ELSEVIER

DRUG DISCOVERY TODAY TECHNOLOGIES

Drug Discovery Today: Technologies

Vol. 7, No. 1 2010

Mechanistic pharmacology, new developments

Epigenetics: tools and technologies

William P. Janzen*, Tim J. Wigle, Jian Jin, Stephen V. Frye

The Center for Integrative Chemical Biology and Drug Discovery, The University of North Carolina at Chapel Hill, Genetics Medicine Building, Campus Box 7363, Chapel Hill, NC 27599-7363, USA

Epigenetics refers to heritable changes that control how the genome is accessed in different cell-types and during development and differentiation. Even though each cell contains essentially the same genetic code, epigenetic mechanisms permit specialization of function between cells. The state of chromatin, the complex of histone proteins, RNA and DNA that efficiently package the genome, is largely regulated by specific modifications to histone proteins and DNA, and the recognition of these marks by other proteins and protein complexes. The enzymes that produce these modifications (the 'writers'), the proteins that recognize them (the 'readers'), and the enzymes that remove them (the 'erasers') are crucial targets for manipulation to further understand the histone code and its role in biology and human disease.

Introduction: epigenetics

Multicellular organisms have evolved elaborate mechanisms to enable cell-type specific expression of genes. Epigenetics refers to heritable changes that control how the genome is accessed in different cell-types and during development and differentiation [1]. Even though each cell contains essentially the same genetic code, epigenetic mechanisms permit specialization of function between cells. Over the last decade, the cellular machinery that creates these heritable changes has been the subject of intense scientific investigation as there is no area of biology or indeed, human health where epigenetics may not play a fundamental role [2].

The template upon which the epigenome is written is chromatin – the complex of histone proteins, RNA and DNA that efficiently package the genome within each cell. The basic building block of chromatin structure is the nucleosome – an octomer of histone proteins (associated dimers of H3 and H4 capped with dimers of H2A and H2B) around which 147 bp of DNA are wound. The amino-terminal tails of histone proteins project from the nucleosome structure and are subject to more than 100 post-translational modifications (PTM) [2]. The state of chromatin, and therefore access to the genetic code, is largely regulated by specific modifications to histone proteins and DNA, and the recognition of these marks by other proteins and protein complexes [3,4]. The enzymes that produce these modifications (the 'writers'), the proteins that recognize them (the 'readers'), and the enzymes that remove them (the 'erasers', Fig. 1) are crucial targets for manipulation to further understand the histone code and its role in biology and human disease [5,6]. Indeed, small molecule inhibitors of histone deacetylases have already proven useful in the treatment of cancer [7,8] and the role of lysine acetylation is rivaling that of phosphorylation in importance as a PTM that regulates protein function [9,10]. While histone phosphorylation plays a significant role in epigenetics, the technologies underlying kinase activity measurement are well understood and the impact of ubiquitination and sumoylation are as yet nascent, so this review will focus on tools and techniques associated with methylation and acetylation.

*Corresponding author.: W.P. Janzen (bjanzen@email.unc.edu)

1740-6749/$ DOI: 10.1016/j.ddtec.2010.07.004

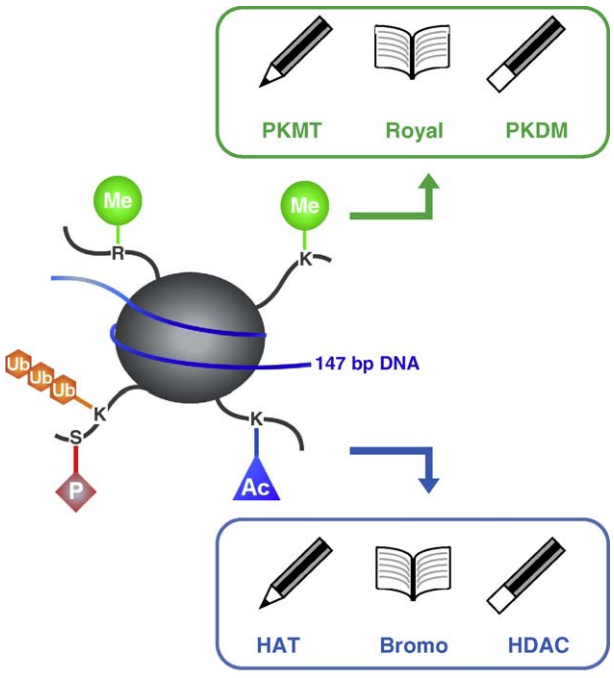


Figure 1. Nucleosomes are octomers of associated dimers of histone H3 and H4 proteins capped by dimers of H2A and H2B, and this protein core is surrounded by ~147 bp of double-stranded DNA. The physical spacing between repeating nucleosomal subunits controls the level of DNA condensation and the access of transcription factors and replication machinery to the genetic information. Post-translational modifications to the flexible N-terminal tails that protrude from the nucleosomal core controls the level of DNA packaging, and influences the temporal and spatial expression of genes. The most commonly studied modifications are the acteylation of lysine, which is 'written' and 'erased' by histone acetyltransferases and histone deacetylases, and lysine methylation which is 'written' and 'erased' by protein methyltransferases and protein demethylases. The marks are 'read' by two major families of proteins: Bromodomains bind to and recognize acetylated lysine, while the Royal family of proteins recognize and bind to methylated lysine. Other important histone post-translational modifications include the methylation of arginine, phosphorylation, and ubiquitination.

Overview of histone methylation – tools and technologies

Since the discovery of the first histone lysine methyltransferase in 2000 [11], the study of histone methylation in the context of drug discovery has experienced exponential growth because of its essential function in many biological processes [12]. Now, a decade later, there are >50 protein lysine methyltransferases (PKMTs) and >10 protein arginine methyltransferases (PRMTs) known [12–14] and, depending on the identity of the enzyme, varying degrees of methylation can be attained; lysine can be mono-, di- or tri-methylated, while arginine can be monomethylated, symmetrically dimethylated or asymmetrically dimethylated. Among the PKMTs, all but one enzyme, DOT1L, contain an evolutionarily conserved catalytic subunit of ~130 amino acids called a SET domain [15,16] and the PRMTs are divided into type I and type II families that respectively catalyze the formation of asymmetric or symmetric ω-N^G,N^G-dimethylarginine tails [17]. All PKMTs and PRMTs transfer a methyl group from the cofactor *S*-adenosylmethionine (SAM) to the target residue through a bimolecular S_N2-like mechanism and produce *S*-adenosylhomocysteine as a by-product [14]. Due to their functional similarity to protein kinases, protein methyltransferases (PMTs) may represent a novel and highly tractable target-family for drug discovery.

Recognition of methyl-lysine marks has been associated with the 'Royal Family' of proteins including those containing Tudor, Chromo, maligant brain tumor (MBT), PWWP, and plant Agenet domains, the plant homeodomain (PHD) family and the WD40 repeat protein WRD5. These motifs all have structurally related binding pockets defined by an aromatic electron-rich cage and H-bond donors that interact with the lysine cation [18]. Methyl-lysine binding proteins can directly influence the structural state of chromatin [19] or act as scaffolding for other proteins that are involved in chromatin remodeling [20]. In addition, many chromatin-acting enzymes, including a vast number that modify histones, contain methyl-lysine recognition domains or can often be found in complexes with proteins that do, recruiting the catalytic domains to the appropriate site of action [21].

Until recently, histone methylation was thought to be a stable and irreversible PTM, but the isolation of the first known histone demethylase in 2004 [22] and the subsequent identification of >30 demethylating enzymes since has suggested that histone methylation is a highly dynamic and complex process. All protein demethylases (PKDMs) oxidize the carbon of the targeted methyl group, which degrades to release formaldehyde. Among the demethylases, there are flavin-dependent monoamine oxidases like LSD1 that utilize an FAD^+ cofactor to catalyze oxidation of mono- and dimethyl-lysines, and the JmjC domain demethylases that utilize iron and α-ketoglutarate cofactors to hydroxylate mono-, di or trimethyl-lysines [23].

As epigenetic targets involved in writing, reading and erasing histone methylation continue to find a place in drug discovery pipelines, the assay technologies available to support high-throughput screening and compound profiling have become more advanced and sophisticated. For the enzymes that alter the methylation state of histone proteins, there are two major strategies for measuring activity: (1) detecting the formation or depletion of methylated substrate, and (2) monitoring the rate of cofactor usage by the enzymes.

While it is more practical to perform *in vitro* activity assays on peptide substrates, it can be advantageous to consider the use of whole nucleosomes as substrates in enzymatic reactions. For example, the enzyme DOT1L only shows catalytic activity in the context of whole nucleosomes and requires

contact with ubiquitinated histone H2B to stimulate its catalytic activity toward the H3K79 residue, which is part of the core nucleosome rather than the amino-terminal tail [24]. In addition, allosteric regulators of PMTs and PDMs that do not bind near the lysine binding channel or the SAM-binding pocket may be overlooked when using peptide substrates. However, most histone-modifying enzymes, particularly those that act on the flexible amino tails, are often amenable to the use of peptide substrates.

In the substrate-based assay strategy, synergy can be obtained between the methyltransferases and demethylases, as assays configured to monitoring the methylation status of the substrate are applicable to both classes of enzymes. The use of antibodies against specific methyl-lysine histone marks and a secondary anti-IgG antibody with a reporter molecule are frequently employed in small molecule screening efforts. The secondary antibody can be conjugated to an enzyme such as horseradish peroxidase (ELISA) [25], to lanthanide metals such as Europium (DELFIA) [26] for a time resolved fluorescence (TRF) signal or to an AlphaScreen acceptor bead. In the latter, a second AlphaScreen donor bead is coupled to the peptide substrate and a binding event that brings the beads into close proximity (within 200 nm) will allow singlet oxygen molecules to be transferred from the donor to the acceptor bead, generating a chemiluminescent signal [27]. Success using the antibody-based detection method is heavily reliant on the use of high quality antibodies, and selecting an antibody for the proper mark. For example, G9a activity can produce both mono- and dimethyl-lysine, but functional assays have only been performed with an antibody against dimethyl-lysine [26]. Another technique takes advantage of the fact that endoproteinase-LysC, which cleaves peptide bonds C-terminal to lysine, is unable to do so if the lysine is methylated. When coupled to microfluidic capillary electrophoresis using the Caliper Life Sciences Labchip™ Technology, this methylation-sensitive proteolysis permits the detection of the ratio of methylated to unmethylated peptides from a 384-well plate, and enzymatic activity can be quantitated accurately and precisely [28], allowing it to be used for both HTS and quantitative enzymology in the generation of K_i's. Alternatively, measuring the incorporation of a radioactive methyl group from ^{3}H-SAM to substrates anchored to microplates is a proven, inexpensive and sensitive method that is compatible with both synthetic peptides and whole nucleosomes [29,30]. However, radioactivity is inherently hazardous to the assay operator and the necessity for disposal of bulk reagents and decontamination of liquid handling equipment usually make it an assay of last resort.

The second strategy, measuring cofactor usage, is PKMT- or PKDM-specific. In the case of PKMTs, the conversion of SAM to SAH has been measured using an enzyme-coupled assay that uses SAH hydrolase (and adenosine deaminase) to produce inosine and homocysteine, the latter of which can be detected using the Thioglo reagent, which fluoresces strongly when its maleimide moiety reacts with a thiol [31]. Caution must be exercised when using the assay to avoid reducing agents such as DTT in the assay buffer, and to keep the PKMT and any thiol-containing substrates at concentrations that do not saturate the Thioglo emission. In addition, several other enzyme coupled assays for PKMTs have been reported [32,33]. PKDMs produce formaldehyde and peroxide as by-products of catalysis, both of which can be detected using enzyme-coupling systems. Formaldehyde dehydrogenase reduces formaldehyde to formic acid, and this can be coupled stoichiometrically to the reduction of NAD^+ to NADH, which has an absorbance maximum of 340 nm [34]. The formaldehyde dehydrogenase coupling assay is quite robust, and has recently been miniaturized to a 1536-well format to enable μHTS [35]. Another method compatible for screening PKDMs is the detection of the peroxide formed using one of several known peroxidase coupled assays [36,37].

Isolated methyl-lysine binding proteins (KMe readers) bind their cognate histone peptides with low affinity; however, the combinatorial effect of multiple interactions on various lysine marks leads to high binding affinity and specificity *in vivo* [38]. Biochemical techniques such as fluorescence polarization [39], isothermal titration calorimetry [39,40], surface plasmon resonance [41] and nuclear magnetic resonance [42] have indicated that, *in vitro*, the K_d of KMe readers for a single methyl-lysine histone mark on a synthetic peptide is in the 25–200 μM range. As a result, it is challenging to subject individual reader proteins to biochemical screening assays amenable to HTS because the requirement for protein to run assays would be too great (*i.e.*, >50 μM protein per well). To circumvent this problem, AlphaScreen has been employed in screening for inhibitors of methyl-lysine recognition [43,44]. In these assays a biotinylated peptide containing the desired methyl-lysine modification is bound by the KMe reader containing a hexahistidine or glutathione S-transferase purification tag, and streptavidin-coated donor beads and nickel- or glutathione-coated acceptor beads are added. As described above, a chemiluminescent signal can be generated when the beads are brought into proximity due to a binding interaction between the peptide and protein. The requirement for protein in the AlphaScreen assay is in the low nanomolar range, as opposed to the micromolar range for other techniques. This can be attributed to the phenomenon of bead avidity, where each bead has multiple sites for the capture of ligands, and binding affinities are the sum of multiple interactions. The transduction of the AlphaScreen signal is sensitive to singlet oxygen quenchers, organometallic compounds and metal-chelating agents, and a counter-screen should be performed to purge these compounds from subsequent follow-up studies. AlphaScreen is a very promising tool for hit discovery but the caveat should be noted that these same advantages for use in primary screening

complicate compound profiling and rank-order potency determination, and alternate biophysical methods may be more appropriate for lead optimization once the hits have been identified with the primary AlphaScreen assay.

Chemical tools for protein lysine methyltransferases

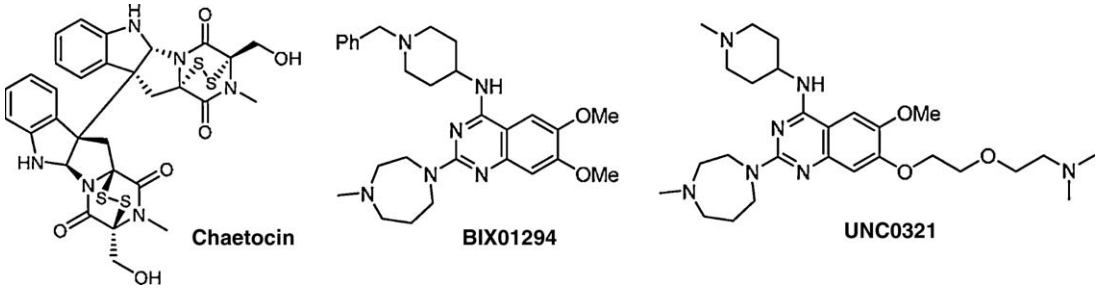
Growing evidence suggests that PKMTs play crucial roles in the development of various human diseases including cancer [15,45,14,46], inflammation [47], drug addiction [48], and mental retardation [49]. For example, G9a, also known as EHMT2, is over expressed in human cancers and knockdown of G9a inhibits cancer cell growth [50,51]. In addition, G9a catalyzes dimethylation of lysine 373 (K373) of p53, a tumor suppressor [52]. The dimethylation of p53 K373 results in the inactivation of p53 [52].

To date, 3 selective small molecule PKMT inhibitors have been reported [26,53–55]. Chaetocin, a fungal mycotoxin, was identified as the first selective small molecule inhibitor of H3K9 PKMT SU(VAR)3-9 (IC_{50} = 0.6 μM) via screening of 2967 compounds [54]. Chaetocin also inhibited SUV39H1 (IC_{50} = 0.8 μM), the human ortholog of SU(VAR)3-9, and was selective for H3K9 PKMTs over other PKMTs that do not target H3K9, for example, EZH2, SET7/9, and SET8/PR-SET7 [54]. Mechanistically, chaetocin is a SAM competitive inhibitor that was reported to be cellularly active and not toxic to cells at up to 0.5 μM [54]. Cells treated with 0.5 μM chaetocin show a marked reduction of dimethylation and trimethylation of H3K9 without affecting the methylation state of H3K27, H3K36, and H3K79 [54].

BIX-01294 is a small molecule inhibitor of G9a and GLP (a H3K9 PKMT that shares 80% sequence identity with G9a in their respective SET domains) that was discovered via screening of a library of 125,000 synthetic compounds [26]. BIX-01294 is selective for G9a and GLP over several H3K9 PKMTs including SUV39H1 and ESET, other KMTs such as SET7/9, and the arginine methyltransferase PRTM1 [26]. The X-ray crystal structure of GLP and BIX-01294 confirmed that BIX-01294 bound to the histone peptide binding pocket but failed to interact with the lysine binding channel [59]. Cells dosed with BIX-01294 at 4.1 μM were characterized by reduced H3K9me2 levels in several cell lines but toxicity to cells at >4.1 μM was observed [26]. More importantly, BIX-01294 at 4.1 μM reduced the H3K9me2 levels at several G9a target genes including *mage-a2*, *Bmi1*, and *Serac1* and the inhibitor effects were reversible and restored upon removal of the inhibitor [26].

Design and synthesis based on the GLP and BIX-01294 X-ray co-crystal structure in combination with structure–activity relationship (SAR) exploration led to the discovery of UNC0321 and closely related analogs as potent and selective G9a inhibitors [55–57]. UNC0321 (Morrison K_i = 63 pM) possessing a 7-dimethylaminoethoxyethoxy chain was >250-fold more potent compared to BIX-01294 (Morrison K_i = 16 nM) in the G9a microfluidic capillary electrophoresis assay [56]. UNC0321 showed similar potency versus GLP, but was more than 1,000-fold selective for G9a over SET7/9,SET8/PRSET7, PRMT3, and JMJD2E [56]. A high resolution X-ray co-crystal structure of G9a and UNC0224, which is a closely related analog of UNC0321 [56], confirmed that the 7-amino alkoxy side chain of UNC0224 indeed occupied the lysine binding channel of G9a thus explaining the higher potency of UNC0224 and UNC0321 compared to BIX-01294 [57]. The combination of high potency and good selectivity makes UNC0321 a potentially useful tool compound for the biomedical research community to further investigate the biology of G9a and its role in chromatin remodeling and other post-translational modifications [58].

Discovering and developing high quality chemical probes [60] of PKMTs is gaining momentum in both the academic research community and the pharmaceutical industry. Although PKMTs inhibitors are clinically unprecedented, they hold great promise as effective mono-therapies or synergizing agents in combination with existing therapeutics [61].



Overview of histone acetylation – tools and technologies

The acetylation state of histones is primarily controlled by 2 families of enzymes; histone acetyl transferases (HAT) and histone deacetylases (HDAC) [62–64]. As their names imply, the former add a terminal ε-acetyl function to lysines and the latter remove this modification. Both families have been

extensively studied [9,10], and in particular, there have been multiple successful drug discovery campaigns for inhibitors of HDACs [7,65,66] that eventually yielded several marketed drugs.

There are currently 18 known human HDAC isoforms that are commonly grouped into 4 families based on their homology to yeast HDACs, subcellular localization and enzymatic mechanism [67,68]. Class I HDACs (HDAC 1, 2, 3, and 8) are homologous to the yeast RPD3 protein, act as zinc-dependent enzymes and are predominantly localized in the nucleus. These enzymes are ubiquitously expressed in human tissue. Class II HDACs (HDAC 4, 5, 6, 7, 9, and 10) share homology with the yeast Hda I protein, are also zinc dependent and in comparison to class II HDACs, are known to translocate between the nucleus and the cytoplasm. Two HDACs (6 and 10) are unique within this class because they have two deacetylase domains [69,70]. HDAC6 is also distinctive in that it targets non-histone substrates [71,72]. The class III HDACs are also known as sirtuins (SIRT 1–7) for their homology with the yeast SIR2 protein. These enzymes also have a unique enzymatic mechanism and require NAD^+ for their activity. HDAC11 is the lone class IV HDAC. While it shares some sequence homology with both classes I and II, HDAC 11 is not zincdependent [62].

Similar to the familiar enzymatic classes of kinases and phosphatases that add or remove phosphate groups from amino acids, the opposing nature of HDACs and HATs means that these two enzyme families can largely be monitored by the same technologies. Any technology that measures the relative presence of an acetyl group will be able to monitor either the addition or loss of acetyl moieties. While there are, of course, many radioactive methods for monitoring this activity, they are complicated by the need for pre-acetylated substrates for HDAC enzymes and the obvious drawbacks of waste disposal and safety [73,74].

The first technique developed for the non-radioactive measurement of HDAC activity was the Fluor-de-Lys® assay developed by Biomol (now Enzo Life Sciences). This assay is based on the deacetylation of a short lysine-containing peptide substrate that is developed by trypsin digestion, which releases a proprietary fluorescent dye that fluoresces upon cleavage. Later versions of this assay are sold as a kit and use a green shifted (485ex/530em) dye which avoids some of the interference problems associated with small molecule compounds. The Fluor-de-Lys® assay came to prominence when it was reported that the compound resveratrol was a potent activator of the HDAC SIRT1 [75]. However, this finding has been disputed by later research that demonstrated that the purported activation was solely due to substrate specific interactions with the bulky dye group and the SIRT1 enzyme [76–78].

Other homogeneous assay technologies have been developed, for example a linked luminescent assay from Promega wherein deacetylation of a prolumigenic peptide substrate allows proteolytic cleavage and a subsequent luminescent readout. This offers a lower level of interference from compound libraries and increased sensitivity. Linked assays using luciferase to monitor NAD^+ production in class III enzymes have also been reported [73].

Caliper Life Sciences Labchip™ technology can also be used to quantitatively measure the acetylation or deacetylation of a fluorescently labeled peptide substrate. In this system, the charge to mass ratio change associated with acetylation allows microfluidic, capillary electrophoresis-based separation and quantification of product and substrate from an HDAC or HAT enzymatic reaction [73,79]. The ability to monitor both product and substrate makes this technique extremely precise and less prone to compound interference.

Table 1. Target classes and technologies involved in methylation and acetylation PTM of histones

Target class	Detection method	Assay
Protein methyltransferases	Colorimetric	ELISA [25]
	TRF	DELFIA [26]
	Chemiluminescence	AlphaScreen [27]
	Fluorescence	Microfluidic capillary electrophoresis [28]
	Radioactive	Incorporation of radioactive methyl groups [29,30]
	Enzyme-coupled fluorescence	Thioglo chromophore [31] or Ellman's reagent [32,33]
Protein demethylases	Enzyme-linked colorimetric	Formaldehyde dehydrogenase coupled reaction [35]
	Colorimetric	Peroxide production [36,37]
Histone-binding proteins	Chemiluminescence	AlphaScreen [43,44].
Histone acetyl transferases and histone deacetylases	Fluorescence	Fluor-de-Lys® assay [75]
	Luminescence	Prolumigenic peptide
	Fluorescence	Microfluidic capillary electrophoresis [73,79]

Conclusions

While the field is relatively new, there have been great strides in developing assays to monitor the PTM of histones through methylation and acetylation (Table 1). Given the growing importance of epigenetics in our understanding of human biology and the tractability of enzymes that target PTM of proteins for drug discovery, we can anticipate rapid development of new technologies to monitor the writers and erasers of the histone code. While the readers of the code are also of interest, their tractability for discovery of potent and selective small molecules is unproven. We can however be confident that new understanding of biology and new therapeutic agents will emerge as technology drives scientific understanding in epigenetics.

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