53, SMC1, SMC3 and KAP1, all of which are involved in the activation of cell cycle checkpoints and DNA repair pathways [17–20].

The phosphorylation of histone 2AX, an early event mediated by ATM activation and widely used as an indicator of DDR [21], seems to be equally increased when Daudi cells are exposed to [Gem+Clo], [Gem+Flu] or [Clo+Flu] in the absence or presence of Bu (Figure 2). Phosphorylation at Ser19 of CHK2, a protein involved in cell cycle checkpoint activation [22], increases in the presence of [Gem+Clo], [Gem+Flu], or [Clo+Flu]. Addition of Bu to this regimen further enhances CHK2 phosphorylation except for the [Gem+Flu] combination.

Another ATM substrate involved in cell cycle checkpoint and DNA repair regulation is the p53 protein. Exposure of Daudi cells to individual drugs shows negligible phosphorylation of p53 at Ser20. However, exposure of cells to [Gem+Clo] or [Gem+Clo+Bu] dramatically increases p53 phosphorylation (Figure 2). Less dramatic phosphorylation of p53 was observed when cells were exposed to [Gem+Flu], [Gem+Flu+Bu], [Clo+Flu] and [Clo+Flu+Bu] (Figure 2).

SMC1 (structural maintenance of chromosomes 1) and SMC3 are components of the cohesin complex 1, which is involved in sister chromatid cohesion [23] and DNA recombination during double-strand break (DSB) repair [24]. Exposure of Daudi cells to two nucleoside analogs in the presence or absence of Bu increases the phosphorylation of SMC1 and SMC3 proteins, suggesting the activation of a DNA repair response (Figure 2).

ATM-dependent DDR is accompanied by chromatin relaxation which facilitates DNA repair; this pathway is mediated by the ATM-catalyzed phosphorylation of KRAB-associated protein 1 (KAP1) at Ser824 [20]. Exposure of Daudi cells to [Gem+Clo], [Gem+Flu] or [Clo+Flu] increases the phosphorylation of KAP1 and addition of Bu to these drug combinations, except with [Gem+Flu], further enhances this effect (Figure 2). This

phosphorylation of KAP1 further suggests the activation of DNA repair pathways when Daudi cells are exposed to these drug combinations.

We also examined the effects of each drug combination on the activation of the ATR (ATM and RAD3 related)-CHK1 pathway in Daudi cells. Unlike the autophosphorylation of ATM at Ser1981 as a marker for ATM activation, none of the known modifications of ATR has proven to be a reliable indicator of ATR activation [25]. However, monitoring downstream events such as the phosphorylation of CHK1 at Ser317 or Ser345 appears to be a reasonable indicator of ATR activity [25]. As shown in Figure 2, phosphorylation of CHK1 at Ser317 was readily apparent with all combinations of [2 NAs ± Bu] but not following treatment with any of the drugs individually. Phosphorylation of ATR at Ser428 was apparent in control cells as well as in all of the drug-treated samples (Figure 2).

Drug combinations trigger apoptosis and down-regulate pro-survival genes

The extensive activation of the ATM-CHK2 and ATR-CHK1 pathways and cell cycle checkpoint proteins discussed above is indicative of a sequential cellular response to high levels of complex genomic injury that may eventually lead to activation of apoptosis. Flow cytometric analysis of Daudi cells exposed individually to 60 μ M Bu, 0.1 μ M Clo, 20 μ M Flu, or 0.004 μ M Gem for 48 hr shows 2% – 4% of cells with sub-G1 DNA content (Figure 3A). Combination of two nucleoside analogs such as [Gem+Clo], [Gem+Flu] or [Clo+Flu] at these low concentrations increases these cell fractions to 8% – 11% and addition of Bu further increases cells with sub-G1 DNA content to 12% – 14%, suggesting that exposure of Daudi cells to [2 NAs+Bu] increases the proportion of cells undergoing apoptosis (Figure 3A). This observation is supported by the cleavage of PARP1 (poly-ADP ribose polymerase-1), activation (by cleavage) of caspase 9, and upregulation of pro-apoptosis proteins such as XAF1 and NOXA (Figure 3B). Although the status of p53 in Daudi cells is controversial [26, 27], its activation through phosphorylation at Ser15 is greater in cells exposed to various drug combinations, which at least partially parallels the observed upregulation of p21 (Figure 3B), a contributing factor in cell cycle checkpoint activation and apoptosis [28, 29].

The growth inhibitory activities of these drug combinations may also be attributed to down-regulation of the ubiquitous pro-survival transcription factor c-MYC [30]. Exposure of Daudi cells to various drug combinations decreased the level of c-MYC protein and consequently down-regulated the expression of proteins encoded by its target genes, including *RhoA* and *CDK4* (Figure 3C). The decrease in the levels of c-MYC, RhoA and CDK4 protein may contribute to the cytotoxicity mediated by these drug combinations, although this effect may also in part be a consequence of perturbation of the cell cycle.

Drug combinations increase histone 3 methylations that correlate with down-regulation of histone lysine demethylases

We previously showed that exposure of AML cells to [Clo+Flu+Bu] resulted in increased histone 3 methylation which may contribute to chromatin relaxation [13]. We wished to determine whether similar changes would be observed in lymphoma cells exposed to combined Bu and nucleoside analogs and if so identify its possible cause. Exposure of Daudi cells to two- or three-drug combinations greatly increased methylations of histone 3 at Lys4, with [Gem+Flu+Bu] showing the greatest effect (Figure 4). The largest increase in methylation of histone 3 at Lys9 and Lys27 was observed when cells were exposed to [Gem+Clo+Bu] and [Clo+Flu+Bu] (Figure 4).

The level of histone methylations may be dictated by balanced enzymatic activities between histone lysine methyltransferases and demethylases [31]. Immunostaining analysis of

extracts from Daudi cells exposed to various drug combinations generally shows a decrease in the protein levels of JMJD1B, JARID1A and JARID1C/SMCX (Figure 4), which are all histone lysine demethylases [31], compared with controls or single-agent treatment. We did not observe a concomitant increase in the level of expression of histone lysine methyltransferases including ASH2L, SET7/9, G9A, ESET and EZH2 (data not shown). These results suggest that the observed increase in histone 3 methylations might be due to down-regulation of histone lysine demethylases by the combined drug treatment.

SAHA provides a synergistic cytotoxicity when combined with [2 NAs+Bu]

Of the three cell lines analyzed in this study, J45.01 exhibited the highest resistance to the growth-inhibitory effects of [2 NAs+Bu] combinations (Figure 1). We hypothesized that increasing histone modifications would sensitize J45.01 cells to these drug combinations. This hypothesis is based on our model that nucleoside analogs modulate histone 3 methylations which in turn results in chromatin relaxation and increased susceptibility of the genomic DNA to alkylators. Higher levels of modified histones would suggest more relaxed chromatin. We, therefore, examined the effect of increasing chromatin relaxation through histone acetylations using SAHA, a known HDAC inhibitor [32]. Exposure of J45.01 cells to low concentrations of [2 NAs+Bu] for 48 hr resulted in negligible effects on cytotoxicity (Figure 5A) and proportion of cells with sub-G1 DNA content (Figure 5B). Addition of 0.8 μ M SAHA to these formulations inhibited cell proliferation by as much as 60% and increased the proportion of cells with sub-G1 DNA content to as high as 53% (Figures 5A and 5B). Similar results were observed when Daudi cells were exposed to the same combinatorial formulations (data not shown). These results suggest that addition of SAHA to [2 NAs+Bu] results in a synergistic cytotoxicity in lymphoma cells.

To further confirm the efficacy of SAHA-containing formulations, we determined their effects on the phosphorylation of known ATM substrates. Phosphorylations of histone 2AX, SMC1 and KAP1 were all elevated when cells were exposed to [2 NAs+Bu+SAHA], suggesting robust activation of the DDR network (Figure 5C). The observed cytotoxicity (Figure 5A) is consistent with a decrease in the level of pro-survival c-MYC and an increase in apoptosis as suggested by the cleavage of PARP1 (Figure 5C). The efficacy of SAHA in inhibiting HDACs is shown by a corresponding increase in the acetylations of histone 3 at Lys9 (AcH3K9) and Lys14 (AcH3K14) and of histone 4 at Lys5 (AcH4K5) (Figure 5C). The observed increase in histone acetylations further correlates with a decrease in the level of HDAC4 and HDAC5 without a significant change in the level of HDAC1, suggesting a degree of specificity (Figure 5C). This SAHA-mediated decrease in HDAC4 and HDAC5 levels might be due to their sumoylation and subsequent proteosomal degradation, as was previously reported for breast cancer and osteosarcoma cell lines [33]. The observed increase in the methylations of histone 3 at Lys4 (3MeH3K4) is consistent with the previously reported interaction between histone lysine deacetylases and methyltransferases [34]. Overall, these results suggest that activation of the DDR network and post-translational modifications of histones contribute to the efficacy of [2 NAs+Bu+SAHA] in lymphoma cells.

DISCUSSION

To expand the benefit of HSCT to patients with advanced, chemotherapy-refractory lymphoma the development of more efficacious pre-transplant conditioning regimens is highly desirable. The present study expands our previous observation that synergistic cytotoxicity was achieved with double nucleoside analog combinations in AML, and highlights the general mechanisms underlying the effects observed with [2 NAs + Bu] to include combinations such as [Gem+Clo], [Gem+Flu] and [Clo+Flu] in lymphoma cell lines (Figure 1). Addition of a bifunctional DNA alkylating agent, Bu, to these combinations

synergistically enhances the cytotoxic effects (Figures 1A and 1C). These drugs may be combined with a HDAC inhibitor (e.g., SAHA) to further augment the cytotoxicity (Figure 5). Since similar responses were obtained in cell lines derived from B-cell as well as T-cell lymphomas, our findings suggest that [2 NAs+Bu] may be useful in pre-transplant conditioning regimens for most lymphoma patients, and further, that inclusion of SAHA, which in itself has very limited side effects, may be safely used for improving disease-control in patients who had a suboptimal response to previous chemotherapy.

The present results are consistent with our previous preclinical and clinical studies on the efficacy of [Bu+Clo+Flu] combinations in AML cell lines and AML patients undergoing HSCT [11, 13]. Our results provide a preclinical basis for further investigating the use of Bu, Clo, Flu, Gem and SAHA for lymphoma patients undergoing HSCT. In fact, clofarabine (Clolar®) is approved for treatment of pediatric ALL, and gemcitabine (Gemzar®) shows efficacy in different forms of lymphoma, both when used as a single agent and in combination with other drugs, as well as when included in conditioning therapy for HSCT [11, 12, 35–39]. Moreover, SAHA (Vorinostat®) is approved for the treatment of cutaneous T-cell lymphoma [40].

We previously reported strong activation of the components of the DDR and apoptotic pathways in AML cells exposed to low concentrations (equivalent to IC_{20}) of [Bu+Clo+Flu] combinations [13]. We obtained similar results when Daudi and U937 lymphoma cells were exposed to [2 NAs+Bu] (Figure 2 and data not shown). Addition of SAHA to this combination augments the effects in the same activated pathways in less sensitive J45.01 lymphoma cells (Figure 5). An apparent difference between our findings with lymphoma cells and our earlier studies with AML cells [13] relates to the extent of ATR-CHK1 activation by these drug combinations. The ATM-CHK2 and ATR-CHK1 pathways are usually both activated in cells exposed to most cytotoxic chemotherapy agents [25, 41], as was the case here with Daudi cells exposed to [2 NAs+Bu]. In our earlier studies with Bu-resistant KBM3/Bu250⁶ AML cells, the ATR-CHK1 pathway was only weakly activated by these drugs alone or in combination [13]; however, both phospho-ATR Ser428 and phospho-CHK1 Ser317 showed fairly strong signals even in the untreated control cells, possibly a reflection of ongoing genetic instability in KBM3/Bu250⁶ cells and/or the fact that these cells have acquired Bu resistance following repeated genotoxic stress. Thus, DDR pre-activation in control cells may have obscured the ATR-CHK1 activation by [2 NAs+Bu] seen in the earlier study.

The observed phosphorylation of KAP1 in Daudi cells (Figure 2) is notably relevant to our proposed mechanism of action of [Bu+Clo+Flu] whereby the nucleoside analogs mediate the relaxation of chromatin, increasing the susceptibility of the genomic DNA to formation of Bu-mediated DNA crosslinks [13, 15]. A previous report showed that DDR involves ATM-dependent chromatin relaxation which is effected by phosphorylation of KAP1 [20]. This suggests that exposure of lymphoma cells to the two nucleoside analogs activates ATM-KAP1 signaling, relaxes chromatin and enhances DNA alkylation. KAP1 phosphorylation may work in concert with histone modifications in mediating chromatin relaxation. Further, exposure of lymphoma cells to two nucleoside analogs causes histone 3 lysine methylations (Figure 4). These modifications of KAP1 and histone 3 and their impact on chromatin structure may subsequently affect DNA-related cellular transactions such as transcription, replication and repair, as well as apoptosis [42, 43] and likely contribute to the down-regulation of pro-survival proteins and activation of apoptosis (Figure 3).

The synergistic effects of SAHA may be partly attributed to increased histone modification by lysine acetylation, which is consistent with the above-mentioned mechanism of chromatin relaxation. Although SAHA, a pan-HDAC inhibitor, may also increase

acetylation of other proteins (such as transcription factors, molecular chaperones and cytoskeletal proteins), the observed increase in histone 3 and 4 acetylations correlates with its observed synergistic cytotoxicity with [2 NAs+Bu] and down-regulation of HDAC4 and HDAC5 (Figure 5).

In summary, our findings suggest that the cytotoxicity of [2 NAs+Bu+SAHA] combinations in lymphoma cells can be mechanistically attributed to the chromatin relaxation mediated by nucleoside analogs and SAHA and thus to an increased susceptibility of genomic DNA to Bu crosslinking. Although their efficacy in clinical transplantation remains to be investigated, the observed *in vitro* activity in inhibiting the proliferation of lymphoma cells and inducing apoptosis suggest that [2 NAs+Bu±SAHA] combinations should be evaluated as an alternative conditioning therapy for HSCT in patients with advanced, refractory lymphomas, analogous to our myeloablative, reduced-toxicity, conditioning program(s) for myeloid leukemia.

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References

1. Murawski N, Pfreundschuh. New drugs for aggressive B-cell and T-cell lymphomas. *Lancet Oncol.* 2010; 11:1074–1085. [PubMed: 21051020]
2. Swerdlow, S. International Agency for Research on Cancer, World Health Organization. WHO classification of tumours of haematopoietic and lymphoid tissues. 4. Lyon: IARC; 2008.
3. Coiffier B, Lepage E, Briere J, et al. CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. *N Engl J Med.* 2002; 346:235–342. [PubMed: 11807147]
4. Pfreundschuh M, Truemper L, Osterborg A, et al. MabThera International Trial Group. CHOP-like chemotherapy plus rituximab compared with CHOP-like chemotherapy alone in young patients with good-prognosis diffuse large B-cell lymphoma: a randomized controlled trial by the Mabthera International Trial (MInT) Group. *Lancet Oncol.* 2006; 7:379–391. [PubMed: 16648042]
5. Salit RB, Bishop MR, Pavletic SZ. Allogeneic hematopoietic stem cell transplantation: Does it have a place in treating Hodgkin lymphoma? *Curr Hematol Malig Rep.* 2010; 5:229–238. [PubMed: 20730513]
6. Srivastava S, Jones D, Wood LL, et al. A Phase I Trial of high-dose clofarabine, etoposide, and cyclophosphamide and autologous peripheral blood stem cell transplantation in patients with primary refractory and relapsed and refractory non-Hodgkin lymphoma. *Biol Blood Marrow Transplant.* 2010 Oct 20. Epub ahead of print.
7. Suyani E, Sucak GT, Aki SZ, Ye in ZA, Ozkurt ZN, Ya ci M. Gemcitabine and vinorelbine combination is effective in both as a salvage and mobilization regimen in relapsed or refractory Hodgkin lymphoma prior to ASCT. *Ann Hematol.* 2011; 90:685–691. [PubMed: 21072518]
8. Russell JA, Tran HT, Quinlan D, et al. Once-daily intravenous busulfan given with fludarabine as conditioning for allogeneic stem cell transplantation: study of pharmacokinetics and early clinical outcomes. *Biol Blood Marrow Transplant.* 2002; 8:468–476. [PubMed: 12374451]
9. de Lima M, Couriel D, Thall PF, et al. Once-daily intravenous busulfan and fludarabine: clinical and pharmacokinetic results of a myeloablative, reduced-toxicity conditioning regimen for allogeneic stem cell transplantation in AML and MDS. *Blood.* 2004; 104:857–864. [PubMed: 15073038]
10. Andersson BS, de Lima M, Thall PF, et al. Once daily i.v. busulfan and fludarabine (i.v. Bu-Flu) compares favorably with i.v. busulfan and cyclophosphamide (i.v. BuCy2) as pretransplant conditioning therapy in AML/MDS. *Biol Blood Marrow Transplant.* 2008; 14:672–684. [PubMed: 18489993]

11. Andersson BS, Valdez BC, de Lima M, et al. Clofarabine ± fludarabine with once daily i.v. busulfan as pretransplant conditioning therapy for advanced myeloid leukemia and MDS. *Biol Blood Marrow Transplant.* 2011; 17:893–900. [PubMed: 20946966]
12. Farag SS, Wood LL, Schwartz JE, et al. Phase I trial and pharmacokinetic study of high-dose clofarabine and busulfan and allogeneic stem cell transplantation in adults with high-risk and refractory acute leukemia. *Leukemia.* 2011; 25:599–605. [PubMed: 21252987]
13. Valdez BC, Li Y, Murray D, Champlin RE, Andersson BS. The synergistic cytotoxicity of clofarabine, fludarabine and busulfan in AML cells involves ATM pathway activation and chromatin remodeling. *Biochem Pharmacol.* 2011; 81:222–232. [PubMed: 20933509]
14. Plunkett W, Gandhi V. Purine and pyrimidine nucleoside analogs. *Cancer Chemother Biol Response Modif.* 2001; 19:21–45. [PubMed: 11686015]
15. Valdez BC, Andersson BS. Interstrand crosslink inducing agents in pretransplant conditioning therapy for hematologic malignancies. *Environ Mol Mutagen.* 2010; 51:659–668. [PubMed: 20577993]
16. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods.* 1983; 65:55–63. [PubMed: 6606682]
17. Kastan MB, Lim DS. The many substrates and functions of ATM. *Nat Rev Mol Cell Biol.* 2000; 1:179–186. [PubMed: 11252893]
18. Zhou BB, Elledge SJ. The DNA damage response: Putting checkpoints in perspective. *Nature.* 2000; 408:433–439. [PubMed: 11100718]
19. Kim S-T, Xu B, Kastan MB. Involvement of the cohesion protein, Smc1, in Atm-dependent and independent responses to DNA damage. *Genes Dev.* 2002; 16:560–570. [PubMed: 11877376]
20. Ziv Y, Bielopski D, Galanty Y, et al. Chromatin relaxation in response to DNA double-strand breaks is modulated by a novel ATM- and KAP-1 dependent pathway. *Nat Cell Biol.* 2006; 8:870–876. [PubMed: 16862143]
21. Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem.* 1998; 273:5858–5868. [PubMed: 9488723]
22. Reinhardt HC, Yaffe MB. Kinases that control the cell cycle in response to DNA damage: Chk1, Chk2, and MK2. *Curr Opin Cell Biol.* 2009; 21:245–255. [PubMed: 19230643]
23. Hirano T. SMC-mediated chromosome mechanics: A conserved scheme from bacteria to vertebrates? *Genes Dev.* 1999; 13:11–19. [PubMed: 9887095]
24. Stursberg S, Riwar B, Jessberger R. Cloning and characterization of mammalian SMC1 and SMC3 genes and proteins, components of the DNA recombination complexes RC-1. *Gene.* 1999; 228:1–12. [PubMed: 10072753]
25. Cimprich KA, Cortez D. ATR: an essential regulator of genome integrity. *Nat Rev Mol Cell Biol.* 2008; 9:616–627. [PubMed: 18594563]
26. Gaidano G, Ballerini P, Gong JZ, et al. p53 Mutations in human lymphoid malignancies - association with Burkitt lymphoma and chronic lymphocytic leukemia. *Proc Natl Acad Sci USA.* 1991; 88:5413–5417. [PubMed: 2052620]
27. Johnstone RW, Wei W, Greenway A, Trapani JA. Functional interaction between p53 and the interferon-inducible nucleoprotein IFI 16. *Oncogene.* 2000; 19:6033–6042. [PubMed: 11146555]
28. Liu G, Lozano G. p21 stability: linking chaperones to a cell cycle checkpoint. *Cancer Cell.* 2005; 7:113–114. [PubMed: 15710322]
29. Abbas T, Dutta A. p21 in cancer: intricate networks and multiple activities. *Nat Rev Cancer.* 2009; 9:400–414. [PubMed: 19440234]
30. Grandori C, Cowley SM, James LP, Eisenman RN. The Myc/Max/Mad network and the transcriptional control of cell behavior. *Annu Rev Cell Dev Biol.* 2000; 16:653–699. [PubMed: 11031250]
31. Varier RA, Timmers HT. Histone lysine methylation and demethylation pathways in cancer. *Biochim Biophys Acta.* 2010; 1815:75–89. [PubMed: 20951770]
32. Xu WS, Parmigiani RB, Marks PA. Histone deacetylase inhibitors: molecular mechanisms of action. *Oncogene.* 2007; 26:5541–5552. [PubMed: 17694093]

33. Scognamiglio A, Nebbioso A, Manzo F, Valente S, Mai A, Altucci L. HDAC-class II specific inhibition involves HDAC proteasome-dependent degradation mediated by RANBP2. *Biochim Biophys Acta*. 2008; 1783:2030–2038. [PubMed: 18691615]
34. Vaute O, Nicolas E, Vandel L, Trouche D. Functional and physical interaction between the histone methyl transferase Suv39H1 and histone deacetylases. *Nucleic Acids Res*. 2002; 30:475–481. [PubMed: 11788710]
35. Marchi E, Alinari L, Tani M, et al. Gemcitabine as frontline treatment for cutaneous T-cell lymphoma: phase II study of 32 patients. *Cancer*. 2005; 104:2437–2441. [PubMed: 16216001]
36. Duvic M, Talpur R, Wen S, Kurzrock R, David CL, Apisarnthanarax N. Phase II evaluation of gemcitabine monotherapy for cutaneous T-cell lymphoma. *Clin Lymphoma Myeloma*. 2006; 7:51–58. [PubMed: 16879770]
37. Zinzani PL, Venturini F, Stefoni V, et al. Gemcitabine as Single Agent in Pretreated T-cell Lymphoma Patients: Evaluation of the Long-term Outcome. *Annals Oncol*. 2010; 21:860–863.
38. Aribi M, Mesli N, Remla N, et al. Gemcitabine and treatment of diffuse large B-cell lymphoma in relapsed or refractory elderly patients: A prospective randomized trial in Algeria. *J Cancer Res Therap*. 2010; 6:41–46. [PubMed: 20479546]
39. Nieto Y, Anderlini P, Popat U, et al. Gemcitabine, busulfan and melphalan (GemBuMel) is a new high-dose chemotherapy (HDC) regimen with high activity in refractory Hodgkin's lymphoma (HL) patients receiving an autologous stem-cell transplant (ASCT): A contemporaneous comparison with BEAM and busulfan/melphalan (BuMel) [abstract]. *Blood*. 2010; 116(Supplement):Abstract 690.
40. Younes A. Beyond chemotherapy: new agents for targeted treatment of lymphoma. *Nat Rev Clin Oncol*. 2011; 8:85–96. [PubMed: 21151205]
41. Smith J, Tho LM, Xu N, Gillespie DA. The ATM-Chk2 and ATR-Chk1 pathways in DNA damage signaling and cancer. *Adv Cancer Res*. 2010; 108:73–112. [PubMed: 21034966]
42. Wang GG, Allis CD, Chi P. Chromatin remodeling and cancer, part I: covalent histone modifications. *Trends Mol Med*. 2007; 13:363–372. [PubMed: 17822958]
43. van Attikum H, Gasser SM. Crosstalk between histone modifications during the DNA damage response. *Trends Cell Biol*. 2009; 19:207–217. [PubMed: 19342239]

Figure 1.

Cytotoxicity of combinations of Bu and nucleoside analogs towards lymphoma cells. Cells were continuously exposed to drugs alone, or in combination, for 96 hr and analyzed by the MTT assay. Bu or B: busulfan; Clo or C: clofarabine; Flu or F: fludarabine; Gem or G: gemcitabine.

Figure 2.

Combinatorial formulations activate the DNA damage response. Daudi cells were continuously exposed to drugs alone, or in combination, for 48 hr and changes in protein expression or phosphorylation status were determined by immunoblot analysis. Bu or B: busulfan; Clo or C: clofarabine; Flu or F: fludarabine; Gem or G: gemcitabine.

Figure 3.

Drug-mediated activation of apoptosis. Daudi cells were continuously exposed to the various drugs alone, or in combination, for 48 hr and analyzed for apoptosis based on cell fractions in sub-G1 as determined by flow cytometry (A) and expression/activation of apoptosis- and survival-related proteins (B and C). Bu or B: busulfan; Clo or C: clofarabine; Flu or F: fludarabine; Gem or G: gemcitabine.

Figure 4.

Drug-mediated methylation of histone 3. Daudi cells were continuously exposed to the various drugs alone, or in combination, for 48 hr and analyzed for methylation of histone 3 at lysines 4, 9 and 27 and for expression of the histone demethylases JMJD1B, JARID1A and JARID1C/SMCX. Bu or B: busulfan; Clo or C: clofarabine; Flu or F: fludarabine; Gem or G: gemcitabine.

Figure 5.

Synergistic effects of SAHA with [2 nucleoside analogs + Bu]. J45.01 cells were continuously exposed to various drug combinations (as indicated) for 48 hr and analyzed for cytotoxicity by the MTT assay (A), cells with sub-G1 DNA content by flow cytometry (B), and protein levels, cleavage or phosphorylation/methylation/acetylation status by Western blot (C). Bu or B: busulfan; Clo or C: clofarabine; Flu or F: fludarabine; Gem or G: gemcitabine.

Table I

List of primary antibodies, their sources and dilutions

Antigen	Source/Cat. #	Clone type*	Dilution**
AcH4K5	Active Motif/39170	pAb	3000
AcH3K9	Active Motif/39138	pAb	3500
AcH3K14	Active Motif/39616	pAb	3000
ATM	Santa Cruz Biotech/23921	mAb	750
ATR	Cell Signaling/2790	pAb	2500
Caspase 9	Cell Signaling/9502	pAb	1500
CDK4	Cell Signaling/2906	mAb	2500
CHK1	Cell Signaling/2345	pAb	2000
CHK2	Cell Signaling/2662	pAb	2500
c-MYC	Cell Signaling/9402	pAb	2500
HDAC1	Cell Signaling/5356	mAb	3000
HDAC4	Cell Signaling/5392	mAb	3000
HDAC5	Cell Signaling/2083	pAb	2500
Histone 3	Abcam/1791	pAb	3000
JARID1A	Bethyl Laboratories/A300-897A	pAb	3000
JARID1C/SMCX	Santa Cruz Biotech/98701	pAb	700
JMJD1B	Cell Signaling/2621	pAb	2500
KAP1	Bethyl Laboratories A300-774A	pAb	3000
NOXA	Abcam/13654	mAb	2000
p21	Upstate Biotech/05-345	mAb	1000
p53	Santa Cruz Biotech/126	mAb	1000
PARP1	Santa Cruz Biotech/8007	mAb	1000
P-ATM (Ser1981)	Rockland/200-301-4000	mAb	2000
P-ATR (Ser428)	Cell Signaling/2853	pAb	2500
P-CHK1 (Ser317)	Cell Signaling/2344	pAb	2500
P-CHK2 (Ser19)	Cell Signaling/2666	pAb	2500
P-KAP1 (Ser824)	Bethyl Laboratories A300-767A	pAb	1500
P-p53 (Ser15)	Cell Signaling/9284	pAb	2000
P-p53 (Ser20)	Cell Signaling/9287	pAb	2000
P-SMC1 (Ser957)	Novus Biolog/NB100-205	pAb	2000
P-SMC3 (Ser1083)	Bethyl Lab, Inc/A300-480A	pAb	2000
RhoA	Cell Signaling/2117	mAb	2500
SMC1	Cell Signaling/4802	pAb	2500
SMC3	Millipore/AB3914	pAb	4000
XAF1	Santa Cruz Biotech/19193	pAb (goat)	500
β-ACTIN	Sigma/A5316	mAb	10000
γ-H2AX	Upstate Biotech/05-636	mAb	3000
3MeH3K27	Active Motif/39155	pAb	3500
3MeH3K4	Active Motif/39159	pAb	3000

Antigen	Source/Cat. #	Clone type*	Dilution**
3MeH3K9	Active Motif/39161	pAb	3500

* 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