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Novel Scaffolds of Cell-Active Histone Demethylase Inhibitors Identified from High-Throughput Screening

Wei Wang[1], Laura J. Marholz[1], and Xiang Wang[1]

Abstract

Jumonji C domain-containing histone demethylases (JHDMs) are epigenetic proteins capable of demethylating methylated lysine residues on histones proteins and for which high-quality chemical probes and eventual therapeutic leads are highly desirable. To expand the extent of known scaffolds targeting JHDMs, we initiated an unbiased high-throughput screening approach using a fluorescence polarization (FP)–based competitive binding assay we recently reported for JHDM1A (aka KDM2A). In total, 14,400 compounds in the HitFinder collection v.11 were screened, which represent all the distinct skeletons of the Maybridge Library. An eventual three compounds with two new scaffolds were discovered and further validated, which not only show in vitro binding for two different JHDMs, JHDM1A and JMJD2A (aka KDM4A), but also induce hypermethylation of their substrate in cells. These represent novel scaffolds as JHDM inhibitors and provide a basis for future optimization of affinity and selectivity.

Keywords

epigenetics, fluorescence polarization competition assay (FPCA), Jumonji C domain-containing histone demethylase (JHDM), immunofluorescence, high-throughput screening (HTS)

Introduction

Epigenetic modifications of histone proteins are an essential component in the regulation of gene expression through mechanisms such as chromatin remodeling and the recruitment or obstruction of reader proteins.[1] One such covalent modification is the methylation of different lysine or arginine residues, which can be either activating or deactivating depending on their positions and methylation states within the histone.[2] The lysine methyl marks are reversible, and their removal is regulated by the epigenetic-modifying proteins called histone demethylases. Because histone methylation is central to understanding many biological processes and disease states, these proteins are attractive targets for the development of selective small-molecule inhibitors.[3,4] Histone demethylases are divided into two families of proteins depending on how they catalyze the demethylation reaction. The first family (KDM1) uses flavin adenine dinucleotide (FAD) as a cofactor, while the second family (KDM2-8) is divided into seven classes, which all contain a Jumonji C catalytic domain dependent on Fe(II) and α-ketoglutarate (αKG).[3] The members of the second family, also known as JHDMs, are known to have strict substrate scopes depending on the position of the lysine within the histone and its methylation state (mono-, di-, or trimethyl). Many of these enzymes have been reported to possess unique cellular functions and roles in disease, and hence there is a strong need for highly selective inhibitors that can differentiate between members/classes of JHDMs.[5]

Currently, most JHDM inhibitors either mimic their substrate (lysine)/cofactor (αKG) or are metal chelators capable of binding the Fe(II) in the active site.[6–8] These approaches are limited because any enzyme that contains a Jumonji C domain or are Fe(II)/αKG dependent may be inhibited. These include hypoxia-inducible factors (HIFs), prolyl 4-hydroxylases (PHDs), and 5-methylcytosine hydroxylases (TETs). Among these, there is considerable evidence that while the Fe(II) and αKG binding sites tend to be more or less structurally conserved, the substrate binding sites tend to be more flexible in scope and therefore more

[1]Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO, USA

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Corresponding Author:
Xiang Wang, Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO 80309, USA.
Email: xiang.wang@colorado.edu

easily exploited to possibly heighten JHDM class and isoform selectivity.[9,10] However, success in actually developing specific inhibitors has been extremely limited so far. Presently in the literature, few examples have been reported, such as the KDM6 subfamily selective inhibitor GSK-J1 and its analogues.[11] Removal of this molecule's iron-chelating ability rendered the inhibitor inactive, however, demonstrating again dependence on metal chelation for JHDM inhibition. Completely novel inhibitor scaffolds of JHDMs are greatly needed to increase the specificity and consequently the value of small-molecule inhibitors for the studies of the cellular functions of these enzymes.

Our methodologic approach toward these ends was the application of our previously reported[12] fluorescence polarization (FP)–based competition assay for screening small-molecule binders of the protein JHDM1A, a member of the KDM2 family capable of demethylating mono- and dimethylated histone H3 lysine 36 (H3K36me1/2). An initial high-throughput screen of 14,400 compounds from the commercially available HitFinder collection v.11 (Maybridge, Thermo Fischer Scientific, Waltham, MA) against JHDM1A was conducted in duplicate in a 384-well plate format. From the complete collection, an eventual three hit compounds were discovered that comprised two novel scaffolds never before seen in demethylase inhibitors. These three were then subjected to additional investigation to both verify and define their efficacy. The half maximum inhibitory concentration (IC_{50}) and dissociation constant (K_i) toward JHDM1A and JMJD2A were determined and compared in individually generated binding curves. Further studies revealed the activity of these novel JHDM inhibitors in cells. These compounds and the methodology of their discovery offer a means toward the eventual goal of class- and isoform-selective JHDM inhibitors. This is the first reported application of a high-throughput FP-based screen to discover histone demethylase inhibitors. These novel scaffolds lack the chelating group to the Fe(II) cofactor and provide a foundation for further study and optimization of their structure-activity relationships to enhance activity and selectivity.

Materials and Methods

Protein Expression and Purification

Recombinant JHDM1A (1−517 aa) and JMJD2A (1−359 aa) were expressed as 6XHis fusion proteins using the pNIC28 and pET28b expression vectors, respectively. The coding regions were verified by sequencing, and the plasmids were transformed into BL21 *Escherichia coli*. Following expression, JHDM1A and JMJD2A were purified using Nickel Agarose Beads (High Density; Gold Biotechnology, St. Louis, MO) by gravity chromatography according to the manufacturer's instructions. The purified proteins were exchanged into assay buffer (Tris 25 mM, NaCl 100 mM, $NiCl_2$ 50 μM, pH 7.5 for JHDM1A and pH 6.8 for JMJD2A), flash frozen in liquid nitrogen, and stored at −80 °C.

Compound Library

The High-Throughput Screening Core Facility in the University of Colorado Boulder has purchased a drug-like diversity library, HitFinder collection v.11, from Maybridge, which contains 14,400 distinct compounds. This compound library was selected as the diversity set from the vendor's entire compound collection, representing all the skeletons available. The compound library was reformatted to the standard 384-well plates after purchase for high-throughput screening (HTS).

FP-Based HTS

In total, 200 nL of each compound (10 mM in DMSO) was transferred from 384-well compound plates to a black 384-well low-volume microplate (Corning 3677; Corning, Corning, NY) containing 16 μL of 300 nM JHDM1A in FP assay buffer (Tris-HCl 25 mM [pH 7.5], NaCl 100 mM, $NiCl_2$ 50 μM) by a Cybi-Well 384-channel simultaneous pipettor (CyBio, Jena, Germany). After incubation for 30 min, 4 μL methylstat^{fluor} (1 nM in assay buffer) was added to each well by a MicroFill liquid dispenser (BioTek, Winooski, VT). The plates were incubated at room temperature for 4 h before signals were recorded by an EnVision MultiLabel plate reader (PerkinElmer, Waltham, MA) using a standard light polarizer and filters.

Counterscreen

For the counterscreen plate, 16 μL assay buffer instead of JHDM1A was added to a black 384-well low-volume microplate, and 200 nL compounds was transferred from the compound plate to achieve final compound concentrations of 100 μM. After a 30-min incubation, 4 μL methylstat^{fluor} (1 nM in assay buffer) was added using the MicroFill liquid dispenser (BioTek). The plates were read after incubating for 4 h at room temperature using an EnVision MultiLabel plate reader (PerkinElmer).

FP Competitive Binding Assay

Four concentrations (20, 10, 5, and 2.5 μM) of each of the 100 cherry-picked compounds were prepared as 100× solutions in DMSO. Then, 16 μL JHDM1A (300 nM in assay buffer) was added to each well, to which 200 nL of the above compound solutions was added. The mixtures were incubated at room temperature for 30 min prior to addition of 4 μL methylstat^{fluor} (1 nM in assay buffer). The experiment was performed in duplicate. The assay plates were incubated at room temperature for 4 h before signals were recorded on an EnVision MultiLabel plate reader (PerkinElmer).

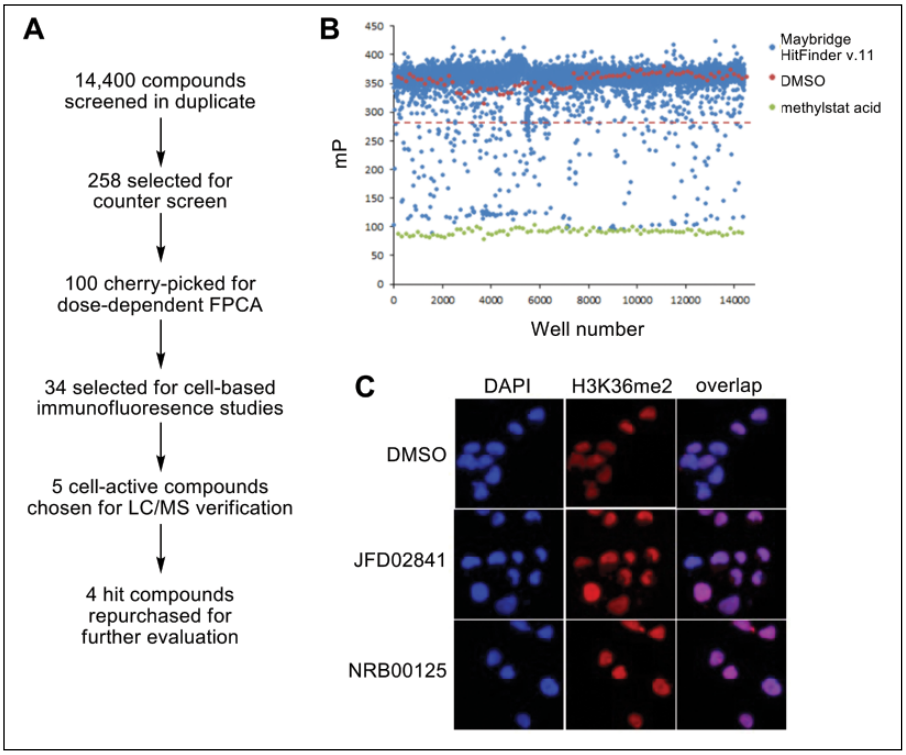

Figure 1. (**A**) Scheme of workflow identifying novel scaffolds of cell-active Jumonji C domain-containing histone demethylase (JHDM) inhibitors. (**B**) High-throughput screen of the Maybridge HitFinder collection v.11 (14,400 compounds) in a 384-well plate format. DMSO was used as a negative control (red), and methylstat acid was used as a positive control (green). Compounds were screened in duplicate at a final concentration of 100 μM. The orange dashed line indicates the cutoff mP value of 280 for selecting positives. (**C**) Representative immunofluorescence assay results for two positives demonstrating cellular activity through an increase in the H3K36me2 epigenetic mark. HeLa cells were treated with individual compounds with a series of concentrations (image shows 1.56 μM for JFD02841 and 0.39 μM for NRB00125) for 48 h before visualization.

The half maximum inhibitory concentrations (IC_{50}) toward JHDM1A of the final four hit compounds were determined as previously described,[12] and the inhibitor dissociation constants (K_i) were calculated using the online K_i calculator.[13] Another FPCA toward JMJD2A was optimized with an alternative fluorophore (**Suppl. Fig. S1**), and the binding data were calculated as before.

Immunofluorescence Assay

HeLa and MiaPaCa2 cells were seeded in 96-well optical bottom tissue culture plates (165305; Thermo Scientific, Waltham, MA) in Dulbecco's modified Eagle's medium (DMEM). Six hours later, 1 μL of a serial dilution of compounds was added in each well (50 μM to 25 nM). Similar dilutions of methylstat acid[14] and DMSO were used as positive and negative controls, respectively. After incubation for 48 h, cells were fixed in 4% paraformaldehyde in Dulbecco's phosphate buffered saline (D-PBS), permeabilized, blocked with bovine serum albumin (BSA), and then treated with methylation-specific antibodies, rabbit polyclonal anti-H3K36me2 (ab9049; Abcam, Cambridge, MA), or rabbit polyclonal anti-H3K9me3 (07-442; Millipore, Billerica, MA), respectively, followed by Alexa Fluor 488–labeled goat–anti-rabbit secondary antibody (Invitrogen, Carlsbad, CA) and Hoechst 33258 stain (Sigma, St. Louis, MO). Images were captured and analyzed using the ArrayScan VTI High Content Screen (HCS) Reader (Thermo Scientific).

Results and Discussion

Quantitative HTS of the Maybridge HitFinder Collection v.11

The initial HTS was performed with the optimized and miniaturized FP-based competition assay as described. This entailed testing all 14,400 distinct compounds in duplicate at a single final concentration of 100 μM for the ability to competitively bind to JHDM1A in the presence of methylstat^{fluor}, our fluorescent tracer. The polarization of each assay was calculated, and the results are visualized in **Figure 1B**, which shows the quantitative polarization value of each compound in comparison with DMSO (negative control) and methylstat acid (positive control). Of the entire collection, 258 compounds were selected because their reported mP values were below the cutoff threshold mP of 280.

Confirmation of Positives

The counterscreen was designed to assist in narrowing the list of positives by eliminating false positives due to aggregation or autofluorescence. False positives were identified when the reported mP value did not change in the presence versus absence of the JHDM1A protein. Of these 258 positives, 100 compounds were then "cherry-picked" to undergo another round of confirmation FP assay analysis using four concentrations (20, 10, 5, and 2.5 μM). A smaller subset of

34 compounds was able to display concentration-dependent competitive binding to JHDM1A and was selected for the first initial round of immunofluorescent studies to test for cellular activity.

Initial Cell-Based Study of Positives Identified by HTS

To identify compounds that were cell active, we next performed immunofluorescence assays to test for the ability to increase cellular levels of the H3K36me2 epigenetic mark. This was chosen because JHDM1A is a known H3K36me1/2 demethylase. HeLa cells were treated with various concentrations of the 34 compounds for 48 h before visualization (data not shown). A final subset of five hit compounds presented the ability to induce hypermethylation of H3K36me2 in cells (see representative images in **Fig. 1C**). The average increase in comparison with the DMSO control was around twofold, with cell death observed at high concentrations (>50 μM). These five hits were verified by high-performance liquid chromatography/mass spectrometry (HPLC/MS) analysis for purity and molecular mass. Curiously, only four of the five expected masses were able to be identified. These four compounds were designated the final hits and repurchased from Maybridge for more intensive follow-up investigations.

In Vitro Profiling of Final Hits vs. Demethylases JHDM1A and JMJD2A

An accurate determination of the IC_{50}s and K_is was performed with a serial dilution of the four hit compounds versus JHDM1A according to our established protocol.[12] In addition, we wished to test whether these compounds had any ability to selectively inhibit one demethylase over another. To this end, we developed another FP-based competition assay for JMJD2A, a known H3K36me2/3 and H3K9me2/3 demethylase. This new assay uses a different fluorophore, which is similarly based on the design of methylstat. Its dissociation constant (K_d) was determined (**Suppl. Fig. S1**) and used, along with the individual IC_{50}s, in the calculations of the K_is of the four hits toward JMJD2A. The binding curves and their associated data are shown in **Figure 2**. Compound names were taken from their designations in the Maybridge collection. Compounds JFD02841 and NRB00125 share a similar scaffold and showed strong binding toward both proteins, with K_is of 0.42 and 0.28 μM toward JHDM1A and 0.52 and 0.42 μM toward JMJD2A, respectively (**Table 1**). Interestingly, the third hit, JFD00263, did not have any binding affinity whatsoever for either protein at any concentration. This compound that survived the initial HTS screen, counterscreen, and other initial testing was ultimately a false positive. The last compound, HTS12214, was the third most potent, with a K_i of 1.50 μM toward JHDM1A and 3.02 μM toward JMJD2A. In comparing the K_is, it is clear that these inhibitors have no selectivity between JHDM1A and JMJD2A. This might be unsurprising given that both proteins have similar substrates in H3K36 and therefore could be structurally similar. It is notable that this is the first time that binding constants between two different JHDMs were able to be directly compared. Further development of more FP-based competition assays toward an entire panel of JHDMs is highly desirable to further explore selectivity among JHDM classes and individual isoforms.

In Vivo Analysis of Final Hits Using an Immunofluorescence Assay in MiaPaCa2 Cells

We next wished to further validate and test the cellular activity of the three remaining hits. For this, we again used an immunofluorescence assay to test induction of histone hypermethylation in cells when treated with these compounds. This time, a more extensive series of concentrations was tested to determine the half maximum effective concentration (EC_{50}). Two different epigenetic marks were tested, H3K36me2 and H3K9me3, using methylation-specific antibodies, which are the substrates of JHDM1A and JMJD2A, respectively. Human pancreatic ductal adenocarcinoma MiaPaCa2 cells were used in these studies,[15] and methylstat was used as a positive control and benchmark. The complete dose curves and EC_{50} data for the three hits and methylstat are shown in **Figure 3**. All compounds tested showed concentration-dependent increases (from two- to sixfold) in both epigenetic marks, validating their cellular activity. The lowest half maximum effective concentration was seen by NRB00125 with 0.28 μM toward H3K36me2 and 0.66 μM toward H3K9me3. Intermediate activity was displayed by HTS12214 with an EC_{50} of 0.93 and 0.90 μM toward H3K36me2 and H3K9me3, respectively. These values are comparable with those seen by methylstat (0.96 and 1.18 μM). The highest values were presented by JFD02841, with 1.26 and 3.96 μM, respectively. It is interesting that the most structurally similar hits (JFD02841 and NRB00125), which also had the highest in vitro activity, had the widest variation in EC_{50}s. Their structures suggest that the dimethylamino moiety may resemble and therefore compete with the substrate of JHDM, methyllysine, and the modification of their pyridinium fragments may induce different cellular effects. In addition, growth inhibition data were collected via a CellTitre-Glo luminescent cell viability assay (Promega, Madison, WI) using the MiaPaCa2 cells. The GI_{50}s varied quite widely, with 1.9 μM for JFD02841, 0.071 μM for NRB00125, and >200 μM for HTS12214.

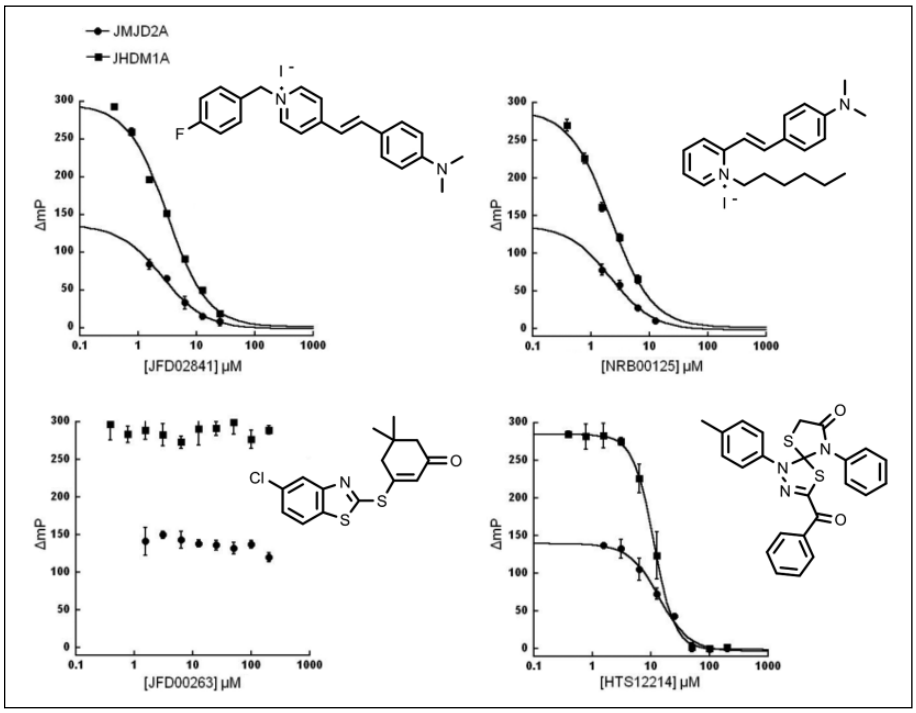

Figure 2. Fluorescence polarization competition curves of hit compounds versus demethylases JHDM1A and JMJD2A. JFD00263 displayed no binding affinity for either protein and was therefore designated a false positive.

Table 1. Fluorescence Polarization Competition Results of Top Four Hits.

	JHDM1A		JMJD2A	
ID	IC_{50} (μM)	K_i (μM)	IC_{50} (μM)	K_i (μM)
JFD02841	3.17 ± 0.37	0.42 ± 0.05	2.58 ± 0.25	0.52 ± 0.06
NRB00125	2.16 ± 0.28	0.28 ± 0.03	2.16 ± 0.26	0.42 ± 0.06
JFD00263	>200	>200	>200	>200
HTS12214	11.2 ± 0.2	1.50 ± 0.03	13.9 ± 1.2	3.02 ± 0.27

No correlation was seen between growth inhibition and hypermethylation. In summary, albeit the lack of selectivity among JHDMs, the final three hits have demonstrated potent activity against JHDM1A and JMJD2A both in vitro and in cells.

Here we report the first application of our FP-based competition assay adapted for HTS. A screen of the 14,400 compounds comprising the Maybridge HitFinder Collection v.11 was performed. This assay was able to successfully identify compounds that bind to the JHDM1A active site and quantify their binding affinity. After a series of confirmatory tests, a final three hits were recognized as potent inhibitors of not only JHDM1A but also JMJD2A and displayed cellular activity by their ability to induce hypermethylation in cells. Most important, these compounds represent novel scaffolds that expand the set of those known to block the JHDM active site.

Currently, the most common JHDM inhibitors are those based on substrate or cofactor mimics or are substrate-cofactor conjugates. These, while often active in biochemical enzyme inhibition assays, frequently show poor cellular activity due to the presence of many off-target proteins that either contain the JmjC domain or are also dependent on Fe(II)/αKG for their enzymatic activity. The ultimate goal of JHDM selectivity, JHDM class selectivity, and JHDM isoform selectivity will be greatly enhanced by inhibitors that contain completely new skeletons and scaffolds from which potent and bioactive inhibitors can be optimized. HTS so far offers the best system to efficiently test a vast number of scaffolds.

This report proves the utility of our FP-based assay toward these goals. This technique has many unique advantages over the biochemical enzyme inhibition assays because those are often highly dependent on the quality of the protein, assay conditions, and the use of a JHDM substrate or cofactor for detection. FP allows a direct measurement of dissociation constants without the need for separation of bound versus free ligand, allowing completely perturbation-free measurements. The widely available commercial instruments and increasing

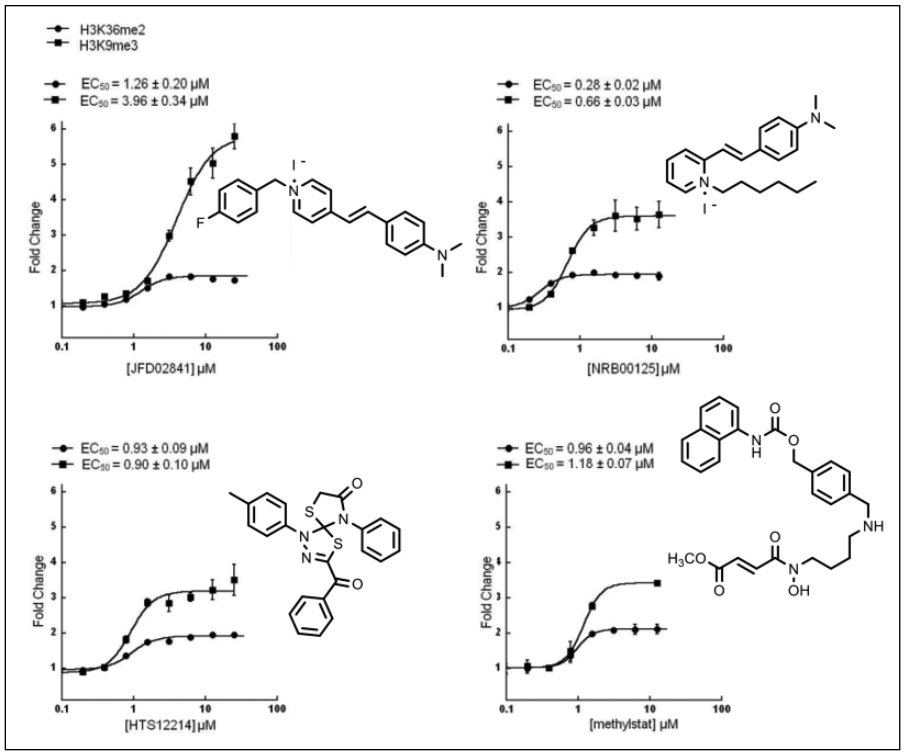

Figure 3. Immunostaining dose-response curves of MiaPaCa2 cells toward the H3K36me2 and H3K9me3 epigenetic marks in the presence of the final three hits and methylstat. Cells were dosed with a series of concentrations (50 μM to ~25 nM in twofold serial dilutions) and incubated for 48 h before visualization. Compounds JDF02841 and NRB00125 had the widest difference in EC_{50} despite having both the highest in vitro activity and structural similarity.

numbers of fluorescent probes available for JHDMs prove this is an attractive technique to compare binding affinities in vitro. The newly developed JMJD2A FP-based competitive binding assay allowed direct comparison of the selectivity of the inhibitors among different JHDMs using their dissociation constants, which is a novel and important development for the characterization of specificity of new inhibitors in this field. Development of a full panel of JHDM FP assays for systematic characterization of the in vitro selectivity of JHDM probes is under way and will be reported in due course. In addition, the newly discovered scaffolds reported here provide a foundation from which additional selectivity and affinity can be optimized through medicinal chemistry approaches.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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