Epigenetic Chemical Probes

Susanne Müller[1] and Peter J. Brown[2]*

[1]Structural Genomics Consortium, University of Oxford, Old Road Campus, Roosevelt Drive, Oxford, OX3 7DQ, UK.
[2]Structural Genomics Consortium, University of Toronto, 101 College Street, Toronto, ON M5G 1L7, Canada,

Epigenetic control of gene expression occurs at two distinct levels; DNA methylation and histone modification. Over the last ten years the discovery of epigenetic targets has accelerated to the point where over 400 domains have been identified which are either involved in DNA methylation, the modification of histones (and some non-histones) or translation of these modifications into changes in gene expression.

One mechanism of epigenetic signaling occurs through the covalent modification of chromatin which is comprised of DNA wrapped around a histone core (nucleosomes). Fig. 1 indicates the different classes of epigenetic proteins which are referred to as the writers (adding a covalent modification), erasers (deleting a covalent modification), and readers (binding to a covalently modified histone). The field of epigenetics offers a plethora of orphan proteins with over 400 domains available for study.[1] The effects of epigenetic enzyme inhibition or chromatin binding antagonism in cells can be delineated using chemical probes which are potent, selective, and cell-active ligands (inhibitors or antagonists).[2] Protein knockdown or knockout experiments can offer hints of consequences of inhibiting/antagonizing various proteins, however, many epigenetic targets contain multiple domains of different character and thus small molecule inhibition/antagonism may show different effects compared to protein knockdown/out experiments. While there are many well-documented covalent modifications of histones, this review will focus on methyl and acetyl modifications as space restricts a meaningful discussion of citrullination, ubiquitinylation, phosphorylation, and other modifications recently reviewed.[3]

Histone methyltransferases (HMTs)

The HMTs have garnered much attention as oncology targets and are responsible for methylating histone lysines or histone arginines in addition to a growing list of non-histone proteins. Methylation occurs by the transfer of a methyl group from S-adenosylmethionine (SAM) to the substrate peptide, so structurally the SAM-binding pockets and peptide-binding pockets are in close proximity. HMTs can add up to three methyl groups to a lysine residue and up to two methyl groups to an arginine

residue, however, some HMTs are only capable of partially methylating a histone residue indicating that full methylation is only possible by the action of multiple HMTs. EHMT1 and its close homolog, EHMT2, (otherwise known as GLP and G9a) act as a heterodimer to mono and dimethylate histone 3 lysine 9 (H3K9). BIX-01294 was the first published inhibitor of G9a/GLP (IC_{50} 700 nM) and was shown to decrease H3K9me2 levels in mouse ES cells by mass spectral analysis of extracted histones.[4] A related analog, UNC0638, was developed which was more potent (IC_{50} 19 nM) and less toxic, resulting in a better profile for use in experiments where maximum inhibition of methylation is required.[5] Further optimization of this chemical template is underway to find analogs which have better pharmacokinetic properties, suitable for whole animal studies. Disruptor of Telomeric Silencing 1-like (DOT1L) is the only methyltransferase which does not contain a SET domain common to all the other lysine methyltransferases, and resides in the arginine methyltransferase branch of the HMT phylogenetic tree. This protein catalyses the dimethylation of H3K79 and is implicated in leukemogenesis. EPZ004777 was the first potent and selective inhibitor of DOT1L (IC_{50} 0.6 nM) published in an elegant manuscript which shows that EPZ004777 selectively inhibits proliferation of MLL-rearranged cell lines and also extends the survival of NSG mice injected with MV4-11 transformed cells.[6] SMYD2 is an H3K36 HMT which also methylates p53 and Rb resulting in repression of the tumor suppressing activity of these proteins making the SMYDs intriguing oncology targets. AZ505 is the only potent (IC_{50} 120 nM) and selective inhibitor of SMYD2 to date whose structure and kinetics indicate that AZ505 is a competitive inhibitor of the peptide substrate, however, cellular activity has not been demonstrated.[7] GSK has discovered a class of inhibitors of EZH2 (IC_{50} 4 nM) which catalyses the methylation of H3K27, and have been shown to be active in cells.[8] An intriguing aspect of this target is the identification of EZH2 mutants in tumors which have a different substrate profile than the wild-type protein.[9] BMS[10] and Methylgene[11] have published potent, selective inhibitors of CARM1 (PRMT4), a potential oncology target due to its role in transcriptional activation.

Histone Lysine Demethylases (KDMs)

Methylation of histone lysines has long been believed to be permanent.. However, in 2004 the first histone lysine demethylase was discovered and later work showed that KDMs are grouped into two classes by their enzymatic mechanisms. The first identified KDM was the flavin-containing oxidase LSD1 that uses FAD as a co-factor. LSD1 is over-expressed in a variety of cancers regulating proliferation and expression of pro-survival genes. In addition to demethylation of histone H3 marks H3K4me2 and H3K4me1, LSD1 has also non-histone substrates such as DNA methyltransferase 1,

p53, STAT3, E2F1, and MYPT1. Several inhibitors of LSD1 have been identified, which were developed based on inhibitors against the related monoamine oxidase (MAO) A and B.[12] An example of MAO inspired inhibitors is the recently identified gamma-pyrone Namoline, a selective reversible cell-active LSD1 inhibitor. Specificity of Namoline has been characterized by screening against the closely related MAO A or B, spermine oxidase and polyamine oxidase against which the compound is inactive at the IC_{50} concentration determined for LSD1 (50 μM).[13] Another series of cell-active inhibitors against LSD1 has been developed by structure-based design. These peptide-inspired inhibitors (eg. CBB1007) have low μM cellular and *in vitro* activity and do not inhibit LSD2 or JARID1A.[14] The second family of histone lysine demethylases, the Jumonji (JmjC) family of demethylases, belongs to the superfamily of 2-oxoglutarate (2-OG) dependent oxygenases which utilize Fe(II) ions and α-ketoglutarate as co-factors. Members of the JmjC family have been suggested as therapeutic targets for cancer and for a variety of other diseases. JARID1B is upregulated in prostate cancer and the related JARID1A regulates cell proliferation and tumorigenesis through its interaction with the tumor-suppressor retinoblastoma-associated protein 1 (RB1). Also, members of the JMJD2 family are over-expressed in a variety of human tumors. In some cases their role in oncogenesis is due to regulation of tumor suppressors such as p53 (JMJD2A and JMJD2D) and pRB (JMJD2C). In other cases tumorigenesis is regulated at the transcriptional level e.g. JMJD2B positively regulates expression of cyclin-dependent kinase 6, CDK6. The H3K27 demethylase JMJD3 also regulates tumor suppressor genes at the transcriptional level but has mostly been characterized for its role in inflammation. JMJD3 is inducibly expressed in macrophages in response to inflammatory signals and regulates expression of inflammatory genes. Also FBXL11 influences the inflammatory response by negatively regulating the pro-inflammatory transcription factor, NFkB. Most inhibitors of the 2-OG family are 2-OG-competitive mimicking the Fe(II)-ion chelating binding mode of alpha keto-glutarate. These compounds include widely used 'pan inhibitors' such as N-oxalyl amino acids, 2,X-pyridinedicarboxylates and more specific substituted hydroxamic acids. Sub-family specificity has been obtained by functionalization of these scaffolds but a wider assessment of inhibitor selectivity is often missing.[15] The recently reported Methylstat is a bivalent inhibitor combining a methyl-lysine mimic and cofactor mimic of alpha ketoglutarate. Methylstat is the cell-active ester of the *in vitro* characterized compound and has been tested in a variety of cell assays. The compound has also been profiled against the wider 2-OG panel *in vitro* including JMJD2A, E, and C, the H3K27me3 demethylase JMJD3, PHF8, the HIF hydroxylases PHD1, 2 and 3, FIH and inhibits the various members with potencies between 4.3 μM and 83 μM. No or low inhibition was observed for LSD1 and HDACs.[16] The first specific inhibitor of the 2-OG dependent demethylases is a

bipyridine which selectively inhibits JMJD3, and to a lesser extent UTX. The compound inhibits JMJD3 with an IC_{50} of 60 nM and is highly selective with only weak cross-reactivity against JMJD1 (41 μM). The compound has been extensively characterized against the wider family of 2-OG dependent demethylases and related enzymes. Cellular activity of a pro-drug reduced TNF-alpha production in macrophages derived from patients suffering from rheumatoid arthritis, making a compelling case for targeting JMJD3 for the treatment of this disease.[17]

Histone acetyltransferases (HATs)

While the HMTs generally show significant substrate selectivity, the HATs show little substrate specificity and acetylate a broad spectrum of histone lysines. HATs catalyze the transfer of an acetyl group from acetyl CoA (AcCoA) to the substrate histone lysine (arginines are not acetylated). In contrast to the HMTs, few chemical probes for HATs have been discovered to date and P300/CBP is the only HAT for which potent and selective inhibitors have been reported. Bisubstrate inhibitors contain structural elements of both the peptide and AcCoA substrates and span both binding sites. Lys-CoA was the first P300/CBP bisubstrate inhibitor reported with K_i=20 nM[18] however, this compound was not cell permeable unless conjugated with a TAT peptide.[19] Cole also described C646 as a potent inhibitor of P300/CBP (IC_{50} 400 nM) which is competitive with AcCoA.[20] C646 was also shown to significantly reduce global levels of histone acetylation in WM983A cells.

Methyllysine Binders

Despite the numerous methyllysine binding domains which have been purified and crystallized to date (126 in PDB) only a single small molecule ligand has been reported for this family. The MBT family of proteins binds peptides containing mono-and di-methylated lysines, and UNC0669 is the first L3MBTL1 antagonist with Kd of 5 μM and shows a minimum selectivity of 6 over other MBT members.[21] Efforts are underway to optimize this template for L3MBTL1 and cross-screen analogs on the whole family.

Bromodomains (Acetyllysine Binders)

Selective targeting of bromodomains has been thought to provide a challenging task. Early antagonists of bromodomains are acetyl-lysine mimic fragments with modest activity such as ischemin. Ischemin, an inhibitor of CBP, has an *in vitro* potency of 19 μM and shows some degree of selectivity against the four bromodomains tested (BRD4-1, BAZ1B, PCAF and BAZ2B).[22,23] The first potent and selective bromodomain antagonists are triazolo-diazepine compounds such as the pan-

BET family inhibitor JQ1[24] and the related compound I-BET.[25] Both BET antagonists show low nM affinity and are highly selective for the BET-subfamily. JQ1 is >300-fold selective over 40 other bromodomains tested. JQ1 has been studied in midline carcinoma, an aggressive subtype of squamous carcinoma where exposure of established cell lines led to cell cycle arrest, terminal differentiation and apoptosis. Moreover, in a patient-derived xenograft model, significant reduction of tumor growth was achieved. JQ1 has been widely distributed in the scientific community and has had further major impact by elucidating the role of BRD4 in regulating c-myc transcription providing a therapeutic strategy targeting the c-Myc oncogene for treatment of cancer.[26] The related compound, I-BET, has been shown to confer protection against lipopolysaccharide-induced endotoxic shock and bacteria-induced sepsis by down-regulating pro-inflammatory genes.[25] In addition, I-BET151, a BET antagonist containing a different chemical template, has shown efficacy against human leukemia cell lines.[27]

While the number of epigenetic chemical probes is small relative to the number of unliganded (orphan) epigenetic targets, the scientific community has started to use these probes to unravel the complex biology of epigenetic signaling. With every new chemical probe developed, an opportunity arises to use a new combination of ligands to help understand the combinatorial nature of epigenetic signaling. Future efforts will target proteins containing multiple domains in an effort to design ligands which interact with multiple domains.

Figure 1

Readers, writers and erasers of histone modifications. Proteins modifying lysine or arginine residues in the histone tail by acetylation or methylation are referred to as writers (adding a modification) or erasers (removing a modification). Specific domains binding to these modifications and interpreting them are called readers. Examples of each class of proteins are shown.

Figure 2

Chemical structures of inhibitors and antagonists of epigenetic targets.

Table 1

Summary of epigenetic chemical probe properties.

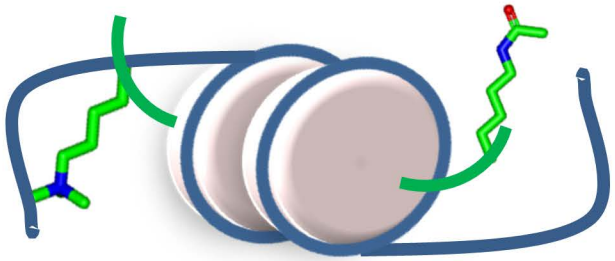
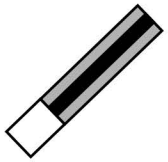
Acknowledgements

The Structural Genomics Consortium is a registered charity (number 1097737) that receives funds from Canadian Institutes of Health Research, Eli Lilly Canada, Genome Canada, GlaxoSmithKline, the Ontario Ministry of Economic Development and Innovation, the Novartis Research Foundation, Pfizer, Abbott, Takeda and the Wellcome Trust.

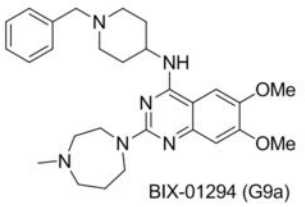
References

1. http://apps.thesgc.org/resources/phylogenetic_trees/.
2. Frye, S.V. The art of the chemical probe. *Nature Chemical Biology* **6**, 159-161 (2010).
3. Dawson, M.A. & Kouzarides, T. Cancer Epigenetics: From Mechanism to Therapy. *Cell* **150**, 12-27 (2012).
4. Kubicek, S. *et al.* Reversal of H3K9me2 by a small-molecule inhibitor for the G9a histone methyltransferase. *Mol. Cell* **25**, 473-481 (2007).
5. Vedadi, M. *et al.* A chemical probe selectively inhibits G9a and GLP methyltransferase activity in cells. *Nature Chemical Biology* **7**, 566-574 (2011).
6. Daigle, S.R. *et al.* Selective killing of mixed lineage leukemia cells by a potent small-molecule DOT1L inhibitor. *Cancer Cell* **20**, 53-65 (2011).
7. Ferguson, A.D. *et al.* Structural basis of substrate methylation and inhibition of SMYD2. *Structure* **19**, 1263-1273 (2011).
8. Duquenne, C. *et al.* Indazoles. *WO Patent* 2011/140325 Nov. 10, 2011.
9. Sneeringer, C.J. *et al.* Coordinated activities of wild-type plus mutant EZH2 drive tumor-associated hypertrimethylation of lysine 27 on histone H3 (H3K27) in human B-cell lymphomas. *Proc. Natl. Acad. Sci.* **107**, 20980-20985 (2010).
10. Huynh, T. *et al.* Optimization of pyrazole inhibitors of Coactivator Associated Arginine Methyltransferase 1 (CARM1). *Bioorg. Med. Chem. Lett.* **19**, 2924-2927 (2009).
11. Allan, M. *et al.* N-Benzyl-1-heteroaryl-3-(trifluoromethyl)-1H-pyrazole-5-carboxamides as inhibitors of co-activator associated arginine methyltransferase (CARM1). *Bioorg. Med. Chem. Lett.* **19**, 1218-1223 (2009).
12. Lohse, B. *et al*. Inhibitors of histone demethylases. *Bioorg. Med. Chem*. **19**, 3625-3636, (2011).
13. Willmann, D. *et al*. Impairment of prostate cancer cell growth by a selective and reversible LSD1 inhibitor. *Internat. J. Cancer*., doi:10.1002/ijc.27555 (2012).
14. Wang, J. *et al.* Novel histone demethylase LSD1 inhibitors selectively target cancer cells with pluripotent stem cell properties. *Cancer Res.* **71**, 7238-7249, (2011).

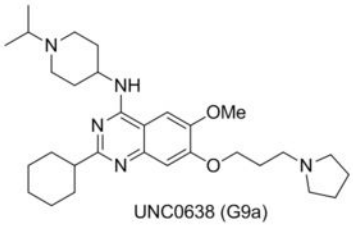
15. Rose, N.R., McDonough, M.A., King, O.N., Kawamura, A. & Schofield, C.J. Inhibition of 2-oxoglutarate dependent oxygenases. *Chem. Soc. Rev*. **40**, 4364-4397 (2011).
16. Luo, X. *et al*. A selective inhibitor and probe of the cellular functions of Jumonji C domain-containing histone demethylases. *J. Amer. Chem. Soc*. **133**, 9451-9456 (2011).
17. Kruidenier, L. *et al.* Selective H3K27 Jumonji demethylase inhibitor modulates the proinflammatory macrophage response. *Nature* in press (2012).
18. Lao, O.D. *et al*. HATs off: Selective Synthetic Inhibitors of the Histone Acetyltransferases p300 and PCAF. *Mol. Cell* **5**, 589-595 (2000).
19. Zheng, Y. *et al.* Synthesis and Evaluation of a Potent and Selective Cell-Permeable p300 Histone Acetyltransferase Inhibitor. *J. Amer. Chem. Soc*. **127**, 17182-17183 (2005).
20. Bowers, E.M. *et al.* Virtual Ligand Screening of the p300/CBP Histone Acetyltransferase: Identification of a Selective Small Molecule Inhibitor. *Chem. & Biol*. **17**, 471-482 (2010).
21. Herold, J.M. *et al.* Small-Molecule Ligands of Methyl-Lysine Binding Proteins. *J. Med. Chem*. **54**, 2504-2511 (2011).
22. Muller, S., Filippakopoulos, P. & Knapp, S. Bromodomains as therapeutic targets. *Exp. Rev. Mol. Med.* **13**, e29 (2011).
23. Borah, J.C. *et al*. A small molecule binding to the coactivator CREB-binding protein blocks apoptosis in cardiomyocytes. *Chemistry & Biology* **18**, 531-541, (2011).
24. Filippakopoulos, P. *et al*. Selective inhibition of BET bromodomains. *Nature* **468**, 1067-1073 (2010).
25. Nicodeme, E. *et al*. Suppression of inflammation by a synthetic histone mimic. *Nature* **468**, 1119-1123, (2010).
26. Delmore, J.E. *et al*. BET bromodomain inhibition as a therapeutic strategy to target c-Myc. *Cell* **146**, 904-917 (2011).
27. Dawson, M.A. *et al.* Inhibition of BET recruitment to chromatin as an effective treatment for MLL-fusion leukaemia. *Nature* **478**, 529-533 (2011).

		 Writer	 Reader	 Eraser
	Acetyl	HAT	Bromo, PHD	HDAC
	Methyl	KMT	Tudor, MBT, Chromo, PWWP, PHD	KDM

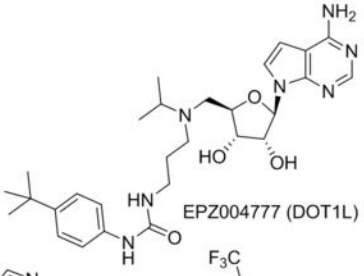
HMT



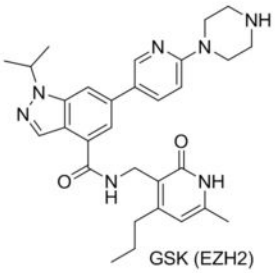
BIX-01294 (G9a)



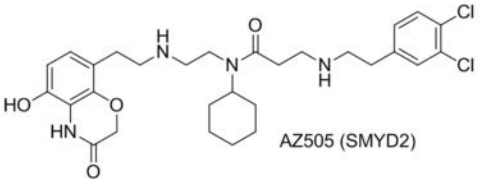
UNC0638 (G9a)



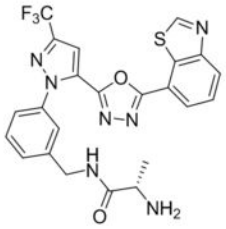
EPZ004777 (DOT1L)



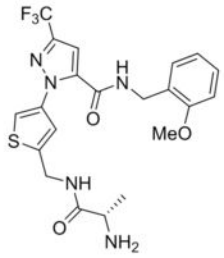
GSK (EZH2)



AZ505 (SMYD2)

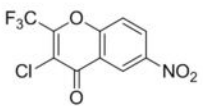


BMS (CARM1)

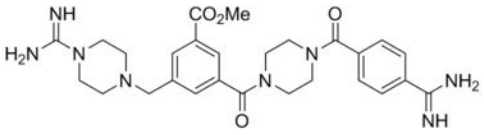


Methylgene (CARM1)

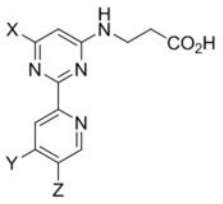
KDM



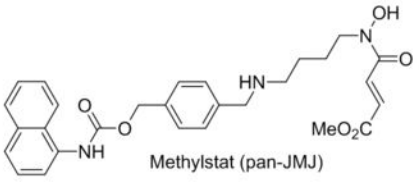
Namoline (LSD1)



CBB1007 (LSD1)

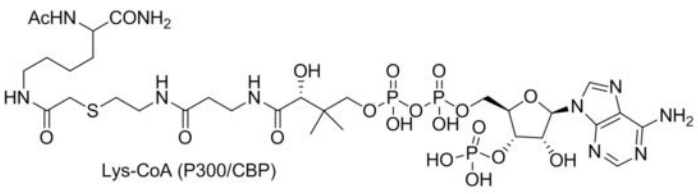


GSK (JMJD3)

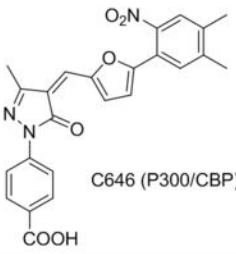


Methylstat (pan-JMJ)

HAT

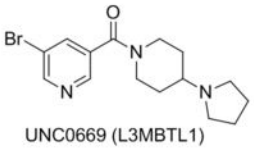


Lys-CoA (P300/CBP)



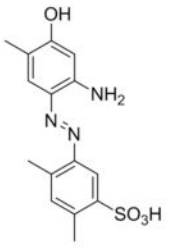
C646 (P300/CBP)

MeLys

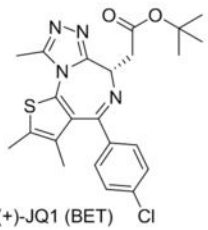


UNC0669 (L3MBTL1)

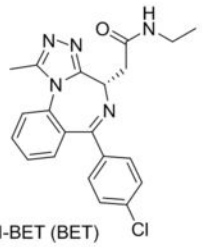
BRD



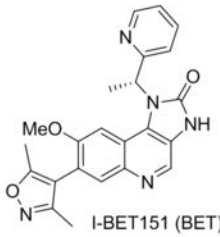
ISCHEMIN (CREBBP)



(+)-JQ1 (BET)



(+)-I-BET (BET)



I-BET151 (BET)

Biological Target	Molecule	Potency (IC50 or Kd)	Selective?	Cell Active?	Ref	Disease Association
G9a/GLP	BIX-01294	700 nM	Y	Y	4	Oncology
G9a/GLP	UNC0638	19 nM	Y	Y	5	Oncology
DOT1L	EPZ004777	0.6 nM	Y	Y	6	Oncology
SMYD2	AZ505	120 nM	Y	N	7	Oncology
EZH2	GSK	4 nM	Y	Y	8	Oncology
CARM1	BMS	40 nM	Y	N	10	Oncology
CARM1	Methylgene	60 nM	Y	N	11	Oncology
LSD1	Namoline	51 μM	Y	Y	13	Oncology
LSD1	CBB1007	5 μM	Y	Y	14	Oncology
Pan-JMJ	Methylstat	3-43 μM	NA	Y	16	Oncology, Inflammation, Neurology
JMJD3	GSK	90 nM	Y	Y	17	Inflammation
P300/CBP	Lys-CoA	20 nM	Y	N	18	Oncology
P300/CBP	C646	400 nM	Y	Y	20	Oncology
L3MBTL1	UNC0669	5 mM	Y	N	21	Unknown
CREBBP	Ischemin	19 μM	N	Y	22, 23	Ischemia
BET BRD	(+)-JQ1	30 nM	Y	Y	24, 26	Oncology
BET BRD	(+)-I-BET	50 nM	Y	Y	25	Inflammation
BET BRD	I-BET-151	20 nM	Y	Y	27	Oncology