Abstract: The protein methyltransferases (PMTs) constitute a large and important class of enzymes that catalyze site-specific methylation of lysine or arginine residues on histones and other proteins. Site-specific histone methylation is a critical component of chromatin regulation of gene transcription - a pathway that is often genetically altered in human cancers. Oncogenic alterations (e.g., mutations, chromosomal translocations and others) of PMTs, or of associated proteins, have been found to confer unique dependencies of cancer cells on the activity of specific PMTs. Examples of potent, selective small molecule inhibitors of specific PMTs are reviewed that have been shown to kill cancers cells bearing such oncogenic alterations, while having minimal effect on proliferation of non-altered cells. Selective inhibitors of the PMTs DOT1L and EZH2 have entered phase 1 clinical studies and additional examples of selective PMT inhibitors are likely to enter the clinic soon. The current state of efforts towards clinical testing of selective PMT inhibitors as personalized cancer therapeutics is reviewed here.
Background

Post-translational modifications of histone proteins play a critical role in defining the local structure of chromatin (the complex of DNA and histone proteins that make up chromosomes) and thereby control entire programs of gene transcription within cells (1). Both reversible small molecule (e.g., acetylation, methylation and phosphorylation) and protein (e.g., ubiquitination, sumoylation) modifications of histone are known to affect nucleosome compacting within chromatin and to also serve as recognition loci for binding of transcription factors, polymerases and auxiliary proteins that either facilitate or antagonize gene transcription (2). Among these various histone modifications (Figure 1A), methylation of lysine and arginine residues appears to play a particularly important role in control of gene transcription programs (3-5). These modifications are catalyzed by a class of group-transfer enzymes known as the protein methyltransferases (PMTs) (3, 6). The PMT enzyme class is composed of two distinct families of enzymes, based on their active site structures and on the amino acid to which they transfer methyl groups: the protein lysine methyltransferases and the protein arginine methyltransferases (6). There is one exception to this general structural bifurcation of the PMT class. The enzyme DOT1L is biochemically a lysine methyltransferase, but its active site structure is most closely aligned with that of the protein arginine methyltransferases (6). Based on active site structure, a number of enzymes that had been previously annotated as RNA methyltransferases can also be included within the family of PRMTs and some of these enzymes have
subsequently been shown biochemically to catalyze both RNA and protein methylation\(^\text{6, 7}\). Thus, the PMT class is composed of 96 enzymes within humans. Reaction fidelity is variable among the PMTs. In some cases, a single enzyme is responsible for site-specific methylation of a unique histone location (e.g., DOT1L methylation of H3K79, vide infra). In other cases, several PMTs appear to methylate a common histone site, but may do so in a gene-specific manner (Figure 1B). In yet other cases, a single enzyme may methylate multiple protein substrates.

The PMTs constitute an interesting target class for cancer therapeutics both because of the critical role these enzymes play in controlling cellular programs of gene transcription and because increasingly, bioinformatic surveillance of cancer genome databases are demonstrating that a number of these enzymes are implicated in specific human cancers\(^\text{5, 8}\); in a recent review, for example, Copeland (2012) listed over 10 PMTs for which genetic alterations are found in specific human cancers.

PMTs may play critical roles in cancer cells in general, but their greatest value as therapeutic targets may be in cases where the oncogenic alterations of PMTs (or of other proteins that impact PMT activity, vide infra) associated with specific human cancers have been demonstrated to confer to cancer cells a unique dependency on the enzymatic activity of the altered PMT for proliferation and/or survival (i.e., an addiction to PMT activity). Hence, a reasonable working hypothesis is that selective inhibition of the affected PMT would result in cell killing of cancer cells bearing the genetic alteration with minimal affect on non-altered cells. This would be expected to provide a significant therapeutic index in vivo, with potent
elimination of affected tumor cells and limited attendant safety concerns.

Preclinical animal studies of potent, selective inhibitors of two PMTs, DOT1L (9) and EZH2 (10) (vide infra) have borne out these expectations and have paved the way to clinical testing of PMT inhibitors in cancer patients.

Active Site Structure and Enzymatic Mechanism of Methyl Transfer

All PMTs utilize S-adenosyl methionine as a universal methyl donor in much the same way that all kinases utilize ATP as a universal phosphate donor (Figure 2).

The crystal structures of a number of PMTs have been solved to atomic resolution and reveal a well-organized S-adenosyl methionine binding pocket which is conformationally distinct between the lysine and arginine methyltransferases (3, 6, 11). In both cases, however, the S-adenosyl methionine binding pocket is juxtaposed to a long channel into which the methyl accepting amino acid side chain binds. This arrangement allows for close proximity and molecular orbital alignment between S-adenosyl methionine and the nitrogen of lysine or arginine for facile methyl transfer. All of these enzymes transfer the methyl group directly from S-adenosyl methionine to the acceptor amino acid, so that the enzymatic reaction requires the simultaneous binding of both substrates for transfer to occur (3).

These features of the enzymatic reaction of PMTs afford multiple opportunities for inhibitor interactions with the enzymes. Indeed, small molecule PMT inhibitors of various binding modalities have been reported: S-adenosyl methionine competitive, methyl acceptor competitive, noncompetitive, etc (5, 8). This diversity of inhibitor...
binding modalities distinguishes the PMTs from other group-transfer enzymes that have been targeted for cancer therapies (e.g., the kinases) and may allow greater flexibility in choosing inhibitors that offer the greatest chance for clinical translation(12).

**Role of PMTs in Regulation of Gene Transcription**

How site-specific methylation of histone lysines and arginines controls gene transcription is incompletely understood at present, but it is clear that methylation at specific histone sites affects downstream transcription in different ways. For example, methylation of histone H3 on lysines 4 (H3K4) and 79 (H3K79) has been implicated in the transcriptional activation of genes. In contrast, methylation at other locations, such as H3K9 and H3K27, are involved in repression of gene transcription(13). These opposing effects of methylation of different histone sites can play a critical role in cancer tumorigenesis when alterations in the location and amplitude of specific site methylation leads to aberrant activation of oncogene transcription and/or transcriptional repression of tumor suppressor genes. It is also becoming clear that the effect of site-specific methylation of histone sites on transcription cannot be understood in isolation, but depends on the context of other modifications of proximal histone sites(14). Finally, there is emerging data to suggest that methylation of one histone site may enhance or antagonize methylation of other histone sites. For example, data in multiple myeloma cells suggests that elevated methylation of H3K36 antagonizes methylation of H3K27, and likewise...
diminution of H3K36 methylation may result in elevation of H3K27 methylation levels(15).

**Genetic Alterations of PMT Activity as Drivers of Cancer**

A variety of genetic alterations are seen in members of the PMT enzyme class associated with specific human cancers. Ongoing surveillance of cancer genome databases are continuing to reveal additional examples of amplifications and mutations within PMTs and within PMT-associated proteins that appear to be specific to individual cancer types. Over the past decade, several examples have emerged of alterations of PMT activity that appear to be critical drivers of genetically defined cancers(16). Some of these will be described here to exemplify the diversity of diver alterations observed to impinge on this enzyme class.

**Indirect Chromosomal Translocation Leading to Ectopic PMT Activity**

*MLL*-rearranged leukemia is a devastating form of acute leukemia that affects patients from infancy through adulthood, and is also prevalent among secondary leukemia associated with etoposide (and other topoisomerase II inhibitors) use (17). This disease is universally associated with a chromosomal translocation (11q23) affecting the *MLL* gene that encodes for the PMT MLL, which normally catalyzes the methylation of the H3K4 residue (18, 19). As a result of the translocation, the MLL protein loses its enzymatic active site, hence PMT activity, and is fused to any of a number of proteins of the AF or ENL families (20, 21). Gene localization is conferred by recognition elements within the N-terminal region of the
MLL protein, and these are retained within the various fusion proteins. Hence, the MLL-fusion proteins localize to the same gene locations as wild type MLL, but lack the ability to catalyze histone methylation at H3K4. It has been recognized for some time that this chromosomal translocation plays a causal role in *MML*-rearranged leukemia, but the molecular mechanism of pathogenesis was not clear. Early attention focused on the loss of PMT activity associated with the fusion proteins that results from the translocation, but this was hard to reconcile with the residual H3K4 methylation activity of the wild type MLL protein resulting from the second, unaffected allele. In 2005 Zhang and coworkers (22) demonstrated that another enzyme, DOT1L, binds to AF10, one of the various fusion partner proteins associated with the *MLL* translocation and thereby recruits DOT1L to ectopic gene locations at which the MLL fusion proteins reside. Subsequent studies revealed that other AF and ENL fusion partners were also able to directly, or indirectly recruit DOT1L to MLL fusion bound genes (23-25). The resulting ectopic localization of DOT1L catalyzes H3K79 methylation of genes otherwise under the control of MLL-catalyzed H3K4 methylation, including pro-leukemogenic genes such as *HOXA9* and *MEIS1* (22, 26, 27). Methylation of H3K79 is a transcriptional activation mark. Thus, the ectopic localization of DOT1L, due to the universal chromosomal translocation in *MML*-rearranged leukemia, results in elevated transcription of a program of leukemogenic genes that in turn drive this proliferative disease. It has been hypothesized that *MML*-rearranged leukemia is uniquely dependent on DOT1L enzymatic activity and that selective inhibition of DOT1L would lead to specific cell killing of *MML*-rearranged leukemia cells. This hypothesis was confirmed by gene
silencing approaches (26) and by the use of potent and selective small molecule inhibitors of DOT1L (vide infra) (28-30).

**Direct Chromosomal Translocations Involving PMTs**

WHSC1 (also referred to as MMSET or NSD2) is a PMT that catalyzes the dimethylation of H3K36 (31, 32). Methylation of this histone site is associated with regions that are transcriptionally active. The t(4;14) chromosomal translocation occurs in a subset of about 15% of multiple myeloma (33, 34) and patients with this translocation represent one of the worst prognostic subgroups of multiple myeloma (35). The t(4;14) translocation results in massive overexpression of WHSC1 and of fibroblast growth factor receptor 3 (FGFR3) due to the placement of the strong IgH intronic Eμ enhancer and 3′ enhancer in the promoter regions of WHSC1 and FGFR3 genes, respectively. While ca. 30% of t(4;14) patients have lost expression of FGFR3, 100% retain overexpression of WHSC1, suggesting that WHSC1, rather than FGFR3, is the primary driver of disease (35). The overexpression of WHSC1 in t(4;14) translocated cells results in significantly elevated levels of dimethylated H3K36, as would be expected from elevation of catalytic enzyme levels (15). Genetic knockdown of WHSC1 or disruption of the translocated allele in t(4;14) myeloma cells results in inhibition of cellular proliferation and of tumorigenicity. As expected, genetic knockdown of WHSC1 demonstrates an accompanying reduction in global levels of H3K36me2 (15).

**Point Mutations Affecting PMT Activity**
The most well studied example of tumorigenic point mutations affecting PMT activity is that of mutations within the catalytic domain of EZH2 in germinal center diffuse large B-cell lymphoma and follicular lymphoma subsets of non-Hodgkin lymphoma (36-38). EZH2 (or the closely related EZH1) is the catalytic subunit of a multi-protein complex known as PRC2 (Polycomb Repressive Complex 2) that is responsible for mono-, di- and tri-methylation of the H3K27 site. Tri-methylated H3K27 is a translational repressive mark, and hyper-trimethylation of H3K27 has always been found to lead to tumorigenesis in various human cancers, presumably due to the transcriptional repression of tumor suppressor genes. Mutations at Tyr641 or Ala677 have been found to occur heterozygously in non-Hodgkin lymphoma patient groups (36, 37) and have been shown to be change-of-function mutations with respect to the substrate specificity of the enzyme(38). The wild type enzyme is most active in placing the first methyl group on to H3K27, and its catalytic efficiency wanes with consecutive methylation cycles. In direct contrast, the mutations associated with non-Hodgkin lymphoma show the exact opposite substrate specificity; they are essentially unable to put the first methyl group on H3K27, but once that first methyl is on, the mutant enzymes are able to put the second methyl on and are extremely efficient at putting the third methyl group on. In the context of disease heterozygosity, the wild type and mutant enzymes work in concert with one another to effect a tumorigenic hyper-trimethylated H3K27 phenotype(38). Two groups have now demonstrated that potent, selective, small molecule inhibitors of EZH2 selectively kill NHL cells bearing these EZH2 mutations.
while having essentially no effect on the proliferation of homozygous EZH2 wild type cells(39, 40).

*Synthetic Lethal Associations with PMT Activity*

In addition to histone methylation, gene expression is also regulated by remodeling of nucleosomes in an ATP-dependent manner. Of the ATP-dependent chromatin remodelers, SWI/SNF complexes are emerging as bona fide tumor suppressors, as specific inactivating mutations in several SWI/SNF subunits are found in human cancers(41). For instance, the INI1 (also known as SMARCB1 or hSNF5) subunit is inactivated in nearly all malignant rhabdoid tumors and atypical teratoid rhabdoid tumors, aggressive cancers of young children with no effective therapy(42). Tumorigenesis in INI1-deficient malignant rhabdoid tumors can be completely suppressed by tissue specific co-deletion of EZH2, suggesting an antagonistic relationship between PRC2 and SWI/SNF activities(43). Recently, Knutson et al.(44) have demonstrated that potent, selective inhibitors of EZH2 are effective in eradicating INI1-deficient malignant rhabdoid tumors in cell culture and in a mouse xenograft model. Hence, although the tumorigenic driver of malignant rhabdoid tumors occurs in a pathway distinct from any PMT, it nevertheless creates a synthetic lethal relationship that confers a unique sensitivity to selective EZH2 inhibition in this disease.

**Clinical-Translational Advances**
Preclinical In Vivo Activity of Selective PMT Inhibitors

Selective inhibitors of several PMTs have been described and compounds targeting two particular PMTs, DOT1L and EZH2, have been reported to show dramatic anti-tumor activity in rodent models of disease (9, 10, 40, 44). The DOT1L tool compound EPZ004777 was the first PMT inhibitor reported to demonstrate anti-tumor effects in a disseminated mouse model of MLL-rearranged leukemia (28). Subsequent pharmacological optimization within this chemotype series resulted in the clinical candidate DOT1L inhibitor EPZ-5676, which was also shown to eradicate MLL-rearranged tumors (≥ 100% tumor growth inhibition) in a rat xenograft model (9, 45). Two distinct EZH2 inhibitors have been shown to display significant, dose-dependent anti-tumor activity in mouse xenograft models of EZH2-mutant bearing non-Hodgkin lymphoma tumors (60-100% tumor growth inhibition in various tumor models) and of INI1-deficient malignant rhabdoid tumors (≥ 100% tumor growth inhibition) (10, 40, 44). In all of these studies, the compounds were well tolerated by the rodents at doses that resulted in significant tumor growth inhibition, with no acute toxicity or weight loss observed. These results provide a strong foundation for the clinical testing of DOT1L and EZH2 inhibitors in genetically defined cancer patients, and also portend the general utility of PMT inhibitors as personalized cancer therapeutics. Also, in all of these studies, the investigators were able to show a correlative relationship between tumor growth inhibition and diminution of the relevant histone methyl mark in tumor tissue, as well as in surrogate tissues such as skin and peripheral blood mononuclear cells.
The ability to measure drug-induced loss of site-specific histone methylation in surrogate tissue by non-invasive means provides a cogent basis for pharmacodynamics assessment of target engagement in clinical trials.

Clinical Trials

At the time of this writing, two PMT inhibitors have entered phase 1 human clinical trials: the DOT1L inhibitor EPZ-5676 and the EZH2 inhibitor EPZ-6438. The primary goals of both of these trials is to assess the safety of the drugs and to establish the maximum tolerated dose for each.

The EPZ-5676 phase 1 trial is divided into two parts: a dose escalation portion to establish maximum tolerated dose and an expansion cohort of patients treated at the maximum tolerated dose. To accelerate dose escalation, eligibility is not restricted to genetically defined patients; patients with hematologic malignancies (mainly AML and ALL) are eligible for this portion of the trial. Once the maximum tolerated dose has been established, the expansion cohort study will begin exclusively in patients with leukemia involving the MLL rearrangement at 11q23. In this manner, the drug may be tested as early as possible in the genetically defined patients for whom it was designed.

The EZH2 inhibitor EPZ-6438 is being studied in a phase 1 trial of relapsed or refractory malignancies that have failed all standard therapy. Again, the primary goal here is to establish the safety and define the maximum tolerated dose of the drug. As with the EPZ-5676 trial, a secondary objective is to test EPZ-6438 as early
as possible in the genetically defined patients for whom the drug was designed.

Hence, once the maximum tolerated dose has been established, a phase 2a trial is planned exclusively in non-Hodgkin lymphoma patients bearing mutations in EZH2.

No data have yet been reported for either of these trials. Nevertheless, these trials represent a true watershed in the development of PMT inhibitors as personalized cancer therapeutics. The cancer community will be looking forward with great interest towards the outcomes of these trials. It is clear that these trials represent the vanguard of a much larger array of PMT inhibitors that will be entering clinic trials in the near future, for testing against genetically defined groups of cancer patients.

**Conflict of Interest**

The author is an employee and stockholder of Epizyme, Inc.

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**Figure Caption**

**Figure 1.** Chromatin modification affect gene transcription. (A) Chromatin consists of histone proteins (red) around which chromosomal DNA (blue) is wrapped. The histone proteins undergo a variety of posttranslational modifications, including methylation of arginine (R) and lysine (K) residues catalyzed by the class of enzymes known as the protein methyltransferases (PMTs). These
posttranslational modifications affect the state of compacting of nucleosomes (histone-DNA units) along the chromosomes. Regions of chromosomes can transition from the open, transcriptionally permissive state referred to as euchromatin to the more compact, transcriptionally repressive state known as heterochromatin. (B) PMTs catalyze methylation of specific lysine and arginine residues on histones H3 and H4. The enzymes that catalyze methylation of a specific histone residue are listed in the red boxes.

Figure 2. Chemical mechanism of PMT catalysis (left) and the products of lysine (upper right) and arginine (lower right) methylation.
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**Euchromatin - Transcriptionally permissive**

Site-specific methylation of histones H3 & H4

**Heterochromatin - Transcriptionally repressive**

**Figure 1 AB:**

**Euchromatin**

- CH3
- PO4R
- K
- Ac
- Ub

**Heterochromatin**

- CH3
- CpG
- K
- K
- K
- K
- K
- K
- K
- K
- C

**PMTs**

- Arginine (RMTs)
- Lysine (KMTs)

**MLL1**

**MLL2**

**MLL3**

**MLL4**

**MLL5**

**SET1A**

**SET1B**

**ASH1**

**SET7/9**

**SUV39H1**

**SUV39H2**

**G9a**

**GLP**

**SETDB1**

**RIZ1**

**PRDM3**

**PRDM16**

**PRMT5**

**CARM1**

**NDS1**

**NSD2**

**NSD3**

**SET2**

**SMYD2**

**EZH1**

**EZH2**

**DOT1L**

**Pr-SET7/8**

**SUV4-20H1**

**SUV4-20H2**

**SET9**

**Pr-SET7**

**SUV4-20**

**NSD1**
Figure 2:

PKMTs

PRMTs

Histone

Mono-methyl lysine

Di-methyl lysine

Tri-methyl lysine

Mono-methyl arginine

Symmetrical di-methyl arginine

Asymmetrical di-methyl arginine

Lysine

Arginine

Methyltransferases (PKMTs and PRMTs) catalyze the transfer of methyl or dimethyl groups to lysine and arginine residues in histones and other proteins, respectively.
Molecular Pathways: Protein methyltransferases in cancer

Robert A Copeland

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