5.1: Discussion

Acetylation of lysines on histones has been shown to be highly dynamic and its regulation is attributed to the opposing action of enzymes from two families, histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Xhemalce et al., 2011). Besides histones, acetylation also occurs on many non-histone proteins thereby regulating their stability, function and localization within the cell (Minucci et al., 2006). The balance between acetylation and deacetylation is altered in the event of carcinogenesis which disturbs their homeostasis. A therapeutically relevant feature of acetylation is its reversible nature (Rasheed et al., 2007). This property of acetylation and deacetylation has been harnessed and its therapeutic window has been exploited for the development of novel HDAC inhibitors. Hyperacetylation of histone and non-histone proteins by HDAC inhibitors promotes growth arrest, differentiation, apoptosis, cell cycle arrest, autophagy and senescence of cancer cells (Marks et al., 2009). HDAC inhibitors have also been implicated in the reactivation of expression of tumor suppressor genes (Kim et al., 2006; Bots et al., 2009) which limits growth of malignant cells and promotes cell death.

HDAC inhibitors are more specific towards cancer cells as compared to normal cells (Kim et al., 2006). The specificity of HDAC inhibitors towards cancer cells is an important pharmacological advantage which has been harnessed for clinical applications. This allows selective elimination of cancer cells with limited toxicity. Moreover, HDAC inhibitors affect diverse cellular phenomena which make HDAC inhibitors more effective therapeutic candidates than anticancer agents which target single gene or protein (Mai et al., 2005). However in spite of recent developments, our understanding of molecular and cellular basis of HDAC inhibition has significant gaps. The simultaneous epigenetic and non-epigenetic effects of HDAC inhibitors make it difficult to decipher their exact mechanism of action (Schrump et al., 2009; Kim et al., 2011). Moreover, HDAC inhibitors have varied HDAC isoform specificities with different functional roles in normal physiology and pathogenesis, which lead to differences in their mechanism of action and overall effect (Heltweg et al., 2004; Park et al., 2004; Ahmad et al., 2012). So new chemical entities with isoform specificities are being explored as possible HDAC inhibitors for their role in modulating acetylation, development and progression of cancer (Mai et al., 2005). In
particular, chemical entities with high specificity towards HDAC isoforms are being explored. HDAC inhibitors like SAHA, Romidepsin, Belinostat and Panobinostat target multiple HDAC isoforms which make them toxic and limit their clinical use (Subramanian et al., 2010). Isoform specific HDAC inhibitors may enable designing of anticancer drugs with limited off target undesirable effects. The main pharmacophore of HDAC inhibitors comprises of three regions viz cap, linker and chelator, which perform different functions. Chelator acts as a Zn$^{2+}$ binding group, linker region acts as an arm enabling chelator to bind Zn$^{2+}$ ion located at the bottom of active site while the cap induces conformation change in the HDAC enzyme by interacting with the amino acids located at the rim of active site (Miller et al., 2003; Bradner et al., 2010). Historically, natural products have served as important repositories of anticancer drugs. Two natural products i.e. β-boswellic acid and l-vasicine were explored as potential HDAC inhibitors by employing their derivatives as cap groups (Sharma et al., 2014; Ahmad et al., 2017).

In the present study a library of molecules containing β-boswellic acid and l-vasicine as cap groups were evaluated for their cytotoxicity potential against panel of cells including leukaemic cell lines (THP-1, MOLT-4 and HL-60), colon cancer cell lines (Colo-205 and Caco-2), pancreatic cancer cell line (MIAPaCa-2), prostate cancer cell line (PC-3), lung cancer cell line (A549), breast cancer cell line (MCF-7) and normal cell line (fR2). The aim of the study was to develop selective HDAC inhibitors to alleviate toxicity and increase anti-tumor activity. Out of library of molecules screened for their possible cytotoxicity and potential HDAC inhibition, one novel molecule from β-boswellic acid series as HDAC inhibitor i.e. N$^1$-hydroxy-N$^5$-(3-α-hydroxy-11-oxo-24-norurs-12-en-4-yl)glutaramide (SBAK-GHA) was found to be the lead molecule with selective targeting of class I HDAC isoforms. SBAK-GHA was found to be less toxic and capable of inducing cell death over spectrum of cancer cells of different tissue origins. Effects of HDACi are known to be cell line specific (Glaser et al., 2003). The involvement of HDACs in carcinogenesis of leukemic and breast cancer cell lines and their good response to already known HDACi were the main reasons to choose these cell lines for this study.

Acute promyelocytic leukemia (APL) is the first disease in which mechanistic involvement of HDACs in carcinogenesis was established (Ceccacci and Minucci,
APL is widely used as a model for understanding role of HDACs in carcinogenesis (Marks and Xu, 2009; Sadakierska et al., 2015). It has been reported earlier that knock down of class I HDACs in APL cells induced growth arrest and apoptosis (Vlasáková et al., 2007; Meisenberg et al., 2016). Deregulation of histone acetylation has been found in many neoplasms and particularly in leukemias (Look et al., 1997). A possible novel therapeutic approach to restore the deregulation of histone acetylation was achieved to great extent by introduction of HDAC inhibitors (HDACi). But mechanism of action of these HDACi is still a debatable topic. Various residues of histone H3 and H4 have seen to be playing an important role in defining the state of cell e.g. H3K9 acetylation is associated with active transcription (Glozak et al., 2007). 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 range of leukemic cell lines viz HL-60, MOLT-4, chemoresistant DOXO-K562 and breast cancer cell line MCF-7 using immunoblotting. In HL-60 cells, gain in acetylation at most of the residues occurred as early as 6 h and acetylation was found to be retained for as long as 48 h for both SAHA and SBAK-GHA treated cells. For NaB treated cells acetylation was induced after 24 h that was retained by most of the residues at 48 h. However the most profound and prominent acetylation over the period of time was induced in SBAK-GHA treated cells.

We further extended our study to acute-lymphoblastic-leukemic-cell-line i.e. MOLT-4 to observe acetylation pattern at different lysine residues of H3 and H4. 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 of 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 groups of Ropero et al., 2007, Singh et al., 2010 and Mrakovcic et al., 2017.

It has been well established that loss of monoacetylation at H4K16 and trimethylation at H4K20 are hallmarks of cancer (Fraga et al., 2005). H4K16 is considered to be the main contributor of total H4 acetylation and its association with DNA damage, repair and cell senescence (Dang et al., 2009; Li et al., 2010; Sharma et al., 2010; Krishnan et al., 2011) apart from disruption of higher order chromatin structures is well known (Shogren-Knaak et al., 2006). In recent past it came into fore that loss of H4K16 acetylation is responsible for the tumor progression and sensitivity to chemotherapy (Castro et al., 2017). 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.

Till now what we interpreted from our results is that there is a difference in level of modulation of acetylation at different residues of H3 and H4 upon treatment with SBAK-GHA and SAHA. In order to check whether SBAK-GHA targeted specific HDAC isoforms or was a pan HDAC inhibitor like SAHA, HDAC isoform enzymatic assay was performed. SBAK-GHA was evaluated against HDAC isoforms with SAHA as the positive control. 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 with IC_{50} values 200, 230, 250 and 300 nM 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 (Haberland et al., 2009). After SBAK-GHA was found to be class I HDAC inhibitor, it became imperative to confirm 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 proteins of cancer i.e. H4K16 acetylation and H4K20 (3Me). We have already discussed the importance of H4K16 but H4K20 (3Me) is another important hallmark associated with cancers and is the only lysine in the tail of H4 that is methylated. Trimethylation of H4K20 is a marker of constitutive heterochromatin, gene silencing (Kourmouli et al., 2002; Schotta et al., 2004) and aging (Sarg et al., 2002). Defects in the DNA methylation may be related to the generation of genomic aberrations (Ehrlich et al., 2002; Eden et al., 2003), however the imbalanced and defective pattern of histone modifications still could be reversed. It was observed that knockdown of class I HDAC isoforms 1 and 8 upregulated H4K20 (3Me) but not H4K16 acetylation, where as HDAC 2 upregulated both H4K20 (3Me) and H4K16 acetylation. However no change in H4K20 (3Me) or H4K16 was found upon knockdown of HDAC3. Our results indicated the importance of different Class I HDACs in regulation of H4K16 acetylation and tri methylation at H4K20. For the first time we are reporting that H4K16 acetylation was seen to be affected by knock down of HDAC2 only. It is worth to note that possible involvement of HDAC2 in regulation of H4K16 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 (Sharma et al., 2014; West et al., 2014; Ahmad et al., 2017). Therefore, both HDAC1- and HDAC1/HDAC2-mediated chromatin modifications seemed to be important for cell cycle control and development. They also induced cell cycle arrest either at G1 or G2/M and apoptosis, killing tumor cells (Belyaev, 1996; Senese et al., 2007; Matthews et al., 2015). Usually G1 phase growth arrest induced by HDAC inhibition is attributed to acetylation dependent increase in expression of cyclin dependent kinase inhibitor p21\(^{\text{waf1}}\) while as G2 phase growth arrest is usually attributed to reactivation of checkpoint kinase 1 (Chk1) (Ahinger et al., 2000; Fiskus et al., 2014). The pathway leading to tumor cell death is still unknown, but it is very
unlikely that a single molecular pathway will be involved in different cell types for all HDAC inhibitors. It has been reported that HDAC1 null cells can arrest either at the G\textsubscript{1} 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 (Matthews et al., 2015). 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 found that G\textsubscript{1} arrest due to treatment of HDACi in HL-60 was prominent however shift towards G\textsubscript{2} phase at 72 h was observed in NaB and SBAK-NH2 treated cells. Interestingly shift in growth arrest of cells from G\textsubscript{1} to G\textsubscript{2} and S phase over the period of time could be due to increase in apoptotic population.

Likewise we extended our study to acute-lymphoblastic-leukemic-cell-line MOLT-4 and distribution of cells in different phases of cell cycle was studied. Initially G\textsubscript{1} arrest was found to be prominent in SBAK-GHA treated cells however there was shift in cells from G\textsubscript{1} to S phase arrest in later stages. We also studied cell cycle pattern of DOXO-K562 cells upon treatment with SAHA and SBAK-GHA, singly and in combination, prominent G\textsubscript{2} arrest with increase in time was found. The role of class I HDACs has already been appreciated in cell cycle arrest, as class1 HDAC inhibitor Romidepsin has showed G\textsubscript{1} or G\textsubscript{2}/M arrest and our data is in agreement with already published reports of previous groups (Richon et al., 1996; Rasheed et al., 2007; Eckschlager et al., 2017), 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, role of direct acetylation of histones in the modulation of expression of cyclins is unknown (Gates et al., 2017). In HL-60 and MOLT-4 cells ChIP and qPCR showed that expression of cyclins was indeed controlled by acetylation induced at H3 and H4 lysine residues of promoter regions of different cyclins on treatment with SBAK-GHA. Prominent G\textsubscript{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 indicated that decreased expression of cyclin D1 in SBAK-GHA treated cells immunoprecipitated with various lysine residues especially with H4K16 might be the possible reason for its efficient G1 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 (Cress et al., 2000; Ng et al., 2000). 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 TNFα that promote cell growth (Bolden et al., 2006). Our results for the first time have shown direct relation between histone acetylation and modulation of expression of cyclins.

Generally HDAC inhibitors derived from natural sources have limited retention time and are unstable under in vivo conditions (Ahinger et al., 2000). The clinical use of FDA approved HDAC inhibitors SAHA, Romidepsin, Belinostat and Panobinostat is limited by their toxicity (Sadakierska et al., 2015). Moreover, HDAC inhibitors like TSA, Trapoxin and Depudecin from natural sources have shown toxic effects under in vivo conditions (Johnstone et al., 2002) 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 (Richon et al., 1996). Earlier it has been reported that proportion of G1 and G2 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 (Clausen et al., 1987). 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
Discussion

most of the natural or synthetic Pan HDACi reported earlier. Our findings conclusively demonstrated that SBAK-GHA, class1 HDAC inhibitor has the therapeutic potential against lymphocytic leukemia when used singly or in combination with SAHA.

HDACi have shown successful results in lymphoma but limited response in solid tumors. HDACi as a monotherpic agent in solid tumors does not have much success though in combination it has increased the efficiency of various monotherpic molecules like trastuzumab. Combination of Panobinostat with trastuzumab was seen to potentiate trastuzumabs effect in breast cancer patients (Thurn et al., 2011). However, the use of HDAC inhibitors as monotherapy in solid tumors remains unclear and has largely limited their clinical applications. Though, inhibition of bromodomains protein (BRD4) or Janus kinase (JAK) due to treatment of HDAC inhibitors were shown as possible monotherpic targets (Zeng et al., 2016), but they are still in early stages.

Efficacy of our lead molecule SBAK-GHA as a possible monotherapeutic agent for solid tumor was evaluated using MCF-7 cell line. Acetylation of lysine residues of H3 and H4was found to be regulated by SBAK-GHA in MCF-7 cells, although the level of acetylation was not as prominent as was found in leukemic cells. Our results indicated that SBAK-GHA could possibly regulate chromatin organisation in MCF-7 cells also. It has been reported that upon knock down of HDAC 1, HDAC 3 there was a drastic effect on cell growth and proliferation of MCF-7 cells, similar effect was observed with HDAC 2 when knocked down along with HDAC 1, as compared to MCF10A, the untransformed counterpart of MCF-7, hence greatly supported the observation that HDAC inhibitors have antiproliferative and proapoptotic properties mainly in transformed cells (Senese et al., 2007). Cell cycle arrest at G1 both in HL-60 and MCF-7 cells on treatment with SBAK-GHA, SBAK-NH2 and SAHA underlined the importance of class I HDACs in cell cycle.

Tumor growth inhibition of 84% in Ehrlich Ascites Carcinoma SBAK-GHA and 46.64% in SARCOMA-180 Ascites further suggested that SBAK-GHA can be used to understand mechanism of action of HDACs in leukemia as well as the solid tumor.
Taken together, our findings have conclusively demonstrated that SBAK-GHA, class 1 HDAC inhibitor has the potential to be used as monotherapeutic agent against lymphocytic leukemia or in combination with SAHA, an FDA approved HDAC inhibitor. However pharmacokinetic and pharmacodynamics studies are required to fully establish the therapeutic potential of SBAK-GHA.

5.2: Future perspectives of present study

We have tried to address the role of class 1 specific HDAC inhibitor and its mechanism of action to best possible ambit in current scenario. Largely, we are ignorant of off target effects of HDAC inhibitors and their possible implications on cellular mechanisms. Not only this but also in order to study and establish a particular role of individual HDAC isoforms we need specific knock downs / knock outs for elucidating their functions. We in our study have successfully reported the design and synthesis of a novel natural product based HDAC inhibitor i.e. SBAK-GHA which showed significant cell death against cancer cell lines of diverse origins. Moreover, SBAK-GHA showed significant activity under in vivo conditions unlike most of the natural product based HDAC inhibitors. Though we were able to decipher the potential of SBAK-GHA to a large extent yet specific function of individual HDAC isoforms remains to be ascertained, which will largely be possible with the stable cell line of these HDAC isoforms. Though various HDAC inhibitors have been fast tracked and approved by FDA against various forms of leukemias but nothing concrete has been done to extend their application in solid tumors with few exceptions. During the last decade there has been a lot of progress in the field of HDAC biology but off target effects of pan HDAC inhibitors largely remain a debatable question, yet to be addressed in a convincing manner. Due to non-redundant properties of these HDAC isoforms it is imperative to use stable cell lines through which it will be possible to decipher the differential role of specific HDAC isoforms in cancer in the near future.