Selective Targeting of Class1 Histone Deacetylase (HDAC) Isoforms by a Novel Inhibitor SBAK-GHA Potently Resist Leukemogenesis

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Research

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Abstract

**Background:** Acute promyelocytic leukemia (APL) and acute lymphoblastic leukaemia (ALL) are often presented with loss of H4K16 monoacetylation (ac) and H4K20 trimethylation (3Me) due to increased activity of Class I HDAC’s. In the current study we explored the efficacy and mechanistic basis of a novel Class I HDAC inhibitor SBAK-GHA across different leukemic cell lines and characterised the distinct acetylation pattern on histone H3 and H4.

**Methods:** We initially performed general and class specific HDAC enzyme activity assays to establish the effect of our lead molecule SBAK-GHA. Following, we have probed various acetylation sites to understand a thorough acetylation profile of various leukemic cell lines by immunoblotting. Next, to understand the effect of various Class 1 HDAC isoforms on acetylation levels of hallmark proteins in leukaemia; lentiviral knockdown approach was carried out. In addition, cell cycle analysis was also done to distinguish the pattern of cell cycle phase arrest, followed by Chip-qPCR studies of various cyclins and their relationship with cell cycle arrest. Finally, an *in vivo study was performed* to confirm the anti-leukemic activity of SBAK-GHA by using specific leukaemia models.

**Results:** SBAK-GHA showed class I HDAC inhibitor activity specifically targeting HDAC 2. SBAK-GHA treatment upregulates H4K16 ac and H4K20 me3 in variety of leukemic cell lines. Similar results were found during knock down of HDAC2 in leukemic cell lines. Moreover, we also observed a coherence of events like cell cycle arrest across different cell types of leukemias and modulation in the levels of acetylation across different cyclin promoters. Further on, studies in various *in vivo* cancer models demonstrated SBAK-GHA to be highly selective towards lymphocytic leukaemia.

**Conclusion:** Our data provided a basic overview of relationship between different class I HDAC isoforms and their possible roles in regulation of histone acetylation in pathogenesis of leukaemia. Our study here presented multiple evidences regarding SBAK-GHA as a novel HDAC2 inhibitor. SBAK-GHA resist leukemogenesis mainly by inducing the repressed H4K16 ac and H4K20 me3. Further, the results in present study had established a relationship between class I HDAC isoforms and their possible roles in regulation of histone acetylation in pathogenesis of leukaemia.

**Background**

Histone post-translational modifications are important players in determining the role of normal versus diseased state of the cell. The acetylation at lysine residues is one of the important posttranslational modifications that regulates cellular function and is controlled by the action of acetylase and deacetylase enzymes [1–3]. For decades, it has been appreciated that histone deacetylases (HDACs) play an important role in the cancer development.

Acute promyelocytic leukemia (APL) is the first disease wherein the mechanistic involvement of HDACs was established [4]. APL, a subtype of acute myeloid leukaemia (AML), is widely used as a model for understanding the role of HDACs in carcinogenesis [5, 6]. It has been observed that AML as well as acute
lymphoblastic leukaemia (ALL) pose increased challenges due to resistance to different chemotherapies [7–9]. In the last decade, a lot has been revealed regarding HDAC inhibitors (HDACi) but mechanistic pathways are still far from actual understanding. H4K16 monoacetylation (ac) and H4K20 trimethylation (me3 3Me) has been established as hallmarks of tumours [10]. Loss of acetylation on specific lysine residues of H3 and H4 is one of the key factors thought to be involved in the progression of carcinogenesis. Moreover, the loss of H4K16 acetylation has been shown to influence the tumor progression and sensitivity to chemotherapy [11, 12]. So, in order to combat these cancers it is important to devise new strategies, one such way is by modulating acetylations levels of histones. During the last decade four HDACi, SAHA, Romidepsin, Belinostat and Panobinostat have been approved by US FDA as drugs against various cancers [5, 6]. Besides, many HDACi are at advanced stages of clinical trials against a variety of solid and hematological cancers. However, in spite of clinical advantages like high specificity towards cancer cells and least drug resistance, HDACi are associated with undesirable effects like fatigue, diarrhea, bone marrow toxicity and thrombocytopenia [13]. Importantly the toxicity associated with HDACi in treatment regimen is mainly due to pan-HDAC inhibition activity and may be addressed by designing the class and isoform specific HDAC inhibitors [5]. Based on our earlier results [14], where we have reported the design, synthesis and biological evaluation of a library of natural product based novel HDAC inhibitor molecules against a panel of cancer cell lines along with normal cell line. Out of the library of possible HDAC inhibitors we found one lead molecule i.e. N1-hydroxy-N5-(3-α-hydroxy-11-oxo-24-norurs-12-en-4-yl) glutaramide (SBAK-GHA) highly active against leukemic cells, with class I isoform specificity. This selective targeting of class I HDACs enabled us to understand the changes in the chromatin acetylation induced by class I HDACs and their effect on the expression of cancer associated genes. It has earlier been reported that knockdown of class I HDAC isoforms induces growth arrest and cell death of APL cells [15]. Therefore, in this study, we comprehensively examined the acetylation patterns of lysine residues on H3 and H4 histone proteins upon treatment with our novel class I specific HDAC inhibitor i.e. SBAK-GHA in APL and ALL cells both in vitro and in vivo conditions.

Materials And Methods

Chemicals, antibodies and kits

Growth medium (RPMI-1640), Fetal Calf Serum (FCS), Trypsin, Penicillin, Streptomycin, DMSO, RNase, Proteinase K, RIPA buffer, Acrylamide, Bisacrylamide, SDS, Ammonium persulfate (APS), N, N, N’, N’ Tetramethylethylendiamine (TEMED), 2-Mercaptoethanol, Tris base, Propidium iodide, Protease inhibitor cocktail, Sodium Deoxycholate, EGTA, Salmon sperm DNA, Bovine Serum Albumin, Quanti-Pro BCA assay kit, NaCl, EDTA, Hexadimethrine bromide and Lentiviral constructs were obtained from Sigma Aldrich Pvt. Ltd. (St. Louis, MO, United States). Six well plates were obtained from Iwaki (Japan), Triton X-100, H3K9ac, H3K14ac, H3K18ac, H3K27ac, H4K5 ac, H4K12ac, H4K16ac and H4K20 me3 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Immobilon Western Chemiluminescent HRP Substrate, Protein A Agarose beads, H3 and H4 antibodies were purchased from Millipore (Billerica, MA, USA). Anti-HDAC 1, Anti-HDAC 2, Anti-HDAC3, and Anti-HDAC8 antibodies and HDAC isoform specific
fluorometric drug screening kit were procured from Biovision (Milpitas, CA, USA). SAHA and NaB were purchased from Selleck Chemicals Ltd (Houston, TX, USA).

**Cell culture and growth conditions**

Cell lines HL-60 and MOLT-4 were procured from European Collection of Authenticated Cell Cultures (ECACC), UK. Chemoresistant cell line Doxo-K562 was a kind gift from Dr. Ajay's Lab in CSIR-IIIM, India. All cell lines were maintained in RPMI-1640 medium supplemented with 10% FCS and culture media was supplemented with 1% Penicillin. Cells were cultured in humidified conditions at 37°C in CO2 incubator (New Brunswick, Galaxy 170R, Eppendorff) with internal atmosphere conditions of 95% humidity and 5% CO2.

**Histone extraction and Western blot analysis**

These experiments were performed as described previously [16] with certain modifications. HL-60, MOLT-4 and DOXO-K562 cells were seeded, incubated and harvested at different time points (6-48 h).

**Measurement of HDAC isoform specificity**

This assay was carried out as described previously [16] with certain modifications. SBAK-GHA and SAHA were used in experimental setup.

**DNA content and cell cycle analysis**

Cell cycle analysis was performed as described previously [16] with certain modifications. HL-60, MOLT-4 and DOXO-K562 cells were seeded, incubated and harvested at different time points (6-72 h for HL-60) and (24-48 h for MOLT-4 and DOXO-K562).

**Lentiviral Transduction**

For stable and transient transduction protocol, procedure of Sigma-Aldrich was followed with certain modifications. 1.6 x 10^4 HL-60 cells were added in fresh media to corresponding number of wells for construct in a 96-well plate. Triplicate wells for each lentiviral construct and control were used. 4-6 h of incubation at 37°C in a humidified incubator in an atmosphere of 5-7% CO2 was carried. The number of cells plated was accommodated to a confluency of 70% upon transduction. Hexadimethrine bromide (final concentration 8 mg/ml) to each well was added just before lentiviral addition. Multiplicity of infection (MOI) of different isoforms of Class 1 HDACS i.e. HDAC 1, HDAC 2, HDAC 3, HDAC 8, Non target control (NTC) and Luciferase positive control were standardized and then used singly or in combination for stable and transient transduction. The cells were incubated for 48-72 h before adding Puromycin for selection (kill curve for Puromycin was drawn and 5µg/ml was used) in case of stable cell line formation, Puromycin addition continued every 3-4 alternative days for one month. Knockdown of the selected target gene by stable transduction could not be validated by immunoblotting and microscopy due to the death of cells (data not shown). However, incubation period of 48-72 h for transient transduction was carried
out without Puromycin addition. Cells were harvested and knockdown of the target genes were validated by immunoblotting and microscopic based expression studies.

**Chromatin Immunoprecipitation (ChIP) assay**

Approximately $10 \times 10^6$ HL-60 and MOLT-4 cells were seeded in 100 mm dishes and cultured with different concentrations of SAHA and SBAK-GHA for 48 h. Briefly, cells were cross linked with 1% formaldehyde for 10 minutes at room temperature and cross linking reaction was stopped by adding glycine (125 mM). After centrifugation at 1000 rpm for 5 minutes, the cells were incubated in RIPA buffer in presence of protease inhibitors and sonicated to shear DNA to lengths between 200 and 800 base pairs. After sonication, centrifugation was done at 14,000 g for 20 minutes at 4°C. The supernatant was 10-fold diluted with ChIP dilution buffer (16.7 mM Tris-HCl pH 8.1, 167 mM NaCl, 1.2 mM EDTA, 0.01% SDS, 1.1% Triton X-100). A 10% aliquot of the total chromatin preparation was set aside and designated as the input fraction. Protein A agarose beads were blocked with salmon sperm DNA and 1 mg/ml BSA for 4 h at 4°C under constant agitation. The samples were pre-cleared by incubating with 50 μl of protein A agarose beads. Following centrifugation (1,000 rpm, 1 min, 4°C), sample supernatants were added with prescribed concentration of antibodies overnight at 4°C under constant agitation followed by addition of pre-cleared beads for 6 hours. After this step immuno-precipitates were collected. Washing was done and complexes were eluted with 200-300 μl elution buffer (0.1 M NaHCO3, 1% SDS and 10 mM DTT). After addition of 0.2 M NaCl, all samples, including input, were incubated at 65°C for 4 h to revert cross-linking. Following treatment with 100 μg/ml Proteinase-K, DNA was extracted by phenol-chloroform-isoamyl alcohol method. The immunoprecipitated DNA was quantified by Nano drop and results were analyzed by qPCR method.

**Quantitative real-time PCR**

Immunoprecipitated DNA was analyzed for cyclins by Real-Time PCR as per Khanday et al., (2013) with some modifications. Primers were designed using the online Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primerblast/) and cross-checked for primer self-dimerization and potential hairpin formation using Oligonucleotide Properties Calculator. Details of primers are given in (Table S1).

The quantified DNA was analyzed using Applied Biosystems Step One plus TM Real-Time PCR System under the conditions of 1 cycle of 95°C for 10 min, 40 cycles of 95°C for 15s, primer-specific annealing temperature for 1 min and followed by a melt curve of 95°C for 10s, 60°C for 1 min and 95°C for 15s. The PCR mixture contained 10 μL of SYBR Green master mix (Applied Biosystems, CA, USA), 500 nM of each primer and 1.5 mM of MgCl2 and 1 μl (5-20 ng) of DNA template for 20μl reaction.

The comparative Ct method ($ΔΔCT$) (Livak et al., 2001) was used for quantification of DNA immunoprecipitated with different specific Abs and IgG. Measurement of $ΔCt$ was performed in triplicates.
**In vivo anticancer activity**

SBAK-GHA was evaluated for its in vivo anti-cancer activity against P388 lymphocytic leukemia and L1210 lymphoid leukemia murine models. CD2F1 mice (18-23 g) from an in-house colony were used for this study. The animals were housed under standard husbandry conditions as per guide for the care and use of laboratory animals and fed with standard pellet diet and autoclaved water was given ad libitum. Approval of the Institutional Animal Ethics Committee, Indian Institute of Integrative Medicine, Jammu was sought for the study and number of animals used in all the experiments. P388 and L1210 were obtained from the peritoneal cavity of DBA/2 mice harboring 6-10 days old ascitic tumor. 2.5×10⁶ cells were injected in the peritoneal cavity of each mouse on day 0. Intraperitoneally injected animals were randomized on the next day and assigned to different groups containing six animals in each group. Test group first was treated with SBAK-GHA at a dose of 25mg/kg i.p. test group second was treated with SAHA at a dose of 50mg/kg i.p. test group third was treated with 5-FU at a dose of 20mg/kg i.p. which served as positive control. Tumor bearing control group was administered normal saline (0.2 ml, i.p.). Treatment lasted for nine consecutive days and Median Survival Time was taken as the Parameter of Effect. Median Survival Time can be calculated by the following formula.

\[
\text{Median Survival Time} = \frac{\sum S + 6S_5 - 19N}{S_5 - NT}
\]

Where, \(S_5\) = No. of survivors on Day 5, \(\sum S\) = Sum of daily survivors from day 6 to day 18, \(NT\) = no. of no takes (survivors beyond day 18)

Acceptable Control Median Survival Time: 9-13 days

Minimum criteria of activity: \(T/C < 86\%\) Toxicity

\(T/C \geq 130\%\) Moderate Activity

\(T/C \geq 175\%\) Significant Activity

**Statistical evaluation**

The results of experiments were expressed as the Mean ± S.D. Statistical evaluation was performed using students’ unpaired t-test \(*p \leq 0.05\), \(**p \leq 0.01\), \(***p \leq 0.001\) for each analysis.

**Results**

**SBAK-GHA induces differential acetylation of H3 and H4 at multiple Lysine residues in a time dependent manner**

Acetylation on different lysine residues of H3 and H4 has been found to be deregulated in cancer cells and this property has been harnessed for therapeutic window of epidrugs like various HDAC inhibitors (HDACi). Here a time dependent study was conducted to examine the effect on acetylation of different lysine residues on H3 and H4 upon treatment with different HDACi like SAHA, Sodium Butyrate (NaB),
SBAK-GHA and parent compound of SBAK-GHA i.e. SBAK-NH2 in two different leukemic cell lines HL-60 and MOLT-4. Untreated HL-60 cells (Negative control) showed minimal H3 and H4 acetylation on different H3 residues-K9, K14, K27 and H4 residues-K5, K12 and K16. In comparison to untreated HL-60 cells, SAHA(1 µM ) treated cells showed increase in acetylation at H3; K14, K27 and H4K16 by 82, 60 and 52% by 6 h and 60, 82 and 43 by 24 h, though acetylation levels were retained till 48 h but the levels declined to 15, 18 and 33% respectively(Fig 1A). Cells treated with NaB (0.9 mM) and SBAK-NH2 (6 µM) showed decrease in acetylation by (2-8%) lower than the control at 6 h but by 24 h increase in acetylation (2-50%) on most lysine residues of H3 was found. At 48 h acetylation was retained in the range of 14-25% on all H3 residues except for H3K14, where 10% decrease in acetylation was found in SBAK-NH2 treated cells (Fig 1A). Similarly NaB and SBAK-NH2 treatment over the period of time from 6- 48 h induced acetylation in the range of 11-30% in most of the H4 lysine residues except for H4K12, where decrease of acetylation in the range of 3-10% was found. In comparison to untreated cells, SBAK- GHA treated cells at 1.5 and 6 µM concentrations showed increase in induction of acetylation by 5- 80% on most of the H3 and H4 lysine residues from 6-48 h( Fig 1A and B). The effect of SBAK-GHA on overall modulation of acetylation was more rapid and profound on most of the lysine residues as compared to cells treated with other HDACi (Figure 1 and densitometry analysis shown in Supplementary Figure S1). The change in acetylation at various H3 and H4 lysine residues is given in Supplementary Table S2. Similarly, in MOLT-4 cell line, in comparison to untreated cells (Negative control), increase in acetylation level of cells treated with SBAK-GHA (12 µM) was again found to be more rapid and profound than SAHA (Figure 1 C and D and densitometry analysis shown in Figure S1). The change in acetylation at various H3 and H4 lysine residues is given in Table S3.

In comparison to untreated cells SAHA in combination with SBAK-GHA (1.5 µM) at 24 h showed increase in acetylation in range of 2-61% at H3; K9, K27 and H4; K12, K16 and decrease of acetylation in the range of 13-24% at H3; K14, K18. Similarly at 48 h increase in acetylation in the range of 5-46% at H3; K9, K14, K18 and H4; K12, K16 and decrease in acetylation by 13% at H3K27 was observed. However in comparison to untreated cells, SAHA in combination with SBAK-GHA (6 µM) at 24h showed increase in acetylation in the range of 6-67% at H3; K9, K27 and H4; K12, K16 and decrease in acetylation by 16 and 6% at H3; K14, K18. At 48 h increase in acetylation in the range of 35-73% at H3; K9, K14, K27 and decrease in acetylation by 8% at H3K18 was observed respectively (Figure 2 A and B and densitometry analysis shown in Figure S2 and detailed change in acetylation given in Table S4).

In order to substantiate our results, we further tested our molecule of interest SBAK-GHA singly and in combination with SAHA in chemoresistant leukemic cell line DOXO-K562. In comparison to untreated cells, SBAK-GHA treated cells showed increase in acetylation by 42 and 47% at 24 h and 48 and 12 % at 48 h in comparison to 22 and 22% at 24 h and 22 and 28% at 48 h of SAHA treated cells at H4; K5 and K16 respectively. In comparison to untreated cells, SBAK-GHA in combination with SAHA treated cells showed increase in acetylation by 42 and 20% at 24 h and 52 and 13 % at 48 h at H4; K5 and K16 respectively. It was found that SBAK-GHA increased and retained the level of acetylation at H4; K5 and K16 better than SAHA. Moreover when used in combination it was seen to potentiate SAHA in retaining H4K5 and H4K16 acetylation levels for as long as 48 h (Figure 2 C and densitometry analysis shown in
Figure S2 and detailed change in acetylation given in Table S5). Hence taken together these results indicate therapeutic potential and efficacy of SBAK-GHA.

**SBAK-GHA is a Class 1 HDAC isoform inhibitor**

We have previously reported the HDAC inhibitory properties of SBAK-GHA\(^1\), but the differences in the pattern of acetylation of different lysine residues of H3 and H4 led us to perform HDAC isoform enzymatic assay. SBAK-GHA was evaluated against different HDAC isoforms with SAHA used as a positive control. The results are shown in Table 1. Interestingly SBAK-GHA exhibited HDAC isoform specificity and was found to be most effective against HDAC isoforms 2, 8, 1 and 3 with IC\(_{50}\) values 200, 230, 250 and 300 nM respectively. It is worth noting SBAK-GHA showed more potent inhibition of HDAC 2 and 8 as compared to commonly used HDACi SAHA. Moreover, SBAK-GHA exhibited more than 10-30 fold inhibitory activity towards Class I HDAC isoforms in comparison to Class II HDAC isoforms. These results indicate that SBAK-GHA is more selective towards class I HDAC isoforms as compared to other classes of HDACs.

**Knockdown of HDAC2 up regulates H4K20 (3Me) and H4K16 acetylation in HL-60 cells.**

To further verify that SBAK-GHA is specific to Class I HDAC and the effects seen are due to inhibition of Class I HDACs and not due to off target effects. We decided to silence Class I HDAC isoforms such as HDAC 1, HDAC 2, HDAC 3 and HDAC 8 by shRNA. HL-60 cell lysates were prepared and tested for potential expression of these HDAC Class I isoforms before silencing. Both untreated and treated cells with SBAK-GHA, SAHA and SBAK-NH2 showed the expression of class I HDAC isoforms (Figure 3A).

To knock down HDAC isoforms, HL-60 cells were transduced by lentiviral particles against various isoforms of Class 1 HDAC isoforms was carried, unfortunately it did not result in stable clones of any of the Class I HDAC isoforms, possibly due to their non-redundant nature. In order to overcome this problem transient silencing was carried out for 48-72 h, which successfully led to knockdown of all Class 1 HDAC isoforms in HL-60 cells. Lentiviral constructs containing Turbo Green fluorescent protein (Turbo GFP) was used as a control to confirm transient infection. After incubation for 48-72 h, transduced cells expressed green fluorescence under fluorescent microscope (Figure 3 B), confirming the transduction of cells. The knock down of all Class 1 HDAC isoforms was confirmed by immunoblotting using antibodies against Class 1 HDAC isoforms (1, 2, 3 and 8). We observed that most of the Class 1 HDAC isoforms were knocked down at 48-72 h (Figure 3 C). After the confirmation of transient transduction, the amount of protein lysates obtained was too low to perform detailed studies, therefore two of the epigenetic hallmarks of cancer viz. H4K16 acetylation and H4K20 (3Me) were assayed by using specific antibodies against them by performing immunoblotting. It was observed that knockdown of HDAC1 and HDAC8 genes upregulated H4K20 (3Me) trimethylation but not H4K16 acetylation. On the contrary, knockdown of HDAC2 upregulated both H4K20 (3Me) and H4K16 acetylation. However, no change was observed upon knockdown of HDAC3 (Figure 3 D). It is worth mentioning that SBAK-GHA was most effective against HDAC2 in in vitro results. Thus corroborating our results that inhibitor is very specific.
Modulation of cell cycle arrest by SBAK-GHA

HL-60 cells were incubated with different concentrations of SBAK-GHA for different time points from 6 to 72 h. 1 µM SAHA and 0.9 mM NaB were used as a positive controls. The distribution of cells in different phases of cell cycle was analyzed by flow cytometry. We observed that the inhibition by SBAK-GHA modulated the relative proportion of cells in different phases of cell cycle (Figure 4 A- 4 B). At 6 h apart from NaB treated HL-60 cells which showed 83% G₁ arrest there was not significant change with other molecules. However at 12 h we observed that cells treated with SAHA showed prominent G₁ arrest with 93%, NaB 80%, SBAK-NH2 77% and so did SBAK-GHA at 6 µM with 77%. However SBAK-GHA at 1.5 µM did not showed any prominent G₁ arrest though cells at same concentration shifted towards S phase arrest with 49%. As time progressed we observed that SAHA treated cells were still showing G₁ arrest but number of cells dropped from 93% to 62%, while as cells treated with NaB were still exhibiting 84% G₁ arrest. Interestingly cells treated SBAK-NH2 showed decrease in G₁ arrest from 77% to 47% and cells shifted towards S phase with 53%. However SBAK-GHA at both 1.5 µM and 6 µM showed prominent G₁ arrest with 76% and 93% G₁ arrest respectively. Similarly SAHA at 48 h showed 65.3% G₁ arrest and SBAK-GHA at both 1.5 µM and 6 µM showed prominent G₁ arrest with 74.5% and 92% G₁ arrest respectively. With increase of time period as long as 72 h cells treated with NaB and SBAK-NH2 cells shifted from G₁ to G₂ phase with 91% and 86% G₂ arrest respectively. But cells treated with SAHA at 72 h were still showing G₁ arrest of 56%. Interestingly SBAK-GHA at both 1.5 µM and 6 µM was still showing prominent G₁ arrest with 78% and 77% respectively (detailed % inhibition are given in Table S6). Shift in growth arrest of cells from G₁ to other phases like G₂ and S phase over the period of time could be due to increase in apoptotic population.

Likewise MOLT-4 cells treated with SAHA, NaB and SBAK-GHA showed prominent G₁ arrest (Figure S3 and Table S7) with slight increase in cell number in S phase at late hours.

However, G₂ arrest was found to be prominent in DOXO-K562 cells treated with SAHA and SBAK-GHA singly as well as in combination (Figure S4 and detailed % inhibition of cells in different phases are given in Table S8). Here it is worth to note the change in pattern of cell shift from G₁ to G₂ phase unlike other cell lines like MOLT-4 and HL-60, where G₁ arrest was prominent.

SBAK-GHA modulates acetylation levels at the chromatin associated with cyclins A1, D1 and E gene promoters

Since cell cycle is regulated by the interaction between cyclins and cyclin dependent kinases (cdks), hence the promoter acetylation of cyclins was examined. HL-60 cells were incubated with 6µM SBAK-GHA and 1µM SAHA for 48 hours and subjected to chromatin immunoprecipitation. Chromatin fragments were immunoprecipitated by H3K9, H3K14, H3K27, H4K5 and H4K16 acetylation antibodies. DNA was isolated from immunoprecipitated fragments and promoter regions of cyclin A1, D1 and E were amplified using qPCR. We found that in comparison to negative control (IgG), SBAK-GHA treated cells showed 4, 7
and 3 fold enrichment of H3K9, H3K14 and H3K27 acetylation respectively in the promoter region of cyclin A1 using H3K9, H3K14 and H3K27 acetylation antibodies respectively. 10 and 2 fold decrease in enrichment of H3K9 and H3K27 ac at the promoter sequence of cyclin D1 using anti H3K9 and H3K27 antibodies. However, 7 fold increase in enrichment of H3K14ac at the promoter sequence of cyclin using anti H3K14 ab was found (Figure 5 A and B). For cyclin E, in comparison to negative control (IgG), SBAK-GHA treated cells showed 14, 6 and 9 fold increase in the enrichment of promoter sequences in association with acetylation at H3K9, H3K14 and H3K27 lysine residues. Moreover, in SAHA treated cells for cyclins A1, D1 and E in comparison to IgG, 12, 13 and 25 fold increase in the enrichment of promoter sequences in association with acetylation at H3K9 lysine residue was observed. Further, cells treated with SAHA, 5 fold decrease, 2 and 3 fold increase in enrichment of promoter sequence of cyclins A1, D1 and E promoters respectively in association with acetylation at H4K5 were observed. However in cells treated with SBAK-GHA 3 and 5 fold decrease in enrichment of promoter sequence of cyclins A1, D1 and approximately no change in cyclin E was observed. Moreover 2 fold increase in cyclins A1 and E and 2 fold decrease for cyclin D1 enrichment at promoter sequence of H4K16 was found.

The enrichment of promoter region of different cyclins like A1, D1 and E in association with H3K9, H3K27, H4K5 and H4K16 lysine residues of H3 and H4 histones in MOLT-4 cells was also examined (Figure 5(C)). Interestingly, in comparison to untreated cells (negative control), in SBAK-GHA treated cells 15, 20 and 90 fold increase in promoter enrichment of cyclins A1, D1 and E in association with acetylation at H3K9 lysine residues were observed, while 2, 3 and 2 fold decrease in enrichment of promoter region of cyclins A1, D1 and E were observed due to acetylation at H3K27 lysine residue. Also 2 fold decrease and approximately 2 fold increase in enrichment of promoter sequence of cyclin A1 was observed due to acetylation at H4K5 and H4K16 lysine residues. Likewise, 2 fold increase and 10 fold decrease in enrichment of promoter sequence of cyclin D1 were observed due to acetylation at H4K5 and H4K16 lysine residues. More importantly cyclin E was found to be upregulated by 3 and 4 fold at the promoter sequence of H4K5 and H4K16 lysine residues.

Taken together, these results indicated that SBAK-GHA modulated the expression of cyclins at transcriptional level perhaps explaining the cell cycle arrest in different phases, observed earlier.

**Evaluation of SBAK–GHA for its *in vivo* anti-cancer activity**

SBAK-GHA showed very significant activity against lymphocytic leukaemia P388 model and showed median survival time of 18 days with 184.61% tumor control at 25mg/kg i.p and SAHA showed same median survival time of 19 days with 190.5% tumor control at 50mg/kg i.p. Moreover 5-FU showed median survival time of 19 days with 197.9% tumor control at 20mg/kg i.p. However parent compound SBAK-NH2 exhibited cytotoxicity and no mice survived beyond day 8 and mice treated with normal saline also died on day 9 (Table 2A). Importantly in comparison to P388 model, SBAK-GHA showed moderate activity in lymphoid leukemia model (L1210), where median survival time of 18 days with 104.0% tumor control in comparison of median survival time of 19 days with 231.7% tumor control for 5-Fu was
observed (Table 2B). These results were very promising, indicating potential therapeutic efficacy of SBAK-GHA.

**Discussion**

It has been reported that knock down of class I HDACs in APL cells induced growth arrest and apoptosis [7, 12]. Deregulation of histone acetylation has been found in many neoplasms, particularly in leukemia [17]. A possible novel therapeutic approach to restore the deregulated histone acetylation was achieved to a great extent by the introduction of HDAC inhibitors (HDACi). But mechanism of action of these HDACi is still a debatable topic. Acetylation on various lysine residues of histone H3 and H4 has been shown to play an important role in defining the transcriptional state of a cell e.g. H3K9 acetylation is associated with active transcription [18]. It has been observed that induction of acetylation and its maintenance with passage of time varies with different HDACi. In order to understand the potential of different HDACi like SAHA, NaB and our novel Class I HDAC inhibitor SBAK-GHA, we carried out time dependent study to observe the acetylation pattern of the different residues of histone H3 and H4 viz H3K9, H3K14, H3K27 and H4K5, H4K12 and H4K16 across a range of leukemic cell lines viz HL-60, MOLT-4 and chemoresistant DOXO-K562 using immunoblotting. In HL-60 cells, gain in acetylation at most of the residues of H3 and H4 was found to be most profound and prominent due to SBAK-GHA treatment. Similarly in MOLT-4 SBAK-GHA was able to induce early acetylation and retained the acetylation levels till 48 h as was found in HL-60. Combination of SAHA and SBAK-GHA showed synergistic action in HL-60 cells with respect to modulation of acetylation levels of different H3 and H4 lysine residues, especially of H4K16. SBAK-GHA potentiated the activity of SAHA in combination as acetylation was retained till 48 h. Taken together our results indicated that SBAK-GHA modulated the acetylation levels of specific lysine residues on H3 and H4 and had the potential to remodel the heterochromatin to euchromatin which can enhance the expression of the otherwise silenced genes like p53, p21, RB family members and many such genes responsible for cancer progression as shown by others like Ropero et al., 2007, Singh et al., 2010 and Mrakovcic et al., 2017 [19–21].

It has been well established that loss of monoacetylation at H4K16 and trimethylation at H4K20 are hallmarks of cancer [10]. H4K16ac is considered to be the main contributor of total H4 acetylation and its association with DNA damage, repair and cell senescence [22–25] apart from disruption of higher order chromatin structures is well known [26]. In recent past it came into fore that loss of H4K16 acetylation is responsible for the tumor progression and sensitivity to chemotherapy [8]. In order to evaluate the effect of SAHA and SBAK-GHA on chemo-sensitivity, DOXO-K562 cells were treated with these molecules singly and in combination. It was found that SBAK-GHA modulated acetylation singly and in combination better than SAHA. Retention in acetylation of SAHA treated cells was enhanced in combination with SBAK-GHA for as long as 48 h. These results indicated that loss of sensitivity to chemotherapy due to loss of acetylation at H4K16 could be overcome by use of SBAK-GHA. The outcome of this result further supported the therapeutic potential of SBAK-GHA. SBAK-GHA was more active and selective towards class I HDAC isoforms as compared to other classes of HDACs with maximum activity against HDAC isoforms 2, 8, 1 and 3 respectively. Class 1 isoform specific nature of SBAK-GHA may be the reason
behind its efficient regulation of acetylation of different H3 and H4 residues, where specific HDAC isoforms of a given class may be playing a primary role.

Presence of many HDAC isoforms in eukaryotic cells raises a larger question of their redundancy. Deletion of each isoform of class I HDACs in mice led to lethality with defects like improper heart muscle formation, degenerated brain formation and other such defected organ formations, which control vital physiological roles, hence in turn demonstrated the unique roles of these Class 1 HDAC isoforms [27]. Since SBAK-GHA was found to be class I HDAC inhibitor, it became imperative to validate the role of different class I HDAC isoforms on acetylation of different lysine residues of H3 and H4 during carcinogenesis. We successfully knocked down all HDAC class I isoforms in HL-60 cells, and chose to study two hallmark histone modifications in cancer i.e. H4K16 acetylation and H4K20 (3Me). We have already discussed the importance of H4K16ac but H4K20 me3 is another important histone modification associated with cancers and is infact the only lysine in the tail of H4 that is methylated. Trimethylation of H4K20 is a marker of constitutive heterochromatin, gene silencing [28, 29] and aging [30]. Defects in the DNA methylation may be related to the generation of genomic aberrations [31, 32], however the imbalanced and defective pattern of histone modifications still could be reversed. Our results indicate the importance of different Class I HDACs in regulation of H4K16 acetylation and tri methylation at H4K20. For the first time in this paper we are reporting that H4K16 acetylation was affected by knock down of HDAC2 only. It is worth noting that the possible involvement of HDAC2 in regulation of H4K16ac could lead to new drug target in cancer therapeutics.

Numerous transcription factors, including regulators of cell cycle, differentiation and development have been shown to associate directly with HDAC1 and HDAC2 or with HDAC1/HDAC2 complexes, thereby mediating the repression of specific target genes [14–16]. Therefore, both HDAC1- and HDAC1/HDAC2-mediated chromatin modifications seemed to be important for cell cycle control and development. These modifications if dysregulated may induce cell cycle arrest either at G1 or G2/M and apoptosis, killing tumor cells [33–35]. Usually G1 phase growth arrest induced by HDAC inhibition is attributed to acetylation dependent increase in expression of cyclin dependent kinase inhibitor p21waf1, while as G2 phase growth arrest is usually attributed to reactivation of checkpoint kinase 1 (Chk1) [36, 9]. The pathway leading to tumor cell death is still unknown, but it is very unlikely that a single molecular pathway is involved in different cell types for all HDAC inhibitors. It has been reported that HDAC1 null cells can arrest either at the G1 phase of the cell cycle or at the G2/M transition, resulting in the loss of mitotic cells, cell growth inhibition and an increase in the percentage of apoptotic cells [35]. We investigated the effect of inhibition of class I HDACs by SBAK-GHA on growth of leukemic cells in time dependent manner. Like gene knock down of class I HDAC isoforms, SBAK-GHA also induced growth arrest in HL-60 cells. We observed a prominent G1 arrest in HL-60 cells treated with HDACi with slight shift towards G2 phase at 72 h. Likewise in MOLT-4, a prominent G1 arrest was observed in SBAK-GHA treated cells with slight shift in cells from G1 to S phase at later time points. However DOXO-K562 cells prominent G2 arrest was observed with increase in time. The role of class I HDACs has already been appreciated in cell cycle arrest, as class1 HDAC inhibitor Romidepsin leads to G1 or G2/M arrest and our data is in
agreement with others [37–39], further strengthening our observation that SBAK-GHA acts by inhibiting class I HDACs.

It is well known that cell cycle is regulated by cyclin-cyclin-dependent kinase (CDK) interactions and cyclin A1, D1 and E are some of main players involved in cell cycle control. However, the direct role of direct histone acetylation in the modulation of expression of cyclins is unknown [40]. In HL-60 and MOLT-4 cells ChIP-qPCR results showed that the expression of cyclins was indeed controlled by acetylation induced of H3 and H4 in promoter regions of different cyclins on treatment with SBAK-GHA. Prominent G	extsubscript{1} arrest in HL-60 and MOLT-4 cells could be due to decrease in expression of cyclin D1. HL-60 cells treated with SBAK-GHA and immunoprecipitated with H3K9, H4K5 and H4K16 associated with promoter sequence of cyclin D1 showed its decreased expression. Similar results were observed in MOLT-4 cells, where cells treated with SBAK-GHA and immunoprecipitated with H4K16 associated with promoter sequence of cyclin D1 also showed its decreased expression.

Our results that decreased enrichment of cyclin D promoter sequence when immunoprecipitated with various antibodies of active mark especially H4K16 ac, perhaps explaining G	extsubscript{1} arrest. Previous studies have shown that histone deacetylation occurred at promoters of p21CIP1, p27KIP1, pRb genes in cells undergoing terminal growth arrest and senescence phenotype [41, 42]. Moreover, a well-known HDAC inhibitor butyrate has also been reported to mediate histone deacetylation and repression of various genes like TRAIL, DR5, Fas, Fas-L and TNF\textalpha that promote cell growth [43]. Our results for the first time have shown direct relation between histone acetylation and modulation of expression of cyclins important for cell growth.

Generally HDAC inhibitors derived from natural sources have limited retention time and are unstable under in vivo conditions [36]. The clinical use of FDA approved HDAC inhibitors SAHA, Romidepsin, Belinostat and Panobinostat is limited by their toxicity [3]. Moreover, HDAC inhibitors like TSA, Trapoxin and Depudecin from natural sources have shown toxic effects under in vivo conditions [44], which may be mainly attributed to their pan HDAC inhibition. Two of the most well characterized murine models to study leukemia are the P388 lymphocytic leukemia and the L1210 lymphoid leukemia models [37]. Earlier it has been reported that proportion of G	extsubscript{1} and G	extsubscript{2} cells increased at the expense of a reduced S phase fraction in the P388 Leukemia, whereas only small changes in cell cycle distribution were seen with time after inoculation of L1210 cells [45]. So we evaluated activity of SBAK-GHA in these model systems and it showed very significant and comparable activity to SAHA and 5-FU against lymphocytic leukemia P388 model, whereas moderate activity in lymphoid model L1210 was observed. However, the correlation between different HDAC inhibitors on cell cycle arrest in different phases with their possible dependency on acetylation of cyclin promoter, under in vivo models remains a challenge. Interestingly low toxicity in SBAK-GHA treated animals indicated that specific inhibition of class I HDAC isoforms may alleviate toxicity associated with most of the natural or synthetic Pan HDACi reported earlier.

Conclusions
Taken together, our findings conclusively demonstrate that SBAK-GHA, class1 HDAC inhibitor could be used as possible therapeutic agent against lymphocytic leukemia or could be used in a combinatorial approach with SAHA to yield better results. Moreover, further insights are needed to fully establish the mechanistic paradigm of SBAK-GHA for future pre-clinical development.

**Abbreviations**

APL: Acute promyelocytic leukemia; AML: Acute myeloid leukaemia; ALL: Acute lymphoblastic leukaemia (ALL)

APS: Ammonium persulfate

FCS: Fetal Calf Serum

HDAC: Histone deacetylase; HDACi: HDAC inhibitors

SBAK-GHA : N^1^-hydroxy-N^5-(3-α-hydroxy-11-oxo-24-norurs-12-en-4-yl) glutaramide

SAHA: Suberoylanilide hydroxamic acid

TEMED: N, N, N', N' Tetramethyl ethylenediamine ,

qPCR: Quantitative real-time PCR

**Declarations**

**Ethics approval and consent to participate**

Not applicable

**Consent for publication**

All authors consent for the publication of the current work

**Availability of data and materials**

Not applicable

**Competing interests**

None

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Authors' contributions

Study design and data collection: JAB, NJD, MA, MJ, AH, NC, DMM and AH; JAB and NJD mainly performed the experiments. Data analysis: SS, BAS, MIK and AH; Manuscript preparation: JAB, NJD and AH. All authors read and approved the final manuscript.

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References

1. Millard CJ, Watson PJ, Celardo I, Gordiyenko Y, Cowley SM, Robinson CV, Fairall L, Schwabe JW. Class I HDACs share a common mechanism of regulation by inositol phosphates. Molecular cell. 2013 Jul 11;51(1):57-67.
2. Cohen I, Poręba E, Kamieniarz K, Schneider R. Histone modifiers in cancer: friends or foes?. Genes & cancer. 2011 Jun;2(6):631-47.
3. Sadakierska-Chudy A, Filip M. A comprehensive view of the epigenetic landscape. Part II: Histone post-translational modification, nucleosome level, and chromatin regulation by ncRNAs. Neurotoxicity research. 2015 Feb 1;27(2):172-97.
4. Marks PA, Xu WS. Histone deacetylase inhibitors: Potential in cancer therapy. Journal of cellular biochemistry. 2009 Jul 1;107(4):600-8.
5. Jones PA, Baylin SB. The epigenomics of cancer. Cell. 2007 Feb 23;128(4):683-92.
6. Ceccacci E, Minucci S. Inhibition of histone deacetylases in cancer therapy: lessons from leukaemia. British journal of cancer. 2016 Mar;114(6):605-11.
7. Vlasáková J, Nováková Z, Rossmeislová L, Kahle M, Hozák P, Hodný Z. Histone deacetylase inhibitors suppress IFNα-induced up-regulation of promyelocytic leukemia protein. Blood. 2007 Feb 15;109(4):1373-80.
8. Castro PG, van Roon EH, Pinhancos SS, Trentin L, Schneider P, Kerstjens M, Te Kronnie O, Pieters R, Stam RW. The HDAC inhibitor panobinostat (LBH589) exerts in vivo anti-leukaemic activity against MLL-rearranged acute lymphoblastic leukaemia and involves the RNF20/RNF40/WAC-H2B ubiquitination axis. Leukemia. 2018 Feb;32(2):323-31.
9. Fiskus W, Sharma S, Shah B, Portier BP, Devaraj SG, Liu K, Iyer SP, Bearss D, Bhalla KN. Highly effective combination of LSD1 (KDM1A) antagonist and pan-histone deacetylase inhibitor against human AML cells. Leukemia. 2014 Nov;28(11):2155-64.
10. Fraga MF, Ballestar E, Villar-Garea A, Boix-Chornet M, Espada J, Schotta G, Bonaldi T, Haydon C, Ropero S, Petrie K, Iyer NG. Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. Nature genetics. 2005 Apr;37(4):391-400.
11. Barbetti V, Gozzini A, Cheloni G, Marzi I, Fabiani E, Santini V, Dello Sbarba P, Rovida E. Time-and residue-specific differences in histone acetylation induced by VPA and SAHA in AML1/ETO-positive leukemia cells. Epigenetics. 2013 Feb 1;8(2):210-9.

12. Meisenberg C, Ashour ME, El-Shafie L, Liao C, Hodgson A, Pilborough A, Khurram SA, Downs JA, Ward SE, El-Khamisy SF. Epigenetic changes in histone acetylation underpin resistance to the topoisomerase I inhibitor irinotecan. Nucleic acids research. 2017 Feb 17;45(3):1159-76.

13. Subramanian S, Bates SE, Wright JJ, Espinoza-Delgado I, Piekarz RL. Clinical toxicities of histone deacetylase inhibitors. Pharmaceuticals. 2010 Sep;3(9):2751-67.

14. Sharma S, Ahmad M, Bhat JA, Kumar A, Kumar M, Zargar MA, Hamid A, Shah BA. Design, synthesis and biological evaluation of β-boswellic acid based HDAC inhibitors as inducers of cancer cell death. Bioorganic & medicinal chemistry letters. 2014 Oct 1;24(19):4729-34.

15. West AC, Johnstone RW. New and emerging HDAC inhibitors for cancer treatment. The Journal of clinical investigation. 2014 Jan 2;124(1):30-9.

16. Ahmad M, Aga MA, Bhat JA, Kumar B, Rouf A, Capalash N, Mintoo MJ, Kumar A, Mahajan P, Mondhe DM, Nargotra A. Exploring derivatives of quinazoline alkaloid L-vasicine as cap groups in the design and biological mechanistic evaluation of novel antitumor histone deacetylase inhibitors. Journal of medicinal chemistry. 2017 Apr 27;60(8):3484-97.

17. Look AT. Oncogenic transcription factors in the human acute leukemias. Science. 1997 Nov 7;278(5340):1059-64.

18. Glozak MA, Seto E. Histone deacetylases and cancer. Oncogene. 2007 Aug;26(37):5420-32.

19. Ropero S, Esteller M. The role of histone deacetylases (HDACs) in human cancer. Molecular oncology. 2007 Jun 1;1(1):19-25.

20. Singh BN, Zhang G, Hwa YL, Li J, Dowdy SC, Jiang SW. Nonhistone protein acetylation as cancer therapy targets. Expert review of anticancer therapy. 2010 Jun 1;10(6):935-54.

21. Mrakovcic M, Kleinheinz J, Fröhlich LF. Histone deacetylase inhibitor-induced autophagy in tumor cells: Implications for p53. International journal of molecular sciences. 2017 Sep;18(9):1883.

22. Dang W, Steffen KK, Perry R, Dorsey JA, Johnson FB, Shilatifard A, Kaeberlein M, Kennedy BK, Berger SL. Histone H4 lysine 16 acetylation regulates cellular lifespan. Nature. 2009 Jun;459(7248):802-7.

23. Li X, Corsa CA, Pan PW, Wu L, Ferguson D, Yu X, Min J, Dou Y. MOF and H4 K16 acetylation play important roles in DNA damage repair by modulating recruitment of DNA damage repair protein Mdc1. Molecular and cellular biology. 2010 Nov 15;30(22):5335-47.

24. Sharma GG, So S, Gupta A, Kumar R, Cayrou C, Avvakumov N, Bhadra U, Pandita RK, Porteus MH, Chen DJ, Cote J. MOF and histone H4 acetylation at lysine 16 are critical for DNA damage response and double-strand break repair. Molecular and cellular biology. 2010 Jul 15;30(14):3582-95.

25. Krishnan V, Chow MZ, Wang Z, Zhang L, Liu B, Liu X, Zhou Z. Histone H4 lysine 16 hypoacetylation is associated with defective DNA repair and premature senescence in Zmpste24-deficient mice. Proceedings of the National Academy of Sciences. 2011 Jul 26;108(30):12325-30.
26. Shogren-Knaak M, Ishii H, Sun JM, Pazin MJ, Davie JR, Peterson CL. Histone H4-K16 acetylation controls chromatin structure and protein interactions. Science. 2006 Feb 10;311(5762):844-7.

27. Haberland M, Montgomery RL, Olson EN. The many roles of histone deacetylases in development and physiology: implications for disease and therapy. Nature Reviews Genetics. 2009 Jan;10(1):32-42.

28. Kourmouli N, Jeppesen P, Mahadevhaiah S, Burgoyne P, Wu R, Gilbert DM, Bongiorni S, Prantera G, Fanti L, Pimpinelli S, Shi W. Heterochromatin and tri-methylated lysine 20 of histone H4 in animals. Journal of cell science. 2004 May 15;117(12):2491-501.

29. Schotta G, Lachner M, Sarma K, Ebert A, Sengupta R, Reuter G, Reinberg D, Jenuwein T. A silencing pathway to induce H3-K9 and H4-K20 trimethylation at constitutive heterochromatin. Genes & development. 2004 Jun 1;18(11):1251-62.

30. Sarg B, Koutzamani E, Helliger W, Rundquist I, Lindner HH. Postsynthetic trimethylation of histone H4 at lysine 20 in mammalian tissues is associated with aging. Journal of Biological Chemistry. 2002 Oct 18;277(42):39195-201.

31. Ehrlich M. DNA hypomethylation, cancer, the immunodeficiency, centromeric region instability, facial anomalies syndrome and chromosomal rearrangements. The Journal of nutrition. 2002 Aug 1;132(8):2424S-9S.

32. Eden A, Gaudet F, Waghmare A, Jaenisch R. Chromosomal instability and tumors promoted by DNA hypomethylation. Science. 2003 Apr 18;300(5618):455-

33. Belyaev ND, Keohane AM, Turner BM. Differential underacetylation of histones H2A, H3 and H4 on the inactive X chromosome in human female cells. Human genetics. 1996 May 1;97(5):573-8.

34. Senese S, Zaragoza K, Minardi S, Muradore I, Ronzoni S, Passafaro A, Bernard L, Draetta GF, Alcalay M, Seiser C, Chiocca S. Role for histone deacetylase 1 in human tumor cell proliferation. Molecular and cellular biology. 2007 Jul 1;27(13):4784-95.

35. Matthews GM, Mehdipour P, Cluse LA, Falkenberg KJ, Wang E, Roth M, Santoro F, Vidacs E, Stanley K, House CM, Rusche JR. Functional-genetic dissection of HDAC dependencies in mouse lymphoid and myeloid malignancies. Blood, The Journal of the American Society of Hematology. 2015 Nov 19;126(21):2392-403.

36. Ahringer J. NuRD and SIN3: histone deacetylase complexes in development. Trends in Genetics. 2000 Aug 1;16(8):351-6.

37. Richon VM, Webb Y, Merger R, Sheppard T, Jursic B, Ngo L, Civoli F, Breslow R, Rifkind RA, Marks PA. Second generation hybrid polar compounds are potent inducers of transformed cell differentiation. Proceedings of the National Academy of Sciences. 1996 Jun 11;93(12):5705-8.

38. Rasheed WK, Johnstone RW, Prince HM. Histone deacetylase inhibitors in cancer therapy. Expert opinion on investigational drugs. 2007 May 1;16(5):659-78.

39. Eckschlager T, Plch J, Stiborova M, Hrabela J. Histone deacetylase inhibitors as anticancer drugs. International journal of molecular sciences. 2017 Jul;18(7):1414.
40. Gates LA, Shi J, Rohira AD, Feng Q, Zhu B, Bedford MT, Sagum CA, Jung SY, Qin J, Tsai MJ, Tsai SY. Acetylation on histone H3 lysine 9 mediates a switch from transcription initiation to elongation. Journal of Biological Chemistry. 2017 Sep 1;292(35):14456-72.

41. Cress WD, Seto E. Histone deacetylases, transcriptional control, and cancer. Journal of cellular physiology. 2000 Jul;184(1):1-6.

42. Ng HH, Bird A. Histone deacetylases: silencers for hire. Trends in biochemical sciences. 2000 Mar 1;25(3):121-6.

43. Bolden JE, Peart MJ, Johnstone RW. Anticancer activities of histone deacetylase inhibitors. Nature reviews Drug discovery. 2006 Sep;5(9):769-84.

44. Johnstone RW. Histone-deacetylase inhibitors: novel drugs for the treatment of cancer. Nature reviews Drug discovery. 2002 Apr;1(4):287-99.

45. Clausen OP, Bolstad KG, Mjelva E. In vivo growth kinetics of P388 and L1210 leukemias. Virchows Archiv B. 1987 Dec 1;54(1):278-83.

**Tables**

Due to technical limitations, table 1, 2 is only available as a download in the Supplemental Files section.

**Figures**
Figure 1

Effect of HDAC inhibition on Post translational modification of acetylation on different Lysine residues of H3 and H4 histones upon treatment of SBAK-GHA, NaB, SAHA and SBAK-NH2 in HL-60 and MOLT-4 cell lines. Cells were treated with different HDACi, lysed and harvested at different time points i.e. 6h, 24h and 48 h. Molecular weight of all H3 lysine residues are 18kDa and H4 residues are 11kDa (A-B) Immunoblotting of Lysine residues of H3 and H4 in HL-60 cells over the spectrum of time period of 6-48 h
(C-D) Immunoblotting of Lysine residues of H3 and H4 in MOLT-4 cells over the spectrum of time period of 24-48 h.

**FIGURE 1**

(A)

| Treatment                | 6h  | 24h | 48h |
|--------------------------|-----|-----|-----|
| SAHA (1 μM)              | -   | +   | -   |
| NaB (0.9 mM)             | -   | -   | +   |
| SBAK-NH2 (6 μM)          | -   | +   | -   |
| SBAK-GHA (1.5 μM)        | -   | -   | +   |
| SBAK-GHA (6 μM)          | -   | -   | -   |

(B)

| Treatment                | 6h  | 24h | 48h |
|--------------------------|-----|-----|-----|
| SAHA (1 μM)              | -   | +   | -   |
| NaB (0.9 mM)             | +   | -   | -   |
| SBAK-NH2 (6 μM)          | +   | -   | -   |
| SBAK-GHA (1.5 μM)        | -   | +   | -   |
| SBAK-GHA (6 μM)          | -   | -   | +   |

(C)

| Treatment                | 24h | 48h |
|--------------------------|-----|-----|
| SAHA (1 μM)              | +   | -   |
| NaB (0.9 mM)             | -   | +   |
| SBAK-GHA (12 μM)         | -   | -   |

(D)

| Treatment                | 24h | 48h |
|--------------------------|-----|-----|
| SAHA (1 μM)              | +   | -   |
| NaB (0.9 mM)             | -   | +   |
| SBAK-GHA (12 μM)         | -   | -   |

**Figure 1**

Effect of HDAC inhibition on Post translational modification of acetylation on different Lysine residues of H3 and H4 histones upon treatment of SBAK-GHA, NaB, SAHA and SBAK-NH2 in HL-60 and MOLT-4 cell lines. Cells were treated with different HDACi, lysed and harvested at different time points i.e. 6h, 24h and
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**FIGURE 2**

(A) Evaluation of Combinatorial effect of HDACi SBAK-GHA and SAHA in HL-60 and DOXO-K562 cells (A-B) Evaluation of Combinatorial effect of SBAK-GHA+ SAHA on Post translational modification of acetylation on lysine residues of H3 and H4 histones using immunoblotting. Acetylation was found be up regulated and retained with increase in time. Molecular weight of all H3 lysine residues are 18kDa and H4 residues are 11kDa (C) Combinatorial and singular effect on Post translational modification of acetylation on different Lysine residues of H4 histones upon treatment of SBAK-GHA + SAHA in Chemoresistant Doxo-K562 cells were studied by immunoblotting. SBAK-GHA was found to upregulate the acetylation status of lysine residues of H4, when used singly and in combination with SAHA.

Figure 2
Figure 2

Combinatorial effect of HDACi SBAK-GHA and SAHA in HL-60 and DOXO-K562 cells (A-B) Evaluation of Combinatorial effect of SBAK-GHA+ SAHA on Post translational modification of acetylation on lysine residues of H3 and H4 histones using immunoblotting. Acetylation was found be up regulated and retained with increase in time. Molecular weight of all H3 lysine residues are 18kDa and H4 residues are 11kDa (C) Combinatorial and singular effect on Post translational modification of acetylation on different Lysine residues of H4 histones upon treatment of SBAK-GHA + SAHA in Chemoresistant Doxo-K562 cells were studied by immunoblotting. SBAK-GHA was found to upregulate the acetylation status of lysine residues of H4, when used singly and in combination with SAHA.
Figure 3

Transient silencing by lentivirus encoding shRNA against class I HDAC isoforms in HL-60 cell line (A) Expression of different class I HDAC isoforms upon treatment with different HDAC inhibitors before silencing (B-C) knock down of class I HDAC isoforms using lentiviral transient transduction was confirmed by (B) Assessment of Turbo GFP property of lentiviral constructs in successful transient transduced cells by using fluorescence microscopy (C) Immunoblotting with different antibodies against Class I HDAC isoforms after transient transduction (D) Tri methylation at H4K20 (3Me) and monoacetylation at H4K16 were evaluated after successful knock down of different isoforms of Class I HDACs. It was found that knock down of HDAC 2 up regulated both H4K20(3Me) and H4K16(ac) while as knock down of HDAC 1 and HDAC 8 up regulated H4K20(3Me) only and knock down of HDAC3 had no effect on either proteins. Moreover the numerical numbering in panel indicates individual isoforms of the respective knock down genes of Class I HDACs
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Figure 4

Evaluation of modulation of different phases of cell cycle upon the treatment of SBAK-GHA, SBAK-NH2, SAHA and NaB in HL-60 cell line. Cells were treated and incubated with said molecules for different time points (6-72 h) and cell lysates were collected at their respective time points and cell cycle analysis were performed accordingly. (A) Different phases of cell cycle arrest from (6-12 h) are depicted here and G1 arrest was found to be prominent throughout. (B) Graphs in this panel depict different phases of cell cycle arrest at (24, 72 h), G1 arrest remained prominent, though with increase in time apoptotic population increased and in NaB treated cells there was a huge shift from G1 to G2 arrest at 72 h.
Evaluation of modulation of different phases of cell cycle upon the treatment of SBAK-GHA, SBAK-NH2, SAHA and NaB in HL-60 cell line. Cells were treated and incubated with said molecules for different time points (6-72 h) and cell lysates were collected at their respective time points and cell cycle analysis were performed accordingly. (A) Different phases of cell cycle arrest from (6-12 h) are depicted here and G1 arrest was found to be prominent throughout. (B) Graphs in this panel depict different phases of cell cycle arrest at (24, 72 h), G1 arrest remained prominent, though with increase in time apoptotic population increased and in NaB treated cells there was a huge shift from G1 to G2 arrest at 72 h.
SBAK-GHA modulated the acetylation level of histones at the chromatin associated with promoter regions of cyclins. (A-B) Histograms represent the relative quantification of immunoprecipitated DNA of Cyclin promoters of different Lysine residues of H3 and H4 histones recovered upon treatment with SBAK-GHA and SAHA in HL-60 cells. (C) Histograms represent the relative quantification of immunoprecipitated DNA of Cyclin promoters of different Lysine residues of H3 and H4 histones recovered upon treatment with SBAK-GHA in MOLT-4 cells. Data are representative of three similar experiments.
Figure 5

SBAK-GHA modulated the acetylation level of histones at the chromatin associated with promoter regions of cyclins. (A-B) Histograms represent the relative quantification of immunoprecipitated DNA of Cyclin promoters of different Lysine residues of H3 and H4 histones recovered upon treatment with SBAK-GHA and SAHA in HL-60 cells. (C) Histograms represent the relative quantification of immunoprecipitated DNA of Cyclin promoters of different Lysine residues of H3 and H4 histones recovered upon treatment with SBAK-GHA in MOLT-4 cells. Data are representative of three similar experiments.

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- SupplementaryDaretal.pdf
