Drug Discovery Today: Technologies
Vol. 18, No. nullC 2015

Profiling used in lead optimization and drug discovery

Challenges in profiling and lead optimization of drug discovery for methyltransferases

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The importance of epigenetics in the initiation and progression of disease has attracted many investigators to incorporate this novel and exciting field in drug development. Protein methyltransferases are one of the target classes which have gained attention as potential therapeutic targets after promising results of inhibitors for EZH2 and DOT1L in clinical trials. There are many technologies developed in order to find small molecule inhibitors for protein methyltransferases. However, in contrast to high throughput screening, profiling against different methyltransferases is challenging since each enzyme has a different substrate preference so that it is hard to profile in one assay format. Here, different technologies for methyltransferase assays will be overviewed, and the advantages and disadvantages of each will be discussed.

Section editor:
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Introduction

Epigenetic modifications are dynamic and reversible processes that establish normal cellular phenotypes, but also contribute to human diseases [1]. Thus scientists have tried to understand the landscape of epigenetic modifications and their relationship with diseases [2]. Some epigenetic enzymes have been long time therapeutic targets such as Histone deacetylases; however, Histone methyltransferases, especially, have gained attention after promising results of inhibitors for EZH2 and DOT1L in clinical trials [review]; [3–6]. Recently, other protein methyltransferases including PRMT1, PRMT5, and PRMT7 are also considered as potential drug targets [7–13]. In order to promote and accelerate drug discovery activities, biochemical assays for profiling and lead optimization of drug discovery are essential after lead identification by high-throughput screening (HTS).

Histone methyltransferases (HMTs) and Protein arginine methyltransferases (PRMTs) belong to the enzyme class which transfers a methyl group from S-adenosyl-L-methionine (SAM or AdoMet) to a lysine residue or arginine residue in the substrate protein such as histones, a member of chromatin, and produces S-adenosyl-L-homocysteine (SAH). Each HMT has specific target lysine residue(s) for methylation; for example, EZH2 complex methylates Histone H3 Lysine 27, mono-, di-, and tri-methylation. On the other hand, PRMTs methylate arginine residue mono-methyl, symmetrical di-methyl (type-II) or asymmetrical di-methyl (type-I), depending on type [14–18], to many proteins including histones. Because of such complexity, making antibodies against specific residue and specific methylation state (mono-, di-, etc.) is challenging. In addition, newly identified non-histone substrate proteins for PRMTs have been explored [19].

In addition to methylation state, each methyltransferase has substrate specificity. For example, DOT1L methylates

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 http://dx.doi.org/10.1016/j.ddtec.2015.10.010

Histone H3 Lysine 79, however, peptide or histone H3 protein does not serve as a substrate; only nucleosomes work as substrates in *in vitro* assays. Similarly, NSD1–3 also use only nucleosomes as substrate. On the other hand, EZH2 complex prefers histone octamers rather than nucleosomes, as well as histone H3 protein and peptides. As mentioned below, some assay formats only allow use of peptides as substrates while other assay formats cannot use peptide. Therefore, profiling compounds against a large panel of methyltransferases in one assay format is demanding, and comparing compound potency against one enzyme to another in different assay formats is difficult. Since *Km* values are very different between peptide and protein substrate for methyltransferases in general, selectivity of the substrate competitive compound shown for an enzyme with peptide substrate against enzymes using protein substrate may not be accurate. In fact, BIX019, reported as a potent inhibitor against G9a and GLP using peptide as substrate, showed weak inhibition when using protein substrate [20,21]. Other factors such as buffer conditions also need to be kept in mind since compound solubility may be different in different buffers. If the buffer is different for each enzyme assay, compounds could be selective by solubility rather than potency; one buffer contains detergent, for example, which solubilizes the compound, but another buffer is absent of detergent resulting in a false positive or negative. Factors such as pH, salt, DTT or TCEP, etc. also affect compound behavior, depending on structure. Thus compound selectivity must be carefully evaluated for true selectivity against enzymes rather than the difference in assay conditions.

Another challenge of profiling against a large panel of methyltransferases is collection of enzymes; one needs to collect or produce a large number of targets. However, enzyme resources are limited and not many vendors offer many targets. In this review, we will focus on commercially available products and assay technologies for profiling.

Detection of methylation on peptide substrate

Assay formats using peptide as substrate are good for finding peptide (or substrate) competitive inhibitors since the *Km* for peptide is usually much higher than the *Km* for protein or histone complex substrates. In addition, in order to save reagents, most assays use peptide substrate concentration much lower than *Km* values. The most popular detection is antibody-based detection in conjunction with fluorescence labeling such as AlphaLisa technology (Perkin Elmer (PE), Waltham, MA) or HTRF (Homogeneous Time Resolved Fluorescence: Cisbio Bioassays, Bedford, MA). The enzyme reaction produces methylation on biotinylated substrate peptide, and acceptor- (or donor-) labeled antibody binds to the methylation site which will be detected by a streptavidin-tagged donor (or acceptor) binding to biotinylated peptide and energy transfer occurring between donor and acceptor in close proximity. Antibody choices are against mono-, di-, or tri-methylation on specific residues of histones. Specificity of antibody recognition is essential; however, sometimes specificity makes signal windows narrow. For instance, if the antibody is specific for mono-methylation of a specific residue but the enzyme can methylate mono-, di-, and tri-methylation, the enzyme titration curve will not be linear; the signal will increase when mono-methylation is increased but then decrease when di- and tri-methylations are increased. Thus the enzyme titration curve will be bell-shaped and the optimal signal window is narrow. Antibodies are widely available from many vendors; however, it is substantial work to test specificity for available antibodies. A wide variety of different methylation levels and sites of histone peptides are available from AnaSpec (Fremont, CA) with biotin-labeling for antibody testing as well as for substrates. Assay signals also depend on labels or FRET (fluorescence resonance energy transfer) pairs. AlphaLisa is proprietary technology by Perkin Elmer (PE), and its detection requires a special instrument; excitation at 680 nm and emission at 615 nm. However, high signals result in a signal/background ratio of more than 100 easily so that this assay is suitable for HTS application. Although false-positive rates are relatively high, a counter assay is available. Other labeling choices are LANCE TR-FRET from PE (Eu donor and ULight acceptor) and HTRF from Cisbio Bioassays (Eu or Tb donor and XL665 acceptor). These vendors are also offering antibodies coupled with labeling. Other labeling is also available from many vendors such as Life Technologies (Thermo Fisher Scientific; Grand Island, NY) and Rockland Immunochemicals Inc. (Limerick, PA). These antibody-based fluorescence detections are also applied for demethylase assays. A major drawback of antibody-based assays is the requirement of multiple antibody sets for each methylation site by different HMTs for profiling.

Radioisotope-based detections are considered as a gold standard; this also applies for peptide detection with tritiated SAM. The most popular format is scintillation proximity assay (SPA) format; however, substrate peptides have to be labeled with biotin for capture by streptavidin-coated SPA beads or FlashPlate (both from PE). In addition, the peptide binding capacity of SPA beads or FlashPlate is limited. Therefore it is necessary to determine substrate concentration carefully in the reaction or detection when using SPA. In fact, peptide substrate concentrations above 0.5 μM lose linearity when using streptavidin-FlashPlate without dilution. PE also offers SPA imaging beads which emit light in the red region of the visible spectrum, making them ideally suited for use with any CCD-based imager such as ViewLux (PE). The CCD-based imagers are sensitive which enables ultra high-throughput screening with even 1536-well plate format.

Mass spectrometry based detections are reliable and can distinguish between mono-, di-, and tri-methylated products.

Since antibody-detection detects one population depending on antibody specificity or total methylation by radioisotope-based detections, mass spectrometry based detection is excellent for kinetic studies as well as HTS applications, depending on instrumentation [22].

Detection of methylation on protein substrate

Several protein methyltransferases cannot use peptides as substrates in *in vitro* assays. These enzymes require histone protein(s), histone octamer (core histones) or nucleosomes as substrates (see Table 1: The substrate preference in this table is based on our experiences. It may be different in different assay formats.). For many antibodies, it is hard to recognize methylation on histone proteins or nucleosomes in native structures, and it requires special treatment for detection. PE developed a DOT1L assay, for example, in AlphaLisa format in which nucleosomes are dissociated with high salt buffer for antibody detection (PE Tech Note AlphaLISA #25). However, if you want to develop your own, a lot of effort is needed to optimize the detection conditions for each antibody in

Table 1. HotSpot substrates with RBC proteins.

Enzyme	Methylation sites*	Preferred substrate	Alternative substrate
ASH1L	H3K36	Nucleosomes	N/A
DOT1L	H3K79	Nucleosomes	N/A
EZH1 complex	H3K27	Core histones	H3 protein, H3 peptide
EZH2 complex	H3K27, Jarid2 [40]	Core histones	H3 protein, H3 peptide
G9a	H3K9, p53K373, many	H3 protein	Core histones, H3 peptide
GLP	H3K9, p53K373, many	H3 protein	Core histones, H3 peptide
MLL1 complex	H3K4	Nucleosomes	Core histones, H3 protein, H3 peptide
MLL2 complex	H3K4	Core histones	H3 protein, H3 peptide
MLL3 complex	H3K4	Core histones	H3 protein, H3 peptide
MLL4 complex	H3K4	Core histones	H3 protein, H3 peptide
NSD1	H3K36	Nucleosomes	N/A
NSD2	H3K36	Nucleosomes	N/A
NSD3	H3K36	Nucleosomes	N/A
PRDM9	H3K4	H3 protein	Core histones, H3 peptide
SETD1B complex	H3K4	Core histones	H3 peptide
SETD2	H3K36	Nucleosomes	N/A
SETD7	H3K4, p53K372, TAF10K189	Core histones	H3 protein, H3 peptide
SETD8	H4K20, p53K382	Nucleosomes	H4 peptide
SETMAR [41]	snRNP70 K130	N/A	N/A
SMYD1	H3K4	Core histones	H3 protein, H3 peptide
SMYD2	H3K4, H3K36, p53K370, RB1K860	H4 protein	H4 peptide
SMYD3	MAP3K2 [42,43], H4K20 [44]	N/A	N/A
SUV39H1	H3K9	H3 protein	H3 peptide
SUV39H2	H3K9	H3 protein	H3 peptide
SUV420H1	H4K20	Nucleosomes	N/A
PRMT1	H4R3, many, Twist1R34 [7]	H4 protein	H4 peptide
PRMT3	Many	H4 protein	H4 peptide
PRMT4	H3R17, many	H3 protein	H3 peptide
PRMT5	H2AR3, H4R3, H3R8, many	H2A or H4 protein	H2A or H4 peptide
PRMT5/MEP50	H2AR3, H4R3, H3R8, many	H2A or H4 protein	H2A or H4 peptide
PRMT6	H3R2, H2AR3, H4R3, many	H3 protein	GST-GAR
PRMT7 [45]	H2AR3, H4R3, H2B, many	GST-GAR	H2B peptide
PRMT8	Many	H4 protein	H4 peptide

* Information from UniProtKB unless citation given.

addition to the choice of antibody conjugation with acceptor or donor. Both PE and Cisbio offer an Epigenetic tool box to develop assays on your own.

SPA-based radioisotope assays do not need to expose methylation sites, but do require biotinylated protein substrates. Biotinylated histones or recombinant biotinylated nucleosomes are available from Active Motif (Carlsbad, CA), Reaction Biology (Malvern, PA), and BPS Bioscience (San Diego, CA). A traditional TCA precipitate-glass fiber-filter capturing format for TopCount measurement is good for all protein substrates including nucleosomes; however, peptide substrates may be hard to collect by filter especially at low concentrations, and relatively large amounts of reagents are required in addition to the HTS-unfriendly washing steps. For outsourcing, Reaction Biology's HotSpotTM format, which was originally developed for kinase assays and applied for methyltransferase assays [21,23], is essentially the filter-binding method but in a miniaturized format. Thus it requires minimal reagents, and any type of substrates, from peptides to nucleosomes, can be used. This is the ideal format for profiling since one assay format can be used for all HMTs and PRMTs even with non-histone proteins without any modifications.

The advantages of Mass spectrometry based detections are not limited to distinguishing between mono-, di-, and tri-methylated products stoichiometrically. They also include identifying the sites of lysine and arginine methylation in the human proteome [24]. Mass spectrometry is a powerful tool for global identification of protein methylation beyond histones and proteomics [25–27]. It is also useful in *in nucleo* assays to determine methylation state and sites [28].

Detection of SAH product

Instead of direct measurement of methylation on substrates, the product of SAM, SAH, is measured. This assay format has an advantage since any substrate can be used, thus it is often called a 'universal' assay. The SAH detection can be applied for any enzyme assays that produce SAH as a product including DNA methyltransferases and N- or O-methyltransferases. The simplest format is direct measurement by Mass spectrometry or HPLC. Medium to high throughput mode can be applied although instruments are expensive if set up new. Thus Mass spectrometry has been used by pharmaceutical companies in HTS set up as well as profiling [29].

Another direct method for detecting SAH formation is a competitive fluorescence polarization (FP) assay [30] in which anti-SAH antibody binds free SAH and releases the fluorescent tracer (fluorescent-SAH conjugate) resulting in FP change. The key of this assay is specificity of antibody against SAH selective from SAM that limits assay sensitivity. Similar but in TR-FRET format, Cisbio offers EPIgeneousTM methyltransferase assay kit which uses Tb-labeled SAH antibody and SAH probe: The resulting TR-FRET signal is inversely proportional to the concentration of SAH in the calibrator or in the sample.

There are many coupling enzyme systems available to detect SAH (Fig. 1). SAH hydrolase catalyzes SAH into Adenosine and Homocysteine (HCy) which can be detected by a thiol-sensitive fluorophore, such as the ThioGlo® from Covalent Associate Inc (Corvallis, OR) or ThioFluor from Cayman Chemical (Ann Arbor, MI), the CPM (7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin) [31,32], or DTNB (5,5′-Dithiobis(2-nitrobenzoic acid) for color alternative. Adenosine can be further metabolized by Adenosine kinase

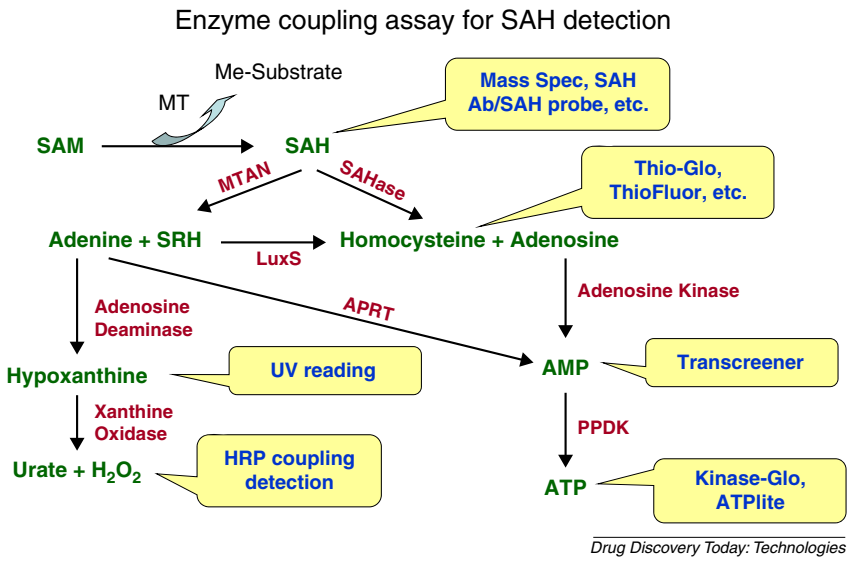

Figure 1. Enzyme coupling assays for SAH detection. *Abbreviations*: SAHase: SAH hydrolase; MTAN: 5′-methylthioadenosine nucleosidase; LuxS: S-ribosylhomocysteine lyase; APRT: adenine phosphoribosyl transferase; PPDK: pyruvate orthophosphate dikinase; SRH: S-Ribosyl-L-homocysteine.

and ATP to produce AMP and ADP. The AMP is detected by Transcreener® AMP/GMP assay from BellBrook Labs (Madison, WI), and Transcreener EPIGEN kit includes coupling enzymes. Now BellBrook offers Transzyme Methyltransferase assay kits with Reaction Biology's Methyltransferase enzymes that are validated and optimized for ready to use [33]. Using a similar pathway, Drake *et al.* [34] measured the amount of ATP remaining by using a luminescent assay kit, Kinase-Glo from Promega (Madison, WI), during the process of SAH converting enzymatically to AMP by Adenosine kinase and ATP.

Another coupling enzymes pathway uses 5′-methylthioadenosine nucleosidase (MTAN) which converts SAH to adenine and S-Ribosyl-L-homocysteine (SRH). The adenine can be converted into two different products with different enzymes. The first pathway is to convert adenine to AMP by adenine phosphoribosyl transferase (APRT), then the AMP is further converted to ATP by PPDK (pyruvate orthophosphate dikinase) [35]. The ATP is then quantified by commercially available luciferase assay kits, such as ATPlite from PerkinElmer or Kinase-Glo from Promega.

The second pathway after MTAN is to convert adenine to hypoxanthine with adenine deaminase, which is associated with a decrease in absorbance at 265 nm that can be monitored continuously with a spectrophotometer [36]. Hypoxanthine can be further converted to urate and hydrogen peroxide (H_2O_2) by xanthine oxidase. H_2O_2 can then be measure by fluorescent or colorimetric reagents that are available from many commercial resources, such as Amplex Red/HRP coupling (Life technologies), Hydrogen peroxide detection kits (Enzo Lifesciences), and xanthine oxidase kits (Cayman Chemicals). A methyltransferase assay kit combining MTAN, adenine deaminase, xanthine oxidase and resorufin/HRP for H_2O_2 detection is offered by G-Biosciences (St. Louis, MO).

Finally, the MTAN pathway also provides an alternative route to generate Hcy, which can then be detected as mentioned above. The enzyme *S*-ribosylhomocysteinase (LuxS) cleaves the thioether bond in *S*-ribosylhomocysteine (SRH) to produce homocysteine and 4,5-dihydroxy-2,3-pentanedione [37,38].

A minor drawback of SAH detection is that SAH cannot be used as a positive control inhibitor. Since many methyltransferases do not have good reference inhibitors, it is inconvenient that one cannot use SAH, a broad methyltransferase inhibitor. In addition, the involvement of many coupling enzymes makes it hard for kinetic studies, and counter assays are required for compound profiling to distinguish real methyltransferase inhibition.

Other detections

Wigle and coworkers [39] have employed a methylation-sensitive endoproteinase strategy to separate methylated peptides from unmethylated peptides by a capillary electrophoresis system (Caliper LC3000). This method requires special sequences for cleavage by Endo-LysC proteinase, thus applicable methyltransferase targets are limited resulting in suitability for HTS.

Recently, Cayman Chemical offers fluorescence probe competition assay, SAM-Screener™, developed and patented with University of Michigan. This probe binds SAM-binding pocket in SET domain-containing methyltransferases, and the outcome is a change in fluorescence polarization. Currently the assay can be applied only for SET domain-containing methyltransferases and the probe is an unknown proprietary small molecule. Thus it is convenient for HTS application to find SAM competitive inhibitors, but it may miss new scaffolds different from probe or SAM noncompetitive inhibitors. Cayman Chemical is developing the second-generation of the probe which competitively binds to SAM-binding sites of a different set of methyltransferases.

Conclusions

Compound profiling against a large panel of enzymes is important for understanding target specificity and lead optimization of drug discovery. An ideal profiling is using the same assay format for all targets. Antibody-based assays may be suitable for HTS, but they need an exhaustive collection of antibodies for entire targets for profiling. Universal SAH detections have the advantage that any substrates can be used; however, most assays require more than one coupling enzyme that need counter assays. Mass spectrometry based assays are the most reliable, but they need costly instrumentation. In addition, collecting or producing a large panel of enzymes is another challenge. If you are thinking of outsourcing, there are several vendors offering assay services, but most only have a few targets available and are more suitable for screening. Among them, Reaction Biology will be the best choice for profiling because of gold standard radioisotope-based assays in one assay format, HotSpot℠ which can use any substrates, and the largest collection of active methyltransferase enzymes for profiling as well as HTS.

Conflict of interest

The author confirms that this article content has no conflict of interest.

Acknowledgements

I thank Dr. Konrad T. Howitz and the Protein Production Group of Reaction Biology Corp. for producing methyltransferase proteins and substrate proteins, and Joseph J. Ferry and Daniel Kelly for performing methyltransferase enzyme assays.

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