Overcoming off-target changes in gene expression by targeting epigenetic enzyme inhibitors to oncogenes using LNA-drug bioconjugates

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Abstract

Inhibition of epigenetic drug targets is growing into a powerful tool to regulate gene expression for the treatment of many diseases. This has become the new frontier of candidate therapies for cancer treatment. However, epigenetic enzymes, such as histone modifiers, are critical regulators of gene expression throughout the genome and there is currently no way to target an inhibitor to a particular diseased gene. Here we show the histone deacetylase (HDAC) inhibitors vorinostat, entinostat and mocetinostat (current and candidate treatments for lymphoma) inhibit the expression of lysine demethylases, and in the case of mocetinostat, results in the off-target increase of total histone lysine methylation. We identified that inhibition of key regulatory elements results in the alteration of other histone-modifiers that results in unpredictable, cascading changes in gene expression. We also show that in addition to HDAC, inhibitors of the disruptor of telomere silencing 1-like (DOT1L) and the lysine specific demethylase 1 (LSD1) (candidate treatments for leukemia) lead to similar off-target and unanticipated effects on the expression of histone-modifying enzymes, depending on cell type and dose. These approaches in epigenetic therapy often lead to the discontinuation of treatment due to the nature of their off-target effects. Therefore these inhibitors should be targeted to a particular diseased gene. We propose this may be achieved by conjugating the inhibitors to single stranded locked nucleic acid (LNA) oligonucleotide mixmers that are complementary to the genes those inhibitors are intended to target. Our experiments show that such LNA-drug conjugates increase the potency of DOT1L inhibitors by 16-fold.

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Chapter 1.

1.1 Introduction[1]

Epigenetics describes the molecular processes of heritable changes in gene expression that are not derived from changes in DNA sequence. These processes are including, but not limited to, non-coding microRNA, methylation of DNA and post-translational modifications to histones. Although the former cannot be discounted, our discussion herein is limited to the modifications to histones to keep this text succinct. Eukaryotic DNA is packaged with clusters of histones to produce chromatin, which in its extended form resembles beads on a string. Each "bead", known as a nucleosome, is composed of ~147 base pairs wrapped around the core histones in an octameric structure comprised of two copies of histones H2A, H2B, H3 and H4 with linker DNA between nucleosomes which binds histone H1. Chromatin forms higher order structures that culminate on a larger scale into chromosomes. On a localized level, chromatin can become highly condensed producing heterochromatin or less condensed producing euchromatin. The less condensed euchromatin can serve as a template for transcription resulting in gene expression, whereas heterochromatin is too condensed to allow access to the transcriptional machinery resulting in little or no gene expression. The level of condensation, and hence the state of eu- or heterochromatin is, in-part dictated by the epigenetic modifications to histones (*1*). Therefore on one level, histone modifications play a key role in the regulation of transcription by controlling regions of transcriptionally active euchromatin and silent heterochromatin (*2*).

Common histone modifications include mono-, di- and trimethylation of lysine, mono-, symmetric and asymmetric dimethylation of arginine, lysine and serine acetylation, as well as serine, threonine and tyrosine phosphorylation, oxidation of arginine to citruilline, and lysine ADP ribosylation, sumoylation and ubiquitination among others (*3*). The types of modifications

and the histone residues upon which they occur are not random, but appear in patterns that dictate gene expression. The consistency of these modifications suggests that there is a pattern that has been called the histone code (*4*). The term histone code suggests that it is like the genetic code but it has since been realized that the histone code is much more complex. The histone code must be understood in the context of other epigenetic mechanisms such as DNA methylation and miRNA so some now use the term "nucleosome code".

In addition to their roles in regulating eu- and heterochromatin, histone modifications also alter gene expression by acting as recognition binding sites for the recruitment of transcription factors, co-activators, or co-repressors and by directly modifying the contacts to DNA thereby remodeling chromatin. Some modifications increase, while others decrease transcription depending on the type, sequence position and context of modifications already present. Chromatin remodeling can activate or silence gene transcription depending on the interactions between DNA and histones.

Given the potential for epigenetic modifications to regulate gene expression it should come as no surprise that they play a role in disease (*5*). Modifications to histone lysine have been studied as a major contributing factor in cancer (*6*), neurodevelopmental disorders, neurodegenerative diseases, autoimmune disease, addiction, inflammatory disease, metabolic disease, psychological disorders (*5, 7-11*) and parasitic infections such as malaria (*12*), leading to drug inhibitors targeting lysine modifying enzymes as therapeutic interventions. Drug development has focused on specifically targeting enzymes, but any drug that targets these enzymes cannot be gene specific. Assuming that a disease is caused by aberrant epigenetic enzyme activity at one or a few genes, inhibition of that enzyme activity is only required at those disease causing genes. Therefore, even specific inhibition of an epigenetic enzyme will inhibit

that enzyme at off-target genes and could lead to alterations in expression of those genes. In fact, the overall effect of drugs targeting epigenetic enzymes will be a combination of all the effects of inhibiting that enzyme at all the genes where it is active. Considering this, it is important for newly designed inhibitors to not only be enzyme subtype-selective, but also be selective for the intended target gene when evidence shows that one or a few genes are involved in the disease.

The following section details the mechanisms of enzymes that catalyze modifications to histone lysine and serves as an introduction to the rationale for inhibitors that target these enzymes. For the sake of simplicity the discussion will focus on the modifications to lysine (*13*).

1.2 Inhibitors of enzymes catalyzing modifications to histone lysine residues: Structure function and activity[1]

Histone modifying enzymes contribute to the regulation of chromatin remodeling by dictating the site, extent and genomic loci of their respective histone modifications. Histone modifiers or those that recognize and bind histone modifications have been categorized as readers, writers or erasers (*14*). Writers of lysine modifications are those enzymes that add post-translational modification to histones, among these are lysine acetyltransferases (KAT) and lysine methyltransferases (KMT), while erasers are enzymes that remove these marks including histone lysine deacetylases (HDAC) and lysine demethylases (KDM). Readers of lysine modifications are those proteins that contain domains that recognize these marks; bromodomains (BRD) bind acetyl-lysine, while chromodomains (CRD), Tudor and WD40 domains, among others, bind methylated histones (*15*). The plant homeodomain (PHD) fingers bind methylated histone H3 depending on the methylation and acetylation state of neighboring arginine and lysine residues, respectively (*16*). Often, chromatin writers and erasers also contain reader domains or are within a reader complex in order to direct their activity to specific histone residues (*17*).

1.2.1 Histone lysine acetylation

Lysine acetylation is catalyzed by a broad number of KATs (*18*) with two major families: General Control Nonderepressible-5 (GCN5)-related N-acetyltransferases (or GNATs) and the

[1] Republished (adapted) with permission of Newlands Press Ltd from Ryan Lillico, Nicholas Stesco, Tina Khorshid Amhad, Claudia Cortes, Mike P Namaka and Ted M Lakowski. Inhibitors of Enzymes Catalyzing Modifications to Histone Lysine Residues: Structure, Function and Activity. *Future medicinal chemistry* **2016**, 8, 879-897. Copyright 2016 Newlands Press Ltd; permission conveyed through Copyright Clearance Center, Inc.

MYST family. These enzymes require an unmodified lysine residue within a protein and acetyl-CoA as substrates producing CoA-SH and acetylated protein lysine as products (**Figure 1.1**).

KATs can be, "transcription-related" which acetylate histones assembled in chromatin, or "deposition/replication related" which acetylate newly translated histones prior to chromatin assembly (*19*). Like other epigenetic enzymes, KATs have a catalytic domain and usually a reader domain, such as BRD or CRD, that can assist in directing histone residue specificity (*20*).

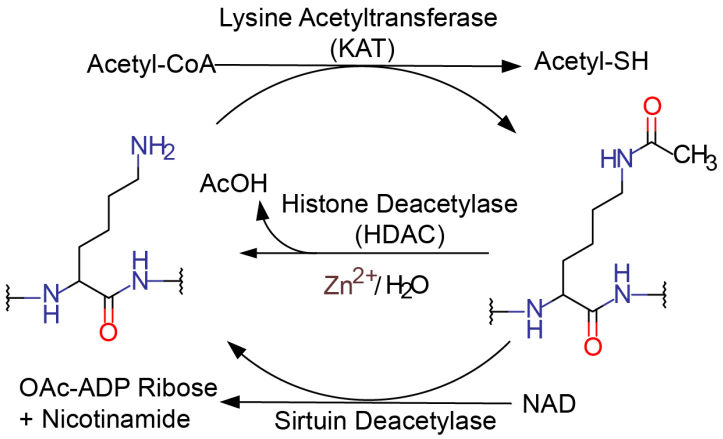

Figure 1.1 The mechanisms of acetylation by KAT and deacetylation by HDACs: KATs require Acetyl-CoA as a source of acetyl groups. Two groups of HDACs exist, NAD dependent Sirtuin deacetylases and zinc dependent.

The GNATs include CREB-binding protein (CBP), p300, PCAF (p300/CBP-Associated Factor) and GCN5. CBP/p300 are components of transcription factor co-activator complexes that appear at most promoter regions of genes (*21, 22*). It was originally thought that the activities of these KATs were redundant due to homology between catalytic KAT domain and BRD, but recently CBP and p300 have been shown to have some subtle substrate specificity and selectivity differences. For example, CBP exhibits higher specificity constant (k_{cat}/K_m) for H3K18 compared to p300, while p300 has the highest specificity towards H4K16 (*23*).

The MYST family of KATs was originally named for the founding members Morf, Ybp2, Sas2 and Tip60, derived from yeast and mammals (*24*). There are five important human KATs in the MYST family: MOZ, MORF, HBO1, MOF and Tip60 (*25*). Similar to GNATs, the MYST family members have catalytic KAT domains and BRD, but are distinguished by their CRD and PHD fingers. Illustrating the importance of this difference, a recent study found that a double PHD finger domain in MOZ promotes H3K9 and H3K14 acetylation unless H3K4 is trimethylated (*26*). Although the focus of this work is the epigenetic implications of histone lysine modifications, it must be pointed-out that KATs (and the other aforementioned epigenetic enzymes) also show specificities to non-histone proteins. Therefore, lysine acetylation has many functions in the cytosol and nucleus that are not related to epigenetics or gene expression (*27*).

1.2.2 Histone lysine deacetylation

Lysine acetylation is removed by HDACs (**Figure 1.1**) and four classes have been identified in humans consisting of 11 classical deacetylases and 7 sirtuin deacetylases. The classical HDACs (class I, II and IV) contain a zinc cofactor, while the sirtuins (class III) bind NAD and show distinct structural contrast compared to classical HDACs (*28*). HDAC classification is based on sequence homology, substrate specificity and sub-cellular localization (*29*). The status of lysine acetylation on chromatin is dynamic and due to the tandem interactions between KATs and HDACs on histones, where HDACs often directly compete for chromatin binding with KATs in a mutually exclusive manner (*30*). Moreover, HDAC activity is attenuated by becoming acetylated itself by p300 (*31*), further demonstrating the KAT/HDAC relationship. Aberrant HDAC activity has been associated with many types of cancer (*32*) making both classical and sirtuin deacetylases an important target for therapeutics (*33*), this being further discussed in Chapter 2. From the perspective of interactions between histones and DNA, HDAC

activity promotes chromatin condensation to heterochromatin, which would normally lead to gene silencing. However, HDAC activity also results in removal of binding sites for reader proteins and therefore the HDAC effect on the expression of any individual gene can vary. Although HDACs have activity on non-histone proteins, (otherwise known as KDAC or lysine deacetylases) we have chosen to use the abbreviation HDAC because it is commonly used throughout the literature.

1.2.3 Histone lysine methylation

Histone lysine methylation exerts its effects on gene transcription by serving as a recognition site for chromatin readers. Unlike acetylation, lysine methylation does not significantly modulate the charge of the lysine residue and therefore does not significantly affect the electrostatic interaction between histones and DNA. Rather, chromatin reader domains recognize methylated lysine resulting in recruitment of transcription factors, co-activators and co-repressors leading to changes in transcription. In particular, histones are methylated by KMTs on multiple residues, where the residue and the histone involved determine the effect on transcription. Histone lysine methylation is catalyzed by KMTs requiring two substrates, S-adenosyl-L-methionine (AdoMet) and a histone lysine residue. The corresponding products are S-adenosyl-L-homocysteine (AdoHcy) and a methylated histone lysine (**Figure 1.2**).

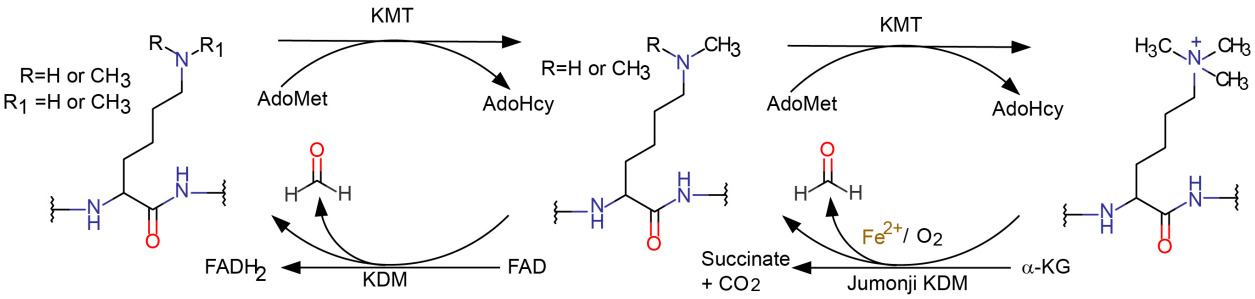

Figure 1.2 The mechanisms of methylation by KMTs and demethylation by KDMs: KMT can add one to three methyl groups to the lysine εNH_2. Both classes of KDM can demethylate mono- and dimethyl-lysine but only Jumonji domain containing KDMs can demethylate trimethyl-lysine.

All KMTs contain a conserved SET domain with the exception of the non-SET domain containing KMT, disruptor of telomeric silencing (DOT1) and its homologs, further discussed in Chapter 3. The SET domain KMTs are divided into families based on function and substrate specificity: the Su(var), Enhancer-of-zeste homologue, Trithorax (SET), Retinoblastoma protein-interacting zinc finger (RIZ), SET/MYND (SMYD), G9a/GLP and Mixed Lineage Leukemia (MLL) families (*34*). G9a (EHMT2) and RIZ are established H3K9 methyltransferases associated with heterochromatin and transcriptional repression (*35, 36*). SET2 and SMYD2 are H3K36 methyltransferases (*37, 38*), which help to direct deacetylation by recruiting HDAC complexes to H3K36 trimethylation, ultimately inhibiting transcriptional initiation (*39*). Su(var)4-20 methylates H4K20 within pericentric heterochromatin (*40*). Many SET domain containing methyltransferases methylate H3K4 promoting gene transcription including SMYD1-3, MLL, SET1 and SET7/9 (*41*). The non-SET domain DOT1 family are the only enzymes known to methylate H3K79 which is in the globular domain of histone H3 (*42*). The human homolog, DOT1-like (DOT1L) catalyzes the formation of H3K79 mono-, di- and trimethylation (*43*) depending on the ubiquitination status of H2B (*44*). The DOT1L mediated enrichment of H3K79 methylations are generally found in regions of actively transcribed genes (*45*).

1.2.4 Histone lysine demethylation

Histone lysine demethylation is catalyzed by KDMs. These enzymes require a histone methyl-lysine substrate and an oxidative co-substrate, either flavin adenine dinucleotide (FAD) or molecular oxygen, iron, and alpha-ketoglutarate producing a demethylated histone lysine, reduced FAD or succinate and formaldehyde as products (**Figure 1.2**). The two classes of KDM are defined by the reducible co-substrate used by the enzyme. The lysine specific demethylases (LSD1 and 2) belong to the FAD dependent amine oxidases and share sequence homology with

the monoamine oxidases (MAO). LSD1 removes mono- and dimethylation on H3K4, and has shown a preference for H3K4 versus H3K9 (*46*). LSD1 is unable to demethylate trimethyl-lysine as the FAD dependent mechanism requires a lone pair of electrons in the formation of the iminium intermediate (**Figure 1.3**). LSD1 associates with HDAC1/2, CoREST and other co-repressors to form its active complex (*47*), where the demethylation of H3K4 and deacetylation activity of the complex operates synergistically to increase heterochromatin and decrease gene expression. There is also some evidence that LSD1 participates in H3K9 demethylation albeit to a lesser extent (*48*) and it's differential selectivity towards gene-activating H3K4 versus gene-suppressing H3K9 methylation marks depends on the association with other KDMs (*49*).

The second class of KDM is the jumonji C (jmjC) domain containing KDMs, which demethylate mono-, di- and trimethyl-lysine using alpha-ketoglutarate (α-KG), Fe(II) and molecular oxygen as cofactors (**Figure 1.3**) (*50*). The jmjC family is subcategorized by activity, substrate specificity and the presence of other domains. These KDMs have activity at both gene activating and repressing marks H3K4, H3K9, H3K27, H3K36, and H4K20 (*51*). JmjC KDMs are dependent on cellular oxygen and hypoxic cells show global increases in lysine methylation due to their inhibition (*52*).

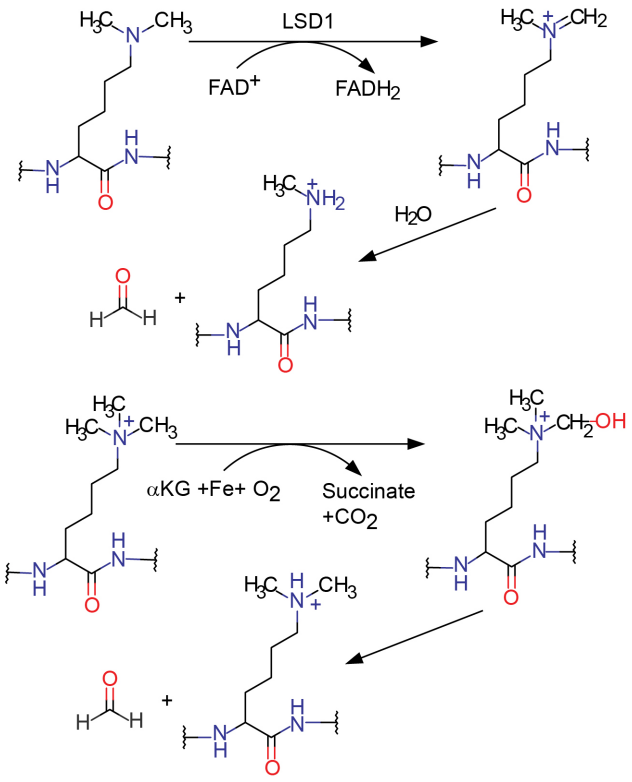

Figure 1.3 The detailed mechanism of demethylation by LSD1. A lone pair of electrons is required to form the iminium intermediate, which decomposes into formaldehyde and the free εNH_2 of lysine. It is for this reason that LSD1 cannot demethylate trimethyl-lysine.

1.2.5 Epigenetic modifications and human disease

Aberrant epigenetic activity can result in changes in gene expression causing disease. The dysregulation of many epigenetic marks are associated with neurological dysfunction and cancers (*9*). Ectopic regulation or mutation of lysine methyltransferases KMT1D and 3B and demethylase KDM5C have been linked to mental retardation (*53-55*) as well as autism (*56*) and schizophrenia (*57*). Reduced activity of CBP/p300 resulting in hypo-acetylation, is associated with the polyglutamine pathogenesis of Huntington's disease (*8*) and increased expression of HDAC3 causing hypo-acetylation in multiple sclerosis (*58*). Improper expression of HDAC is likewise a cause of disease. For example, HDAC1 is overexpressed in many lymphocytic cell

lines and primary lymphoid tumors (*59*) and HDAC8 has been linked to cancers (neuroblastoma), X-linked retardation and parasitic infection (*60*).

Class specific HDAC inhibitors are effective in treating cancer, including cutaneous T-cell lymphoma, Hodgkin's Lymphoma as well as myeloid leukemia and solid tumors (*61*). Four HDAC inhibitors are have been approved by the United States Food and Drug Administration (*62*): vorinostat, is used for cutaneous T-cell lymphoma and is being explored for treatment of other cancers (*63*), romidepsin is used for peripheral and cutaneous T-cell lymphoma (*64*), belinostat is used for the same lymphomas but can be used in combination with other drugs to treat ovarian cancer (*65*) and most recently, panobinostat has been approved for use in combination with bortezomib for recurrent multiple myeloma (*66*).

Despite the promise of epigenetic enzyme inhibitors it must be noted that any given epigenetic enzyme might be active at multiple promoters. As such, it is inevitable that at least some of those affected genes will not be involved in disease and their normal expression may be needed for proper cellular function. Therefore even the most exquisitely selective epigenetic enzyme inhibitor may have off target effects by altering expression of off-target genes. Understanding this "crosstalk" between epigenetic machineries will highlight the potential off target effects of these drugs that can lead to secondary complications associated with their use (*63*). It has been noted that targeting epigenetic enzymes can lead to alterations in the expression of other epigenetic enzymes (*67*). This is most evident with HDAC inhibitors, but can be extended to all epigenetic enzyme inhibitors. Often, determining the changes in genome-wide expression is time-consuming and cost prohibitive, for this reason it is rarely evaluated. Without considering the expression of all genes simultaneously during treatment, the off-target effects of epigenetic therapies remain unknown. Future therapies targeting histone modifications must

therefore be directed to diseased genes that have clearly defined epigenetic targets to ameliorate potential side effects associated with non-gene specific inhibition. On the other hand it is also conceivable that changes in expression of multiple genes could fortuitously converge favorably altering the expression of one or a few disease causing genes. In this case many changes in expression could be induced by the therapeutic, some being beneficial while the rest would be considered off-target.

1.2.6 Hypotheses and objectives

This thesis consists of three major objectives. 1) To develop a validated method to quantify histone modifications, 2) measure the effects of inhibitors of histone lysine modifying enzymes on histone modifications and detail their effects on the expression of off-target histone modifying enzymes, and 3) to develop a means to overcome these off-target effects.

We hypothesize that inhibition of any histone-modifying enzyme will lead to off-target changes in gene expression, including other histone-modifier genes. We extend this hypothesis to suggest these off-target effects may be overcome by targeting the histone-modifying enzyme inhibitor to a particular gene. We suggest this may be achieved by conjugating the histone-modifying enzyme inhibitors to locked nucleic acid (LNA) oligonucleotides that are complementary to the target gene. The rationale is presented in the next two chapters as we outline the off-target effects of HDAC, DOT1L and LSD1 inhibitors, which is followed in Chapter 4 by the introduction of our technology under development using LNA-drug bioconjugates to target DOT1L inhibition to the HOXA9 oncogene.

Chapter 2.

2.1 HDAC inhibitors induce global changes in histone lysine and arginine methylation and alter expression of lysine demethylases[2]

HDAC and HKMT inhibitors are emerging treatments for cancer, the HDAC inhibitor vorinostat is approved for use in cutaneous T cell lymphoma (*68*) and others are in clinical trials. HDAC inhibition leads to rapid acetylation of histones and transcription factors resulting in changes in gene expression (*69*), and stimulating growth inhibition and apoptosis (*70*). HDAC inhibitors can also influence histone modifications other than lysine acetylation possibly contributing to their cytotoxicity. Some examples of these off-target effects are with the HDAC inhibitor entinostat, shown to increase repressive H3K9 methylation (*71*), while other HDAC inhibitors increase H3K4 methylation by recruitment of HKMTs (*72*) or by decreasing expression of particular KDMs (*73*). Therefore, in order to evaluate the total effect of HDAC inhibitors, one must measure the changes in multiple histone modifications in addition to the expected increases of histone acetyl-lysine.

Studies accurately quantifying changes in multiple histone modifications simultaneously are infrequent because of the dearth of analytical techniques to quantify marks on histones. Although few methods attempt such quantification, most that do, use antibodies or proteomics. Antibodies frequently cross-react with unintended targets (*74*) (www.histoneantibodies.com is an interactive database that outlines specific vendors antibodies and their cross-reactivity with other histone modifications (*75*)) and antibody blocking from neighboring histone modifications often results in false negative or positive results. To address this issue, Fuchs et al. developed a high-

[2] Reprinted (adapted) from Ryan Lillico, Marina Gomez Sobral, Nicholas Stesco and Ted M Lakowski. HDAC Inhibitors Induce Global Changes in Histone Lysine and Arginine Methylation and Alter Expression of Lysine Demethylases. *J Proteomics*. **2016**, 133, 125-133.

throughput peptide array containing single or combinatorial histone modifications to test various vendors antibodies for cross reactivity and blocking (*76*), finding many instances of both and varying depending on the vendor. For instance, they show antibody recognition of H3K4Me3 can be blocked by modifications to H3R2 (methylation and citrullination) and/or phosphorylation of H3T6 as well as major cross-reactivity of H3K14Ac-directed antibodies with H3K36Ac. Finally, in addition to the issues with antibodies, the alternative traditional proteomic methods almost never cover 100% of the sequence leaving sites of modification unquantifiable. Therefore, these methods can only quantify modifications at one or a few histone residues and may miss the effects on other histone residues that an inhibitor might have, thereby failing to accurately measure the total activity. The result is activity or inhibition that is difficult or impossible to completely quantify using current techniques.

Here we developed an LC-MS/MS method to quantify total histone modifications in cells to simultaneously measure the total effects of HDAC inhibitors, based on previous work (*77-80*). Rather than quantifying modifications within the context of the histone sequence, the histone is digested into amino acids and the modified amino acids quantified using commercially available standards. This technique cannot provide the context of protein sequences from which the modifications were derived, however, it can measure the total amounts of a given modification on all histones isolated from cells treated with histone-modifying enzyme inhibitors. In this respect the total effectiveness and some off target effects of those inhibitors can be measured. We found the HDAC inhibitors vorinostat, entinostat, and mocetinostat, induced changes in histone lysine and arginine methylation along with the expected hyperacetylation of histone lysine. Mocetinostat exhibited dose dependent increases in lysine methylation, which were correlated to decreases in the expression of seven lysine demethylases (KDM). Vorinostat

produced small increases in lysine methylation and was correlated with decreased expression of a few KDM while others were unaffected. Entinostat treatment was correlated with decreased LSD1 expression, while other KDMs were unaffected or increased expression.

2.2 Materials and Methods

2.2.1 Cell culture

HEK293 cells were generously donated by the laboratory of Spencer Gibson at the Manitoba Institute of Cell Biology and were originally sourced from ATCC (CRL-1573) and authenticated by morphology under microscopic examination with reference to the cell micrograph from ATCC. K562 cells were generously donated by the laboratory of Brian Hasinoff at the University of Manitoba, College of Pharmacy. The cells were originally sourced from ATCC (CCL-243) and authenticated by morphology under microscopic examination with reference to the cell micrograph from ATCC. K562 cells were further authenticated via gene expression analysis for the HOXA9 gene with quantitative PCR using previously defined methods (*74*). Previous studies have shown that K562 cells do not express HOXA9 (*81*). We found that the K562 cells have undetectable expression of HOXA9 in comparison to a positive control cell line MOLM-13 obtained from DSMZ (ACC 554 ref number is A1408102-1) (*74*).
HEK 293 cells were cultured in EMEM supplemented with 1% penicillin/streptomycin and 10% FBS. K562 cells were cultured in alpha-MEM (Life Technologies Cat. 1200-002) supplemented with 2.2 g/L NaHCO3, 20 mM HEPES, 1% pen/strep, pH adjusted to 7.1-7.4, sterile filtered then FBS (10%) was added. Cells were incubated at 37 °C in 5% CO_2, replenishing media every 72 hours. Exponentially growing cells were divided and banked upon receipt, and cultured cells were passaged no more than 30 times.

2.2.2 HDAC inhibitor dose response studies for analysis by LC-MS/MS

HDAC inhibitors vorinostat, mocetinostat, and entinostat (Appendix **Figure A1**) were diluted in sterile filtered DMSO to make appropriate concentrations. Cells were washed with PBS and seeded 1:5 at 80% confluence and allowed to grow 24 h or until a cell density of 10^5 -

10^6/mL. Cells were treated with 0-100 μM of HDAC inhibitors and a total concentration of DMSO less than 1%, for 24h and harvested by centrifugation.

2.2.3 MTS assay HDAC inhibitor dose response studies

Cells were seeded in 96 well plates in 100 μL aliquots of complete media containing 6000 cells incubated for 24 hours then treated with 0-100 μM HDAC inhibitors maintaining total concentration of DMSO less than 1%. Treated cells were incubated for 72 hours. 15 μL of MTS (Promega) dye was added to each well and incubated for an additional 3 hours. The absorbance of each well was measured at 480 nm.

2.2.4 Histone isolation and proteolysis

Treated and untreated cells were pelleted, and histones isolated with an Epiquik (Epigentek) kit according to the manufacturer's instructions, omitting DTT in the buffer. Histones were precipitated over night at 4 °C with 4% perchloric acid. Precipitated histones were pelleted by centrifugation at maximum RCF for 1 h, washed with 4% perchloric acid, aspirated, washed with 0.2% HCl in acetone, aspirated, washed with acetone and dried. Histones were resuspended in 100μL of water, quantified at 280 nm using calf thymus histones (Sigma) as an external standard, confirmed using the extinction coefficient 3960 $Lmol^{-1}cm^{-1}$. Histone solutions were diluted with water to 1mg/mL and stored at -80 °C. Histones were digested with 1:1 (w/w) Pronase (Sigma) in 50mM ammonium bicarbonate (pH 8) with 0.5 mM calcium chloride at 37°C for 72 h. After digestion, Pronase was filtered out using 10 kDa cutoff centrifugal filters and the flow-through with free amino acids was collected and dried in a vacuum centrifuge. The digested sample was reconstituted in 100 μL of 0.05% formic acid for analysis of acetyl-lysine (AcK) using LC-MS/MS.

2.2.5 Vapor phase acid hydrolysis

Methylated lysine and arginine residues were liberated from histones using acid hydrolysis. Modified histones were dried in a vacuum centrifuge in 300 µL HPLC inserts and placed in an ELDEX vacuum hydrolysis vessel with 250 µL of 6N HCl and hydrolysis performed in the vapor phase at 110 °C for 24 h. After the reaction period the inserts were removed and dried. The hydrolyzed sample was reconstituted in 100 µL of 0.05% formic acid for analysis of methylated lysines and arginines by LC-MS/MS.

2.2.6 LC-MS/MS

A Nexera UHPLC connected to an 8040 triple quadrupole mass spectrometry system (Shimadzu) was used for analysis. Chromatographic separation was achieved using a Primesep200 (Sielc) HPLC column heated to 40 °C, at a flow rate of 0.4 mL/min using mobile phases (A) 0.05% formic acid in water and (B) 1% formic acid in 50% aqueous acetonitrile. Initial conditions were 0% (B) for 1.5 minutes, increasing to 85% over 30 seconds and held for 3 minutes. The column was washed with 100% (B) for 2 minutes and reconditioned for 3 minutes with 0% (B) for a total run time of 10 minutes. Analytes were detected in positive multiple reaction monitoring (MRM+) mode using DUIS (ESI-APCI) ionization. The nebulizing gas was set to 2 L/min, drying gas was 15 L/min, desolvation line temperature was 250 °C and the heating block 400 °C. The precursor and product ion *m/z* values were initially estimated with Q1 and product ion scans in the expected *m/z* ranges, respectively. The initial values were then optimized using the software (**Table 1**). The assay quantifies lysine (K), acetyl-lysine (AcK), mono-(MeK) di-(Me2K) and trimethyl-lysine (Me3K), monomethyl-arginine (MeR) asymmetric-(aMe2R) and symmetric dimethyl-arginine (sMe2R) with the corresponding standards.

Table 1. Mass Spectrometry parameters: MRM transitions and collision energies

Modified Amino Acids	Retention Time (min.)	MRM Transition	Collision Energy (eV)
AcK	2.03	189.2>126.0	-14
K	3.27	146.9>130.0	-14
MeK	3.34	161.2>84.0	-18
Me2K	3.42	174.8>84.0	-20
Me3K	3.5	189.2>84.0	-22
MeR	3.41	188.8>70.2	-24
sMe2R	3.46	202.8>172.9	-14
aMe2R	3.46	203.0>45.9	-14

2.2.7 Validation

Recombinant histones with a single modified residue (ActiveMotif) were used to calculate recovery of the hydrolysis reactions. 500 nM of H3K9Ac, H3K9Me, H3K9Me2 and H3K9Me3 were used to validate Pronase proteolysis and 500 nM of H3K4Me, H3K4Me2, and H3K4Me3 were used to validate acid hydrolysis. The concentrations of the recombinant histones were confirmed by UV at 280 nm using the extinction coefficient of 3960 $Lmol^{-1}cm^{-1}$. The results were normalized to the amount of lysine recovered to control for variations in total histone, incorporation of modification and completeness of hydrolysis. The experimental ratio was measured and divided by the theoretical ratio of the modified histone to report the recovery percentage. Briefly, the expected number of lysines on histone H3 was determined from the sequence. The recombinant modified histones were only modified at a single lysine residue so the number of modified residues could be determined. The peak area of modified lysine was divided by the peak area of unmodified lysine and this ratio was divided by the expected ratio claimed by the supplier to calculate percentage recovery.

2.2.8 Histone H3 Modification Multiplex Assay

Histone H3 lysine mono- di- and trimethylation was measured at histone H3, K4, K9, K27, K36 and K79, and acetylation was measured at histone H3, K4, K14, K18 and K56 using a Histone H3 Modification Multiplex Assay Kit (Abcam ab185910). Histone samples from the above dose response curve for the 24 h treatment of 10 μM mocetinostat in K562 cells were used at 50 ng/well of histone extracts in duplicate according to the manufacturer's instructions. The results were compared to a no-treatment control to calculate a percent change in histone modification with mocetinostat treatment.

2.2.9 qPCR gene expression

K562 cells were treated with increasing concentrations of HDAC inhibitors similar to above and RNA isolated and purified using an Ambion Purelink RNA mini kit (life technologies). RNA was quantified using a spectrophotometer at 260 nm and 1.5-2 μg RNA was reverse transcribed to cDNA using Super Script Vilo master mix (life technologies) according to manufacturer's instructions. Quantitative PCR was performed using a ViiA 7 Real-Time PCR (qPCR) System (Life Technologies) and a custom TaqMan gene expression array for lysine demethylases (Appendix **Table A1**). PCR reactions were prepared in 10 μL volumes using 1 μL of cDNA and TaqMan fast Universal master mix (Life Technologies). Thermocycling conditions were as follows, 50 °C for 2 minutes, 95 °C for 20 seconds followed by 40 cycles of 95 °C for 1 second then 60 °C for 20 seconds. Relative gene expression was quantified by comparative CT (ΔΔCT) to no treatment control and normalized using 18s rRNA housekeeping gene.

2.2.10 Data analysis

For the LC-MS/MS data, HDAC inhibition was measured as a function of total acetyl-lysine in the cell hydrolysates that was normalized as a ratio of the concentration of lysine to account for total protein. Controls with no inhibitor were used to establish baseline levels of acetyl-lysine. 100% inhibition is determined relative to the maximal level of acetylation in each cell group. Each curve was normalized to its own maximal response. The curves were fit to a four-parameter logistic regression to calculate each HDAC inhibitors IC_{50} using Sigma Plot 11. The number of methyl groups at each HDAC inhibitor concentration was used to calculate a weighted total of methyl-lysine and methyl-arginine based on the sum of each methyl species.

2.3 Results

2.3.1 Histone hydrolysis and method validation

To measure the modifications to histones derived from cells treated with HDAC inhibitors, histones were extracted from cells and hydrolyzed into modified amino acids for measurement using LC-MS/MS. The hydrolysis and the LC-MS/MS method were validated using recombinant histones with a single site of modification. Regardless of the method of hydrolysis used, the recoveries of modified amino acid from the corresponding recombinant histones were found to be greater than 80% with a standard deviation of less than 20%, which we considered quantitative. We found vapor phase acid hydrolysis of recombinant modified histones with methyl-lysines to be superior with respect to mean recovery and standard deviation (**Table 2**). Recovery of methyl-arginines via acid hydrolysis has already been validated (*82*). Acid hydrolysis of recombinant acetylated histones gives an expected recovery of 0% because the acetylation is removed during the acid hydrolysis procedure. Therefore we used proteolysis with Pronase to measure acetyl-lysine and acid hydrolysis to measure methyl-arginines (MeR, aMe2R and sMe2R) and methyl-lysines (MeK, Me2K and Me3K). This method is the first to validate quantitative recovery and measurement of lysine modifications from histones.

Table 2. Recoveries of epigenetic modifications from modified recombinant histones.

Histone	**H3K4Me**	**H3K4Me2**	**H3K4Me3**	**H3K9Ac**	**H3K9Me**	**H3K9Me2**	**H3K9Me3**
Modification	*MeK*	*Me2K*	*Me3K*	*AcK*	*MeK*	*Me2K*	*Me3K*
Hydrolysis	% recovery of epigenetic modification from recombinant histone						
Acid	83.8±6.9†	117.4±8.4	103.9±5.0	0.0‡	N/A	N/A	N/A
Proteolysis	N/A	N/A	N/A	90.7±15.8	87.1±8.5	100.2±18.1	86±19.1

‡The expected recovery of all acetyl-lysine modifications during acid hydrolysis is 0.
†The values are mean % recovery and standard deviation of 10 samples.

2.3.2 Histone hyperacetylation correlates with cell viability

To measure the effect of HDAC inhibitors on histone acetylation in cells, we used the LC-MS/MS assay to measure changes in acetyl-lysine derived from histones from cells treated with the HDAC inhibitors vorinostat, entinostat, and mocetinostat (**Figure 2.1**). K562 cells were used because HDAC inhibitors are being studied for their potential use for treatment of chronic and other forms of myeloid leukemia (*83*). HEK293 cells are a non-leukemic cell line used for comparison. The IC_{50} calculated for global histone acetylation and growth inhibition by the MTS method were similar for each cell and drug group (**Table 3**). The no-treatment control levels of histone acetylation are lower in K562 than in HEK293 which agrees with a recent report showing higher expression of HDAC in K562 cells leading to increased effect of HDAC inhibitors (*84*). This also agrees with our observed higher potency for all HDAC inhibitors tested in K562 compared to HEK293 cells (**Table 3**). The decrease in the viability of K562 cells by HDAC inhibitors is directly correlated with histone acetylation by comparison of IC_{50} values measured in both MTS and lysine acetylation via our LC-MS/MS assay. The agreement of these values suggests that in K562 cells, histone hyperacetylation by HDAC inhibitors is related to cell viability. However, with respect to the MTS assay we cannot differentiate between decreased cell growth and cell death by apoptosis or any other mechanism of cell death, so for the purpose of this study we use the term decreased cell viability. HDAC IC_{50} values calculated for vorinostat, entinostat and mocetinostat were similar, but entinostat and mocetinostat showed higher potency than vorinostat (**Figure 2.1**).

Table 3. HDAC inhibitory potency

HDAC IC_{50} μM				AcK/K (10^{-3})			
Cell type	entinostat	mocetinostat	vorinostat	control	10μM entinostat	10μM mocetinostat	10μM vorinostat
K562	0.67±0.05 (0.97±0.04)‡	0.48±0.06 (0.54±0.01)	1.3±0.3 (1.4±0.2)	4.5±0.9	35.9±6	37.3±6	36.5±5
HEK293	1.0±0.2 (5.4±0.2)	0.8±0.1 (1.95±0.02)	1.7±0.2 (2.7±0.2)	9.3±2	38.1±6	40.1±9	50.4±7

HDAC inhibition and growth inhibition as measured using LC-MS/MS

‡MTS IC_{50}, in parenthesis.

AcK/K absolute quantity of acetyl-lysine (μM) normalized to lysine (μM).

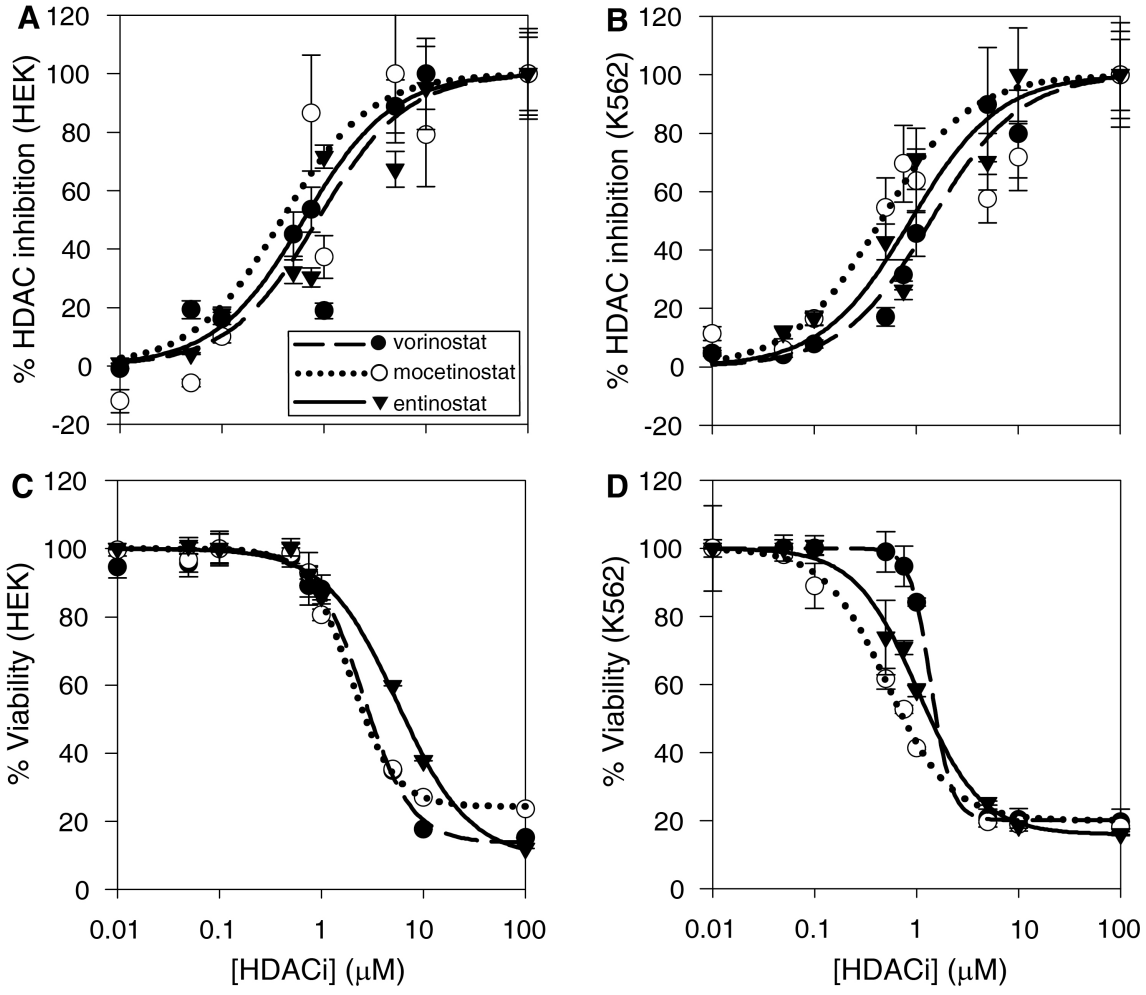

Figure 2.1 Acetyl-lysine dose response curves from 24 hour HDAC inhibition from HEK293 cells (A) and K562 cells (B) measured using LC-MS/MS. Increases in acetyl-lysine during treatment were measured with respect to no treatment controls. 100% inhibition was determined by the maximum increase in acetyllysine achievable for each HDAC inhibitor. MTS Growth inhibition curves from 72 hour HDAC inhibition from HEK293 cells (C) and K562 cells (D).

2.3.3 Time dependent changes in histone acetylation and methylation

To measure the effects of HDAC inhibitors over time, K562 cells were treated with a single 2 μM dose of vorinostat and histones were isolated from the cells up to 72h (**Figure 2.2A**). The maximum histone acetylation occurs 8 hours post vorinostat treatment and decreases from 400 – 200% over three days. This may be because of cellular metabolism of the drug or some compensatory mechanism (such as increased HDAC or decreased KAT expression). Methylation of lysine and arginine were also measured and a sustained decrease in aMe2R was noted, as well as an initial increase in MeK at 4h followed by a steady decrease up to 72h (**Figure 2.2A**).

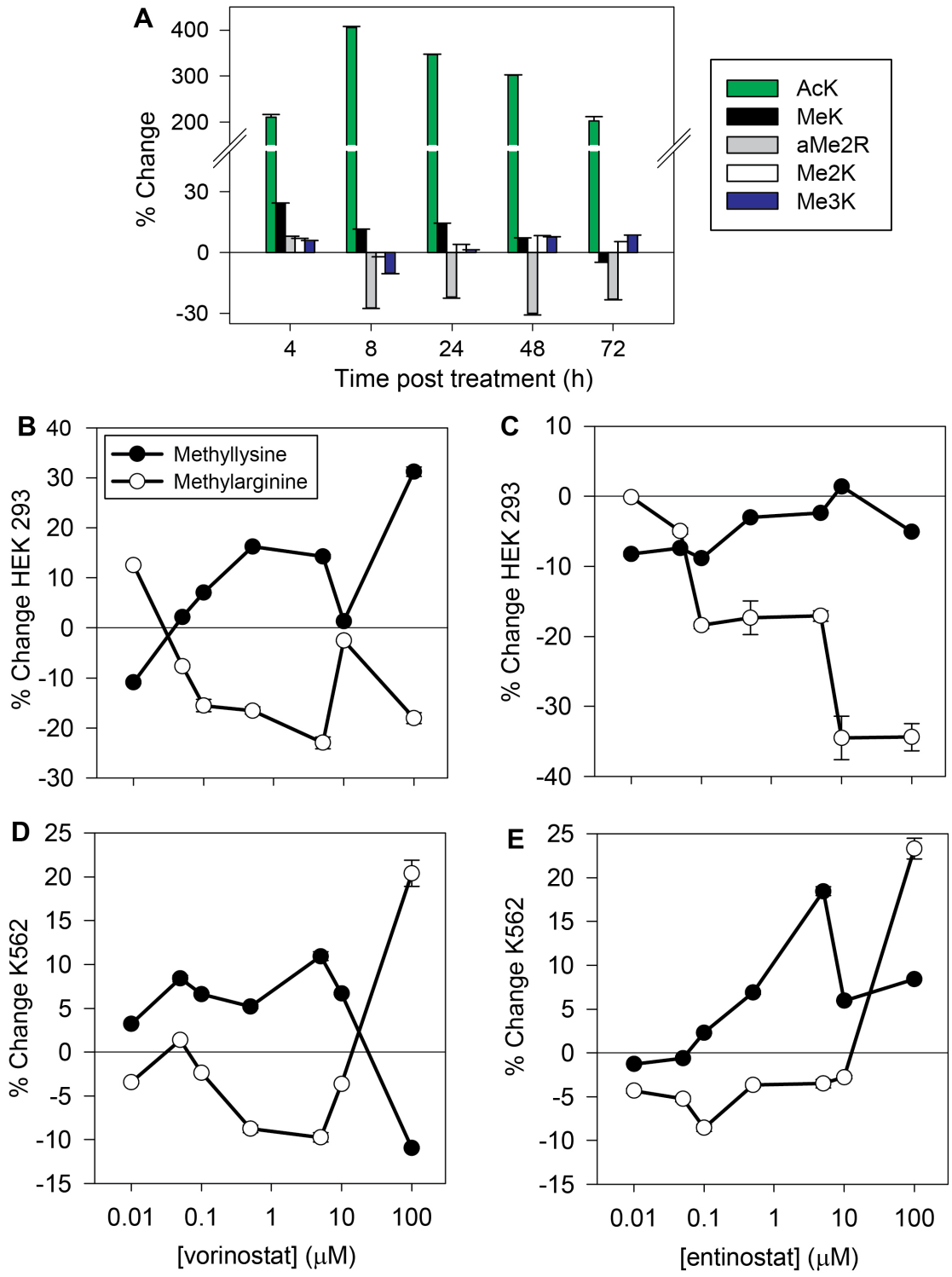

Figure 2.2 The effect of vorinostat and entinostat on global histone modifications in the indicated cell lines. The effects of a single 2 μM dose of vorinostat over 72 h calculated as the percent change in epigenetic modifications relative to a no treatment control (A). Total methyl-lysine and methyl-arginine percent change relative to no treatment control with increasing dose of vorinostat are displayed for HEK293 cells (B) and K562 cells (D), and increasing entinostat dose for HEK293 cells (C) and K562 cells (E).

Histone lysine and arginine methylation were measured as a function of HDAC inhibitor dose in both K562 and HEK293 cells and it was found that changes in total lysine and total arginine methylation varied with dose (**Figure 2.2B, C, D and E**). With vorinostat, a general trend of decreasing arginine methylation and increasing lysine methylation was observed with HEK293 cells (**Figure 2.2B**). In K562 cells, histone lysine methylation increases slightly and arginine methylation decreases slightly until the highest dose, at which arginine methylation trends up and lysine methylation trends down (**Figure 2.2D**). Treatment with entinostat in HEK 293 cells produced little change in lysine methylation but a nearly 40% reduction in arginine methylation at the highest doses (**Figure 2.2C**). This effect is produced almost exclusively by reductions in aMe2R with increasing entinostat dose yielding an IC_{50} of 0.65±0.2 μM in HEK 293 cells (**Figure 2.3**). In K562 cells, entinostat produced small increases in both histone lysine, and arginine methylation at the highest dose (**Figure 2.2E**).

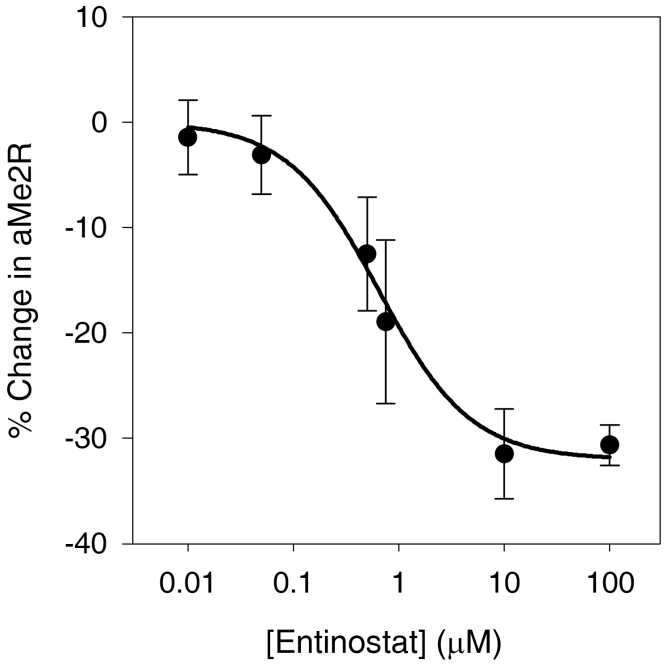

Figure 2.3 Dose response for decrease in global histone asymmetric dimethylarginine (aMe2R) with increasing entinostat dose. The percent decrease in aMe2R with entinostat dose yields an IC_{50} of 0.65±0.2 μM in HEK 293 cells.

2.3.4 Mocetinostat increases lysine methylation and is correlated with decreased expression of lysine demethylases in K562 cells

A correlation between mocetinostat dose and increasing lysine methylation was also observed in K562 cells (**Figure 2.4A**) but not in HEK 293 cells (**Figure 2.5**). All methyl-lysine species increase with dose although MeK was less affected. Methyl-lysine species were plotted against mocetinostat concentration (**Figure 2.4A**) and a dose response effect was observed with IC_{50} values of 4.3±0.6, 3.7±0.7, 4.1±0.2 and 2.7±0.6 μM for increasing, MeK, Me2K Me3K and weighted total lysine methylation, respectively (**Figure 2.4B**). Mocetinostat was the only HDAC inhibitor tested to produce such clear dose response effects on lysine methylation. No consistent effect of mocetinostat on arginine methylation was observed in either HEK 293 or K562 cells (**Figure 2.5**).

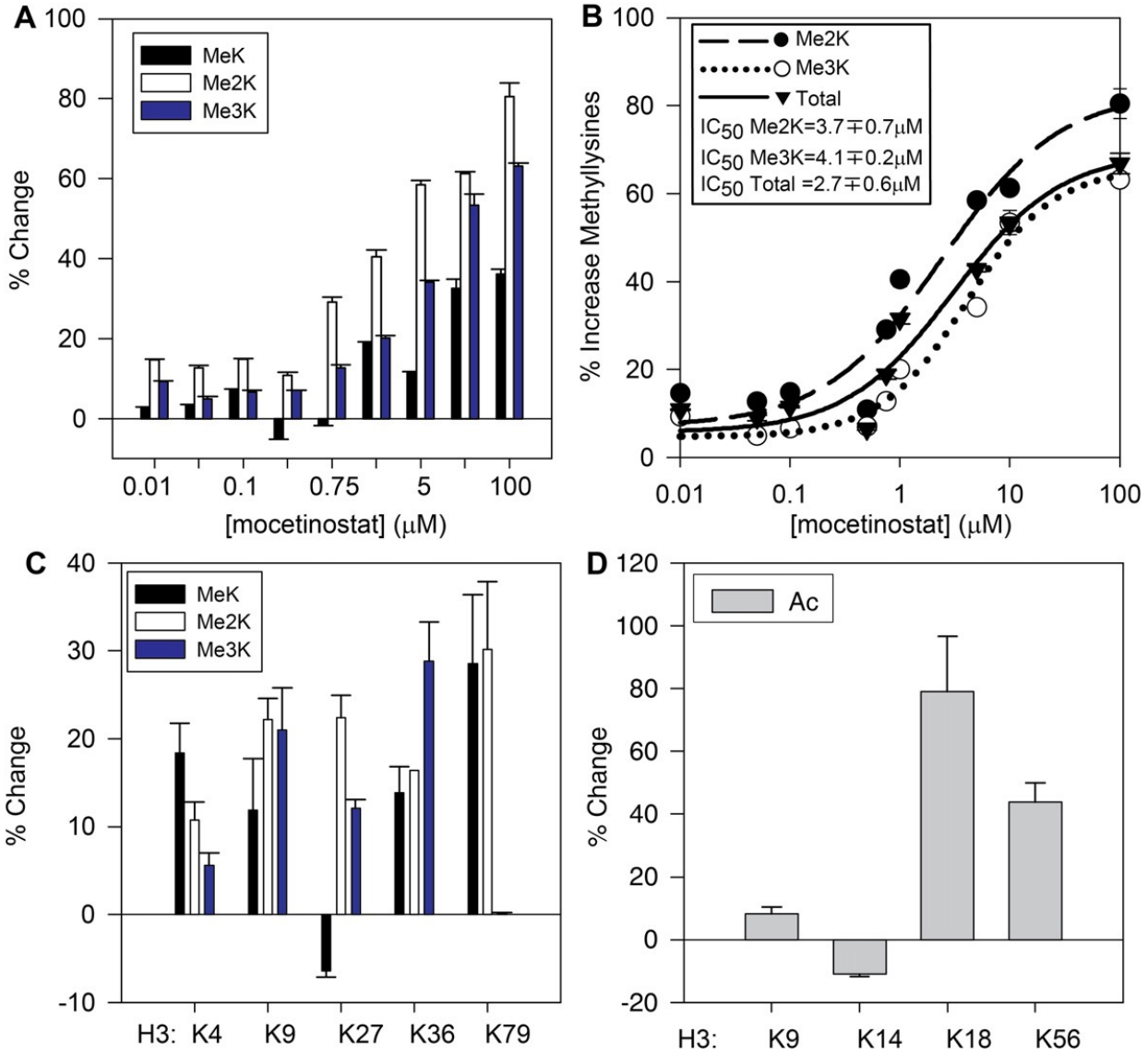

Figure 2.4 The effects of mocetinostat on lysine methylation. Changes in lysine methylation from 24h mocetinostat treatment in K562 cells (A). Dose response curves for increasing Me2K and Me3K relative to no treatment control (B). The fit IC_{50} values for increasing, MeK, Me2K Me3K and total lysine methylation with increasing mocetinostat are, 4.3±0.6, 3.7±0.7, 4.1±0.2 and 2.7±0.6 μM respectively. The total lysine methylation is a weighted sum of methyllysines (ie MeK+2Me2K+3Me3K) relative to no treatment control. Changes in histone H3 lysine methylation (C) measured via histone H3 modification multiplex assay kit are recorded for the listed histone H3 lysine residues, also shown is the changes in lysine acetylation (D) at the listed residues.

Histone samples from the 10 μM mocetinostat treatment group were analyzed for changes in histone H3 K4, K9, K27, K36 and K79 mono- di- and trimethylation compared to a no treatment control (**Figure 2.4C**). The results show elevated methylation for most lysine

tested. These results were consistent with an overall increase in histone lysine methylation that was also observed using our LC-MS/MS assay. Consistent increases in all types of lysine methylation were observed for H3K4, H3K9, and H3K36 but the magnitude of increase for mono- di- and trimethylation was highest for H3K9 and H3K36. The increases in histone H3 acetylation were primarily on H3K18 and H3K56 (**Figure 2.4D**).

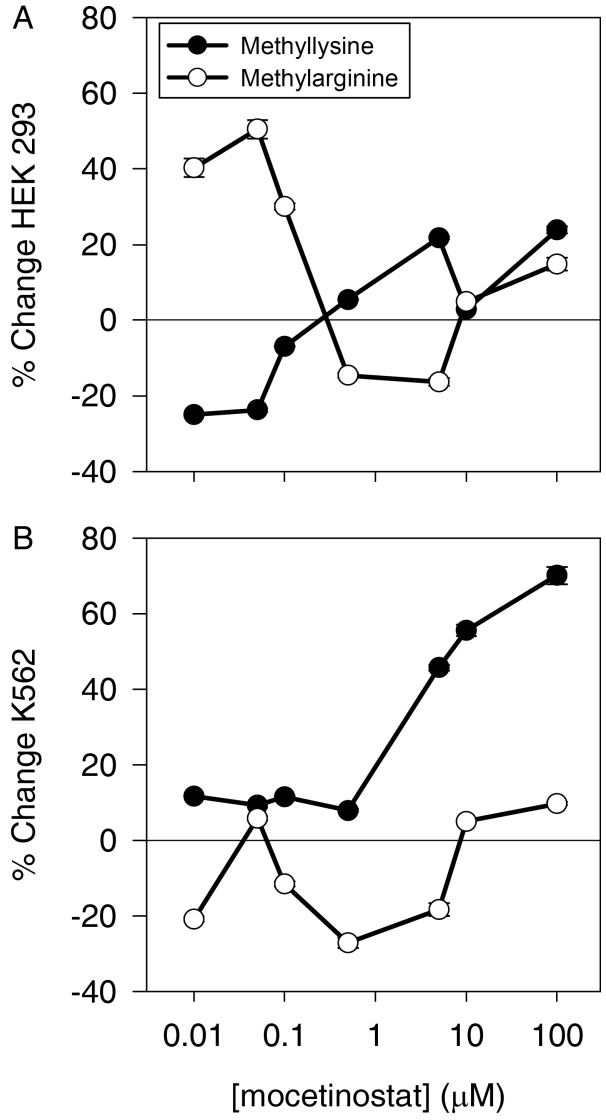

Figure 2.5 Effects of mocetinostat dose on total histone arginine and lysine methylation in HEK 293 (A) and K562 (B) cells. The trace in B for lysine methylation is similar to part of Figure 3B but has fewer concentrations of mocetinostat.

We wanted to investigate whether the prominent increases in histone lysine methylation produced by mocetinostat were a result of direct KDM inhibition. Using a KDM4A (JMJD2A) inhibitor screening kit (Cayman) we tested the inhibitory activity of mocetinostat using the KDM inhibitors IOX1 and JIB04 as positive controls. Mocetinostat was found to interfere with the fluorescence detection. Therefore, to evaluate the effects of mocetinostat we took the contents of each well, hydrolyzed them and quantified the amounts of MeK, Me2K, and Me3K in each reaction well using the LC-MS/MS assay. The positive control wells from JIB04 and IOX1 produced minor amounts of Me2K confirming their demethylase inhibitory activity while mocetinostat provided similar amounts of Me2K as the negative control and other HDAC inhibitors indicating negligible direct lysine demethylase inhibitory activity (**Figure 2.6**).

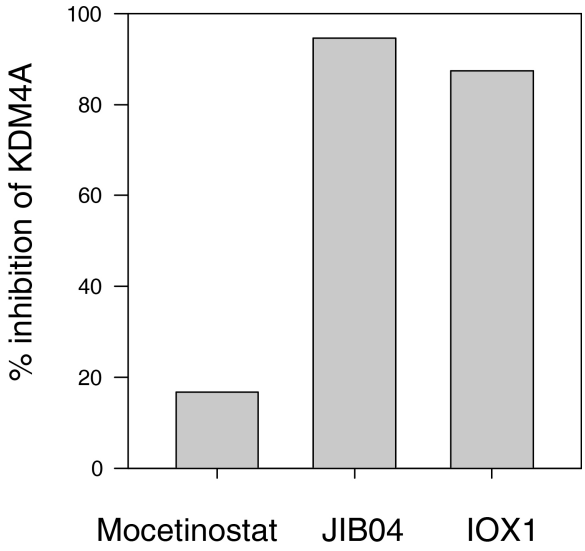

Figure 2.6 Direct KDM4A inhibition by mocetinostat and KDM inhibitors JIB04 and IOX1 at 100μM. Mocetinostat does not directly inhibit KDM4A activity.

To test whether the changes in methyl-lysine species during HDAC inhibitor treatment were a result of changes in KDM gene expression, K562 cells were treated with vorinostat (**Figure 2.7A**) entinostat (**Figure 2.7B**), or mocetinostat (**Figure 2.7C**) for 24 hours and the expression of seven major lysine demethylases was measured using quantitative PCR (qPCR).

Dose dependent decreases in the expression of lysine specific demethylase 1A (KDM1A/LSD1), KDM2A, KDM3A, KDM4A, KDM5A (jumonji/ARID domain containing demethylase 1A (JARID1A)), and KDM6A (ubiquitously transcribed tetratricopeptide repeat protein X-linked (UTX)) were observed resulting in up to a 90% decrease in expression with 100 µM mocetinostat (**Figure 2.7C**). At the 100 µM dose mocetinostat is the only HDAC inhibitor tested that consistently decreases the expression of all KDMs tested (**Figure 2.7D**).

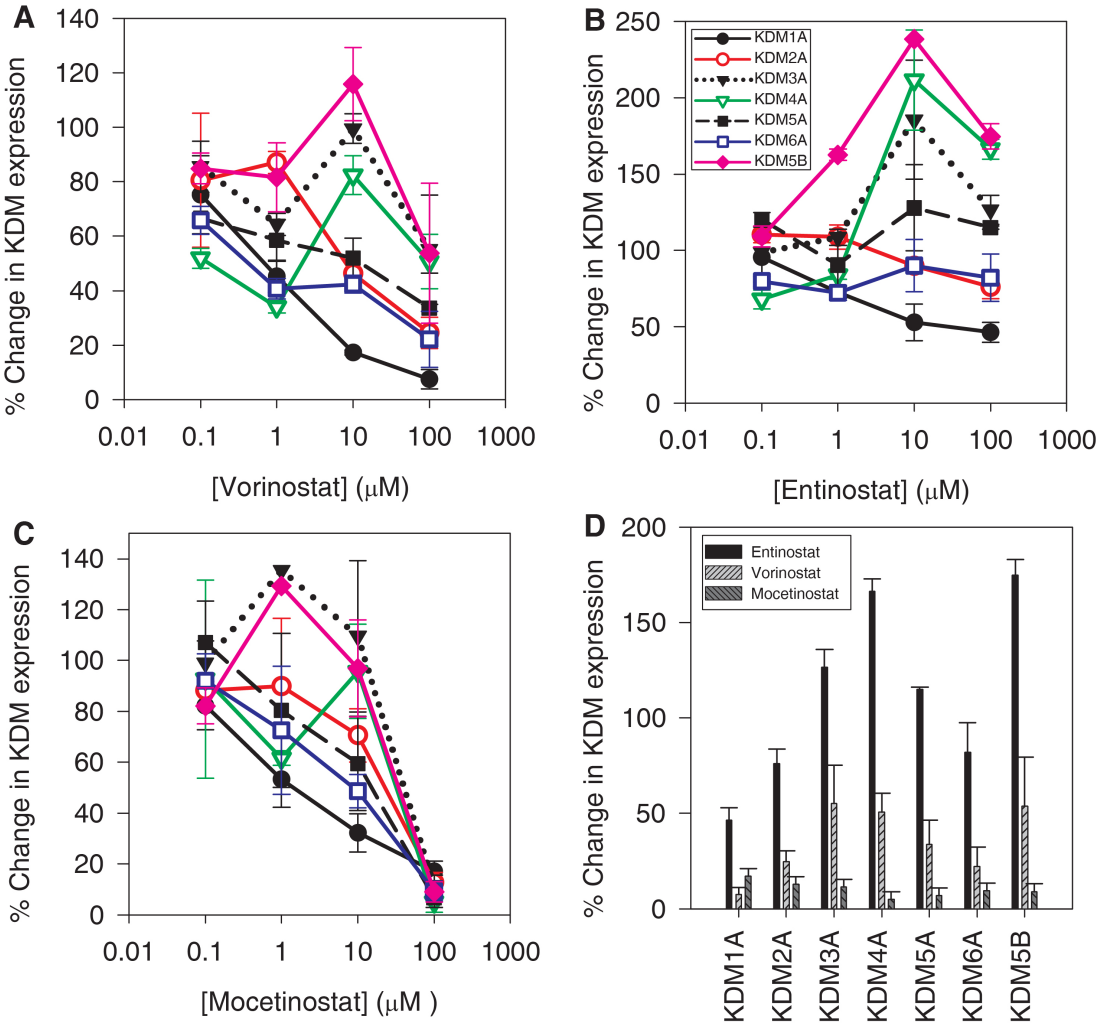

Figure 2.7 The effect of 24 h mocetinostat treatment on expression of lysine demethylases using qPCR. Changing expression of the listed KDM with increasing dose of vorinostat (A), entinostat (B) and mocetinostat (C). The relative KDM expression is compared for each drug at 100 µM (D) demonstrating that only mocetinostat is correlated with decreases in expression of all KDM. The relative gene expression is measured against no treatment control and normalized with s18 rRNA as a housekeeping gene using ΔΔCT quantification.

2.4 Discussion

2.4.1 Proteolysis and acid hydrolysis is valid and quantitative.

Validation of the hydrolysis and LC-MS/MS assay was necessary to ensure that changes in epigenetic modifications were real and quantifiable. To achieve this we used several recombinant histone H3 proteins each with a single unique site of modification and normalized this to total lysine to control for protein concentration similar to previous methods (*79*). Corrected recoveries of methyl-lysines validated by acid hydrolysis were similar to methyl-arginines (*78, 82*). Our validation also demonstrated that methyl-lysines were stable under acid hydrolysis like methyl-arginines (*77-80*). Proteolysis was needed to validate acetyl-lysine within histones because the acetate modification was rapidly hydrolyzed during acid hydrolysis.

The method presented here is the only validated way to completely measure the effects of HDAC inhibitors on histones. Other methods using antibodies and Western blots can detect modifications at specific positions but cannot quantify total amounts of modification. In fact, it is questionable if such techniques are quantitative unless standards of each modified protein are used and this is almost never done. Traditional proteomic strategies often cannot cover the entire sequence of histones leaving some potential sites of modifications unquantifiable. Here we describe a method that quantifies several epigenetic modifications on histones simultaneously, but without the context of sequence. Similar procedures have been used to hydrolyze proteins to assay for acetyl-lysine but the study did not include methyl-arginines and was not validated. In fact, we found that by following the methods in this study we could not achieve quantitative recovery of any epigenetic modification (*85*).

HDAC inhibitors have previously been shown to decrease cell viability by causing cell cycle arrest and apoptosis (*86*). We observed relationships between decreased cell viability and

increasing histone lysine acetylation during HDAC inhibitor treatment (**Figure 2.1**). The similarities in IC_{50}, as measured by MTS and LC-MS/MS assays (**Table 3**) suggest that the decrease in cell viability produced with HDAC inhibitors may be related to histone hyperacetylation.

2.4.2 HDAC inhibitors induce changes in histone methylation

We have shown that, other than lysine hyperacetylation, changes in lysine and arginine methylation accompany HDAC inhibitor treatment. These changes in lysine and arginine methylation may contribute to the intended cytotoxic effect or side effects of HDAC inhibitors and appear to differ among cell lines, HDAC inhibitor, and dose. Broadly, our results reveal a trend of increasing or stable lysine and decreasing or stable arginine methylation with all HDAC inhibitors in HEK 293 cells. In K562 cells, lysine methylation increases slightly and arginine methylation is stable or increases only at the highest dose. Interestingly, mocetinostat produces a potent dose dependent increase in lysine methylation that was correlated with a dose dependent decrease in expression of seven KDMs. This suggests that the increases in global histone lysine methylation with mocetinostat in K562 cells may be caused by a decrease in expression of several KDM. However, the exact mechanism by which mocetinostat decreases KDM expression was not explored in this study.

In order to compare our results using the LC-MS/MS assay with mocetinostat to other assays measuring modifications on specific histone residues, a histone H3 modification multiplex colorimetric assay kit (Abcam) was used (**Figure 2.4C**). Increases in methylation were observed at all residues measured. These results agree with the LC-MS/MS assay and are also congruent with the generalized decrease in expression of KDM produced by mocetinostat that was observed by qPCR. The largest and most consistent increases in all types of lysine methylation

were observed at H3K9 and H3K36. H3K9 is a generally accepted repressive mark while H3K36 reduces chromatin accessibility and is involved in both DNA repair and splicing (*87, 88*). As both modifications are generally repressive, the increased lysine methylation induced by mocetinostat may ultimately reduce expression of additional genes as a result of its decrease in expression of KDMs. Complicating matters, increases in histone H3K4 acetylation with mocetinostat appear to be much lower than other sites (**Figure 2.4D**), suggesting that the increases in histone acetylation caused by mocetinostat will not prevent permissive methylation of H3K4.

Using antibody based assays like the Histone H3 Modification Multiplex Assay Kit, multiple sites of histone H3 modification are measured, however, many other modifications like arginine methylation and other sights of potential lysine methylation and acetylation are not monitored and therefore not quantified. In particular, at least 12 lysines are methylated on histone H3 and at least 5 more are thought to exist on histone H4 to say nothing of histones H2A, H2B and H1 (*5*). Moreover, we have already observed that many antibodies cross-react with other modifications making quantification difficult or impossible (*74*) and although the multiplex assay used in this study was validated by Abcam we cannot rule out this issue. Thus, the LC-MS/MS assay described in this work is superior for the measurement of total effect of mocetinostat and other drugs inhibiting epigenetic enzymes because it measures all changes in lysine acetylation and methylation and arginine methylation regardless of residue position. In this way the complete effect of the drug on these types of modifications can be measured. In addition, the assay time and cost per sample are significantly lower than other methods.

Although most studies continue quantifying the amounts of modifications on specific histone residues, this information is less useful without more knowledge about what genes are

affected and the context of all other modifications to histones that might be present. However, the experiments needed to garner such information would be labor intensive, cost prohibitive and may still not be quantitative or bring any insight as to the expression of the gene in question that cannot otherwise be derived from qPCR.

Vorinostat and entinostat produced decreases in total histone arginine methylation in HEK 293 cells with a dose dependent decrease in aMe2R with entinostat treatment (**Figure 2.2C, Figure 2.3**). These dose dependent decreases of methylarginine caused by vorinostat and entinostat were not observed in K562 cells, where instead, increased methylarginines were observed at the highest concentrations of inhibitor (**Figure 2.2D,E**). The decreases in arginine methylation in HEK 293 cells caused by entinostat were very potent with an IC_{50} similar to its increase on histone acetylation. However, the observed changes in histone arginine methylation do not represent a general HDAC inhibitor class effect because mocetinostat did not have a consistent dose response effect on arginine methylation in either HEK 293 or K562 (**Figure 2.5**).

Previous studies have shown that some HDAC inhibitors increase lysine methylation on specific histone lysine residues especially H3K4Me2 and H3K4Me3 and our results corroborate this (*89*). This is significant because H3K4Me2 and H3K4Me3 have been associated with increased gene expression upon HDAC inhibition (*90*). For example, the weak pan-HDAC inhibitor valproic acid increases the generally permissive H3K4Me3 mark by the repression of KDM5A (JARID1A) and also reduces the generally repressive H3K27Me3 mark catalyzed by EZH2 (*91, 92*). Together with the expected increases of lysine acetylation by valproic acid, increases in H3K4Me3 and decreases in H3K27Me3 would result in the general increase of gene expression. Further, Huang *et al.* showed increases in H3K4Me2 and H3K4Me3 accompanied by decreases in KDM1A (LSD1) and KDM5B (PLU1) mRNA and protein in LNCaP cells

treated with the HDAC inhibitors entinostat and vorinostat (*73*). Earlier reports show a link between HDAC inhibition and methyltransferase activity where hyperacetylation leads to increased binding sites for the lysine methyltransferase MLL4, resulting in methylation of H3K4 (*72*). Therefore, these studies suggest that increasing histone lysine methylation with treatment by some HDAC inhibitors may be explained by decreased expression of KDM1A and KDM5A or increased KMT activity. Our results show that in addition to increases in H3K4 methylation, mocetinostat increases methylation at H3 K9, K27, K36 and K79, all of which have differing or even opposing gene regulatory signatures (whether they represent gene activation or repression). In fact the greatest increases in methylation appear to be on H3K9 and H3K36 (**Figure 2.4C**). In order to begin to explore our finding of increased lysine methylation with the HDAC inhibitors we tested, we had to determine how this activity was manifested. It is unlikely that the HDAC inhibitors directly bind to other histone modifying enzymes causing direct inhibition of KDMs, and even more unlikely they were directly activating KMTs. There is however, growing body of evidence suggesting that our observed increases in lysine methylation could be a result of reduced expression of KDMs, however the extent and to which KDMs are ultimately affected is not completely clear in the literature. To determine if the HDAC inhibitors tested reduced KDM expression, we measured the expression of a panel of KDM in K562 cells treated with mocetinostat, entinostat and vorinostat by qPCR because K562 cells showed the highest increase in histone lysine methylation with mocetinostat treatment.

Each HDAC inhibitor produced dose-dependent decreases in expression of LSD1 (KDM1A) to some extent. Vorinostat and mocetinostat appear to be most potent at decreasing expression of LSD1 (**Figure 2.7**) but only mocetinostat potently decreased expression of all seven KDM tested. Surprisingly, in addition to a modest reduction in LSD1 expression,

entinostat produced increases in expression of KDM4A and KDM5B. Moreover, entinostat does not produce a greater than 50% reduction in the expression of any KDM tested (**Figure 2.7B**). These results may explain why, despite the fact that entinostat was found to be a potent HDAC inhibitor (**Table 3**) it did not produce the same dramatic increases in lysine methylation that was observed with mocetinostat. Vorinostat was the least potent of the HDAC inhibitors tested. This was also reflected in its effect of decreasing KDM expression, as vorinostat only decreased the expression of LSD1, KDM2A, KDM5A and KDM6A appearing less potent with respect to these effects than mocetinostat. This may explain why vorinostat did not produce the same increases in total lysine methylation that were observed with mocetinostat using LC-MS/MS.

We show that HDAC inhibitors cause changes in other epigenetic modifications including histone arginine and lysine methylation in addition to the expected increase in histone lysine acetylation. Others have noted changes in lysine methylation at specific residues. Here we show that particular HDAC inhibitors have different effects on total histone arginine and lysine methylation that also depend on cell line and are therefore not an HDAC inhibitor class effect. The changes in histone lysine methylation are in many cases correlated with changes in KDM expression presenting a possible explanation. These results show that inhibition of epigenetic enzymes like HDAC can influence other epigenetic modifications by altering expression of enzymes that add or remove those modifications. Such effects may contribute to potency but may also produce off target effects that are a result of selective inhibition and are therefore inseparable from the drugs mechanism of action. The off target effects of HDAC inhibitors observed in this study likely stem from the ubiquitous nature of HDAC enzymes that have activity at multiple genes and therefore likely alter the expression of multiple genes even upon enzyme selective inhibition. However, with the present data we cannot determine if HDAC

inhibitors directly alter KDM expression with their activity at the KDM promoters or if the changes in KDM expression arise as a result of some down-stream effect. For example, it is conceivable that HDAC inhibitors could alter the expression of transcription factors, co-activators or co-repressors that then alter KDM expression.

The implications of changing histone lysine methylation by HDAC inhibitors are difficult to predict based on our results. The inconsistent effect of vorinostat and entinostat on KDM expression may lead to differential methylation effects on specific histone lysines like that observed with valproic acid which increases H3K4Me3 but decreases H3K27Me3 (*92*). The global increases in methylation produced by mocetinostat increase the transcriptionally permissive H3K4 methylation but also increase the repressive H3K9 and H3K36 methylation which reduces chromatin accessibility (*87*). Moreover increases in H3K27 and K79 methylation are also observed, therefore the results on gene expression beyond that caused by its HDAC inhibition activity are difficult to predict.

Our finding with mocetinostat to decrease expression of LSD1 may have therapeutic implications in Acute Myeloid Leukemias (AML), in particular those with MLL-rearrangements (Mixed Lineage Leukemia). Aberrant expression and activity of LSD1 has and continues to be shown to be involved in these types of hematopoietic malignancies (*93*), and is a target for drug intervention. We wanted to evaluate whether the off-target activity of mocetinostat (potent reductions in LSD1 gene expression) could compare to direct LSD1 inhibition in the context of Mixed Lineage Leukemia. We also aim to highlight to a greater extent the off-target activities of mocetinostat in comparison to the more common therapies of MLL-rearranged leukemia (*94*).

Chapter 3.

3.1 Selective DOT1L, LSD1, and HDAC class I inhibitors reduce HOXA9 expression in MLL-AF9 rearranged leukemia cells, but dysregulate the expression of many histone-modifying enzymes[3]

Mixed lineage leukemia is caused by chromosomal translocations or insertions of the *MLL* gene. MLL is a H3K4 methyltransferase and its wild-type activities control expression of the homeobox A cluster (especially HOXA9), which are involved in normal hematopoiesis (**Figure 3.1A**) (*95*). H3K4 trimethylation activates the expression of HOXA9 in progenitor cells and is down-regulated by the polycomb repressor complex to drive differentiation. There are more than 60 forms of mixed lineage leukemia that result from fusions between MLL and a variety of proteins that eliminate the histone lysine methylation activity of MLL but not its DNA binding capacity (*96, 97*) Translocations and insertions at chromosome 11q23 results in fusions of MLL with a variety of partners (among these are MLL-AF9, MLL-AF10, and MLL-AF4) that eliminate its H3K4 methylation activity but retain its target gene DNA binding capacity (*97*). MLL mutants have altered epigenetic activity at the HOXA9 promoter because of the loss of its methyltransferase domain and the new activity introduced by the fused protein. The fusion protein recruits other epigenetic enzymes that can lead to over-expression of HOXA9 and leukemia. For example, The translocation t(9;11)(p22;q23) or insertion ins(11;9)(q23;p22p23) result in the fusion of MLL to AF9, (MLL-AF9). MLL-AF9 eliminates the methyltransferase domain of MLL and recruits another methyltransferase, disruptor of telomere silencing 1-like, eliminating H3K4 methylation (*95, 98*) and replacing it with H3K79 methylation within the

[3] Reprinted (adapted) with permission from Ryan Lillico, Courtney K Lawrence and Ted M Lakowski. Selective DOT1L, LSD1 and HDAC Class I Inhibitors Reduce HOXA9 Expression in MLL-AF9 Rearranged Leukemia Cells, But Dysregulate the Expression of Many Histone Modifying Enzymes. *Journal of Proteome Research*. **2018**, 17(8), 2657-2667. Copyright 2018, American Chemical Society.

HOXA9 gene and promoter (**Figure 3.1B**) (*99*). This sustains the overexpression of HOXA9 resulting in the inability to differentiate, producing a self-renewing hematopoietic progenitor cell population of mixed lineage leukemia (*95*).

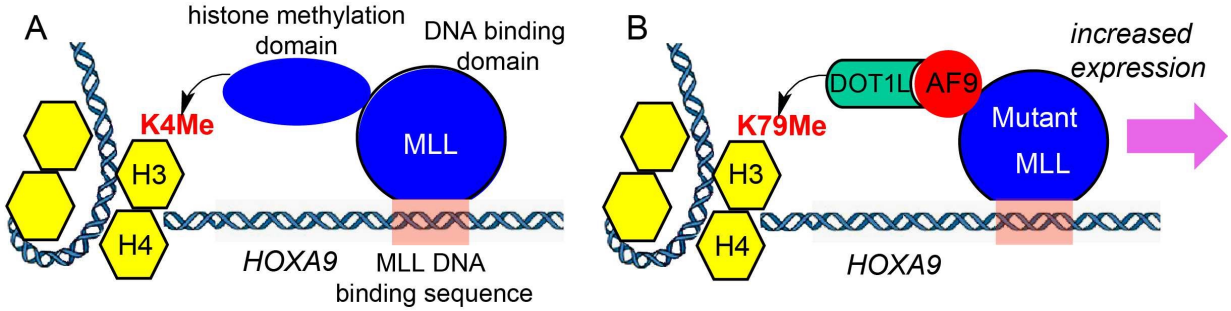

Figure 3.1 Interactions at the normal *HOXA9* promoter and the epigenetic modifications to histones. Depicted are, MLL (blue) bound to DNA (A). MLL-AF9 (blue and red) associates with DOT1L (green) resulting in methylation of histone H3K79, HOXA9 overexpression, and mixed lineage leukemia (B).

The disruptor of telomere silencing 1-like (DOT1L), a histone H3 lysine 79 (H3K79) methyltransferase, is a regulator of gene transcription, somatic reprogramming, DNA damage response, and cell cycle regulation (*100, 101*). DOT1L is the only H3K79 methyltransferase known. It contains a DNA binding domain and only methylates nucleosomal substrates and not free histones (*102*), suggesting that H3K79 methylation is involved in chromatin remodeling rather than nucleosome assembly. H3K79 dimethylation fluctuates through cell-cycle checkpoints and is found mostly in actively transcribed genes, implicating DOT1L as a regulator of euchromatin, transcriptional elongation, and cellular differentiation (*42*).

DOT1L inhibitors are being investigated as treatments for leukemia that result from rearrangements of MLL (*103*). DOT1L inhibitors have been shown to decrease HOXA9 expression and increase cellular differentiation, resulting in the selective killing of MLL-rearranged cells. The DOT1L inhibitor EPZ-5676 is under investigation as a treatment for this

type of leukemia (*104*). However, DOT1L is an important regulator of the expression of multiple genes, therefore even its selective inhibition may lead to unpredictable changes in the expression of multiple genes. We hypothesize that such changes in expression may include other histone modifying enzymes, which would manifest itself as changes in total histone and residue specific histone modifications and this could in turn precipitate down-stream changes in gene expression. It has been shown previously that reducing HOXA9 expression with shRNA knockdown is sufficient on its own to arrest the growth of MLL-AF9 cell lines (*105*). Therefore, changes in the expression of genes that don't ultimately reduce HOXA9 expression, are by definition off-target effects. We and others have observed that histone deacetylase (HDAC) inhibitors, alter the expression of several genes (*67*). It is therefore possible that drugs targeting other histone modifying enzymes may indirectly decrease HOXA9 expression and may be useful as treatments for mixed lineage leukemia as long as other induced off-target changes in histone modifying expression do not result in toxicity.

The lysine specific demethylase 1 (LSD1 or KDM1A) acts on H3K4 and H3K9 and can only demethylate mono- and dimethyl-lysine because of its FAD dependent mechanism (*13*). LSD1 is a partner in co-repressor complexes (CoREST, NuRD, CtBP), co-activator super complexes (MLL, ELL) (*106*) and is important in organogenesis (*107*) and hematopoiesis (*108*). LSD1 is thought to be involved with the MLL-AF9 complex, supporting the activation of HOXA9 thereby preventing differentiation, and as a result increases the leukemic cell population (*109*). Inhibiting LSD1 promotes hematopoietic differentiation (*110*), resulting in toxicity to MLL-rearranged cells, much like DOT1L inhibition. Interestingly, administering LSD1 and DOT1L inhibitors together, synergistically inhibits proliferation of MLL-rearranged leukemia cells (*110*).

DOT1L and LSD1 are cell cycle and proliferation regulators. Inhibition of DOT1L leads to G_0/G_1 arrest (*111, 112*) and accumulation of the cell population in the sub-G_1 phase (*104*), depletion of LSD1 partially stalls cells at the G_2/M checkpoint (*113*) and knockout of either LSD1 or DOT1L in pluripotent cells induces death (*101, 114*). Previously, we have shown that HDAC inhibitors can reduce the expression of LSD1 in addition to other changes in gene expression (*67*). This suggests that HDAC inhibitors may be used for similar indications as LSD1 inhibitors.

In this study, we tested inhibitors of histone modifying enzymes in an acute myeloid leukemia cell line containing the MLL-AF9 fusion that overexpresses HOXA9 (MOLM-13) compared to a chronic myeloid leukemia cell line (BCR-ABL1 fusion) that has undetectable HOXA9 expression (K562). We evaluated the effects of representative selective HDAC class I (mocetinostat), DOT1L (EPZ-5676), and LSD1 (GSK2879552) inhibitors on LSD1 and HOXA9 expression, total and residue specific histone post-translational modifications, cell viability, apoptosis, and the expression of an array of histone modifying enzymes. EPZ-5676 reduced expression of HOXA9 in MOLM-13 cells and reduced viability when treated for 6 days as shown previously (*104*). Despite the fact that it is a lysine methyltransferase inhibitor, EPZ-5676 increased total histone lysine di- and monomethylation, similar to the LSD1 inhibitor GSK2879552. In an effort to explain this activity, we found that EPZ-5676 reduced LSD1 expression. We also confirmed that GSK2879552 decreased HOXA9 expression. EPZ-5676 and GSK2879552 decreased the viability of MOLM-13 cells but had no effect on K562 cells. We have shown previously that the selective HDAC class I inhibitor mocetinostat decreases LSD1 expression in K562 cells. To see if this could produce effects similar to the direct LSD1 inhibition produced by GSK2879552 and the reduction in LSD1 expression produced by EPZ-

5676, we treated MOLM-13 cells with mocetinostat finding that it decreased LSD1 and HOXA9 expression and induced apoptosis in both cell lines. EPZ-5676 is the most effective inhibitor studied at decreasing HOXA9 expression in MOLM-13 cells. Despite the difference in histone modifying enzyme targets, all three compounds directly inhibit, or reduce the expression of HOXA9, DOT1L and LSD1, increase total histone lysine methylation and acetylation and specifically reduce H3K79Me2 and increase H3K14Ac. As predicted, the three compounds changed the expression of several histone-modifying enzymes, which themselves are involved in controlling gene expression. Where this results in reduced HOXA9 expression these changes in expression are advantageous for the treatment of mixed lineage leukemia. However, where such activities do not eventually decrease HOXA9 expression they can be viewed as off target effects.

3.2 Materials and Methods

3.2.1 Cell culture and treatments

MOLM-13 cells carrying MLL-AF9 fusion from ins(11;9)(q23;p22p23) (DSMZ ACC 554 ref number is A1408102-1) were cultured in RPMI 1640 media supplemented with 1% penicillin/streptomycin and 10% FBS. K562 cells (ATCC (CCL-243)) were cultured in IMDM media with 1% penicillin/streptomycin and 10% FBS. Cells were incubated at 37 °C in 5% CO_2 and were passaged every 72 h. Exponentially growing cells were banked upon receipt and cultured cells were passaged no more than 30 times before replaced. Cells ($5x10^4$/mL) were treated for 24, 72, or 144 h with various concentrations of the DOT1L inhibitor, EPZ-5676, the LSD1 inhibitor, GSK2879552, or the HDAC inhibitor mocetinostat (Appendix **Figure A1**) prepared in sterile filtered DMSO with a final concentration in media 0.1% DMSO. For 144 h incubations, cells were counted and diluted back to $5x10^4$/mL in fresh media containing the appropriate concentration of inhibitor after 72 h. MOLM-13 cells derived from the blood cells of a 20-year old male with mixed lineage leukemia were used as our model cell line because they harbor the MLL-AF9 fusion and overexpress HOXA9. The K562 cells were authenticated and confirmed by microscopic examination of morphology and comparison to the cell micrograph from ATCC. K562 cells were further authenticated via gene expression analysis for the HOXA9 gene with quantitative PCR (**Figure 3.2**). Previous studies have shown that K562 cells do not express HOXA9 (*115*). We found that in comparison to MOLM-13, the K562 cells have undetectable expression of HOXA9 and were used as a control cell line.

3.2.2 Cell viability and apoptosis

MOLM-13 and K562 cells were plated $5x10^4$/well in 96-well format and treated with EPZ-5676 for 144 h, GSK2879552 for 72 h, and mocetinostat for 24 h. Cell viability and

apoptosis were then evaluated using the ApoTox-Glo (Promega) assay according to manufacturers instructions. Fluorescence (viability) and luminescence (apoptosis) were measured using the BMG FLUOstar Galaxy microplate reader.

3.2.3 Histone modification analysis

Cells were harvested, and histones isolated using the Epiquik (Epigentek) kit with modifications. Histones were precipitated with 4% perchloric acid at 4 °C overnight, washed with acetone and reconstituted in water to 1 mg/mL. Isolated histone samples (10 μg) were digested using Pronase or hydrolyzed in the vapor phase with 6N HCl and analyzed for acetyl-lysine, methyl-lysines and methyl-arginines by liquid chromatography tandem mass spectrometry (LC-MS/MS) according to previously established protocols (*67*). Briefly, the modified and unmodified amino acids from the histone hydrolysates were separated on a Primesep 200 (Sielc) column with a pH gradient in acetonitrile and water using a Shimadzu Nexera UHPLC. Each analyte was measured in positive MRM mode using a Shimadzu LCMS 8040 mass spectrometer with DUIS ionization and quantified using synthetic standards of acetyl-lysine, mono-, di-, and trimethyl-lysine, monomethyl-arginine, asymmetric dimethyl-arginine and symmetric dimethyl-arginine. Each sample was normalized to the quantity of lysine and each modification was expressed as percent change with respect to controls receiving no treatment.

Specific modifications on histone H3 including H3K4Me1-3, H3K9Me1-3, H3K27Me1-3, H3K36Me1-3, H3K79Me1-3, H3K9Ac, H3K14Ac, H3K18Ac, H3K56Ac, H3Ser10P and H3Ser28P were measured using the Histone H3 Modification Multiplex Assay Kit (Abcam, ab185910) according to the manufacturer's instructions, using 50 ng histone isolate per well. Each treatment was normalized to total H3 and the changes in modifications were calculated as a percentage with respect to a no treatment control.

3.2.4 qPCR gene expression analysis

Total mRNA was isolated and purified from cells using the Ambion Pure Link RNA isolation kit (Life Technologies) and cDNA synthesized using Super Script Vilo master mix (life Technologies). Quantitative PCR (qPCR) was performed using the Applied Biosystems Viia7 real-time PCR system with TaqMan assays (Life Technologies, Appendix **Table A2**). PCR reactions were prepared in 10 or 20 µL volumes using 50-100 ng cDNA in TaqMan fast universal master mix (Life Technologies) following the manufacturers conditions. Relative gene expression (RQ) was quantified by comparative CT (ΔΔCT) to no treatment controls and normalized using either Beta-2-Microglobulin (B2M) or 18 s rRNA housekeeping genes. Dose versus gene expression (response) curves were fit to a 4 parameter logistic regression using SigmaPlot 11 according to the guidelines of the NIH National Center for Advanced Translational Sciences and the IC_{50}, min and max determined (*116*).

3.2.5 In vitro DOT1L methylation assay and LSD1 inhibitor-screening assay

Increasing concentrations of EPZ-5676 were incubated for 1 hour with 120 nM recombinant DOT1L (EpiCypher), 1 µg of recombinant nucleosomes (EpiCypher) and 24 µM S-adenosyl methionine (AdoMet) in 100 µL of a methylation buffer containing 50 mM ammonium acetate, 2.5 mM NaCl, 0.01% Triton X100 and 0.25 mM DTT. The reactions were dried in a vacuum centrifuge and the products hydrolyzed with 6 N HCl vapor for 24 hours and analyzed for methyl-lysines by LC-MS/MS according to previous methods (*67*). DOT1L activity was represented as the weighted sum of all methyl groups transferred to lysine. Measurements of the drug treatments were compared to a DMSO control (100% activity) and no AdoMet control (0% activity). EPZ-5676, mocetinostat and GSK2879552 were screened for LSD1 inhibition using

the LSD1 inhibitor-screening assay (Cayman, Cat. 700120) according to the manufacturer's instructions. Absorbance was measured using the BioTek Synergy HT microplate reader.

3.2.6 Data accessibility

The ChIP-seq data sets used to make **Figure 3.9** were originally published (*117*) and graphics of these data were generated using the Integrative Genomics Viewer 2.3.77 using tracks loaded from the Encyclopedia of DNA elements (ENCODE) (K562 cells) along with the available data sets from the NCBI Gene expression omnibus (GEO) database for MOLM-13 cells https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE76750.

3.3 Results

3.3.1 Inhibition of DOT1L or LSD1 reduces HOXA9 expression in MOLM-13 cells

Previous studies have shown that reversing overexpression of HOXA9 with shRNA in cell lines harboring the MLL-AF9 translocation, is alone sufficient to force differentiation and arrest growth, eventually leading to cell death (*105*). Therefore, reducing HOXA9 expression is a validated target for treatment of MLL-rearranged leukemia and the effectiveness of potential new treatments can, in part be evaluated by measuring reductions in HOXA9 expression. MOLM-13 cells were chosen as a model for mixed lineage leukemia with the MLL-AF9 fusion. Initially the expression of HOXA9 was evaluated using qPCR in MOLM-13 cells that overexpress HOXA9 and compared to K562 cells that express undetectable levels of HOXA9 (*115*), with and without treatment with EPZ-5676, in order to validate the qPCR method (**Figure 3.2**).

MOLM-13 cells were treated with mocetinostat, EPZ-5676, and GSK2879552 and we found that all three reduced HOXA9 expression in a dose dependent fashion (**Figure 3.3A, Table 4**). Similar to previous studies with EPZ-5676 (*104*), the maximum reduction in HOXA9 expression (>90%) occurred at longer incubation periods up to 144 h with 5 μM, while the same dose at 24 h and 72 h reduced HOXA9 expression approximately by 30% and 80%, respectively (**Figure 3.3B**). The IC_{50} for the reduction in HOXA9 expression with a 72 h EPZ-5676 treatment was 150±10 nM, which is within the same range of previously calculated values in MLL-AF4 rearranged MV4-11 cells (**Table 4**) (*104*).

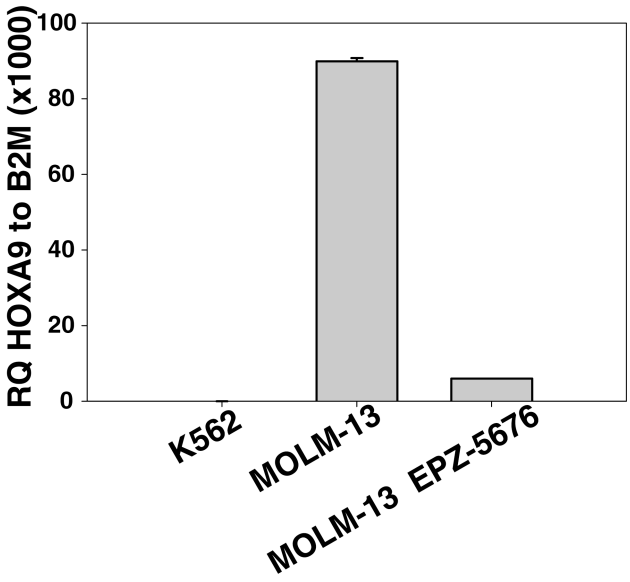

Figure 3.2 HOXA9 expression in K562 cells and MOLM-13 cells. Baseline expression of HOXA9 in K562 cells is not detectable using the standard input cDNA amounts. MOLM-13 has relatively high expression levels compared to K562 which are nearly depleted with a 5 μM treatment with EPZ-5676 for 144 h. These data are represented as the relative quantity (RQ) of HOXA9 expression compared to beta 2 microglobulin.

Table 4. IC_{50} values for reduction in HOXA9 and LSD1 expression, and cell viability upon treatment with EPZ-5676, mocetinostat and GSK2879552.

	EPZ-5676	mocetinostat	GSK2879552
HOXA9 expression IC_{50} (nM)	150±10	105±53	10±3
max/min (% expression)	101±12/22±3	89±5/51±2	90±3/58±1
LSD1 expression IC_{50} (nM)	172±40	127±18	N/A
max/min (% expression)	91±12/57±5	91±2/38±4	N/A
MOLM-13 viability IC_{50} (nM)	196±88	109±17	5±1
max/min (% viability)	98±7/28±7	106±14/19±1	124±14/69±8
K562 viability IC_{50} (nM)	N/A	409±25	N/A
max/min (% viability)	100±2/96±5	104±3/11±1	110±2/95±6

The effects of the LSD1 inhibitor GSK2879552 were evaluated in MOLM-13 cells because such tranylcypromine analogue inhibitors have, (like DOT1L inhibitors) recently been identified as potential treatments for MLL-rearranged leukemia that reduce HOXA9 expression (*109*). GSK2879552 reduced HOXA9 expression with increasing treatment time and dose

producing a maximum ~40% reduction in HOXA9 expression at a 72 h incubation period (**Figure 3.3B**). Longer incubation periods did not result in greater reduction of HOXA9 expression as seen with the DOT1L inhibitor EPZ-5676. Although the maximum decrease in HOXA9 expression by GSK2879552 treatment only resulted in approximately 40% reduction, it did so potently with an IC_{50} of 10±3 nM at 72 h (**Figure 3.3A, Table 4**).

Having confirmed that the LSD1 inhibitor GSK2879552 reduced HOXA9 expression we evaluated whether the Class I HDAC inhibitor mocetinostat could reduce HOXA9 expression because we have previously shown that it reduces the expression of several lysine demethylases in K562 cells, and in particular LSD1 (*67*). Mocetinostat rapidly reduced HOXA9 expression by up to 50% in 24 h in MOLM-13 cells with a similar potency to EPZ-5676 (IC_{50} of 105±53 nM) (**Figure 3.3A, Table 4**). As far as we know this is the first time that any HDAC inhibitor has been shown to reduce HOXA9 expression in an MLL-AF9 cell line.

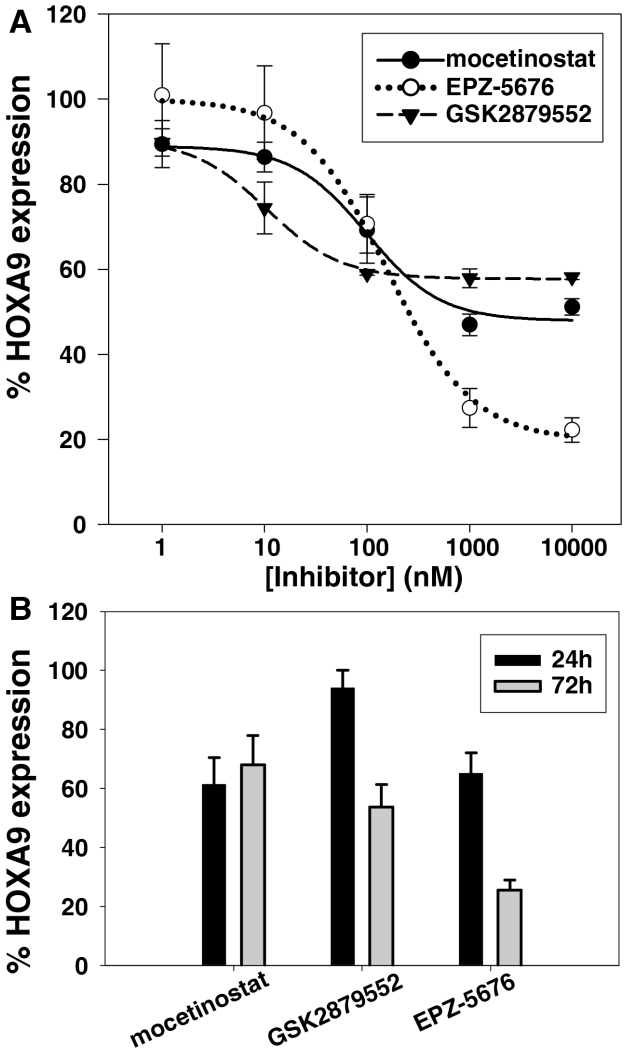

Figure 3.3 Inhibitors of histone modifying enzymes decrease HOXA9 expression depending on treatment time and dose in MOLM-13 cells. Displayed are dose response curves measured by qPCR (N=3) showing decreasing HOXA9 expression with increasing concentration of the LSD1 inhibitor GSK2879552 after 72 h, the HDAC inhibitor mocetinostat after 24h, and the DOT1L inhibitor EPZ-5676 after 72 h (A). Time points were chosen to elicit the maximum effect over the minimum time for one dose of each inhibitor. The maximum reduction of HOXA9 gene expression with a single dose of 1 μM GSK2879552 or 5 μM EPZ-5676 required longer incubation times (72 h) compared to 100 nM mocetinostat, which the maximum reduction on HOXA9 expression occurred at 24 h of treatment (B). Values represent the mean of 3 measurements and SD.

3.3.2 EPZ-5676 and GSK2879552 are toxic to MOLM-13 but not K562 cells

Viability and apoptosis for the selected inhibitors in both MOLM-13 and K562 cells were measured using the ApoTox-Glo assay. EPZ-5676 and GSK2879552 reduced the viability of MOLM-13 cells, but not K562 (**Figure 3.4A**). A 144 hour treatment of EPZ-5676 and a 72 hour

treatment of GSK2879552 did not induce caspase activation in either cell type, suggesting the reductions in viability were through non-apoptotic mechanisms. However, mocetinostat induced apoptosis in both cell types in 24 hours (**Figure 3.4B**). All three inhibitors showed a dose dependent reduction in viability in MOLM-13 cells, but only mocetinostat did so in K562 cells (**Figure 3.4C and D**). Despite not inducing complete reductions in HOXA9 expression, mocetinostat appeared to be more potent and ultimately more effective than EZP-5676 with respect to reductions in MOLM-13 cell viability (**Figure 3.4C and D and Table 4**).

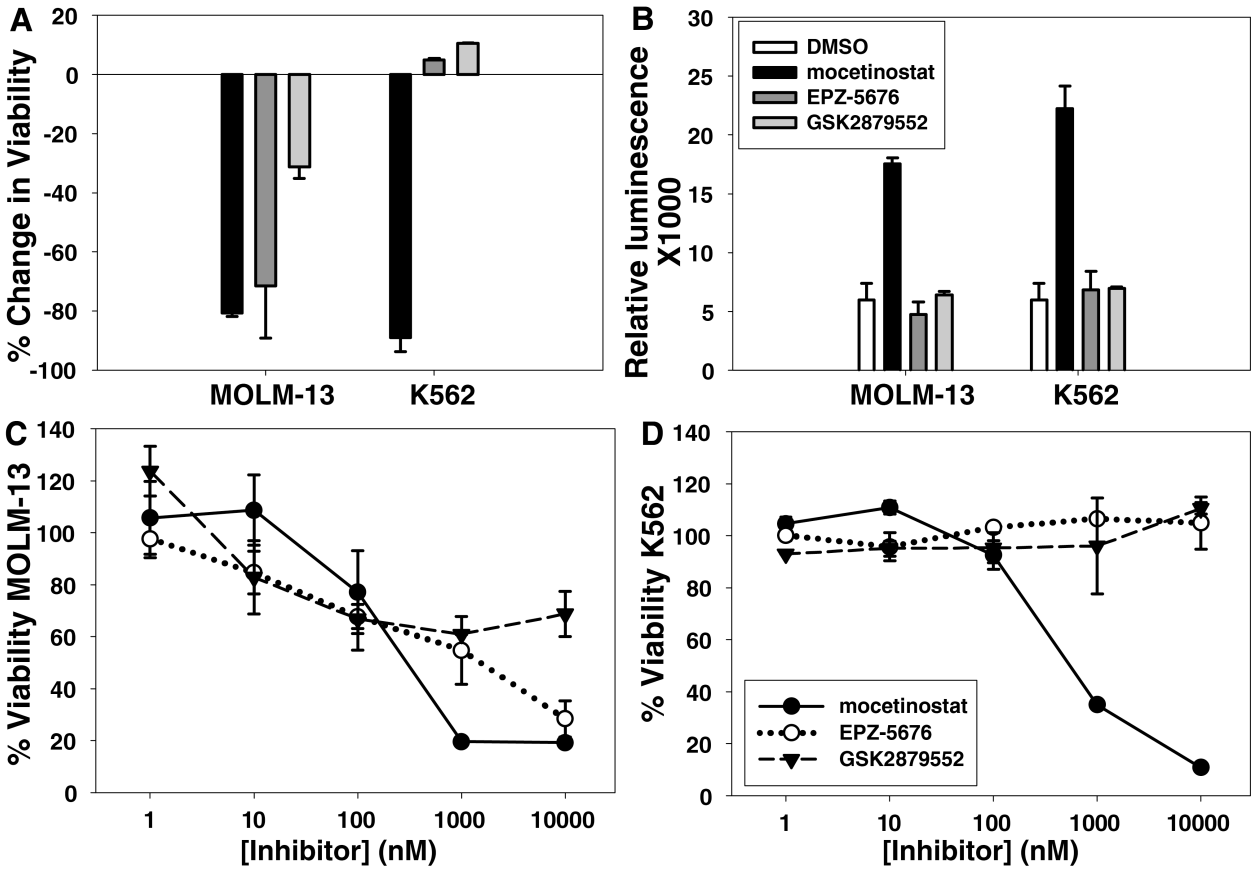

Figure 3.4 EPZ-5676, mocetinostat and GSK2879552 reduce cell growth by different mechanisms. Changes in cell viability are displayed for a 10 μM dose of GSK2879552 for 72 h, mocetinostat for 24 h and EPZ-5676 for 144 h in the MLL-AF9 cell line MOLM-13 and K562 cells (A). Percent changes were measured as a ratio with respect to a no treatment control for both cell lines. Apoptosis was measured by caspase activation with a DMSO control, 1 μM GSK2879552 for 72 h, EPZ-5676 for 144 h or mocetinostat for 24 h. Only mocetinostat appears to induce apoptosis (B). The percentage of viable cells with increasing concentrations of GSK2879552 for 72 h, EPZ-5676 for 144 h and mocetinostat for 24 h in MOLM-13 (C), and K562 cells (D). Viability and apoptosis were measured using the Apo-Tox Glo cell assay (N=3).

3.3.3 Mocetinostat, EPZ-5676, and GSK2879552 produce similar changes in total histone modifications

MOLM-13 cells were treated with the HDAC class I inhibitor mocetinostat, the DOT1L inhibitor EPZ-5676, and the LSD1 inhibitor GSK2879552 to determine if the inhibitors produced changes to total histone modifications (**Figure 3.5A**). Total histone modifications were measured at the time and dose that each inhibitor yields its maximum HOXA9 inhibition (100 nM, 24 h for mocetinostat, 3 μM, 144 h for EPZ-5676 and 1 μM, 72 h for GSK2879552) using our previously developed LC-MS/MS assay (*67*). Surprisingly, we observed similar trends in total histone modifications for all three inhibitors. Given that EPZ-5676 is a methyltransferase inhibitor we expected to see reductions in histone lysine methylation but paradoxically, increased lysine dimethylation, and to a lesser extent monomethylation, were observed (**Figure 3.5A**). Such changes in histone lysine methylation could indicate increasing activity or expression of methyltransferases catalyzing mono- and di-methylation, or decreases in the activity or expression of FAD dependent demethylases such as LSD1 since these enzymes are only capable of demethylating mono and dimethyl-lysine (*13*). Accordingly, the potent LSD1 inhibitor GSK2879552 resulted in a similar pattern of increasing total histone lysine mono- and di-methylation. Our previous results demonstrated that mocetinostat decreases LSD1 expression, and the expression of several other lysine demethylases of the jumonji (JmjC) domain class and as a result we observed increases in all types of histone lysine methylation in K562 cells (*67*). In this study, we found that mocetinostat produced similar increases in all types of histone lysine methylation in MOLM-13 cells. In addition, all compounds increase lysine acetylation at low doses (**Figure 3.5A**) but larger doses eliminated this increase with EPZ-5676, while producing even larger increases with mocetinostat (**Figure 3.5B**).

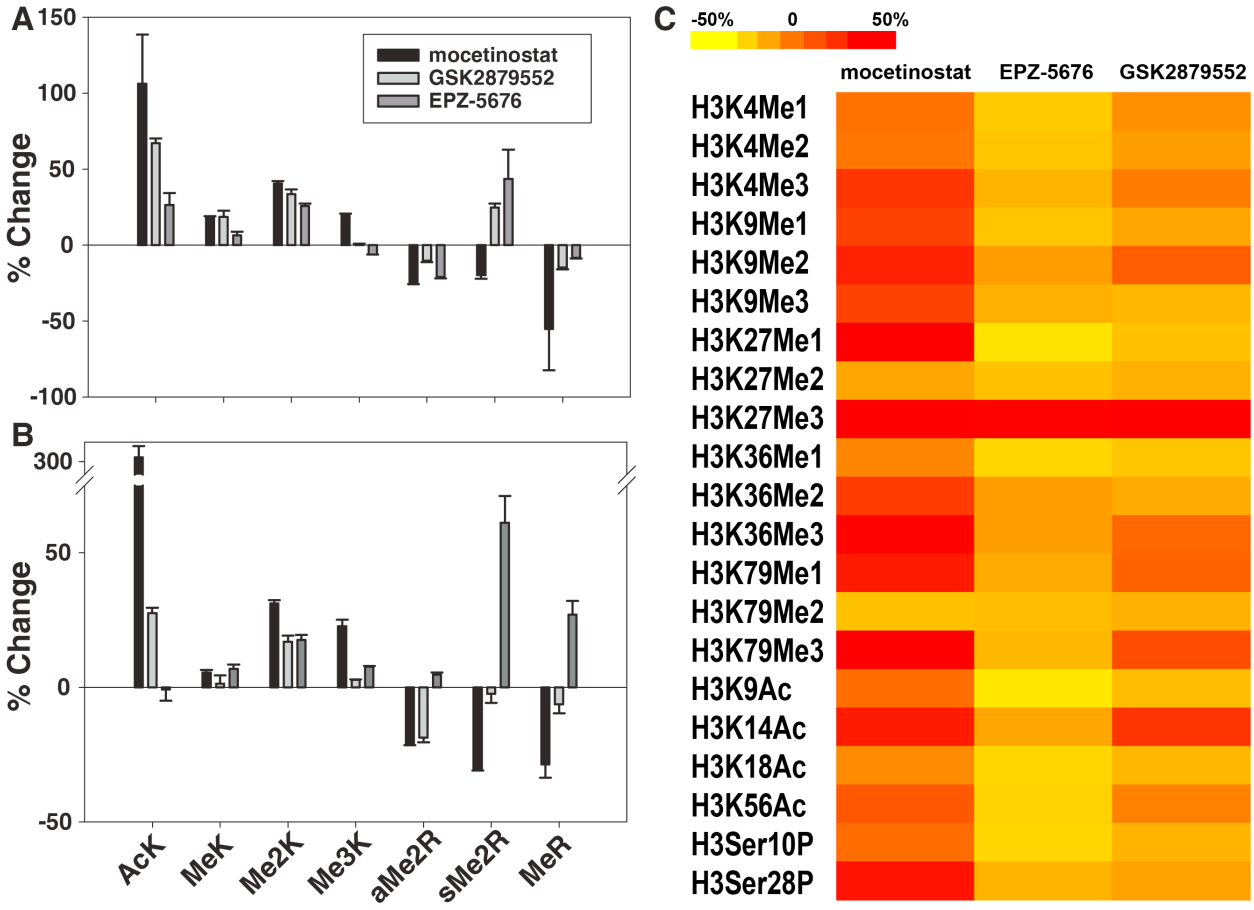

Figure 3.5 The changes in histone modifications in cells treated with GSK2879552, EPZ-5676 and mocetinostat. Shown are the changes in total histone lysine and arginine modifications observed with 100nM treatment of mocetinostat for 24h, 1 μM of GSK2879552 for 72 h and a 3 μM of EPZ-5676 for 144 h in MOLM-13 cells measured using the LC-MS/MS assay described in materials and methods (A). Similar changes in total histone modifications were observed with 1 μM of mocetinostat for 24, 3 μM of GSK2879552 for 72 h and a 5 μM dose of EPZ-5676 for 144 h (B). Bars represent means with SD of 3 measurements. A heat-map of changes to specific histone H3 modifications measured using the Histone H3 Modifications Multiplex Assay Kit (abcam) in MOLM-13 cells using a 5 μM EPZ-5676 100 nM mocetinostat and 1 μM GSK2879552 at the times above (C). Each cell of the heat-map is the mean of 4 measurements. Appendix **Figure A2** shows the same data with the corresponding heat-map of the error expressed a CV% calculated as CV%=(SD/mean)100%.

3.3.4 Specific histone H3 modifications

Although the pattern of total histone modifications can give some insight into the shared effects of mocetinostat, EPZ-5676, and GSK2879552 (which have different histone modifying enzyme targets), histone modifications elicit different context dependent consequences on gene expression with some modifications increasing, while others decrease transcription. For example,

H3K4Me3 increases, while H3K9Me3 decreases transcription, therefore measuring changes in total trimethyl-lysine cannot distinguish the mark from being transcriptionally permissive or repressive (*118*). Moreover, total histone lysine acetylation and methylation were quantified to observe unanticipated changes in histone modification during treatment with inhibitors of histone modifying enzymes, which could then be investigated more thoroughly with additional experiments. With this in mind, we supplemented our total histone modification data with specific histone H3 modifications using a Histone H3 Modification Multiplex Assay Kit (Abcam) (**Figure 3.5C**). Consistent with its total changes in histone modifications, mocetinostat increases lysine methylation and acetylation at a number of residues on histone H3. EPZ-5676 produced a generalized decrease in histone H3 modifications with some exceptions including, H3K27Me3 and H3K14. GSK2879552 did not produce the expected increases in H3K4 methylation (*119*), but rather increased H3K9Me2 and unexpectedly increased H3K79Me1 and 3. Consistent with the total histone acetyl-lysine data, GSK2879552 produced increases in H3K14 and K56 acetylation. Increased H3 acetylation with mocetinostat was expected, however, increases in almost all H3 modifications were also observed. The largest of these were increases in H3K4, H3K9, H3K27 and H3K36 methylation, H3K14 acetylation and H3 serine 28 phosphorylation.

Notably, all inhibitors tested appear to produce decreases in H3K79Me2 in MOLM-13 cells. In the context of the pathology of mixed lineage leukemia, decreased H3K79Me2 most likely results from decreased methylation activity by, or expression of DOT1L, as it is the only enzyme known to methylate this residue. In the HOXA9 promoter of cells with the MLL-AF9 fusion, this would result in decreased HOXA9 expression and is consistent with our finding that all compounds tested reduce HOXA9 expression.

3.3.5 EPZ-5676 reduces LSD1 expression

The increases in total histone mono- and dimethyl-lysine produced by EPZ-5676 suggest that it may be directly inhibiting or decreasing the expression of LSD1 through an epigenetic mechanism since its effects on total histone modifications had a similar trend as seen with direct inhibition of LSD1 by GSK2879552. To ensure there was no unintentional direct inhibition of LSD1, we tested the activity of mocetinostat, EPZ-5676, and GSK2879552 against DOT1L and LSD1 *in vitro* and found that EPZ-5676 was not a direct inhibitor of LSD1, but as expected GSK2879552 was (**Figure 3.6**). Moreover, we found that GSK2879552 did not directly inhibit DOT1L, but similar to other studies, EPZ-5676 potently inhibited DOT1L (*74, 104*). Therefore, we used qPCR to determine if EPZ-5676 could reduce LSD1 expression and we found it did so in a time and dose dependent fashion, similar to mocetinostat (**Figure 3.7A**). Most interestingly, the maximum reduction in expression of LSD1 by EPZ-5676 resulted from the longest treatment period (144 h), which is a similar time-line to its activity in reducing HOXA9 expression (**Figure 3.7B**). Moreover, the magnitude of the IC_{50} is similar to its reduction in HOXA9 expression (**Table 4**). These similarities suggest that the reductions in LSD1 and HOXA9 expression we observed may be causally linked.

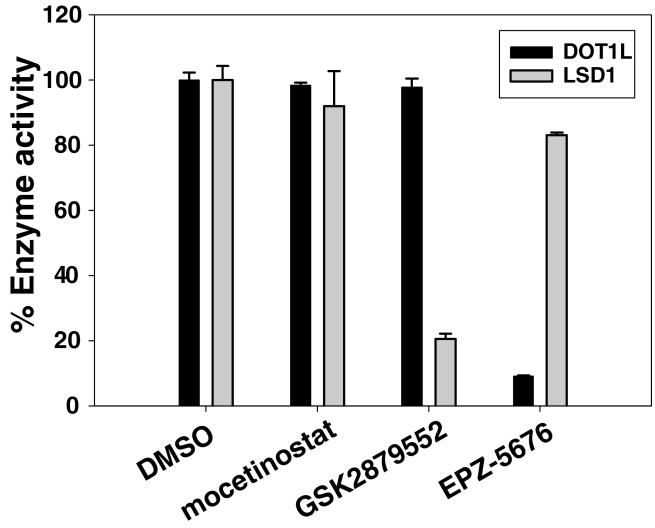

Figure 3.6 EPZ-5676 and GSK2879552 inhibit DOT1L and LSD1 respectively *in vitro.* 10 μM of the inhibitors were used in either the LSD1 inhibitor screening assay (Cayman) or the LC-MS/MS assay adapted to *in vitro* measurement of DOT1L methylation activity as outlined in Materials and Methods. Mocetinostat did not inhibit LSD1 or DOT1L *in vitro*. Values are means and SD for 3 measurements.

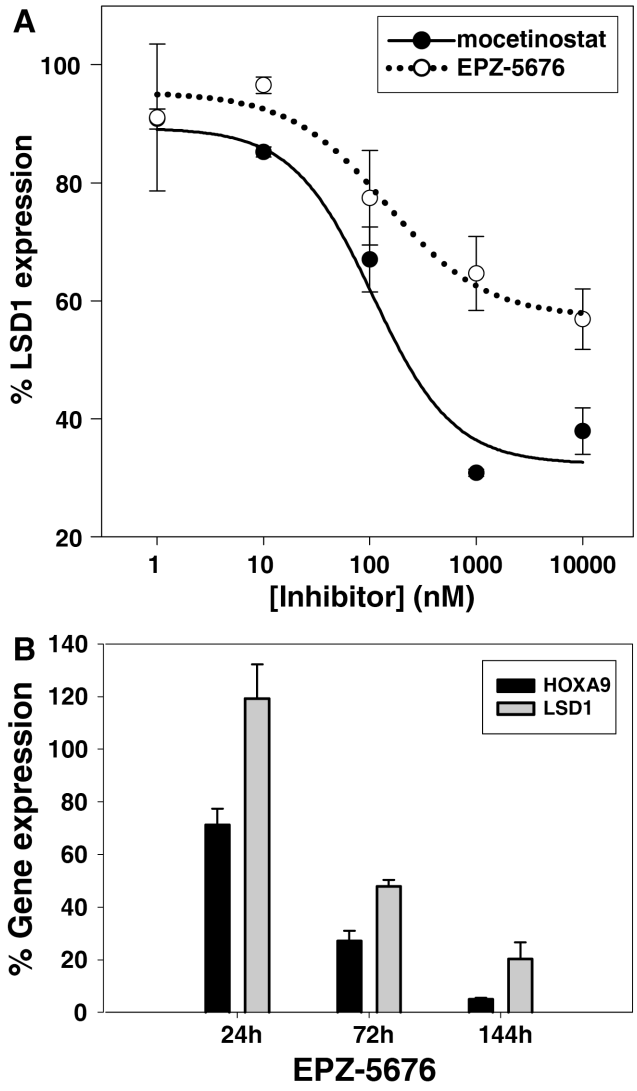

Figure 3.7 EPZ-5676 and mocetinostat reduce LSD1 gene expression in MOLM-13 cells. MOLM-13 cells were treated with increasing doses of EPZ-5676 for 72 h or mocetinostat for 24 h (A) and LSD1 expression was measured by qPCR. The reductions in LSD1 expression induced by EPZ-5676 was measured at varying time-points with maximum reductions of both LSD1 ~80% and HOXA9 expression >90% observed after 144 h (B). Maximum reduction in LSD1 expression induced by mocetinostat appear at 24 h as we have previously shown (*67*). All values are means with SD of 3 measurements.

3.3.6 EPZ-5676 reduces the expression of a panel of histone modifying enzymes in MOLM-13 cells

Establishing that EPZ-5676 decreases many histone H3 modifications, suggested that these changes in histone modifications might be a result of downstream changes in the expression of genes coding for histone modifying enzymes. We therefore measured changes in expression of several histone-modifying enzymes (that are known for their activity as co-activators or co-repressors), in MOLM-13 cells treated with the selected inhibitors (**Figure 3.8**). We chose dosing based on each inhibitors concentration and incubation period that resulted in maximum reductions of HOXA9 expression, this being 100 nM, 24 h for mocetinostat, 5 μM, 144 h for EPZ-5676 and 1 μM, 72 h for GSK2879552. Genes from most classes of histone modifying enzymes including lysine methyltransferases and demethylases, lysine acetyltransferases and deacetylases as well as arginine methyltransferases were selected to correlate the changes in expression with the total and sequence specific changes in histone modifications. Treatment with EPZ-5676 resulted in generalized reductions of nearly all genes evaluated, except KAT5 and HDAC5, which remained unchanged in MOLM-13 cells, while some genes appeared to have elevated expression in K562 cells such as HDAC5. A similar pattern was observed with GSK2879552 where there is generally no change or a slight decrease in expression in MOLM-13 cells but a few genes with elevated expression in K562 cells (**Figure 3.8**). Reductions in gene expression were generally observed in both cell types with mocetinostat treatment, however increases in KDM4A, HDAC5 and 6, and SIRT2 and 6 were observed in K562 cells. There was a common increase in expression of HDAC5 and decreases in SIRT1 in MOLM-13 and K562 cells with mocetinostat treatment. Consistent with the increases in histone lysine mono- and dimethylation (**Figure 3.5A**), EPZ-5676 and mocetinostat cause reductions in

several KDMs but the largest decrease was in LSD1. With respect to mocetinostat, reduction in KDM expression is consistent with our previous results in K562 cells (*67*).

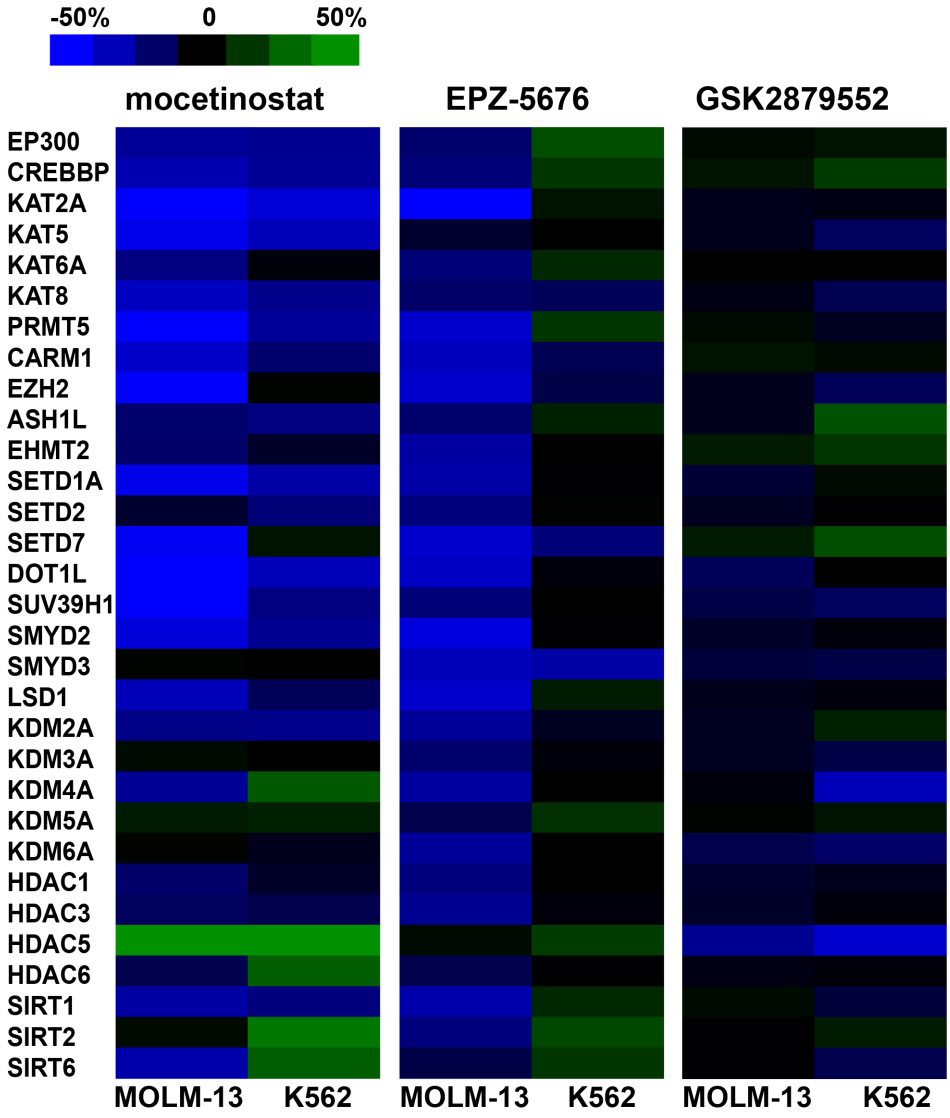

Figure 3.8 The gene expression heat map of selected histone modifying enzymes in K562 and MOLM-13 cells treated with mocetinostat, EPZ-5676, or GSK2879552. Treatments of 100 nM mocetinostat for 24h, 5 μM EPZ-5676 for 144h and 1 μM GSK2879552 for 72h were evaluated in MOLM-13 and the control K562 cells for changes in expression of epigenetic enzymes, measured by qPCR. Each cell of the heat-map is the mean of 3 measurements. Appendix **Figure A3** shows the same data with the corresponding heat-map of the error expressed a CV% calculated as CV%=(SD/mean)100%.

Mocetinostat produces a substantial decrease (83%) in DOT1L expression, which may in part explain the apparent activity of mocetinostat against MOLM-13 cells. In a broad sense, there are many changes in the expression of histone modifying enzymes that are similar upon treatment with EPZ-5676 or mocetinostat. Briefly, lysine acetyltransferase KAT2A, arginine methyltransferases PRMT5, CARM1, lysine methyltransferases EZH2, SET7, SMYD2, DOT1L and lysine demethylase LSD1 all appear to show decreases in expression. In contrast, GSK2879552 shows few changes in the expression of histone modifying enzymes in MOLM-13 cells, however, as with the other compounds there is a notable but modest decrease in DOT1L expression.

3.3.7 Mocetinostat and EPZ-5676 reduce LSD1 expression

The finding that both mocetinostat and EPZ-5676 reduce LSD1 expression, suggests that DOT1L may be involved in regulating the expression of LSD1 in MOLM-13 cells and by extension mixed lineage leukemia caused by the MLL-AF9 fusion. EPZ-5676 reduced HOXA9 and LSD1 expression to similar extents over time suggesting the two events were linked. Considering this we evaluated ChIP-seq data for H3K79Me2 at the HOXA9 and LSD1 genes in MLL-AF9 cells with and without treatment with the DOT1L inhibitor EPZ-4777 (*117*). Inhibition of DOT1L resulted in diminished H3K79Me2 in the HOXA9, LSD1, and DOT1L genes (**Figure 3.9A-C**). When combined with our results that EPZ-5676 reduces the expression of these genes it suggests that DOT1L mediated H3K79Me2, in part, regulates the expression HOXA9, LSD1 and even DOT1L itself (**Figures 3.3, 3.7 and 3.8**).

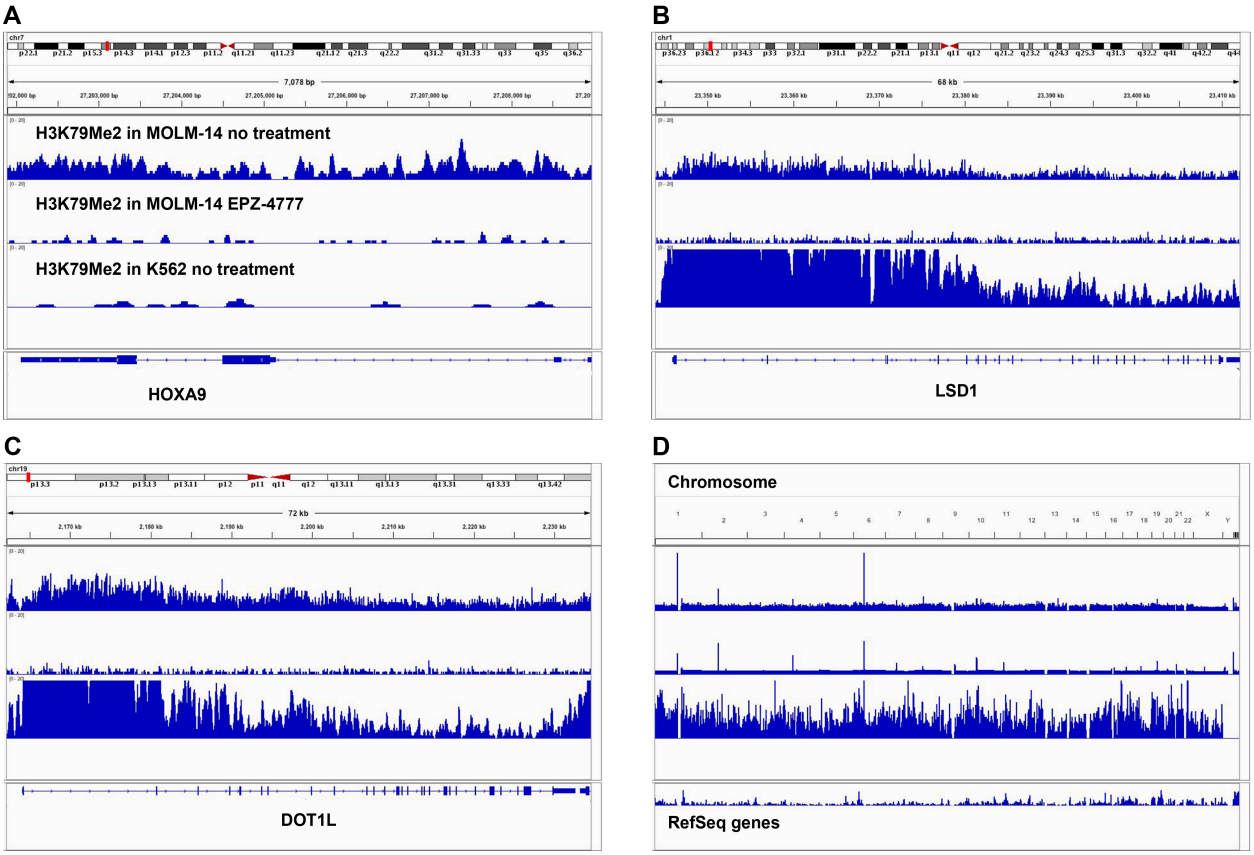

Figure 3.9 H3K79Me2 ChIP-seq data (hg19) for MOLM-14 (a sister cell line MOLM-13 that is derived from the same patient) obtained from the NIH gene expression omnibus (GEO) database and K562 cells from the ENCODE database. Relatively high levels of H3K79Me2 are found at HOXA9 in MOLM-14 cells from the aberrant recruitment of DOT1L to this region (A). Treatment with 3 μM of the DOT1L inhibitor EPZ-4777 (similar to EPZ-5676 but less potent) for 6 days reduces H3K79Me2 levels at HOXA9 to the baseline levels seen in K562 cells. This results in the reduction of HOXA9 expression and cell death (*117*). The same treatment reduces H3K79Me2 at LSD1 (B) and DOT1L (C) in MOLM-14 cells. The genome wide distribution of H3K79Me2 is much higher in K562 compared to MOLM-14 (D).

For mocetinostat, DOT1L and LSD1 expression were simultaneously and substantially reduced in MOLM-13 and K562 cells. In the case of MOLM-13, this resulted in a maximum reduction of ~50% in HOXA9 expression, making it a more effective treatment than GSK2879552. Mocetinostat reduced DOT1L expression by ~83% but the extent to which it reduces HOXA9 expression is not as high as with EPZ-5676. We suspect that the remaining DOT1L activity with mocetinostat treatment is sufficient to sustain some HOXA9 expression. When we knocked-down DOT1L expression using siRNA (**Figure 3.10**) we found that a 70%

knockdown sustained for up to 72 hours was insufficient to significantly decrease HOXA9 expression. Therefore, a small amount of DOT1L activity can sustain elevated levels of HOXA9 in MOLM-13 cells. This may explain why EPZ-5676 was the most effective compound we studied at reducing HOXA9 expression because it both directly inhibits and reduces the expression of DOT1L. Alternately, the short duration of treatment with mocetinostat needed to reduce HOXA9 expression may have been insufficient to allow the potential mechanism of reduction of DOT1L expression to be fully realized, as EPZ-5676 requires 144 h for maximum affect. Unfortunately, treatment of MOLM-13 cells beyond 24h with mocetinostat resulted in reduced cell growth that did not yield sufficient RNA or histones to explore this further.

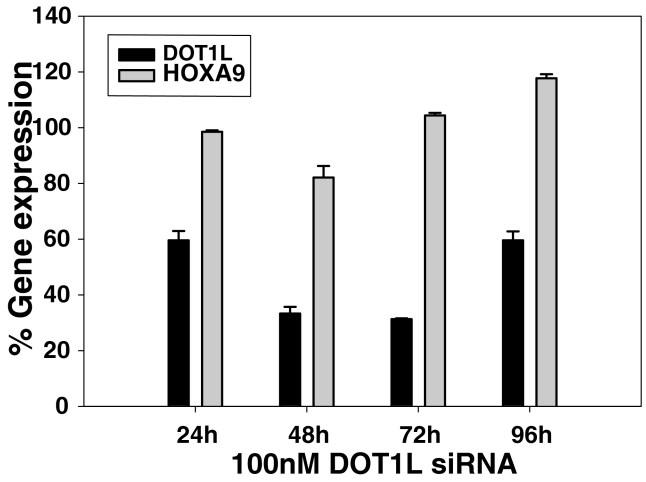

Figure 3.10 DOT1L siRNA knockdown in MOLM-13 cells using electroporation does not reduce HOXA9 expression. MOLM-13 cells were seeded $1x10^5$ and transfected with DOT1L Silencer Select siRNA (Thermo Fisher s39011) using the NEON electroporation system (Thermo Fisher) following the manufacturer's instructions. The electroporation conditions were one pulse, 25 ms width and 1725 V. Transfected cells were incubated for 24 to 96 hours and gene expression quantified as described in materials and methods. For 72h and 96h time points, the cells were transfected a second time after 48h incubation.

3.4 Discussion

3.4.1 Reductions in HOXA9 expression require different concentrations and dosing periods depending on the epigenetic target

Different doses and incubation periods were chosen for each inhibitor based on the doses and times that produced the maximum reduction in HOXA9 expression. This was measured by dose response curves for decreases in HOXA9 expression in MOLM-13 cells at various incubation times from 24 through 144 h. IC_{50} values were determined at incubation times that produced the maximum decrease in HOXA9 (**Table 4**) and these data were used to justify the doses and incubation periods for subsequent experiments. GSK2879552 reached its maximum effect by 72 h and longer incubation periods did not result in greater effects. The decrease in HOXA9 expression induced by EPZ-5676 was ~80% at 72 h, however, there was an additional ~10% decrease in expression up to 144 h, nearly eliminating HOXA9 expression. In order to measure this longer incubation, the cells would need to be passaged, re-seeded to their initial cell number and dosed a second time, which introduced too much error for a small decrease in HOXA9 expression. The reduction in HOXA9 expression produced by mocetinostat plateaued at 24 h, and incubation beyond 48 h at doses higher than 100 nM induced complete cell death in MOLM-13 cells, so examination of the effects beyond 24 h were avoided. This was different from what we observed previously in K562 cells, which were not as sensitive to mocetinostat (*67*).

3.4.2 Common changes in histone modifications and histone modifying gene expression among LSD1, DOT1L and HDAC class I inhibitors.

It is difficult to reconcile the diverse inhibitory activities of mocetinostat, EPZ-5676, and GSK2879552 and their shared effect of decreasing HOXA9 expression, however, we did find some common features among all. Each inhibitor either directly inhibits or decreases the expression of LSD1 (**Figure 3.6 and 3.7**). On a global level, this appears to lead to increases in histone lysine mono- and dimethylation (**Figure 3.5A and 3.5B**), and in particular, the repressive mark H3K9Me2 increases (**Figure 3.5C**). Although EPZ-5676 directly inhibits DOT1L (**Figure 3.6**), EPZ-5676 and all of the other inhibitors we tested also appear to decrease the expression of DOT1L (modestly in the case of GSK2879552) (**Figure 3.8**). This likely leads to the observed decrease in H3K79Me2 (**Figure 3.5C**) produced by all three inhibitors. This is noteworthy as H3K79Me2 is only catalyzed by DOT1L and previous studies have used the H3K79Me2 mark as a measure of the activity of DOT1L (*104*). Therefore, specific decreases in H3K79Me2, even with increases of total histone lysine dimethylation (**Figure 3.5A, B**), are likely a direct effect of inhibition or decreasing expression of DOT1L, which we observed with all compounds. Other studies have suggested that the H3K79Me3 mark is associated with heterochromatin and may be repressive (*120, 121*). The increases in H3K79Me3 and reductions in H3K79Me2 seen with both mocetinostat and GSK2879552 (**Figure 3.5C**) point towards another methyltransferase (or variant of DOT1L) that has yet to be discovered. In fact, we found in our *in vitro* DOT1L methylation assay, we did not detect any trimethyl-lysine even at longer (overnight) incubations (data not shown). Therefore, even with mocetinostat and GSK2879552 reducing the expression of DOT1L, H3K79Me3 can still increase to reduce expression of those target genes. For the case of EPZ-5676 where it reduces all types of H3K79 methylation (**Figure 3.5C**), this suggests that

it may in-fact come from another non-SET domain containing methyltransferase to which EPZ-5676 binds non-specifically. However interesting, further experimentation is needed to draw a factual conclusion and is beyond the scope of the current study.

At lower doses, all three inhibitors appear to generally increase total histone acetylation and in particular, all three result in increases of the generally permissive mark H3K14Ac. This effect was expected with the HDAC inhibitor mocetinostat, but it is an off-target effect for EPZ-5676 and GSK2879552, indicating that these inhibitors may alter the expression of HDACs or lysine acetyltransferases. At low doses all inhibitors decrease arginine mono-methylation and asymmetric di-methylation, however, no consistent effect was seen in CARM1 expression, the only enzyme in the gene expression array capable of causing such changes.

We have previously shown that mocetinostat increases mono- di- and trimethyl-lysine in K562 cells because mocetinostat reduces the expression of at least seven different lysine demethylases including both FAD dependent and JmjC domain containing types (*67*). Here we observed similar increases in total histone mono- di- and trimethyl-lysine and a general increase in most specific histone H3 methylation sites (except H379Me2 and H3K27Me2) produced by mocetinostat in MOLM-13 cells (**Figure 3.5**). This is likely through its mechanism of decreasing the expression of several lysine demethylases (LSD1, KDM2A and 4A) (**Figure 3.8**). On the other hand GSK2879552, and EPZ-5676, increase total histone lysine mono- and di-methylation (**Figure 3.5A and B**) but only increase specific histone H3 lysine methylation such as H3K9Me2 and H3K27Me3 (**Figure 3.5C**).

3.4.3 Mocetinostat is a broad-spectrum inducer of apoptosis

Mocetinostat is under clinical investigation for a variety of solid tumor cancers in combination with other agents (NCT02805660, NCT02236195, NCT02303262), was withdrawn from a study with azacitidine for acute myeloid leukemia (AML) (NCT00666497) and was part of a study for patients with relapsed or refractory Hodgkin's lymphoma (NCT00358982). Recently the HDAC inhibitors panobinostat, romidepsin and mocetinostat in combination with cytarabine have been shown to augment DNA damaging effects of cytarabine for infant acute lymphoblastic leukemia presenting with MLL-rearrangements (*122*). Our results suggest that mocetinostat alone may be sufficient as a potential treatment for mixed lineage leukemia through its activity of reducing LSD1, DOT1L and HOXA9 expression, and potently reducing MOLM-13 cell viability via apoptosis. In fact, we found that mocetinostat inhibits HOXA9 expression similar to the LSD1 inhibitor GSK2879552 that is currently in dose escalation trials for acute myeloid leukemia (NCT02177812). Although less potent, mocetinostat's effect of reducing HOXA9 expression is dose dependent and reaches a maximal effect similar to GSK2879552 in MOLM-13 cells (**Figure 3.3A**). The total reduction in HOXA9 expression in MOLM-13 cells occurred rapidly in 24 h, where GSK2879552 and EPZ-5676, required a minimum of 72 h to observe a substantial reduction in HOXA9 expression (**Figure 3.3B**). While our results show that mocetinostat, EPZ-5676, and GSK2879552 all decrease HOXA9 expression in an MLL-AF9 translocation cell line, all compounds produce changes in the expression of many genes including those coding for enzymes responsible for adding or removing histone modifications and this leads to changes in histone modifications not associated with the direct enzymatic inhibitory activity of the compounds.

With respect to viability, mocetinostat was the most rapid and potent compound we tested, producing apoptosis in both MOLM-13 and K562 cell lines in 24 hours or less. Initial studies with mocetinostat also found it had a broad-spectrum activity inducing apoptosis in colon and lung cancer cell lines (*123, 124*). More recent studies suggest that mocetinostat induces apoptosis in prostate cancer cells by activation of the microRNA miR-31 and by suppressing expression of the transcription factor E2F6 (*125*). Mocetinostat has also demonstrated some success alone or in combination in clinical trials for relapsed lymphoma and some types of leukemia often through the induction of apoptosis (*126-129*). Therefore, our study adds to the growing body of evidence that mocetinostat is a broad-spectrum anticancer agent that induces apoptosis. We find for the first time that mocetinostat is a potent inducer of apoptosis in MLL-AF9 cells.

3.4.4 EPZ-5676 and GSK2879552 reduce MOLM-13 viability through a non-apoptotic mechanism

Whereas mocetinostat reduced viability in both MOLM-13 and K562 cells in 24 h by inducing apoptosis, EPZ-5676 and GSK2879552 at an equivalent dose only affected the viability of MOLM-13. Neither EPZ-5676, nor GSK2879552 induced caspase activation during their 72 h or 144 h dosing period and their reductions in viability over these times were therefore, via non-apoptotic mechanisms. This was an interesting result as previous groups have shown that knockdown of HOXA9 using shRNA reduces viability in cells with the MLL-AF9 translocation, inducing cell death via apoptosis (*105*), and that inhibition of DOT1L with EPZ-5676 for 6 days or more in MV4-11 cells, a MLL-AF4 fusion cell line, leads to apoptosis (*104*). Our results indicated that the inhibition of growth and viability with EPZ-5676 and GSK2879552 in MOLM-13 cells is initially a non-apoptotic mechanism and longer incubation periods may be

required to observe apoptosis. In general apoptotic mechanism of induced cell death are more desirable to non-apoptotic because the latter results in inflammation or mediators of inflammation that may produce damage to neighboring healthy cells (*130*).

3.4.5 Reductions in HOXA9 expression mediated by DOT1L inhibition take multiple cellular generations to appear

As with other groups, we found that there was delayed onset of the effects on gene expression with DOT1L inhibitors. This may be due to the apparent lack of an H3K79 demethylase. Although some studies suggest a H3K79 demethylase exists, it has yet to be identified (*131*). Therefore, in order to effectively reduce H3K79 methylation, cells must undergo nucleosomal turn over and assembly while under persistent DOT1L inhibition to prevent H3K79 methylation on newly assembled chromatin. Treatment with EPZ-5676 must therefore be maintained throughout multiple generations of cells for hypomethylation of H3K79 to occur. Although this may explain the delayed effects observed with EPZ-5676, we cannot yet explain why mocetinostat has similar, yet more rapid effects. Previous studies with mocetinostat in colorectal carcinoma cells show rapid cell cycle arrest after only 16 hours (*123*). However, it is possible that the reduction in HOXA9 expression produced by mocetinostat is mediated by its HDAC inhibition as our previous work has shown that significant increases in histone acetylation are observed in as little as 8 h with mocetinostat (*67*).

3.4.6 Genome wide H3K79 methylation is generally higher in K562 than MOLM-13: consequences for EPZ-5676 toxicity

MOLM-13 cells are particularly sensitive to EPZ-5676 yielding large decreases in the expression of HOXA9, LSD1 and many other genes resulting in potent decreases in cell viability after 144 h of treatment. This phenomenon was only observed in MOLM-13 and not in K562

cells, and may be explained by the overall higher levels of H3K79 methylation in K562 versus MOLM-13 cells. This can be observed when comparing H3K79Me2 ChIP-seq data between the two cell lines, with K562 having far higher counts of this mark throughout its genome compared to MLL-AF9 cells (**Figure 3.9D**). (We assume this comparison to be appropriate, as these two individual data sets have been controlled for total input DNA.) This is also in agreement with the finding that MLL-rearranged leukemias have relatively low levels of H3K79Me2 that are reversed upon differentiation (*132*). Therefore, the lower levels of H3K79Me2 in MOLM-13 cells may make them particularly sensitive to DOT1L inhibitors when compared to K562. Interestingly, the levels of H3K79Me2 within the LSD1 gene in MLL-AF9 cells (**Figure 3.9B**) are abolished upon treatment with DOT1L inhibitors corresponding with the decreased LSD1 expression we observed (**Figure 3.7**). This may not have happened in K562 cells because the local levels of H3K79Me2 in the LSD1 gene are much higher in comparison to those found in MLL-AF9 cells. This may, in part explain the activity of EPZ-5676 in MOLM-13, but not K562 cells.

3.4.7 Concluding remarks

Identifying the off-target effects outlined here reveals the need for a higher level of specificity when using any epigenetic enzyme inhibitor. Not only should the inhibitor be selective to the histone modifier of interest, but also selective to the gene to which inhibition is to take place. The following chapter outlines how we intend to target epigenetic enzyme inhibitors, in particular KMT inhibitors to the HOXA9 gene.

Chapter 4.

4.1 LNA oligonucleotide-drug conjugates as a means of gene-targeted inhibition of epigenetic enzymes

We have shown that inhibitors of HDAC (vorinostat, mocetinostat and entinostat), DOT1L (EPZ-5676) and LSD1 (GSK2879552) induce off-target changes in histone modifications by altering expression of off-target histone modifying enzymes (*67, 94*). We hypothesized that inhibition of any epigenetic target will lead to unpredictable changes in gene expression because the inhibition of a single epigenetic target will affect the expression of many genes including other epigenetic enzymes. We therefore provide evidence that this is true. However, these off-target changes in gene expression are even more significant than they might first appear because they affect so many epigenetic enzymes, which in turn have the potential to produce further down-stream changes in gene expression. To mitigate these problems we hypothesize that any viable therapy that focuses on epigenetic targets, such as HDAC, DOT1L or LSD1, must also be designed to target the pathological gene or genes of interest (*13*). An example of such a gene is HOXA9 and MLL-AF9 rearranged leukemia (*94*) described in Chapter 3. It is known that the MLL-AF9 fusion aberrantly recruits DOT1L to HOXA9 resulting in increased HOXA9 expression and mixed lineage leukemia. Accordingly, reducing the expression of HOXA9 is all that is required to arrest mixed lineage leukemia cell line growth. Therefore, others have validated DOT1L as a therapeutic drug target for this type of leukemia because it reduces HOXA9 expression (*74, 94, 104, 133*). However, as we have shown such DOT1L inhibitors also alter the expression of many histone modifying enzymes that in turn may result in cascading changes in gene expression (Chapter 3). We have selected the MLL-AF9

rearrangement leukemia as a model epigenetic disease state to test the approach of gene selective inhibition of histone modifying enzymes.

In this chapter, we propose that DOT1L inhibitors can be targeted to the HOXA9 promoter by conjugating the inhibitor to locked nucleic acid (LNA) oligonucleotides. Short LNA mixmers (alternating LNA/DNA bases) have the propensity to bind and anchor to genomic DNA in a sequence specific manner and are less susceptible to nucleases yielding longer half-lives in cells than RNA or DNA. Beane *et. al.* show that antigene LNA oligonucleotide mixmers can bind DNA complementary to transcription start sites and inhibit transcription in a gene specific manner by disrupting RNA polymerase binding at their target genes transcription start site (TSS) (*134*). They show that LNA mixmers can invade and base pair with genomic DNA in a sequence specific manner. We use this as our strategy for targeting specific regions in the HOXA9 gene. However, instead of directly targeting MLL or DOT1L binding sequences (like to Beane et al, directly targeting RNApol binding), we will target sequences in HOXA9 promoter where DOT1L mediated H3K79 methylation occurs. Such sequences are derived from publicly available ChIP-seq datasets for H3K79Me2 in the MLL-AF9 cell line, MOLM-14 (*117*) (see Chapter 3**, Figure 3.9**). As DOT1L is the only enzyme known to methylate H3K79, it is reasonable to assume that DOT1L will be concentrated at these sites and by anchoring a DOT1L inhibitor close to these loci; it will increase the probability of interaction between the inhibitor and enzyme. If successful this should have the effect of appearing as a dramatic increase in potency of the targeted, compared to untargeted, DOT1L inhibitor.

The sequences of our LNA mixmer constructs will be determined from H3K79Me2 ChIP data sets described above and the synthetic LNA mixmers can be engineered with a 5' twelve carbon linker with a primary amine in order to chemically conjugate a DOT1L inhibitor using

facile HATU/DIPEA chemistry (*135*) (**Figure 4.1**). However, the inhibitor must have a carboxylic acid to be activated, which poses a major limitation, as very few DOT1L inhibitors are known and even fewer have unhindered carboxylic acid moieties. It is possible to conjugate EPZ-5676 to LNA vial acetal formation with its ribose 2' and 3' OH functionalities. Unfortunately, EPZ-5676 is not the optimal inhibitor for this purpose because it is difficult to conjugate to LNA requiring a multi-step synthesis, and crystal structure data suggests conjugation will prevent binding to DOT1L because it eliminates critical interactions in the active sited with the 2' and 3' ribose OH functionalities of EPZ-5676 (PDB: 4HRA) (*104*).

Rather, we use BIX01338 because 1) it is potent (*136*), 2) we show that it inhibits DOT1L, 3) we can conjugate it to LNA through its carboxylic acid group forming BIX-LNA, and 4) we show that BIX-LNA inhibits nucleosomal methylation by DOT1L (see below). At very low doses, the BIX-LNA concentrates the BIX01338 at the HOXA9 promoter, and the concentration of BIX-LNA elsewhere is too low to produce off target effects.

We designed an *in vitro* methyltransferase assay with recombinant nucleosomes and DOT1L to screen for candidate inhibitors. We measured the total methyltransferase activity of DOT1L by LC-MS/MS using a modified version of the histone modification assay previously developed (*67, 94*). We identified for the first time that BIX01338 is an inhibitor of DOT1L.

We optimized the BIX01338/LNA conjugation synthesis using UHPLC purification and designed the sequence of the LNA mixmer to be complementary to the DNA of the recombinant nucleosome used for the *in vitro* DOT1L inhibition assay. We found that the BIX-LNA conjugate inhibited DOT1L and was 16-fold more potent than unconjugated BIX01338 or the same sequence of unconjugated LNA or random sequence (scramble) LNA.

4.2 Material and Methods

4.2.1 DOT1L methylation assay

Conditions were adapted from the *in vitro* DOT1L methylation assay described in Chapter 3 materials and methods, section *3.2.5*. Recombinant nucleosome core particles (Mononucleosomes/NCP, EpiCypher) contained blank human histone octamer with the Widom 601 strong nucleosome positioning sequence (DNA: CTGGAGAATCCCGGTGCCGAGGCCGCTCAATTGGTCGTAGCAAGCTCTAGCACCGCT TAAACGCACGTACGCGCTGTCCCCCGCGTTTTAACCGCCAAGGGGATTACTCCCTAG TCTCCAGGCACGTGTCAGATATATACATCCTGT) (SKU 16-0009). The lysine methyltransferase inhibitors BRD4770 and BIX01338 were purchased from Sigma Aldrich and EPZ-5676 was purchased from Biovision. Briefly, BIX-LNA_{601}, unconjugated LNA_{601}, scramble LNA, BRD4770, BIX01338, EPZ-5676 or DMSO (no treatment) were incubated with 1 µg of the Widom 601 NCP for one hour at 37 °C. Then, 120 nM (final concentration) of recombinant DOT1L (Epicypher) was added and the methylation reaction initiated with 100 µM S-adenosyl methionine (SAM). The methylation reaction was allowed to proceed for 6 hours and evaporated to dryness in a vacuum centrifuge. The dried reaction products were then hydrolyzed overnight with 6N HCl at 110 °C. Methylated lysine was quantified by LC-MS/MS using the assay described in Chapter 2 Materials and methods except that the histone precipitation procedure was not used. Inhibition curves were generated and the data fit to a 4-parameter logistic regression using Sigma Plot 11 and the IC_{50} Min and Max values calculated.

4.2.2 LNA oligonucleotides

Locked Nucleic Acid oligonucleotides (LNA) were synthesized and HPLC purified by Qiagen (formerly Exiqon) on a 1 micromole scale containing a 5' C12 amino functional group. LNA products were delivered dry and were reconstituted to 1 mM using Milli-Q grade water.

4.2.3 BIX-LNA conjugation synthesis

BIX01338 (Sigma) was solubilized to 200mM in dimethyl formamide (DMF, Sigma), 5% diisopropyl ethylamine (DIPEA, Sigma) and 4 μL of this was combined with 2 μL of 400mM HATU in DMF. BIX01338 and HATU were allowed to form the BIX01338 active ester (BIX-HOAt) for 30 min at RT (**Figure 4.1**). 150 nmole of aqueous LNA was suspended in 400 μL DMF and combined with BIX-HOAt. The reaction was rocked gently for 2 h. The crude reaction was desalted using a PD MiniTrap G-25 size exclusion column (GE Healthcare) pre-equilibrated in deionized water, following the manufacturers instructions.

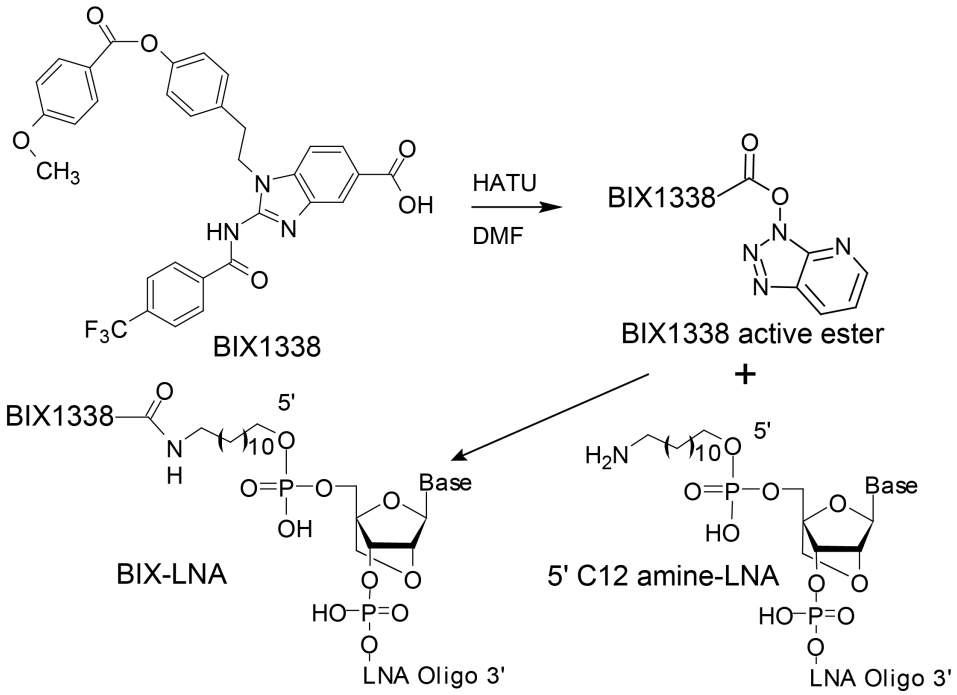

Figure 4.1 The schematic of the synthetic strategy producing BIX-LNA. First, BIX01338 is activated with HATU/DIPEA in DMF. Then, LNA with a 5' primary amine connected to the LNA by a C_{12} linker is added to the BIX01338 activated ester (BIX-HOAt) in DMF. The reaction is gently stirred and progress monitored by LC-MS/MS. Once the reaction is complete the crude product is desalted into water with G25 sephadex spin columns (GE healthcare) and further purified using UHPLC. The purified product is lyophilized and reconstituted in water at the desired concentration.

4.2.4 Identification and purification of BIX-LNA conjugates by UHPLC-MS/MS.

A Shimadzu Nexera coupled to a Shimadzu 8040 triple quadrupole mass spectrometer was used for analysis. A Waters C18 BEH 1.7 μm, 2.1x100 mm column was used with mobile phases (A) aqueous 400mM hexafluoro isopropanol (HFIP), 16mM triethyl amine (TEA) and (B) 75% aqueous methanol with 100mM HFIP, 4mM TEA (75% methanol in mobile phase A). The LC time program begins with 20% B and increased to 80% B over 10 min, and held at 80% B for 2 min. The column was washed with 100% B for 8 min and then stepped down to 20% B to recondition the column for 5 minutes before the next injection. Mass detection was in negative mode using a scan from 1000-2000 *m/z*. The resulting multiple *m/z* peaks were deconvoluted using the Multi Charge Analysis software (LabSolutions v5.72). The peak corresponding to the

correct mass of the BIX-LNA conjugate was identified and correlated to the UV spectrum measured with a Shimadzu SPD-M20A Prominence Diode Array detector. The BIX-LNA conjugate was purified through multiple injections monitoring UV spectrum with the above conditions, and the appropriate fractions were pooled together, concentrated by vacuum centrifugation in a Savant SPD1010 (Thermo Scientific) and the concentrate lyophilized to dryness using the AdVantage 2.0 Bench Top Freeze Dryer / Lyophilizer (SP Scientific). The purified product was dissolved in a minimal amount of water and the concentration was calculated using a NanoDrop 2000 spectrophotometer (Thermo Scientific) at 260 nm. Purity analysis and mass verification was performed using the same UHPLC-MS/MS method with tandem UV detection at 260 nm (Appendix **Figure A4**).

4.3 Results

4.3.1 BIX01338 inhibits DOT1L

Originally, BIX01338 was identified as a SAM competitive broad spectrum lysine methyltransferase inhibitor, shown to inhibit G9a, SUV39H1 and PRMT1 with IC_{50}'s of 4.7, 1.1 and 6 μM respectively (*137*), however no group as far as we know has shown that BIX01338 can inhibit DOT1L. Few selective DOT1L inhibitors are known beyond the EPZ-4777 and EPZ-5676 compounds and only recently through a structure based computational design, two novel DOT1L inhibitors have been discovered (*138*). However, none of the known DOT1L inhibitors also contain a carboxylic acid moiety to which LNA can be easily conjugated. Since BIX01338 is shown to be a SAM competitive inhibitor with activity in the micromolar range for at least three methyltransferases and contains a carboxylic acid that can be used to conjugate LNA and for the reasons enumerated above, we thought it to be the best candidate for further studies. We tested BIX01338 *in vitro* using our previously designed LC-MS/MS methylation assay for DOT1L using mononucleosomes as substrates, using EPZ-5676 and the G9a selective inhibitor BRD4770 (*139*) as controls for DOT1L inhibition (**Figure 4.2A**). We found that, other than EPZ-5676, only BIX01338 could inhibit DOT1L with similar maximum inhibition as EPZ-5676 at the 10 μM level, however, BIX01338 is much less potent than EPZ-5676 when comparing IC_{50} values (**Figure 4.2B, Table 5**). We found BIX01338 to inhibit DOT1L with an IC_{50} of 2.9±0.58 μM, which was within the same range as its reported potency against G9a (IC_{50} of 4.7 μM) (*137*).

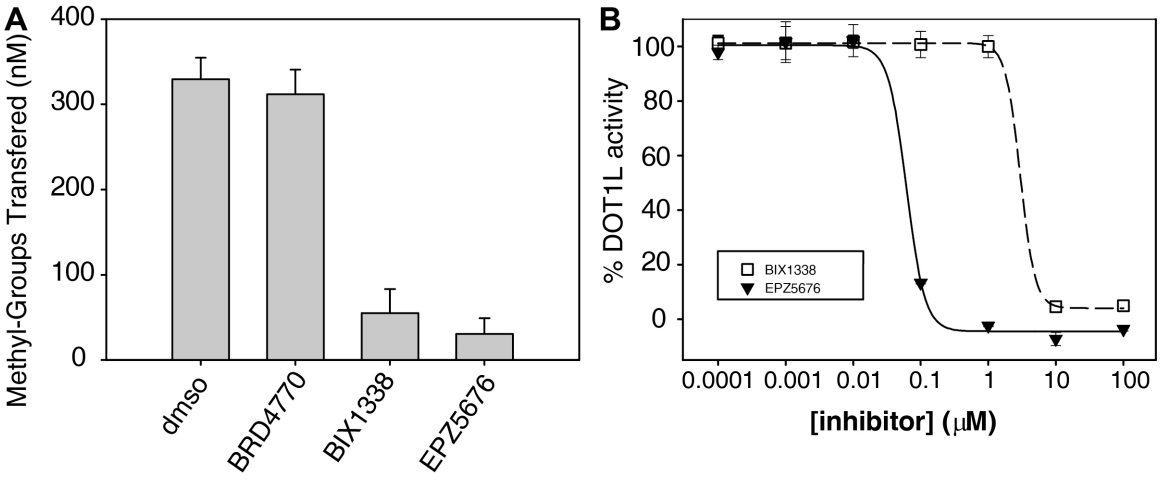

Figure 4.2 Inhibition of recombinant DOT1L *in vitro*. BIX01338 and EPZ-5676 (positive control) fully inhibit DOT1L methylation at 10 μM while BRD4770 (negative control) does not (A). BIX01338 and EPZ-5676 both inhibit DOT1L dependent on dose with an IC_{50} of 2.9±0.58 μM and 45±4.8 nM respectively (B).

4.3.2 BIX-LNA conjugation synthesis

The conjugation synthesis was adapted from Aaronson *et. al.* with minor modifications (*135*) (**Figure 4.1)**. In order to solubilize BIX01338 to the appropriate concentration (200mM), it was necessary to use DMF rather than DMSO as used by Aaronson *et al*, however we found that up to 30% water also need to be added to the reaction in DMF in order to solubilize the LNA. This amount of water might be expected to reverse the activation reaction of BIX-HOAt back to the carboxylic acid, however we did not observe any appreciable loss of the conjugate product. The conjugates were initially verified by mass using the ion pairing reagents HFIP/TEA in order to retain the LNA oligonucleotides to the C18 column. We identified the retention times of the BIX-LNA conjugates and used the same method and column with a PDA detector in order to collect the appropriate peak during purification. Injection volumes were 50 μL and required 10 to 20 injections to process the entire sample on the analytical column. In general, a 30% yield after purification was achieved. This may be attributed to the multi-step purification procedure (size exclusion followed by UHPLC) and the use of an analytical column for the purification. A single

injection of the entire crude onto a semi-preparative column for purification could be used if higher yields are desired.

Using the method described we found a complete conversion of the starting material LNA to conjugated product. We also did not find any major hydrolysis products of the BIX01338 ester or amide moiety using the current conditions. We found the conjugate to be easily traced through the diagnostic wavelength of BIX01338 at 330nm and because its retention time was distinct from that of unconjugated BIX01338.

4.3.3 BIX-LNA$_{601}$ inhibits DOT1L more potently than BIX01338 alone

We designed a BIX-LNA construct to be complementary to the Widom 601 nucleosome position sequence DNA that is commercially available. We carefully chose the sequence of the corresponding LNA mixmer to anchor the nucleosome such that the 5' end with the tethered BIX01338 is in close proximity to the site of H3K79 methylation (**Figure 4.3A**). In fact, the sequence was chosen such that the length of the fully extended conformation 12 carbon linker (~15 Å) excluding BIX01338, was greater than the distance from the complementary DNA sequence 5' O (where the corresponding complementary 5' O of the LNA should be) to the nearest H3K79 εN calculated using the program Chimera (11.3Å). Such a distance allows the 12-carbon linker to adopt several non-fully extended conformations while still positioning the BIX01338 in close proximity to the target H3K79 εN. In this way the BIX01338 is concentrated at the site of DOT1L activity to maximize inhibition.

Using this strategy, the potency of BIX01338 was increased by more than 15-fold, showing complete DOT1L inhibition at 1 μM with BIX-LNA$_{601}$ (**Figure 4.3B**). We found BIX-LNA$_{601}$ to inhibit DOT1L in a dose dependent manner with an IC$_{50}$ of 183±7.8 nM, which was about 16-fold more potent than BIX01338 alone. Several controls were performed including

scramble LNA (random sequence LNA), which showed little to no inhibitory propensity (**Figure 4.3B**). In addition, unconjugated LNA_{601} was evaluated to determine if LNA alone yielded any inhibitory activity against DOT1L methylation. At higher concentrations, unconjugated LNA_{601} inhibited DOT1L methylation with a similar potency as unconjugated BIX01338 (**Figure 4.3C, Table 5**).

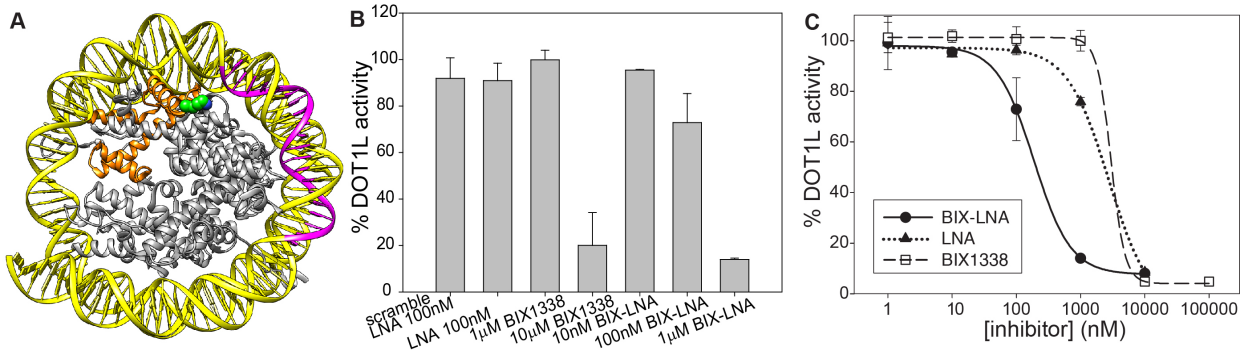

Figure 4.3 BIX-LNA complementary to nucleosomal DNA inhibits H3K79 methylation. Shown is the crystal structure (PDB-3LZ0) of recombinant nucleosome core particle (NCP) with Widom 601 positioning sequence DNA in yellow, histone H3 highlighted in orange with H3K79 in green and the binding site for the LNA_{601} in pink positioning its 5' end adjacent to H3K79 (A). This method of anchoring BIX1338 that is tethered to the 5' end of the LNA_{601} oligonucleotide (BIX-LNA) results in more than 15-fold increase in potency when compared to inhibition by BIX1338 alone (B). Inhibition of DOT1L is noticeable at 100nM and complete at 1 μM BIX-LNA, where 1 μM BIX1338 alone does not inhibit DOT1L any more than the negative control scramble LNA and LNA_{601} at 100 nM. Unconjugated LNA that is complementary to the LNA_{601} inhibits DOT1L methylation at higher concentrations, and has a similar IC_{50} to that of BIX01338 alone (C). BIX-LNA_{601}, unconjugated LNA_{601} and BIX01338 have a dose dependent effect on DOT1L methylation, (**Table 5**).

Table 5. Potencies of DOT1L inhibitors

DOT1L inhibitor	**IC_{50} (nM)***
EPZ-5676	45±4.8
BIX01338	2900±580
LNA	2800±200
BIX-LNA	183±7.8

*IC_{50} values are means of 3 repeats ±SD

4.4 Discussion

We have shown that BIX-LNA conjugates can inhibit DOT1L methylation activity against recombinant nucleosomes and therefore may be developed into a novel treatment for mixed lineage leukemia. The next steps are to identify sequences at the HOXA9 gene that are enriched with H3K79 methylation and design complementary BIX-LNA conjugates to target these regions. We identified candidate sequences based on existing ChIP-seq data in MOLM-14 cells (a cell line with the MLL-AF9 fusion derived from the same individual as the MOLM-13 cell line) (*117*) (**Figure 4.4)** and synthesized BIX-LNA conjugates to target these H3K79 methylation sites (**Table 6, Figure A4**). Peaks were selected based on methylation intensity and numbered them from the HOXA9 transcription start site following the convention that positions 5' or upstream of the TSS are negative and those downstream or 3' to the TSS are positive. Sites of methylation immediately neighboring the TSS and/or with high intensity were suspected to have the most influence over transcription and it is hoped that targeting only one such H3K79 methylation peak will be sufficient to reduce HOXA9 expression. The number of sites of H3K79 methylation within the HOXA9 promoter, suggest that just targeting one peak may be insufficient to produce desirable reductions in HOXA9 expression. Therefore many sites might need to be targeted simultaneously to reduce HOXA9 expression to therapeutic levels.

Table 6. Selected target sequences with respect to the HOXA9 promoter

HOXA9 H3K79 methylation sites*	**BIX-LNA mixmer oligo sequences****
166	BIX--GcGcaTaGcgGcCaaCgCtC
-283	BIX--CcAtaCaCacAcTtcTtAaG
-687	BIX--CaAcgCaGgaTcCgtCcCaA
-2246	BIX--CcCaaAgGttTgGagCcAgA
Widom 601 nucleosome	BIX--GaTtaCtCccTaGtcTcCaG

* Sites are numbered as base pairs from HOXA9 TSS
** Capitol denotes LNA base, lower case denotes DNA base
-- denotes twelve carbon linker

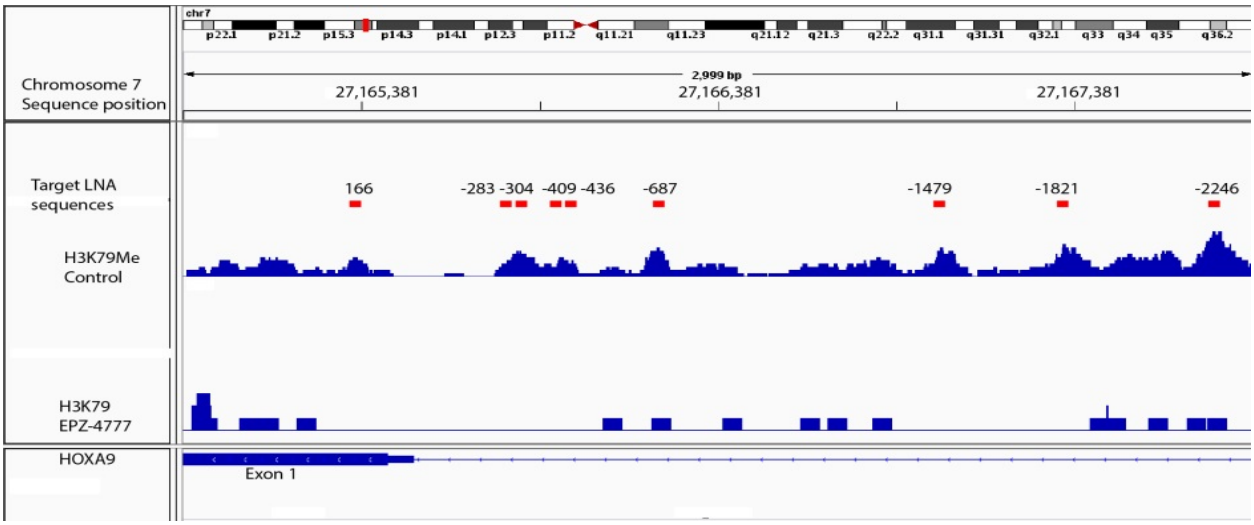

Figure 4.4 A schematic of 3000bp of the HOXA9 promoter and 1st exon derived from H3K79Me2 ChIP-seq (*117*). The HOXA9 gene is found on chromosome 7 starting at position 27165530 to 27162438 (hg19). The red bars show the binding positions of the targeting LNA oligonucleotides and the positions with respect to the transcription start site some of which are represented in (**Table 6**). The levels of H3K79 methylation from ChIP assays are shown without, (above) and with treatment (below) with the DOT1L inhibitor EPZ-4777. This figure was prepared using the Integrative Genomics Viewer (IGV).

The targeting LNA oligonucleotides are composed of ssDNA interspersed with LNA nucleotides (LNA mixmers) complementary to unique sequences within the HOXA9 promoter that are enriched for H3K79 methylation (*117*) (**Figure 4.4**). DOT1L is the only KMT known to produce H3K79 methylation therefore if the BIX-LNA concentrates BIX01338 at these regions BIX01338 will specifically inhibit DOT1L.

LNA mixmer oligonucleotide sequences were chosen based on ChIP assays in a mixed lineage leukemia cell line treated with, and without a DOT1L inhibitor closely related to EPZ-5676 (*117-118*). 20bp HOXA9 promoter sequences that show high intensity H3K79 methylation in the control but not with the DOT1L inhibitor were selected. Methylation at these sequences is important for controlling HOXA9 expression since previous studies have shown that EPZ-4777 reduces HOXA9 expression resulting in reduced MLL-AF9 cell growth (**Figure 4.4 Table 6**). Some of the sequences selected also neighbor MLL-AF9 binding sites (*140-142*), which we confirmed using the programs PROMO and TRANSFAC (*143-145*). This theoretically positions

the BIX-LNA in close proximity to its target DOT1L, which should be bound to AF9 (**Figure 4.3A**)

If the level of H3K79 methylation can be lowered in and around the HOXA9 gene to a comparable level to the DOT1L inhibitors EPZ-4777 or EPZ-5676 (**Figure 4.4**), it may induce region-specific heterochromatin remodeling and turn off the expression of HOXA9 in a similar fashion. This may be evaluated using qPCR for HOXA9 from cells treated with the DOT1L inhibitors or BIX-LNA constructs. Once a reasonable reduction in HOXA9 expression is observed with BIX-LNA, those constructs and doses can be further evaluated with ChIP-PCR at the HOXA9 gene and promoter to measure specific reductions in H3K79 methylation correlated to the observed reduction in HOXA9 expression. Once we find a reasonable reduction in H3K79 methylation at the HOXA9 gene and promoter, we can further evaluate the effect of BIX-LNA throughout the genome using ChIP-seq in comparison to genome wide DOT1L inhibition using EPZ-5676. If we can show that H3K79 methylation can be specifically reduced at HOXA9 using BIX-LNA we can evaluate genome wide expression by RNA-seq as a definitive means of measuring the gene specific effect of BIX-LNA. If successful, this would represent the first instance of gene specific epigenetic enzyme, inhibition that leads to a specific reduction in expression at the intended target gene.

4.4.1 EPZ-5676 is a better measure of genome wide DOT1L inhibition than BIX01338

In order to evaluate treatment success with the BIX-LNA constructs targeted to DOT1L at the HOXA9 promoter, we need to compare to a DOT1L specific inhibitor that has already been validated to reduce HOXA9 and treat mix lineage leukemia. Normally a control like this would be unconjugated BIX01338 to evaluate whether the conjugated LNA serves its intended purpose. However, BIX01338 is not a validated inhibitor to treat mixed lineage leukemia and in

fact, we are the first to show that BIX01338 is an inhibitor of DOT1L *in vitro*. Furthermore, BIX01338 has limited studies in cell culture that determine its cellular permeability, cytotoxicity and genome wide effects on epigenetic enzymes and histone modifications. Finally, since BIX01338 is considered a broad spectrum, SAM competitive methyltransferase inhibitor (*137*), it is not an ideal inhibitor to show genome wide DOT1L inhibition, as it will have additional methyltransferase inhibition activities such as G9a and SUV39H1. For these reasons we used the DOT1L specific inhibitor EPZ-5676 as a positive control for genome wide DOT1L inhibition as well as a measure of therapeutic HOXA9 down-regulation. This comparison is valid since BIX-LNA, when designed to target H3K79 methylation sites acts as a DOT1L specific inhibitor since DOT1L is the only enzyme that catalyzes this type of modification (*131*).

4.4.2 Future initiatives for cellular studies

Due to the chemical nature of BIX-LNA, these constructs must be transfected when evaluating their effects in cell culture. We found MOLM-13 cells difficult to transfect and not compatible with standard, lipid based transfection reagents and many of our initial cellular studies showed poor or no transfection efficiency (Data not shown). This was in part due to the frailty of the MOLM-13 cells when in contact with transfection reagents (lipofectamine/oligofectamine) as they proved to be overtly toxic to the cells. We also attempted to transfect BIX-LNA by electroporation, which achieved better viability, however the transfection efficiency was too variable to assume a consistent dose. We evaluated cellular uptake using a fluorescein-LNA mixmer conjugate (FAM-LNA) with these different transfection techniques using fluorescent microscopy. We were able to show some cellular and nuclear delivery of FAM-LNA using these techniques but the transfection efficiency was poor resulting in nuclear delivery to, at most, 20% of MOLM-13 cells. As it is critical to evaluate the cellular

activity of BIX-LNA, the future initiative for this end will be to develop a nanoparticle formulation of BIX-LNA that is compatible with MOLM-13 cells and provides efficient nuclear delivery.

Chapter 5.

5.1 Conclusions

Based on our research we identified the critical need for gene targeting epigenetic enzyme inhibitors to oncogenes of interest. Since the field of epigenetic enzyme inhibitors targeting histones is continually expanding and becoming more clinically relevant with the case of HDAC and KMT inhibitors, the off-target effects of these inhibitors must be carefully taken into consideration.

We first describe an original, validated method to quantify total histone lysine acetylation, methylation and arginine methylation in cells. We used this method to test the activity of HDAC inhibitors vorinostat, entinostat and mocetinostat on the aforementioned histone modifications. As a result, this study was the first to show the broad effects of several HDAC inhibitors on the expression of many KDM enzymes. We found that the reduction in KDM expression by mocetinostat produces an increase in histone lysine methylation that, although less potent than its HDAC inhibition activity, is still in the low micromolar range. This produces an effect similar to that of a broad spectrum KDM inhibitor. As KDM inhibitors are being explored as potential cancer therapeutics (*146*), this may suggest that mocetinostat has two potential mechanisms of action with respect to cancer chemotherapy: HDAC inhibition and *de facto* broad spectrum KDM inhibition.

Based on previous studies showing that inhibition of DOT1L or LSD1 (a KDM) reduce HOXA9 expression and may be useful treatments for mixed lineage leukemia, we were able to show for the first time that mocetinostat may be used for this indication through its activity as a broad spectrum transcriptional inhibitor of KDMs. We further identified the off-target effects on gene expression with the select DOT1L, LSD1, and HDAC inhibitors. To this end we tested

three compounds with different epigenetic targets (mocetinostat (HDAC class I); EPZ-5676, (DOT1L); GSK2879552, (LSD1)) showing them to reduce MOLM-13 cell viability by directly inhibiting, or reducing the expression of, HOXA9, DOT1L and LSD1. This resulted in increases in total histone lysine methylation and acetylation and specifically reduced H3K79Me2 and increase H3K14Ac. We identified that the efficacy of the DOT1L inhibitor EPZ-5676 may not be solely a function of its DOT1L enzyme inhibition but may be augmented by its reductions in LSD1 and DOT1L expression, which in turn further reduces HOXA9 expression. GSK2879552 directly inhibits LSD1, and yields a modest reduction in DOT1L expression, which further reduces HOXA9 expression. Although not a direct inhibitor of either DOT1L or LSD1, mocetinostat reduces LSD1, DOT1L and HOXA9 expression in MOLM-13 cells and may be another potential treatment for mixed lineage leukemia. As predicted, all three compounds changed the expression of several histone-modifying enzymes which themselves are involved in controlling gene expression. Where this results in reduction of HOXA9 expression these changes in expression are advantageous for the treatment of mixed lineage leukemia. However, where such activities do not eventually decrease HOXA9 expression, they can be viewed as off-target effects. This is a significant contribution to the current literature pertaining to epigenetic enzyme inhibitors because it outlines in great detail the unanticipated off-target effects associated with these inhibitors and as far as we know, this is the first report outlining the potential use of mocetinostat as treatment for MLL-AF9 rearranged leukemia

The off-target effects of the HDAC inhibitors mocetinostat, vorinostat, entinostat, the DOT1L inhibitor EPZ-5676 and the LSD1 inhibitor GSK2879552, reveal some serious issues with inhibition of histone modifying enzymes. In fact, each of these inhibitors was shown to reduce or augment the expression of lysine demethylases, as well as several other histone

modifier genes. The altered expression of histone modifier genes necessarily leads to alterations in the expression of other sets of genes as histone modifications help to control the expression of many genes. Therefore such inhibition leads to unpredictable, cascading changes in gene expression, which we observed in an inhibitor dose, time, and cell type dependent manner. These off-target effects have serious consequences on the utility of these inhibitors as clinical drugs. We suggest that epigenetic enzyme inhibitors could benefit from having a higher level of specificity than just to that of the target enzyme and targeting the inhibitors to specific genomic loci may achieve this end. Our preliminary data suggests that conjugating the inhibitor LNA mixmer sequences that are complementary to the gene intended for the inhibitor to target increases the potency of the inhibitor. We have shown that this targeting strategy increases the potency of the KMT-inhibitor BIX01338 by 16-fold *in vitro* using a BIX-LNA conjugate to inhibit DOT1L. We anticipate a similar effect in future cellular and *in vivo* assays. Our intention for this thesis was to set the groundwork and produce the material required to further explore the idea of gene specific epigenetic enzyme inhibition. When fully developed, the idea of gene specific targeting of epigenetic enzyme inhibitors using drug-LNA bioconjugates may also be used in the general scientific community as a tool to probe the epigenome in a gene specific manner, but more importantly, this method offers the first means to overcome the off-target effects of epigenetic enzyme inhibitors for epigenetic drugs intended to be used clinically.

Author Contributions

R.L. and T.M.L wrote and edited the manuscript and drew the original artwork that was adapted for Chapter 1. N.S., T.K.A., C.C. and M.P.N. edited the original manuscript that was adapted for Chapter 1 (*13*).

R.L. designed experiments, performed experiments, analyzed data, drew original artwork, wrote and edited Chapter 2. T.M.L. designed experiments, analyzed data, drew original artwork, wrote and edited the original manuscript adapted for Chapter 2. M.G.S. assisted in performing experiments described in section *2.2.1, 2.2.4, 2.2.5* and edited the original manuscript adapted for Chapter 2. N.S. edited the original manuscript adapted for Chapter 2 (*67*).

R.L. designed experiments, performed experiments, analyzed data, drew original artwork, wrote and edited the original manuscript adapted for Chapter 3. T.M.L. designed experiments, analyzed data, drew original artwork, wrote and edited the original manuscript adapted for Chapter 3. C.K.L. assisted in performing cell culture maintenance and experiments described in *3.2.1, 3.2.2* and edited the original manuscript adapted for Chapter 3 (*94*).

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Appendix A

Table A1 Genes and corresponding qPCR TaqMan primer and probe identification numbers for lysine demethylase gene expression array plate (ThermoFisher, Invitrogen)

Gene	ID
LSD1	Hs01002741_m1
KDM2A	Hs00957938_m1
KDM3A	Hs00218331_m1
KDM4A	Hs00206360_m1
KDM5A	Hs00231908_m1
KDM5B	Hs00981910_m1
KDM6A	Hs00958902_m1

Table A2 Genes and corresponding qPCR TaqMan primer and probe identification numbers for histone modifying enzyme gene expression array plate (ThermoFisher, Invitrogen)

Gene	ID	Gene	ID
HOXA9	Hs04931836_s1	SETD7	Hs00363902_m1
LSD1	Hs01002741_m1	SUV39H1	Hs00957892_m1
DOT1L	Hs01579928_m1	SMYD2	Hs00220210_m1
EP300	Hs00914223_m1	SMYD3	Hs01585866_m1
CREBBP	Hs00932878_m1	KDM2A	Hs00957941_m1
KAT2A	Hs00904943_gH	KDM3A	Hs00218331_m1
KAT5	Hs00197310_m1	KDM4A	Hs00206360_m1
KAT6A	Hs00198899_m1	KDM5A	Hs00231908_m1
KAT8	Hs01100237_m1	KDM6A	Hs00253500_m1
PRMT5	Hs01047345_g1	HDAC1	Hs02621185_s1
CARM1	Hs00406354_m1	HDAC3	Hs00187320_m1
EZH2	Hs00544830_m1	HDAC5	Hs00608351_m1
ASH1L	Hs00218516_m1	HDAC6	Hs00997427_m1
EHMT2	Hs00198710_m1	SIRT1	Hs01009006_m1
SETD1A	Hs00322315_m1	SIRT2	Hs01560289_m1
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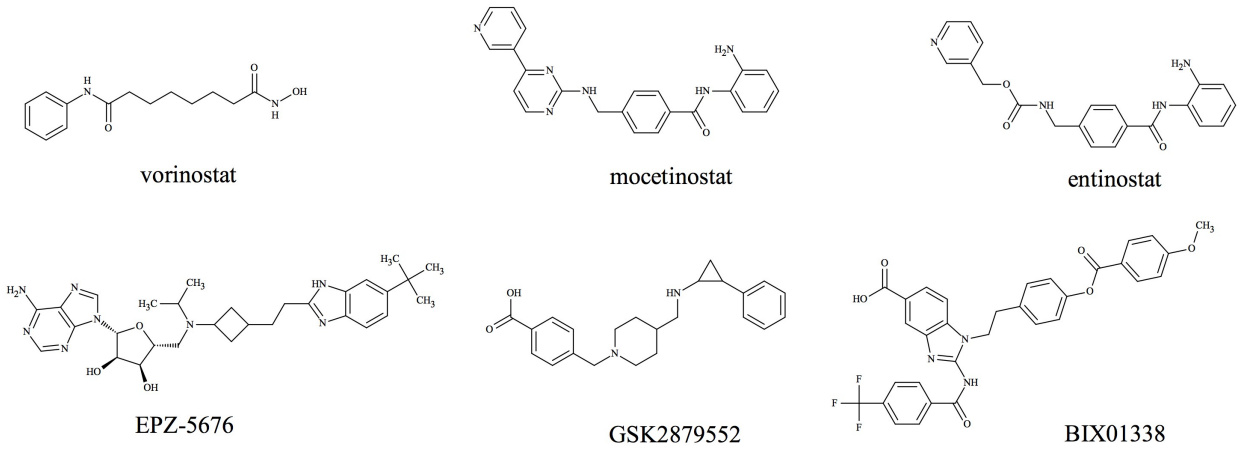

Figure A1. The chemical structures of HDAC inhibitors vorinostat, mocetinostat and entinostat; the DOT1L inhibitors EPZ-5676 and BIX01338; and the LSD1 inhibitor GSK2879552. Vorinostat, entinostat and BIX01338 were purchased from Sigma Aldrich, EPZ-5676 purchased from Biovision, GSK2879552 purchased from Cayman and mocetinostat was purchased from Santa Cruz. All inhibitors were delivered as pure dry powder and stocks were made in 100% sterile filtered DMSO.

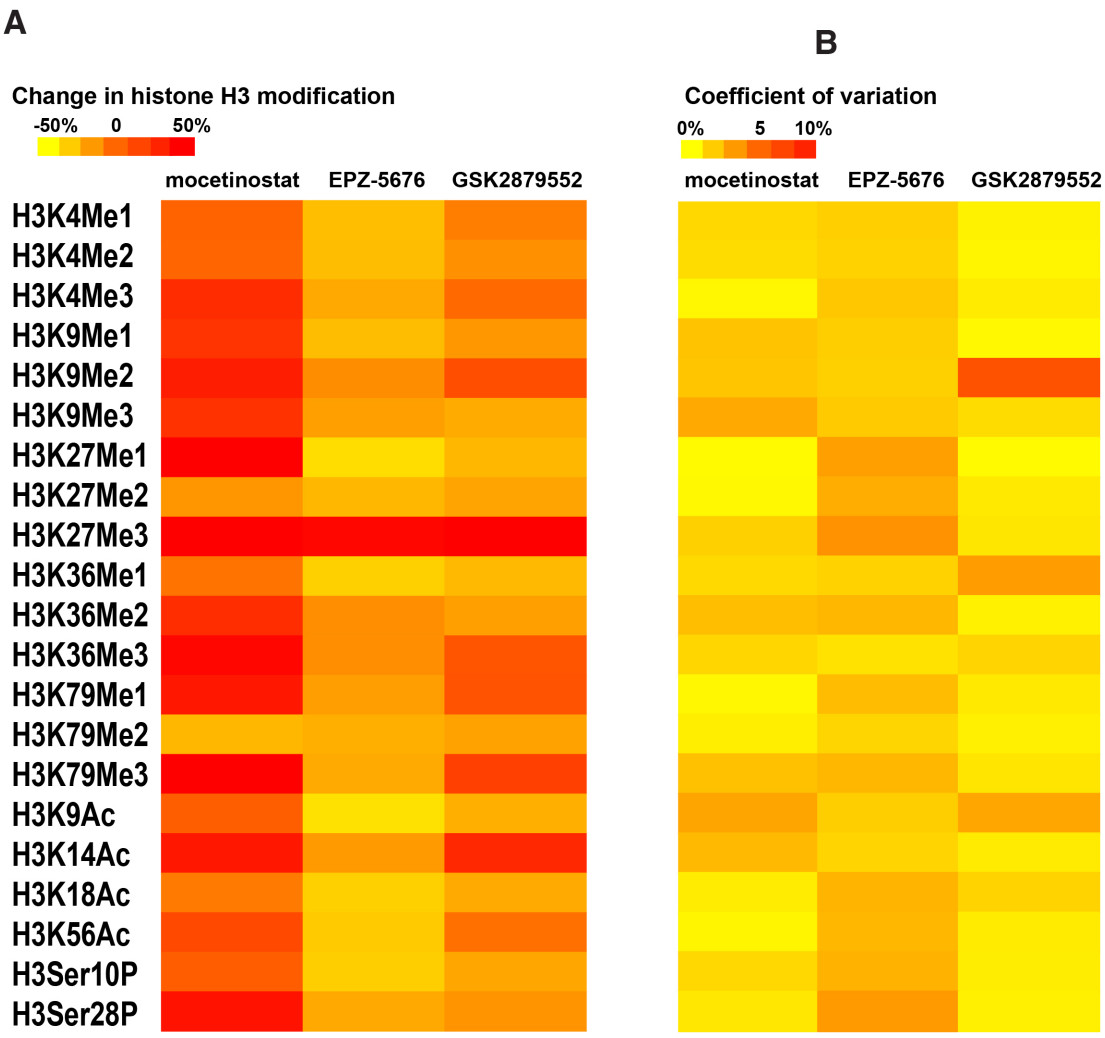

Figure A2. A heat-map of changes to specific histone H3 modifications measured using the Histone H3 Modifications Multiplex Assay Kit (abcam) in MOLM-13 cells using 5 µM EPZ-5676 for 144 h, 100 nM mocetinostat for 24 h and 1 µM GSK2879552 for 72 h (A). Each cell of the heat-map is the mean of 4 measurements. The coefficient of variation percentage (CV%) for the data in (A) is displayed in (B).

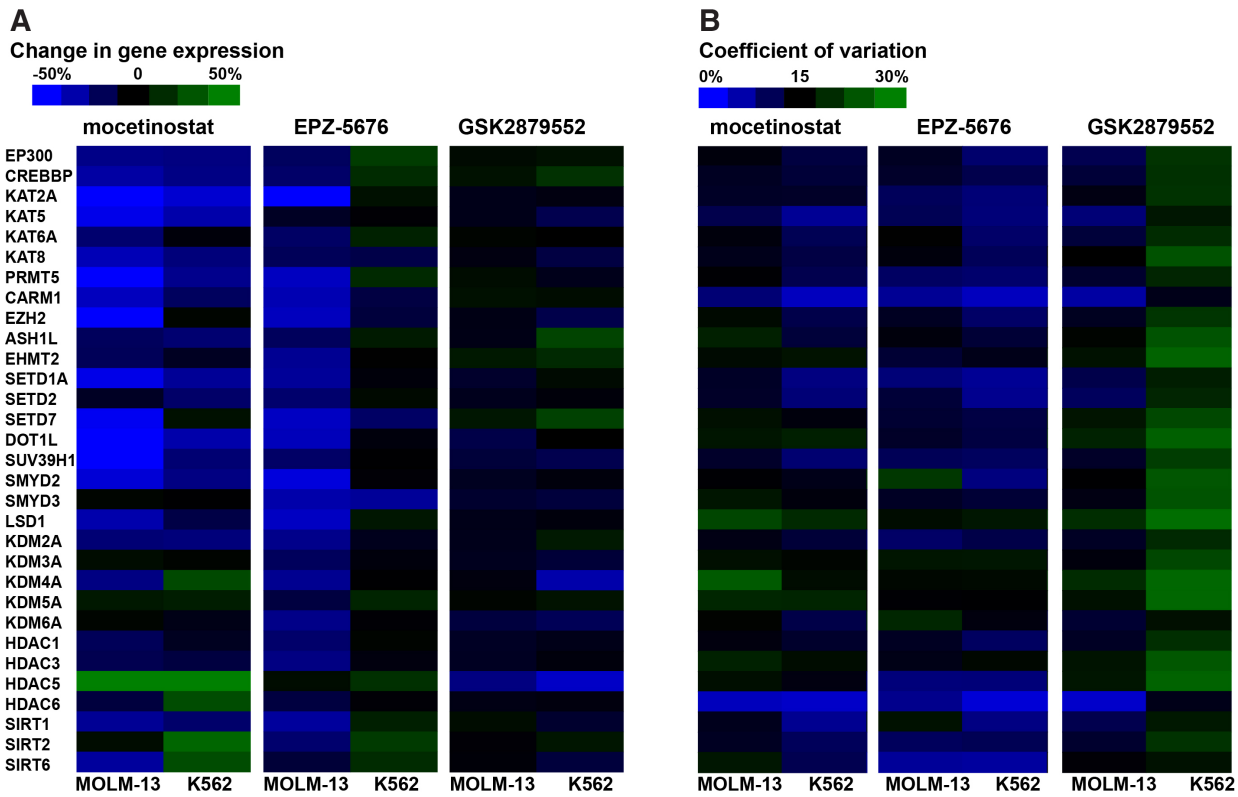

Figure A3. The gene expression heat map of selected histone modifying enzymes in K562 and MOLM-13 cells treated with mocetinostat, EPZ-5676, or GSK2879552. Treatments of 100 nM mocetinostat for 24h, 5 μM EPZ-5676 for 144h and 1 μM GSK2879552 for 72h were evaluated in MOLM-13 and the control K562 cells for changes in expression of epigenetic enzymes, measured by qPCR (A). Each cell of the heat-map is the mean of 3 measurements. The coefficient of variation percentage (CV%) for the data in (A) is displayed in (B).

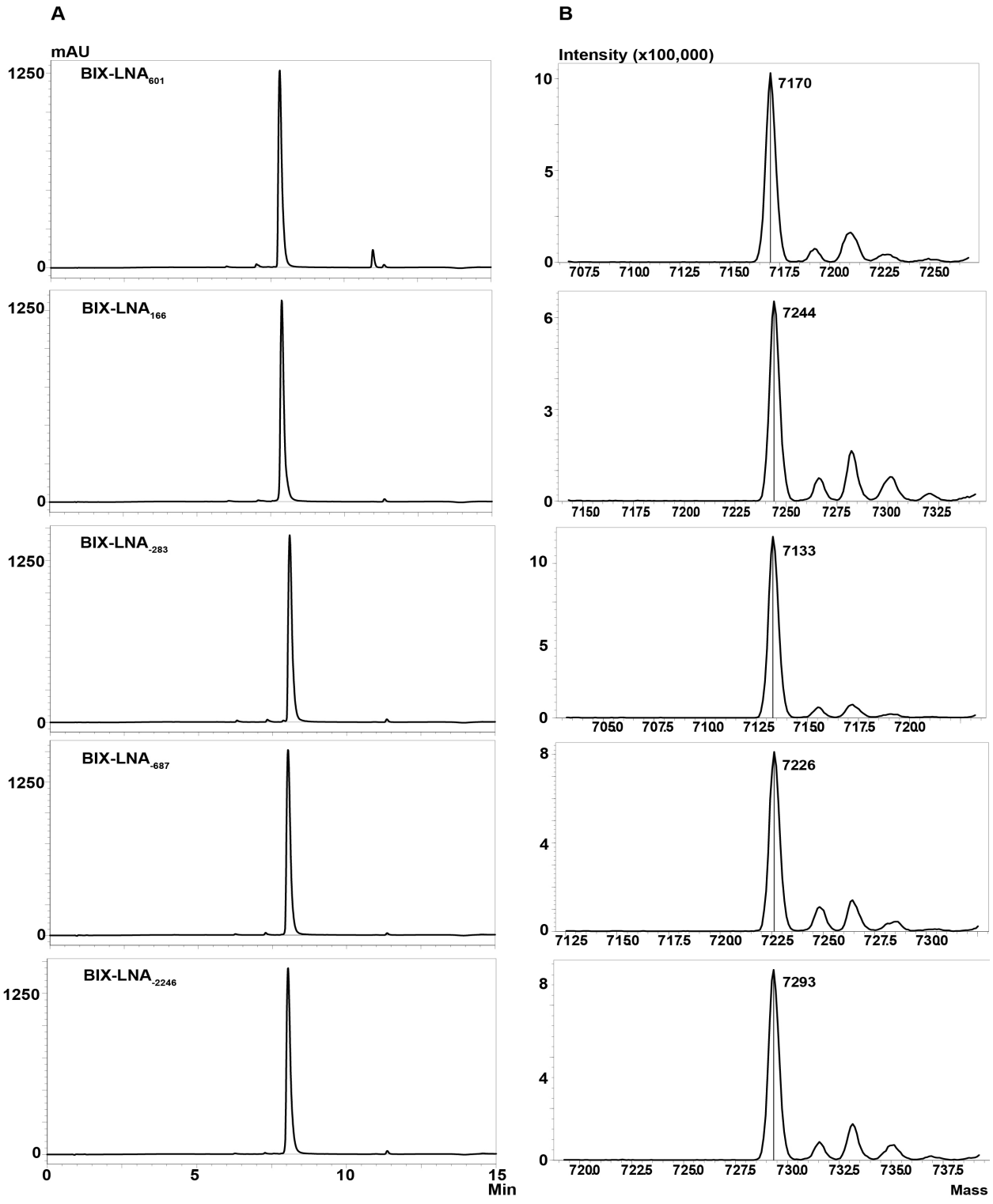

Figure A4. BIX-LNA purity and mass verification. The UHPLC-PDA chromatogram at 260 nm of each BIX-LNA construct is shown after purification on a Waters BEH C_{18} Acquity column. Each BIX-LNA is >95% pure (A). The deconvoluted mass chromatogram is also shown for each BIX-LNA construct (B) to verify correct mass.

Appendix B

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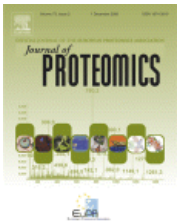

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