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Cell-based assays to support the profiling of small molecules with histone methyltransferase and demethylase modulatory activity

Natalia J. Martinez[a] and **Anton Simeonov**[a,*]

[a]National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD, 20850

Abstract

Histone methylation is a prevalent and dynamic chromatin modification, executed by the action of histone methyltransferases (HMTs) and demethylases (HDMs). Aberrant activity of many of these enzymes is associated with human disease, hence, there is a growing interest in identifying corresponding small molecule inhibitors with therapeutic potential. To date, most of the technologies supporting the identification of these inhibitors constitute *in vitro* biochemical assays which, although robust and sensitive, do not study HMTs and HDMs in their native cellular state nor provide information of inhibitor's cell permeability and toxicity. The evident need for complementary cellular approaches has recently propelled the development of cell-based assays that enable screening of HMT and HDM enzymes in a more relevant environment. Here, we highlight current cellular methodologies for HMT and HDM drug discovery support. We anticipate that implementation of these cell-based assays will positively impact the discovery of pharmacologically potent HMT and HDM inhibitors.

Keywords

cell-based assays; inhibitor profiling; histone methyltransferase; histone demethylase; small molecule inhibitor

Introduction

Small molecule screening campaigns for HMTs and HDMs have been launched both in academia and the private sector. These campaigns are supported by a wide range of approaches, many of which have been translated into robust ready-to-use assay kits. In fact, multiple companies currently commercialize assay kits and tool-box reagents and even offer fee-for-service screening and profiling for many epigenetic factors. The most widely-used

*Corresponding author (asimeono@mail.nih.gov).

Conflict of Interest Statement

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approaches are *in vitro* biochemical assays because of the broad range of available platforms and detection systems well suited for lead identification and profiling for selectivity, potency, and kinetics of inhibition. A drawback of most biochemical assays is that they rely on purified (in many cases truncated) enzymes and isolated histone substrates (mostly short peptides) in a synthetic system and thus cannot measure actual interactions occurring in the nucleus of living cells. Cell-based assays, on the contrary, provide a much more biologically relevant assessment of inhibitor effects on epigenetic marks. Several cell-based platforms have now been designed or adapted for HTMs and HDMs to support lead optimization and structure-activity-relationship (SAR) campaigns. We start with a short introduction on the targeted enzymes, followed by a summary of current cell-based technologies amenable for compound screening/profiling; we evaluate their strengths and weaknesses and when possible, provide examples of their utilization.

Introduction to Histone Methyltransferases (HMTs) and Demethylases (HDMs)

Histone proteins are methylated on lysine (K) or arginine (R) residues by HMTs. At least six lysine and five arginine residues are methylated in the core histones H3 and H4. Lysines can be mono-, di-, or trimethylated, whereas arginines can be mono- or dimethylated. Dimethylated arginine residues arise from monomethylation of both terminal guanidino nitrogens (referred to as symmetric dimethylation) or from dimethylation of one of the terminal guanidino nitrogens (referred to as asymmetric dimethylation). Histone methylation can be either an activating or repressing mark, depending on the site and degree of methylation. For example, methylation on H3K4, H3K36, and H3K79 leads to activation of transcription, whereas methylation on H3K9, H3K27, and H4K20 is associated with gene repression. Comprehensive reviews on histone modifying enzymes are references [1] and [2].

HMTs play important roles in the development of various human diseases, particularly cancer. Either mutations or deregulation of both lysine and arginine HMTs has been associated with numerous forms of cancer. For instance, the lysine methyltransferase G9a is de-regulated in hepatocellular**,** prostate and lung cancer and mutations and rearrangements in the gene that codes for the lysine methyltransferase MLL1, have been reported in leukemias [3, 4]. Aberrant expression of histone arginine methyltransferases have also been documented in numerous cancer types, including leukemia, breast and colon cancer. Similar to HMTs, HDM enzymes are linked to human cancers, validating them as potential therapeutic targets in oncology. Aberrant expression of LSD1 has been shown in bladder, small cell lung, and colorectal cancers. Amplification of genes coding for JmjC-domain demethylases have been documented in several cancer types. For instance, KDM4C (also known as JMJD2C) is amplified in esophageal squamous carcinomas, medulloblastomas, and breast cancers, and KDM4B (JMJD2B) in medulloblastomas (review on the role of HMT and HDM in disease are references [3, 5]).

Anecdotally, HMTs became attractive targets for clinical development not until the discovery of HDMs, which indicated that methylation is a dynamic, reversible regulatory process like other well-established histone posttranslational modifications, including

phosphorylation and acetylation. Multiple chemical probes/inhibitors of HMTs and HDMs have been identified to date, and some are already in preclinical studies. Description of these inhibitors is beyond the scope of this review. Excellent reviews on this topic are references [3-8].

Cell-based assays: advantages and limitations

In vitro biochemical assays, in particular those that detect total enzymatic activity, are amenable to miniaturization and automation and are usually the first choice for routine primary screening [9]. A decisive point in the early stages of HMT and HDM inhibitor development is the identification of compounds that inhibit target's activity in the native cellular context. Although many biochemical assays have been adapted for the use of full histone proteins, octamers and even nucleosomes as substrates, these efforts fall short in recapitulating endogenous conditions. Many epigenetic enzymes are present in cells as complexes of multiple regulatory subunits, so they can be difficult to express as reconstituted functional enzymes. For example, the EZH2 complex has to be co-expressed as a complex of five different proteins (EZH2, EED, SUZ12, RbAp48 and AEBP2) for full activity [10]. In addition, it is not well understood how other adjacent histone posttranslational modification (like acetylation, SUMOylation and phosphorylation) affect methylation of histone tails. Cell-based assays circumvent these limitations by testing histone modifying enzymes in their natural environment and with relevant substrates.

The most potent compounds in biochemical assays are not necessarily the best ones in cell-based assays. Cell-based assays have the advantage of informing not only on target functionality but also on compound cell permeability. For example, the generic inhibitor of JmjC demethylases, 5-Carboxy-8-hydroxyquinoline, which is potent in biochemical assays, suffers from poor cell permeability as demonstrated by a hundred-fold lower efficacy in cellular immunofluorescent assays [11]. Ester derivatives of 5-Carboxy-8-hydroxyquinoline were later shown to improve its cellular permeability and activity [12].

Cell-based assays also inform on compound toxicity. The first potent and selective G9a and GLP inhibitor identified, BIX01294, displays a poor separation between target functionality (as measured by blocking H3K9 di-methylation) and cell toxicity [13]. The inhibitor was later modified to improve the balance between cellular potency and toxicity while maintaining high *in vitro* potency [14].

There are difficulties associated with establishing cell-based methylation and demethylation assays. In contrast to *in vitro* biochemical assays, the determination of direct enzymatic activity is virtually impossible to achieve *in vivo*, due to high endogenous histone background and multiplicity of enzyme family members. Moreover, certain methyltransferases target numerous non-histone protein substrates. Hence, most cellular assays rely on histone methylation specific antibodies and/or labels, which in itself presents technical shortcoming (see below).

Additionally, because of the lack of complete understanding of HMTs and HDMs biology, no good general pathway under HMT/HDM control has been identified that could be used as a read-out in gene-expression and phenotypic assays. Finally, certain cell-based platforms,

like those involving high content imaging discussed below, allow straightforward automation for primary screening of histone modifying enzymes. However, since a myriad of indirect inhibitors can be identified through such an approach, these assays are typically used only in follow-up studies (unless the intended goal is to find epigenetic modulators independent of their exact mechanism of action).

Choosing a cell-based assay for HMT and HDM inhibitors profiling

Due to the diversity of histone methylation sites and states and our incomplete knowledge on HMT/HDM cellular pathways, choosing an assay platform for screening and profiling against HMT and HDMs requires special considerations, including technical aspects such as desired compound throughput, specificity, use of labels, detection system, and protocol complexity. For example, most available cell-based assays that monitor changes in histone modifications utilize antibodies specific to the methylated/demethylated substrate. However, the use of antibodies is constrained by problems such as cost, specificity, availability, limited dynamic range and epitope occlusion triggered by other histone posttranslational modifications. For example, Lau and Cheung showed that an H3K27me3-specific antibody failed to recognize its epitope when the adjacent serine residue (S28) is phosphorylated [15]. Other assays utilize constructs where the histone and/or enzyme are labeled with a tag and/or overexpressed. Careful considerations must be taken before assuming that inhibition of the expressed construct equals inhibition of the endogenous enzyme.

Cell-based platforms currently available to profile HMT and HDM inhibitors and their most prominent advantages and limitations are summarized in Table 1. We note that these assays can be applied to both HMT and HDM profiling; further, we have listed some assays that have not yet been reported in the context of HMT/HDM inhibitor profiling but which could easily be adapted for that purpose.

Antibody-based assays

A traditional antibody-based assay commonly used for the analysis of global cellular levels of specific histone modifications upon compound treatment is nuclear extraction followed by Western blot or a sandwich immunoassay such as enzyme-linked immunosorbent assay (ELISA) using histone methylation-specific antibodies. While being a classical technique, Western blot suffers from low-throughput and is semi-quantitative in nature. The availability of commercial histone methylation ELISA kits, particularly for modifications on H3, provides a moderately sensitive method for detecting global changes in histone methylation levels. A major drawback of these methods is that they require cell lysis followed by acid nuclear extraction, which impacts the throughput level and introduces variability. A more sensitive version of the ELISA is the dissociation-enhanced lanthanide fluorescent immunoassay (DELFIA), which has been modified to quantitate histone methylation levels directly from cells lysates. In this method, cells are grown in 384-well microplates and lysed in an acidic buffer and histones are captured efficiently on a MaxiSorp high binding plate. Following washing and primary antibody binding steps, a secondary antibody labeled with a lanthanide chelate is added, which produces a fluorescent signal upon addition of an enhancement solution (Figure 1a) [16]. A similar DELFIA approach that used fixed and permeabilized cells instead of cell lysates was previously employed to detect global H4R3

upon treatment with selected histone arginine methyltransferase inhibitors. However, this assay was of relatively low-throughput [17].

Other antibody-based cellular assays for the detection of global histone methylated levels include the homogenous cell-based AlphaLISA (PerkinElmer), LanthaScreen (Life Technologies) and EPigeneous HTRF (Cisbio) platforms. AlphaLISA detects changes to epigenetic marks on endogenous histones catalyzed by endogenous enzymes. The assay is currently formatted to investigate methylation of histone 3. In a typical assay, cells are treated with compounds in a 384-well microplate format. Following a homogeneous histone extraction protocol, the histone mark of interest is detected by the addition of a biotinylated anti-histone 3 (C-terminus) antibody and an antibody specific to the methyl mark, which is conjugated to AlphaLISA acceptor beads. The biotinylated antibody is then captured by streptavidin-coated donor beads, bringing the two beads into proximity. Upon laser irradiation, donor beads and acceptor beads in proximity generate an amplified chemiluminescent signal (Figure 1b). Quian *et al.* developed an AlphaLISA assay for the quantification of H3K27me3 levels for the screening and profiling of EZH2 inhibitors [18]. The LanthaScreen assay combines TR-FRET (terbium-based time-resolved fluorescence resonance energy transfer) technology with the BacMam (Baculovirus-mediated gene transduction of mammalian cells) gene delivery system. In this assay, cells are transduced with BacMam to express the histone of interest as a fusion with the green fluorescent protein (GFP). The posttranslational modification is then detected upon cell lysis in the presence of a terbium-labeled antibody specific to the methyl mark of interest. Upon excitation, the level of energy transfer between the two fluorophores, the terbium donor and the GFP acceptor, is quantified (Figure 1c). This technology has been applied to detect several histone H3 site-specific modifications, including H3K4me2, H3K4me3, H3K9me2, and H3K27me3 [18, 19]. The GFP-histone fusion protein can be introduced to different cell lines through transient transfection, offering flexibility in the cellular background. A similar assay format is provided in the EPIgeneous cell based assay (Cisbio), where the specific histone modification of interest is measured by TR-FRET using a sandwich assay format with a primary antibody labeled with europium (donor) and secondary labeled with d2 (acceptor) (Figure 1d). These three methods detect responses that are averaged cell population effects and are, due to an additional cell lysis step, less close to the native cellular state.

Chromatin immunoprecipitation (ChIP) assays that utilize methylation-specific antibodies to monitor histone methylation changes at target genes may also be used. In a standard ChIP assay, cells are treated with inhibitors followed by treatment with a fixing agent (i.e. formaldehyde) to covalently crosslink protein to DNA and lysed. The chromatin is subsequently sheared by sonication or enzymatic fragmentation of DNA and immunoprecipitated utilizing a highly specific antibody to the mark of interest. The DNA target of interest is subsequently detected via qPCR (ChIP-qPCR or ChIP-chip) (Figure 1e). For example, Vedadi *et al.* and Kubicek *et al.* utilized ChIP-qPCR to investigate the decrease in H3K9me2 abundance at specific loci upon treatment with G9a/GLP inhibitors [13, 14]. Advances in liquid handling steps of the immunoprecipitation process currently allows for the processing of multiple samples. In addition, utilization of next generation

technologies (ChIP-Seq) could allow the assessment of inhibitor effects at a genome-wide level.

One of the first methods applied to the *in vivo* profiling of HMT and HDM inhibitors was imaging antibody staining in fixed cells, a method sometimes referred to as “in-cell western” [11, 14, 20]. This assay quantifies the levels of a specific histone mark by normalizing the signal provided by a fluorescently-labeled antibody specific to the desired mark to the cell number determined *via* a nucleic acid dye (Figure 1f). Widely employed, this type of assay was for example utilized to profile the effect of G9a and GLP inhibitors on the di-methylated state of histone H3K9, DOT1L inhibitors on the di-methylated states of H3K79, and pan-histone demethylase inhibitors on H3K9 and H3K4 methylation [14, 20-22]. King *et al.* reported a similar cellular assay to specifically and quantitatively measure the effect of small molecule inhibitors of JMJD2 histone demethylase activity towards a H3K9 tri-methylated substrate [11]. Although these approaches allowed for rapid processing of multiple samples, they were not optimized for large throughputs that could support a primary screen. The throughput issue was recently addresses by the development of automated High Content Screening (HCS) strategies. These HCS imaging platforms use single-cell multiparameter measurements to accurately assess cellular viability and enzymatic activity on specific substrates. Mulji *et al.* recently described a HC imaging approach to prosecute a medium-throughput screen of small molecules to identify inhibitors of the demethylase JMJD3 [23]. By assessing cellular viability and enzyme-dependent demethylation of the H3K27me3 mark by exogenously expressed JMJD3, the authors successfully identified several chemotypes with inherent cellular permeability and good physicochemical properties. A similar approach was reported recently by *Luense et al*., where an HC imaging assay was used to identify inhibitors of the histone methyltransferase EZH2 on H3K27me3 substrate [24]. The high-throughput capacity (384 and 1536-well) of these assays renders them suitable not only for profiling but also for primary HTS of cell-active inhibitors. Finally, a HC imaging assay to quantify the H3K27me3 levels in cells in response to ~6,000 selected chemical probes, was implemented to identify pathway-selective regulators of H3K27me3 in cancer cells [25]. Collectively, these approaches have the added advantage of informing on any cellular toxicity effect of tested inhibitors and are amenable to multiplexing by using different fluorescently-labeled antibodies to detect multiple histone modifications simultaneously. However, a major drawback of these and other antibody-based approaches is the frequently undefined degree of specificity of the antibody for the methylation site and state, which has to be verified *a priori*, and potential influences of other post translational modifications nearby that also need to be considered. Work by Egelhofer and colleagues demonstrated that about 25% of commercially available histone-modification antibodies have specificity or utility issues [26].

Intracellular inhibitor-target binding assays

Cellular ligand binding platforms are powerful tools to determine compound binding to its protein target in a native cellular environment and report on inhibitor cell-permeability, target engagement and intracellular potency. These platforms rely on the well-described attribute of protein targets to change their biophysical properties (i.e. half-life and thermal stability) when bound to small molecules. The “InCELL Hunter” enzyme fragment

complementation (EFC) assay commercialized by DiscoveRx is currently available for testing small molecule binding to G9a and GLP methyltransferases. The cellular thermal shift assay (CETSA) is a recently developed method that also reports on drug-target interactions *in vivo* [27]. Although not yet implemented on epigenetic targets, we believe CETSA merits attention because of its amenability to high-throughput screening and adaptability to potentially all kind of targets.

The EFC platform utilizes two inactive fragments of β-galactosidase (β-gal), the enzyme donor (ED) and the enzyme acceptor (EA), which combine to create an active enzyme. In the InCELL Hunter, a stable cell line is engineered to express the epigenetic enzyme of interest as a fusion protein with the ED. The ED in this case is a small fragment (enhanced ProLabel or ePL tag) that lacks significant tertiary structure and does not alter the half-life of the tagged protein. After compound treatment, protein-ePL fusion is detected by the addition of chemiluminiscent detection buffer including the EA fragment of β-gal enzyme. Thus the level of β-gal activity is directly proportional to the amount of protein-ePL fusion in the cells, which is stabilized by binding of small molecules (Figure 1g) (www.discoverx.com).

CETSA involves the treatment of cells with compounds of interest followed by heating to denature and precipitate proteins, cell lysis, and the separation of cell debris and aggregates from the soluble protein fraction. Whereas unbound proteins denature and precipitate at elevated temperatures, ligand-bound molecules remain in solution. The detection of stabilized endogenous epigenetic target is performed directly in solution by implementing a homogeneous antibody-based assay like AlphaScreen, in which an optimized configuration of antibodies is used to bring donor and acceptor beads into proximity to generate a chemiluminescent signal (similar to AlphaLISA assay described above) (Figure 1h) [28]. Importantly, CETSA can be utilized in both temperature-scan mode and in isothermal conditions.

Mass spectrometry-based assays

Mass spectrometry (MS)-based methods are used to quantify HMT and HDM enzymatic activity in response to small molecule modulation using *in vitro* conditions (i.e. purified proteins/peptides). The label-free mass spectrometry platform RapidFire (Agilent Technologies) enables small molecule screening and inhibitor profiling of histone modifying enzymes in a medium throughput format. For example, screens for LSD1 and JmjD2 histone demethylase inhibitors using RapidFire have been reported [29-31]. However, MS-based methods to quantify HMT and HDM activity in cells have been seldom implemented. MacKeen *et al.* reported a MS-based method for analysis of JMJD2A inhibition in cells by monitoring levels of H3K9me3, the preferred substrate of JMJD2A [32]. In this assay, cells were treated with desired inhibitor followed by acidic extraction of histone proteins, SDS-PAGE separation, chemical derivatization and proteolysis and finally analyzed by ultraperformance liquid chromatography high/low collision switching-MS (UPLC-MS) (Figure 1i). In the report by Kubicek *et al.*, authors determined the effect of G9a/GLP inhibitors on H3K9me2 levels in bulk H3 histone preparations by quantitative MS [13]. This is a sensitive method, but given its multiple steps, it does present capacity limitations.

Gene expression assays

The change in activity of HMT/HDM upon inhibitor treatment can be monitored by changes in mRNA levels of genes known to be regulated by a specific histone methylation state. Several examples of loci under direct control of a specific histone modifying enzyme exist and have been utilized as surrogate for HMT/HDM activity [14, 17, 33, 34]. For example, EZH2 inhibitors have been shown to reduce H3K27me3 levels at the promoter region of TXNIP, thereby increasing TXNIP transcript levels [35, 36]. Transcript levels are usually measured by qRT-PCR, although high-throughput transcription-based profiling is also well suited for these epigenetic targets (Figure 1j) [34]. Two disadvantages of gene expression assays are their throughput limitations and also, in some cases, the fact that modifications in specific histone methyl marks do not lead to transcriptional consequences.

Instead of measuring transcript levels of endogenous genes, assays have been developed to monitor levels of exogenous reporters. High throughput fluorescence-based imaging cellular assays for screening and profiling of epigenetic modulators exploit the susceptibility of viral promoters to epigenetic silencing in mammalian cells [34, 37, 38]. In these assays a viral promoter-reporter constructs with an optical readout is used to determine the effect of candidate compounds on gene silencing (Figure 1j). Vedadi *et al.* investigated the ability of selected G9a methyltransferase inhibitors to reactivate silent retroviral-GFP vectors in mouse embryonic stem cells (a mechanism that requires H3K9 di-methylation) [14]. A broad unbiased cell-based screening strategy, referred to as locus-derepression assay (LDR), was employed by Wang and colleagues and led to the successful identification of broad spectrum Jmj demethylase inhibitors [34]. The LDR assay detected derepression of a stably integrated CMV-GFP reporter that is normally silenced in a murine c127i mammary cell line. Compounds that reactivated GFP expression were identified by enumerating GFP positive cells using a laser-scanning microplate cytometer.

Cellular phenotypic assays

In cellular phenotypic assays, compounds are screened against a cell line of interest for the appearance of the desired phenotype (i.e. cell death, proliferation, differentiation, etc.). This type of assay is employed when there is a body of biological evidence that connects the target to the phenotype. Cellular phenotypic assays have the advantage of identifying disease-relevant modulators; however, since multiple cellular pathways could lead to the same phenotype, the influence of compounds on any of these pathways may be misinterpreted as target enzyme inhibition. The use of pure phenotypic assays has been limited in histone methylation drug discovery primarily because of the relatively poor understanding of the interplay between HMT/HDM activities and cellular environment. However, the level of target validation for certain enzymes supports the implementation of phenotypic assays for follow up studies. For instance, inhibitors of Jarid1B histone demethylase have been shown to inhibit the proliferation of breast cancer cells [39]. Yu *et al.* and Daigle *et al*. demonstrated that specific DOT1L inhibitors selectively kill cells bearing *MLL* chromosomal rearrangements (Figure 1k) [21, 33]. Mannironi and colleagues undertook an *in vivo* phenotypic approach to identify Jumonji C domain-containing HDMs inhibitors in yeast. The screening system tested the effects of candidate inhibitors on an

engineered *S. cerevisiae* strain which requires Jhd2 (JARID1 homolog) demethylase activity to efficiently grow in the presence of rapamycin [40].

Conclusion

To date, several chemical probes/inhibitors of some HMTs and HDMs have been discovered and a few of them are in preclinical and clinical studies (examples in www.clinicaltrials.gov include two LSD1 inhibitors for relapsed or refractory acute myeloid leukemia and small-cell lung carcinoma and one EZH2 inhibitor for advanced solid tumors or B cell lymphomas). However, many of these inhibitors have been discovered on the basis of their structural similarity to enzyme co-substrates or other known inhibitors and suffer from limited specificity and cellular potency. In addition, there are numerous HDM/HTM enzymes with unknown role(s) in human disease for which inhibitors have not been developed yet. Here, we have described numerous cell-based tools that should facilitate chemical screening/profiling of these epigenetic targets to support lead discovery programs in the development of novel chemotypes with increased specificity and cellular potency.

Addition of DELFIA enhancement solution dissociates the Eu^{3+} ion from the immunocomponent allowing the formation of a highly fluorescent chelate in solution. Fluorescence is read with 320/615 nm (Ex/Em) wavelengths. (b) AlphaLISA: two antibodies are utilized, one biotinylated against the C-terminus end of the histone protein and the other against the specific methyl mark (located in the N-terminus of histones), which is conjugated to an AlphaLISA acceptor bead. The biotinylated antibody is then captured by streptavidin-coated donor beads. When the two beads are in proximity, laser irradiation of donor beads at 680 nm produces a short-lived singlet oxygen molecule which reaches the proximal acceptor bead to generate an amplified chemiluminescent signal detected at 615 nm. (c) LanthaScreen: prior to compound treatment, the histone of interest is expressed as a GFP-fusion via BacMam transduction. After cell lysis, the posttranslational modification of interest is detected with a Terbium (Tb)-labeled antibody. When in close proximity, Tb excitation with a 340 nm laser triggers an energy transfer to the GFP fluorophore, which in turns emits at 495 nm. (d) EPIgeneous: two antibodies are used, one specific to the modified H3 labeled with the FRET donor Eu^{3+} and the other specific to H3 labeled with a FRET acceptor d2. When in close proximity, excitation of the donor fluorophore with a light source of 320 nm transfers energy towards the acceptor, which in turn fluoresces at 665 nm. (e) CHIP: after compound treatment, histone-DNA complexes are covalently crosslinked and lysed. The chromatin is subsequently sheared and immunoprecipitated utilizing a highly specific antibody to the mark of interest. Following histone digestion, different gene-specific approaches can be used to determine how much DNA of interest has been precipitated. These approaches include quantitative real time PCR (qPCR), chip or deep sequencing. (f) Imaging staining: after compound treatment, cells are fixed, permeabilized and stained with a fluorescently-labeled antibody specific to the methyl mark of interest. Total cell number is determined by staining with a nuclear dye. *Intracellular inhibitor-target binding assays* (g and h): (g) InCELL Hunter utilizes an engineered cell line that expresses the epigenetic enzyme of interest fused to the ePL tag of β-gal. After compound treatment and lysis, protein-ePL fusion is detected by the addition of detection buffer containing the complementary fragment of β-gal (EA). Reconstituted enzymatic activity is measured by the

substrate cleavage which generates a chemiluminescent signal. (h) CETSA: after compound treatment, cells are heated to denature proteins and lysed. The soluble fraction containing stabilized compound-enzyme complexes is separated and quantified by the AlphaScreen assay with an antibody pair directed against different portions of the targeted enzyme. In this example, one of the antibodies is fused to biotin and has low affinity for protein A (PA). Induced proximity of streptavidin-coated donor and PA-coated acceptor beads upon binding to soluble target enzyme generates a chemiluminescent signal. (i) *Mass spectrometry*. Upon compound treatment, cells are lysed and histone are extracted and separated on a SDS-PAGE followed by chemical derivatization. After proteolysis, histone peptides are identified and quantified by liquid chromatography tandem MS (LC-MS). (j) *Gene expression assays.* The activity of targeted enzyme is indirectly measured by quantifying transcript levels of endogenous genes (top) or a reporter gene (bottom) known to be regulated by a specific histone methylation state. (k) *Cellular phenotypic assays*. This example shows a viability assay upon compound treatment. Specifically, DOT1L inhibitors selectively kill cells bearing *MLL* chromosomal rearrangements.

Compound; histone; streptavidin (SA); antibody; enzyme donor epL tag; enzyme acceptor; epigenetic enzyme; Protein A (PA); nuclear dye (ND).

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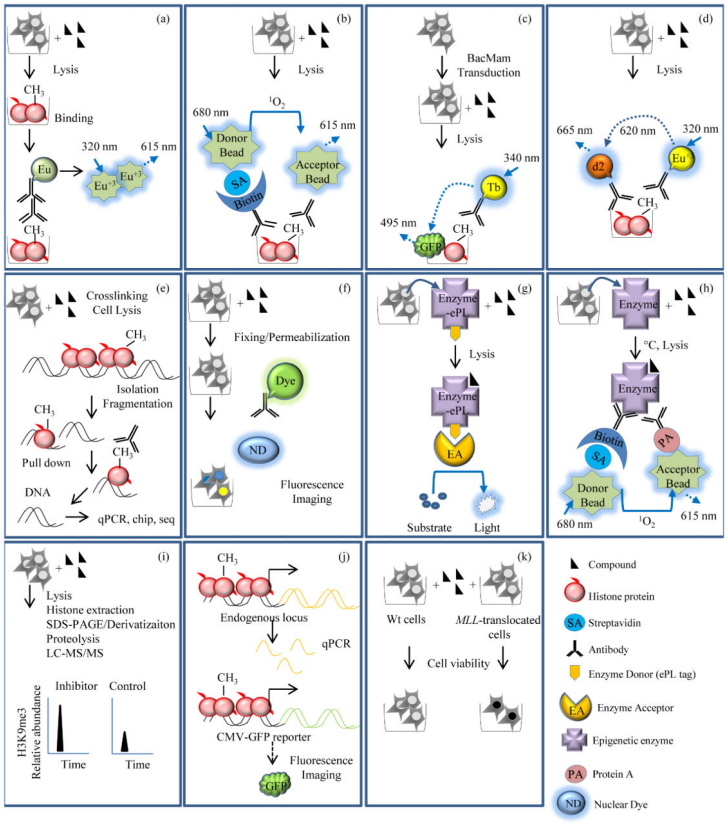

Figure 1.

Antibody-based assays (a, b, c, d, e and f): After compound treatment, cells are lysed and the histone modification of interest is detected using at least one specific antibody in one of the following modalities. (a) DELFIA: histones are directly captured on a high binding plate followed by antibody binding and detection *via* a Europium (Eu)-labeled secondary antibody.

Table 1

Advantages and limitations of cell-based assay platforms for screening and profiling compounds against HMT and HDMs.

Assay	Method	Throughput	Label	Advantages	Limitations	Example	Reference
Antibody-based	DELFIA	Low/Medium	Free		Antibody specificity; cell lysis step	H3K27me3	[16]
	AlphaLISA	Medium	Free	Homogeneous; high sensitivity	Antibody specificity; cell lysis step; potential signal interference	H3K27me3	[18]
	LanthaScreen	Medium	GFP-Histone	Homogeneous; high sensitivity	Antibody specificity; histone is labeled and overexpressed; cell lysis step; potential signal interference	H3K4me2, H3K4me3, H3K9me2, H3K27me3	[18,19]
	Epigeneous HTRF	Medium	Free	Homogeneous; high sensitivity	Antibody specificity; cell lysis step; potential signal interference	H3K4me2, H3K27me3, H3K36me2	
	ChIP (qPCR)	Low	Free	High sensitivity; high specificity	Antibody specificity; cell lysis step; knowledge of target loci required	H3K9me2 at multiple loci in mouse ESC and fibroblasts	[13]
	High Content Imaging	High	Flag-enzyme/ Free	Reports on individual and cell population; multiple methylation status at once	Antibody specificity; indirect effects; labeled and overexpressed enzyme	JMJD3/EZH2/H3K27me3	[23-25]
Binding	InCELL Hunter	Medium	ePL-enzyme	Homogeneous; high sensitivity	Cell lysis step; potential signal interference; stable cell line required	G9a/GLP	
	CETSA-AlphaScreen	Medium	Free	Homogeneous	Cell lysis step, potential signal interference; optimal antibody pair required	Not yet applied to HTM/HDM targets	
Mass Spectrometry	UPLC-MS	Low	Free	High sensitivity, high specificity, multiple methylation status at once	Complex protocol	JMJD2A/H3K9me3	[32]
Gene expression	qPCR	Low/Medium	Free	High sensitivity, high specificity	Knowledge of target loci required; indirect effects	EZH2/H3K27me3/TXNIP gene	[35,36]
	LDR	High	Free	High sensitivity	Indirect effects; reporter cell line required	JmjC/viral promoter-GFP cell line	[34]
Phenotypic	cell viability	High	Free	Disease relevant hits	Knowledge of target-cellular pathway; indirect effects	DOT1L/cells with MLL rearrangements	[21,33]
	yeast cell viability	Low/Medium	Free	Disease relevant hits	Knowledge of target-cellular pathway; indirect effects; hits need to be validated in human cells	Jhd2 (JARID1 homolog)/engineered yeast strain	[40]