Identification and Characterization of Small Molecule Inhibitors of a Plant Homeodomain Finger

Elise K. Wagner,† Nidhi Nath,‡ Rod Flemming,§ John B. Feltenberger,§ and John M. Denu*†⊥

†Department of Biomolecular Chemistry, University of Wisconsin, Madison, Wisconsin 53706
‡Promega Corporation, Madison, Wisconsin 53711
§University of Wisconsin Small Molecule Screening and Synthesis Facility, Madison, Wisconsin 53705
⊥Wisconsin Institutes for Discovery, University of Wisconsin, Madison, Wisconsin 53715

Supporting Information

ABSTRACT: A number of histone-binding domains are implicated in cancer through improper binding of chromatin. In a clinically reported case of acute myeloid leukemia (AML), a genetic fusion protein between nucleoporin 98 and the third plant homeodomain (PHD) finger of JARID1A drives an oncogenic transcriptional program that is dependent on histone binding by the PHD finger. By exploiting the requirement for chromatin binding in oncogenesis, therapeutics targeting histone readers may represent a new paradigm in drug development. In this study, we developed a novel small molecule screening strategy that utilizes HaloTag technology to identify several small molecules that disrupt binding of the JARID1A PHD finger to histone peptides. Small molecule inhibitors were validated biochemically through affinity pull downs, fluorescence polarization, and histone reader specificity studies. One compound was modified through medicinal chemistry to improve its potency while retaining histone reader selectivity. Molecular modeling and site-directed mutagenesis of JARID1A PHD3 provided insights into the biochemical basis of competitive inhibition.

In the nucleus of eukaryotes, genes are organized and compacted into chromatin, which is achieved in part through the wrapping of DNA around histone proteins.1 Histones are enriched for sites of posttranslational modification, which include methylation, acetylation, and phosphorylation.2 Histone modifications both influence chromatin structure and provide ligands for protein domains that recruit gene regulatory complexes to specific loci within the genome.3 These histone “reader” domains are specialized for certain histone modifications. For example, subsets of the PHD finger domain class bind unmodified, methylated, or acetylated lysine side chains.4 These domains generally bind their preferred modification with a high degree of specificity; for example, the PHD finger of ING2 binds histone H3 trimethylated at lysine 4 (H3K4me3), with markedly reduced affinity as the methylation state decreases, and almost negligible binding to the unmodified side chain.5 In addition to PHD fingers, there are other classes of histone binding domains, including bromodomains, chromodomains, tudor and tandem tudor domains, and 14-3-3 domains.6 Altogether, there are hundreds of histone reader domains, which contribute to exquisite control over gene expression.

When misregulated, a number of histone-binding domains are linked to disease, including cancer, autoimmunity, and developmental conditions.7 For example, the third PHD finger of JARID1A (JARID1A PHD3), which binds H3K4me3, is implicated in acute myeloid leukemia (AML).8 In a clinically reported case of AML, the patient expressed a genetic fusion protein containing nucleoporin protein 98 (NUP98) and the C-terminus of JARID1A, which includes its nuclear localization sequence and third PHD finger. A similar fusion protein was reported between NUP98 and the PHD finger of PHF23 in another AML patient.9 Later studies deduced that these genetic fusions caused aberrant transactivation of developmental genes required to maintain the myeloid progenitor state, which resulted in the onset of leukemia.10 The oncogenic properties of the NUP98-PHD finger fusion proteins are directly potentiated by the ability of the PHD finger to bind chromatin. Other examples of histone-binding proteins implicated in cancer include the overexpression of UHRF1 in lung cancer and TRIM24 in breast cancer.11 Because of the role of JARID1A PHD3 and other histone readers in disease, identifying small molecules that inhibit histone binding by these domains is of paramount importance. We predict that epigenetic drugs targeting histone-binding domains represent a new paradigm for the development of cancer therapeutics, which has only recently begun to be explored.12,13

To target histone readers for small molecule inhibition, we developed a 96-well plate assay that uses a HaloTag fusion to
the third PHD finger of JARID1A. HaloTag is a 34 kDa protein fusion tag that forms a specific covalent bond with its synthetic HaloTag ligand.13 HaloTag ligands can be attached to variety of surfaces to allow specific, irreversible, and oriented immobilization of a protein of interest fused to HaloTag.16–18 These features maximize functionality of the protein of interest while allowing stringent washing conditions. To leverage the advantages of HaloTag technology for small molecule screening of JARID1A PHD3, we developed an assay that uses 96-well polystyrene plates activated with HaloTag ligand for covalent and oriented capture of a HaloTag fusion to JARID1A PHD3. Small molecule inhibitors identified from screening were validated biochemically. Specificity studies allowed us to inform chemical modification of one hit compound to increase its potency as an inhibitor of JARID1A PHD3. Predictions made from molecular modeling allowed identification and biochemical analysis of residues within JARID1A PHD3 that contribute to competitive inhibitor binding.

## EXPERIMENTAL PROCEDURES

### General Reagents

Dimethyl sulfoxide (DMSO), tetraethylthiuram disulfide (disulfiram), phenothiazine, and amiodarone HCl were purchased from Sigma Aldrich. Tegaserod maleate, di-N-desethylamiodarone, and desethylamiodarone were purchased from Santa Cruz Biotechnology. TMR HaloTag ligand was purchased from Promega. SuperSignal West Pico ELISA chemiluminescent substrate, high sensitivity streptavidin-HRP conjugate, and high capacity streptavidin-agarose beads were purchased from Pierce. StabilCoat buffer was purchased from Surmodics. AlphaScreen histidine detection kits (nickel chelate) and half-area white 96-well plates were purchased from Perkin-Elmer. Ni-NTA and glutathione sepharose 4B resins were purchased from GE Life Sciences.

### Plasmids

Plasmids for the GST fusions of JARID1A PHD3 (1601–1660), AIRE PHD1 (293–354), ING2 PHD (201–281), BHC80 PHD (486–543), RAG2 PHD (414–487), and JMJD2A double tudor domain (895–1011) were kindly provided by D. Allis (Rockefeller Institute), G. Musco (Dulbecco Teledon Institute), T. Kutateladze (University of Colorado-Denver), Y. Shi (Harvard Medical School), W. Yang (US National Institutes of Health), and R.M. Xu (New York University), respectively. The (HQ)₅-HaloTag N-terminal vector was kindly supplied by Dr. Mike Slater and Dr. Jim Hartnett of Promega Corporation (Madison, WI).

### Peptide Synthesis

Histone H3₁⁻¹¹ peptides were synthesized using solid phase synthesis on the Intavis ResPep SL robot (Intavis, Koeln, Germany). Standard Fmoc/tBu amino acid couplings were used and premodified amino acid building blocks K₅₆₃ and E₈₆₅₇₉-biotin (Novabiochem) were incorporated into positions 4 and 12, respectively, depending on the peptide. Binding peptides contained E₈₆₅₇₉-biotin at position 12 and “unlabeled” competing peptides were unmodified at this position. All peptides (except for fluorescein-labeled species) contained a C-terminal tryptophan for concentration determination by absorbance at 280 nm. Peptide mass was confirmed by MALDI and crude products purified by preparative HPLC (Beckman-Coulter).

Histone H3₁⁻¹⁴ peptides were synthesized as above except for substitution of K(ivDde) (Novabiochem) at position 14. Following synthesis, this lysine was deprotected with 4% v/v hydrazine and labeled with S-carboxyfluorescein (Novabiochem), followed by standard deprotection and purification.

## Protein Expression and Purification

JARID1A PHD3 (1601–1660) was amplified from a GST plasmid and cloned into a bacterial expression vector encoding an N-terminal (HQ)₅₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋_-
**HaloTag Assay for Histone Peptide Binding.** HaloTag fusion protein and peptide were diluted into StabilCoat buffer supplemented with 0.5 mM TCEP. High sensitivity streptavidin-HRP was diluted into SA-AP buffer (30 mM Tris, pH 7.6, 1 M NaCl, 20 mM K/PO4). All steps occurred at ambient temperature, with the microplate sealed to prevent evaporation.

The wells of a 96-well HaloLink microplate were rinsed with 0.2 mL 30 mM HEPES, 150 mM NaCl, pH 7.4, 0.01% v/v Triton X-100 (HBST). (HQ)5-HaloTag-JARID1A PHD3 (50 μL 40 μM) was added to each well and the plate shaken at 200–300 rpm for 2 h at ambient temperature. Wells were aspirated and washed three times with 0.1 mL HBST. Biotinylated histone peptide (50 μL 250 nM) was added to each well and the plate shaken at 200–300 rpm for 1 h. Wells were aspirated and washed three times with 0.1 mL HBST. Streptavidin-HRP (50 μL, 1:2000 dilution) was added to each well and the plate shaken for 20 min at 300 rpm. Wells were aspirated and washed six times with 0.1 mL of HBST. Immediately before detection, 0.1 mL of chemiluminescent substrate was added to each well. Followed by 30 s shaking at slow speed, luminescence was measured (1 s integration time) on a Biotek Synergy H4 multimode plate reader. The initial concentration matrix assay was performed at the University of Wisconsin Carbone Cancer Center Small Molecule Screening Facility. The HaloTag assay was performed as above except for the addition of 1 μL of 10 mM compounds dissolved in DMSO from the NIH Clinical Collection I by a Matrix Hydra liquid handler (Thermo-Fisher) immediately after addition of biotinylated peptide solution, for a final concentration of 200 μM compound. Wash buffer was dispensed by the Matrix Hydra, and all other additions were performed by multichannel pipetting. Screening was performed over two independent experiments with each plate containing DMSO controls for H3K4me3 peptide binding, unmethylated H3 peptide binding, and 7.5 μM unlabeled H3K4me3 peptide competition.

**Secondary Screens, Concentration Dependence, and Counterscreen Studies.** The HaloTag assay and AlphaScreen assays were performed as above, except for the addition of 1 μL of compound dissolved in DMSO to each well immediately after addition of biotinylated peptide. Compounds were assayed at final concentrations between 0 and 200 μM, 2–3% v/v DMSO.

**Affinity Pull Downs.** Streptavidin-agarose beads were equilibrated in HBST, followed by immobilization of biotinylated histone peptides in 1% BSA (w/v)-HBST for 30 min in batch. In all experiments except amiodarone HCl and phenothiazine, approximately 300 pmol of biotinylated peptide per 6.6 μL bed volume resin was immobilized. Approximately 50 pmol of biotinylated peptide was immobilized for studies on amiodarone HCl and phenothiazine due to the poor solubility of these compounds in order to maximize the sensitivity of the assay. Following peptide immobilization, beads were washed three times with 0.5 mL of HBST followed by centrifugation for 1 min at 800g and resuspended in 1% BSA-HBST to form a 50% slurry. (HQ)5-HaloTag-JARID1A PHD3 labeled with TMR HaloTag ligand (10 μM PHD3 labeled with 10 μM TMR ligand in 1% BSA-HBST for 15 min at ambient temperature, covered from light) was dispensed to 0.6-Ml microcentrifuge tubes, followed by 13.2 μL of peptide-linked beads (6.6 μL bed volume) for a final concentration of 100 nM TMR-(HQ)5-HaloTag-JARID1A PHD3 in 1% BSA-HBST. Compounds at varying concentrations (0–500 μM) were added, with a final concentration of 5% v/v DMSO in all samples. Reactions were protected from light and mixed for 45 min at ambient temperature, followed by centrifugation and three 0.5-mL HBST washes. Beads were spun down and all remaining liquid aspirated. Beads were boiled in 20 μL of SDS-sample buffer for 5–10 min before separation by SDS–PAGE (12%, 200 V). Gels were scanned on a Typhoon FLA9000 using the TAMRA setting at 100 μm resolution.

**Fluorescence Polarization Binding Studies.** Increasing concentrations of compounds were added to solutions containing 1 μM GST-JARID1A PHD3 and 3 nM H3(1–14)K4me3K19fluoro in 30 mM HEPES, 150 mM NaCl, pH 7.4, 0.01% v/v Triton X-100. Each condition was measured in triplicate in a black 384-well plate on a Biotek Synergy H4 multimode plate reader. Counterscreen studies utilized 4 μM GST-AIRE PHD1, 4 μM GST-MJD2A DTD, 18 μM GST-UHRF1 TTD, 25 μM GST-RAG2 PHD, 60 μM GST-BHC80 PHD. For AIRE PHD1 and BHC80 PHD, 3 nM H3(1–14)K4unmethylatedK19fluoro peptide was utilized. For UHRF1 TTD, 3 nM H3(1–19)K9me3K19fluoro was utilized. The concentrations of protein for each GST fusion represent 90% fraction bound. From polarization values, fraction bound was calculated by
fraction bound = \frac{P_{\text{obs}} - P_f}{P_b - P_f}
\tag{3}

where $P_{\text{obs}}$ is the observed polarization at a given concentration of compound, $P_b$ is the polarization of free peptide probe, and $P_f$ is the polarization of maximally bound peptide probe.\textsuperscript{20} Dissociation constants ($K_d$) were calculated using the equation:

$$F_b = \frac{[\text{PHD3}]}{K_d + [\text{PHD3}]}
\tag{4}$$

where $F_b$ is fraction bound.\textsuperscript{21}

**Zinc Ejection Studies (PAR Assay).** Zinc was released through the colorimetric reagent, 4-(2-pyridylazo)resorcinol (PAR). In a final volume of 300 μL, 5 μM Histidine-JARID1A PHD3 or (H_{Q})\textsubscript{L}-HaloTag-reader domain was treated with increasing concentrations of disulfiram, methyl methanethiosulfonate, tegaserod maleate, or amiodarone HCl in a solution of 10 mM PAR in 30 mM HEPES, 150 mM NaCl, pH 7.4, 5% v/v DMSO (includes DMSO from compounds). 90 μL of each solution was added in triplicate to a clear 96-well plate. The plate was shaken for 10 min; alternatively, 1 DMSO-only sample was boiled during this time to monitor zinc released due to structural destabilization. Absorbance at 500 nm was read on a Biotek Synergy H4 multimode plate reader. DMSO-only absorbance values were subtracted from each sample.

**Molecular Modeling.** Protein structures were downloaded from RCSB PDB (2KG1, JARID1A PHD3; 2GFA, JMJD2A DTD; 2G6Q, ING2 PHD). Small molecule ligands were prepared using Sybyl (Tripos). Ligands were docked “blindly” onto protein receptors using the AutoGrid function within AutoDock 4, the entire histone-binding domain or lobe (in the case of JMJD2A) was accommodated within the grid.

**Site Directed Mutagenesis of JARID1A PHD3 and Schild Analysis.** Aspartate residues within a GST fusion to JARID1A PHD3 at positions 1624 and 1629 were each mutated to alanine and asparagine, and tryptophan 1625 to alanine, using QuikChange Site-Directed Mutagenesis II kit. JARID1A PHD3 then binds its ligand, a biotinylated histone H3 peptide (± trimethylation at lysine 4) are allowed to bind the immobilized PHD finger. Finally, peptide binding is detected through a streptavidin conjugate to horseradish peroxidase (HRP) that binds biotinylated peptides and emits light when chemiluminescent substrate is added. (B) Initial concentration matrix. Increasing amounts of biotinylated histone H3K4me3 peptides (0.1, 0.5, 1, 10, 15 μM, circles; 15 μM unmethylated biotinylated H3 peptide, open circles) were allowed to bind increasing concentrations of (H_{Q})\textsubscript{L}-HaloTag-JARID1A PHD3 (0–40 μM). (C) Peptide displacement. Unlabeled (no biotin) H3K4me3 peptide (circles) specifically competed away binding of biotinylated H3K4me3 peptides, while a high concentration of unmethylated H3K4 peptide (open circle) did not significantly affect binding signal. (D) Other histone-binding modules within the HaloTag assay. RAG2 PHD, AIRE PHD1, BHC80 PHD, and JMJD2A double tudor domain distinguish the methylation status H3K4 by binding their preferred ligand within the HaloTag assay. Peptide binding can be specifically inhibited via peptide competition (H3K4-unmodified peptide, white; H3K4me3, black; peptide competition, gray).
rigorous washing throughout the assay. Other groups have reported similar detection capabilities with ligand-binding assays.\textsuperscript{23} HaloTag ligand-coated microtiter (HaloLink) plates for the HaloTag assay were generated by reacting amine-reactive 96-well plates with an amine-terminated long chain-pegylated chloroalkane ligand. Capture of HaloTag fusion protein to HaloLink plate is specific and achieves a 10 ng/mL limit of detection using an anti-HaloTag antibody (Figure S1b).

JARID1A PHD3 binds the H3K4me3 peptide with a reported dissociation constant of 0.75 μM and the unmethylated H3 peptide with reduced affinity of 20 μM.\textsuperscript{10} Initial experiments addressed the dynamic range of the HaloTag assay, where concentrations of HaloTag fusion to JARID1A PHD3 and biotinylated histone peptide were simultaneously varied (Figure 1B). Half-maximal binding signal was observed with 1 μM biotinylated H3K4me3 peptide, which agreed well with the reported dissociation constant of JARID1A PHD3.\textsuperscript{10} Sub-K_d concentrations of biotinylated histone peptide maximized the binding range between the methylated and unmethylated histone peptides while minimizing well-to-well error (Figure S1c).

Peptide binding within the HaloTag assay was specifically inhibited via peptide competition, yielding an IC50 value of 2.97 ± 0.35 μM for H3K4me3, while 25 μM unmethylated H3 peptide did not significantly inhibit JARID1A PHD3 (Figure 1C).

The approximately 4-fold difference between the experimental IC50 and the reported K_d for H3K4me3 peptides may be due to an avidity effect caused by the high local concentration of immobilized JARID1A PHD3.\textsuperscript{24} Competitive inhibition was unaffected by the order of addition of the competing H3K4me3 peptide, whether it was added concurrently with the biotinylated peptide or in a separate assay step following binding of the biotinylated peptide (Figure S1d).

The signal for JARID1A PHD3 binding biotinylated H3K4me3 peptide was 200-fold greater than signal observed for HaloTag alone incubated with biotinylated peptides (Figure S1e). The unmethylated histone H3 peptide served as the negative control for statistical calculations even though JARID1A PHD3 can bind it with weak affinity. Importantly, the unmethylated H3 peptide serves as an internal control for the expected binding range of JARID1A PHD3 and establishes greater stringency for statistical calculations. A 10-fold range in binding between the methylated and unmethylated peptides is typically observed within the optimized HaloTag assay, resulting in a Z’ factor of 0.65 ± 0.14 (n = 18 independent experiments with JARID1A PHD3).

The HaloTag assay was readily adapted to other histone-binding proteins, including other PHD fingers (RAG2, BHC80, AIRE) and the double tudor domain (DTD) of JMJD2A.
(Figure 1D). RAG2 PHD and JMJD2A DTD bind H3K4me3. AIRE PHD1 and BHC80 PHD bind the unmodified form of lysine 4 on histone H3. All domains tested demonstrated specificity in binding their preferred form of H3K4 and sensitivity to peptide competition (Figure 1D). In some cases, increasing the concentration of biotinylated peptide to 1–2 μM for low affinity reader domains (e.g., BHC80 PHD, where $K_d > 15 \mu M$) improved assay signal.

**Small Molecule Screening.** The NIH Clinical Collection 1 was screened against JARID1A PHD3 in the HaloTag assay. It contains 446 compounds, all of which have undergone phase I–III clinical trials, and encompasses a broad range of therapeutic indications. The biosafety and bioavailability profiles of compounds within this collection are highly characterized, thus making hits identified from screening candidates for drug repositioning. Compounds were screened at 200 μM and plate-wide controls generated an average Z’ factor of 0.60. Raw screening data are shown sorted by relative binding, where the luminescence counts for the compound-treated well are divided by the luminescence counts for the DMSO-treated H3K4me3-binding control (Figure 2A). Compounds were selected for additional validation if they reduced relative binding by at least three standards of deviation (as calculated from the DMSO-treated H3K4me3-binding controls for individual plates).

From primary screening, 23 compounds were selected for rescreening in triplicate with the HaloTag assay and an AlphaScreen-based histone peptide binding assay (Perkin-Elmer). Similar studies using AlphaScreen technology have been reported for a BET bromodomain and malignant brain tumor (MBT) repeat proteins, and representative binding data for JARID1A PHD3 are shown in Figure S2a. Following secondary screening, compounds were chosen for additional validation based on their ability to reduce binding signal within both the HaloTag and AlphaScreen assays.

**Concentration-Dependence Studies.** Following small molecule screening, we evaluated compound dose dependence (0–200 μM) within the HaloTag assay. Disulfiram, phenothiazine, amiodarone, and tegaserod were the only compounds that significantly reduced signal (by p-value < 0.05) (Figure 2B). The structures of these compounds are shown in Figure 2C. Before proceeding to additional studies, we confirmed that tegaserod was inhibiting JARID1A PHD3, and not maleate (Figure S2b).

Affinity pull downs were performed to detect loss of binding to histone peptides by fluorescently labeled (HQ)₅-HaloTag-JARID1A PHD3. Biotinylated histone peptides were immobilized to streptavidin-conjugated agarose beads, followed by binding of tetramethylrhodamine (TMR)-labeled HaloTag-JARID1A PHD3 to histone peptides, and elution and resolution of bound protein by SDS-PAGE (Figure 3A). Competition with H3K4me3 peptide (Figure S2c) was greatly impaired within this assay ($13.6 \pm 4.5 \mu M$ experimental IC50 versus 0.75 μM expected $K_a$), so compounds were assayed at a high concentration range to detect inhibition. Only disulfiram and tegaserod maleate inhibited JARID1A PHD3 (Figure 3B). Amiodarone and phenothiazine were difficult to test by this strategy due to poor solubility (Figure S2d).

Inhibition of histone binding was also evaluated using a fluorescence polarization binding assay, where a GST fusion to JARID1A PHD3 was allowed to bind fluorescein-labeled H3K4me3 peptide (Figure S2e). When assayed with disulfiram, tegaserod maleate, phenothiazine, and amiodarone, a concentration-dependent loss of polarization corresponding to loss of peptide binding was observed only for tegaserod, with an observed IC50 value of 74 ± 16 μM (Figure 3C). Amiodarone, disulfiram, and phenothiazine did not cause a significant loss in polarization, up to 200 μM (data not shown), consistent with weak inhibition observed in the HaloTag assay.

Next, we sought to evaluate the specificity of these compounds toward JARID1A PHD3. Disulfiram, amiodarone, and tegaserod were screened against AIRE PHD1, BHC80 PHD, RAG2 PHD, and JMJD2A DTD using the HaloTag assay. Significant (by p-value) loss of peptide binding was only observed for the PHD fingers when reader domains were treated with disulfiram, but not JMJD2A DTD (Figure 4A). This result suggested that disulfiram specifically inhibited PHD fingers. RAG2 PHD and JMJD2A DTD, but not AIRE PHD1 or BHC80 PHD, were significantly affected by amiodarone (Figure 4B). Modest reductions in binding were observed for all domains tested when treated with tegaserod, indicative of a nonspecific effect on the HaloTag assay (Figure S3a). When evaluated by fluorescence polarization, only JMJD2A DTD was
inhibited by tegaserod, with an IC50 value of 95 ± 9.8 μM (Figure 4C). The IC50 values for RAG2 PHD, AIRE PHD1, and BHC80 PHD all exceeded the solubility limits of tegaserod.

**Disulfiram Modifies PHD Fingers To Cause Zinc Release.** Disulfiram is a known cysteine alkylating agent, and was shown to eject zinc from a variety of enzymes and transcription factors. PHD fingers are classified by a Cys2-His-Cys4 motif used to engage two zinc ions, so rather than acting as a competitive ligand for PHD fingers, we hypothesized that disulfiram was inhibiting JARID1A PHD3 through modification of structural cysteine residues and subsequent zinc release. To monitor zinc release from JARID1A PHD3, the reagent 4-(2-pyridylazo) resorcinol (PAR) was added to solutions of His6-JARID1A PHD3 and increasing concentrations of disulfiram. Indeed, disulfiram ejected structural zinc from JARID1A PHD3 with a half-maximal zinc loss at 10 μM disulfiram (Figure 5A).

**Figure 5.** Disulfiram ejects structural zinc from JARID1A PHD3. (A) Disulfiram ejects zinc from JARID1A PHD3 in a dose-dependent manner. Zinc release from JARID1A PHD3 was monitored through PAR. Disulfiram (solid curve) causes zinc release at lower concentrations than methyl methanethiosulfonate (MMTS, dotted curve). (B) PHD fingers are differentially sensitive to zinc ejection by disulfiram. AIRE PHD1 (black) and BHC80 PHD (purple) were similarly susceptible to zinc loss as JARID1A PHD3 (blue) within the PAR assay, while RAG2 PHD (green) was more resilient to disulfiram.

The compound methyl methanethiosulfonate (MMTS), which methylates free cysteine side chains and also causes zinc ion ejection, resulted in a half-maximal zinc ion loss at 33 μM. Increasing concentrations of amiodarone and tegaserod added to solutions of JARID1A PHD3 and PAR reagent confirmed that these compounds were not causing zinc release through structural destabilization, suggesting that these compounds were noncovalent ligands for JARID1A PHD3 (Figure S3b). We then investigated whether disulfiram was a general inhibitor of PHD fingers. Upon treatment with disulfiram, zinc release was measured from (HQ)5-HaloTag fusions to JARID1A PHD3, and JARID1A PHD3 (solid blue curve) and JARID1A PHD3 (solid blue curve) within the fluorescence polarization assay, but not RAG2 PHD (solid blue line), ING2 PHD (dotted blue line), AIRE PHD1 (solid green line), or BHC80 (dotted green line).

**Chemical Modification of Amiodarone Improves Potency toward JARID1A PHD3.** Histone reader specificity studies suggested that amiodarone (AMI) preferentially inhibited methyl-lysine readers. To test this hypothesis, we pursued a fragment-based approach to evaluate amiodarone...
Within the HaloTag assay, dronedarone, but not benzbromarone, inhibited JARID1A PHD3 (Figure S3d). These data suggested that the tertiary alkylated amine within amiodarone was an important determinant for binding inhibition. We therefore hypothesized that the alkylated amine chain of amiodarone was a ligand for the aromatic cage of JARID1A PHD3, thereby competitively inhibiting binding of H3K4me3.

To test this hypothesis, we prepared a series of amiodarone derivatives built from 2-butyl-3-(3,5-diiodo-4-hydroxybenzoyl)-benzofuran. Amiodarone is successively de-ethylated by cytochrome P450 in the liver to generate the major metabolite desethylamiodarone (desethyl-AMI) and minor metabolite di-N-desethylamiodarone (di-N-desethyl-AMI). In addition to these derivatives, we synthesized four amiodarone analogues with varied "chain" length of the amine (2 or 3 carbons) and methylation states of the amine (dimethyl or trimethyl). Synthesis of the dimethyl-AMI compounds had been previously reported. As shown in Scheme 1, the synthesis of amiodarone derivatives 4–7 featured the alkylation of (2-butylbenzofuran-3-yl)(4-hydroxy-3,5-diiodophenyl)methanone 1 in the presence of 2-chloro-NN,N-dimethylpropan-1-amine hydrochloride to afford the dimethyl-AMI analog 2, as well as homologue 3 with its chain extended by one methylene unit. With this series of amiodarone derivatives, a range of amine alkylation states could be evaluated.

AMI-based inhibitors were evaluated via fluorescence polarization, utilizing a 90% fraction bound concentration of GST-JARID1A PHD3. Di-N-desethyl-AMI and both trimethyl-AMI analogues inhibited JARID1A PHD3 more potently than did AMI (Figure 6B), with similar IC50 values that ranged between 25 and 40 μM (Table 1). These studies suggest that

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**Figure 6.** Analogues of amiodarone inhibit JARID1A PHD3 binding of H3K4me3 more potently than amiodarone. (A) Amiodarone analogues studied included benzbromarone, dronedarone, metabolites of amiodarone, and four synthetic analogues. (B) Amiodarone analogues inhibit JARID1A PHD3 more potently than amiodarone. Of the compounds assayed, the metabolite di-N-desethylamiodarone (black curve) and the synthetic trimethyl-amiodarone analogues (blue curves; n=2 solid, n=3 dotted) inhibit JARID1A PHD3 within the fluorescence polarization assay, but not amiodarone (black, solid), desethylamiodarone (black, dotted) or dimethyl-amiodarone synthetic analogues (green; n=2 solid, n=3 dotted).

**Scheme 1. Synthesis of Amiodarone Analogues**

Synthesis of amiodarone derivatives 4–7. Reagents and conditions: (a) 1, K2CO3, toluene/H2O (2:1), 60 °C, then 2-chloro-NN,N-dimethylpropan-1-amine hydrochloride, reflux 22 h, 66% (n=1); (b) 1, K2CO3, toluene/H2O (2:1), 60 °C, then 3-chloro-NN,N-dimethylpropan-1-amine hydrochloride, reflux 17 h, 92% (n=2); (c) 2, conc. HCl, toluene, rt, 1 h, 63%; (d) 3, conc. HCl, toluene, rt, 1 h, 50%; (e) 2, MeI, CH2Cl2, rt, 17 h, 76%; (f) 3, MeI, CH2Cl2, rt, 2 h, 81%.

**GST-JARID1A PHD3.** Di-N-desethyl-AMI and both trimethyl-AMI analogues inhibited JARID1A PHD3 more potently than did AMI (Figure 6B), with similar IC50 values that ranged between 25 and 40 μM (Table 1). These studies suggest that
Table 1. IC50 Values of Amiodarone Analogues towards JARID1A PHD3a

<table>
<thead>
<tr>
<th>compound</th>
<th>IC50</th>
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<tr>
<td>amiodarone</td>
<td>NM</td>
</tr>
<tr>
<td>desethylamiodarone</td>
<td>NM</td>
</tr>
<tr>
<td>di-N-desethylamiodarone</td>
<td>26 ± 15 μM</td>
</tr>
<tr>
<td>WAG-003</td>
<td>30 ± 14 μM</td>
</tr>
<tr>
<td>WAG-004</td>
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<td>WAG-006</td>
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“IC50 values calculated from the fluorescence polarization assay, where GST-JARID1A PHD3 was treated with increasing concentrations of amiodarone and its analogues. NM: Not measurable within solubility limit of compound.

di-N-desethylAM1 and trimethyl-AMI analogues are at least 10-fold more potent than amiodarone. Neither dimethyl-AMI analogue nor desetyl-AMI significantly inhibited JARID1A PHD3 within the concentration range tested. We performed anion exchange chromatography on trimethyl-AMI derivatives to generate sodium salts, which confirmed that the iodide counterion was not responsible for binding inhibition (data not shown).

Amiodarone analogues were then assayed against a number of histone reader domains, including JMJ2A DTD, RAG2 PHD, ING2 PHD (H3K4me3), AIRE PHD1, BHC80 PHD (H3K4unmodified), and UHRF1 tandem tudor domain (TTD, H3K9me3) within the fluorescence polarization assay.3,35 The results for trimethyl-AMI (n = 2) and di-N-desethyl-AMI are shown in Figure 7 and Table 2. Trimethyl-AMI (n = 2) and di-N-desethyl-AMI inhibited JARID1A PHD3 the most potently, followed closely by JMJ2A DTD. These data confirmed that amiodarone derivatives retain specificity for trimethyl-lysine reader domains. Interestingly, we observed a hyperpolarization effect when AIRE PHD1 or BHC80 PHD is treated with either compound, but the significance of these data is unclear since fluorescence intensity values were unaffected (data not shown).

Fluorescence polarization studies suggested that amiodarone analogues were inhibiting JARID1A PHD3 independently of the aromatic cage. To identify possible sites on JARID1A PHD3 where inhibitors might be binding, molecular modeling was performed using AutoDock software,36 where the entire JARID1A PHD3 for the H3K4me3 peptide. Consistent with a hydrogen bond interaction, an alanine mutation at 1624 decreased the bond interaction, an alanine mutation at 1624 decreased the

Figure 7. Amiodarone analogues are specific for trimethyl-lysine reader domains. (A, B) Di-N-desethylamiodarone (A) and trimethyl-amirodarone (n = 2) (B) are most potent against specific H3K4me3-reader domains. JMJ2A DTD (dotted blue curve) is similarly inhibited as JARID1A PHD3 (solid blue curve), followed by ING2 PHD (dashed blue curve). AIRE PHD1 (solid green line), BHC80 PHD (dotted green line), RAG2 PHD (solid blue line), and UHRF1 TTD (purple line) were not inhibited by these compounds.

Table 2. IC50 Values of AMI Analogues Towards Lysine Reader Domainsa

<table>
<thead>
<tr>
<th>reader domain</th>
<th>WAG-003</th>
<th>N-desethylAMI</th>
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<tbody>
<tr>
<td>JARID1A PHD3</td>
<td>30 ± 14 μM</td>
<td>26 ± 15 μM</td>
</tr>
<tr>
<td>JMJ2A DTD</td>
<td>34 ± 14 μM</td>
<td>72 ± 26 μM</td>
</tr>
<tr>
<td>ING2 PHD</td>
<td>105 ± 33 μM</td>
<td>97 ± 41 μM</td>
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<tr>
<td>AIRE PHD1</td>
<td>NM</td>
<td>NM</td>
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<tr>
<td>BHC80 PHD</td>
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</tr>
<tr>
<td>UHRF1 TTD</td>
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</tr>
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</table>

“IC50 values calculated from the fluorescence polarization assay, where GST fusions to reader domains were treated with increasing concentrations of trimethyl-amiodarone (WAG-003) or di-N-desethylamiodarone. Fusion proteins were assayed at a 90% fraction bound protein concentration. NM: Not measurable within solubility limit of compound.

Fluorescence polarization (Table 3, Figure 8E). An aromatic cage mutant, W1625A, which was reported to completely abolish histone binding, was generated for comparison.36 As expected, this mutation severely impaired histone peptide binding. On the basis of the crystal structure of JARID1A PHD3, glutamine 5 on histone H3 forms a hydrogen bond to aspartate 1624, and the side chain of H3R2 forms an ionic bond with aspartate 1629 (Figure 8D). We therefore predicted that mutating either site would decrease the affinity of JARID1A PHD3 for the H3K4me3 peptide. Consistent with a hydrogen bond interaction, an alanine mutation at 1624 decreased the
affinity of JARID1A PHD3 for H3K4me3 peptides by 2-fold compared to an asparagine mutation, which would still permit hydrogen bonding (Table 3, Figure 8E). Compared to a wild-type affinity of approximately 0.3 μM for the H3K4me3 peptide, the D1624A and D1624N mutations decreased affinity by four- and 2-fold, respectively (Table 3). Mutating D1629 was more damaging to histone binding and caused a greater than 10-fold defect in affinity for the H3K4me3 peptide, independent of the amino acid substitution at this site (Table 3, Figure 8E). These data are consistent with the formation of a charge–charge ionic bond between D1629 and the side chain of H3R2.

Because D1624 and D1629 mutants exhibited defects in histone binding, standard IC50 analyses would be influenced by protein concentration within fluorescence polarization binding studies. We therefore assayed the ability of amiodarone analogues and tegaserod to inhibit JARID1A PHD3 using a dose ratio analysis, as described in the methods. For both di-N-desethylamiodarone and tegaserod, increasing the fixed concentration of inhibitor caused an increase in EC50 values for wild-type (WT) JARID1A PHD3 (Figure 9A,G). The dose ratios values for WT JARID1A PHD3 increase linearly as the concentration of inhibitor is increased (Figure 9F,L). EC50 values for the D1624 mutants also increased with increasing concentrations of di-N-desethylamiodarone or tegaserod, though the dose ratios were less pronounced than for WT JARID1A PHD3. In contrast, the EC50 values for D1629 mutants increased very little with increasing either di-N-desethylamiodarone or tegaserod concentrations, resulting in dose ratios that remained near one. Our data therefore support
Table 3. Dissociation Constants of JARID1A PHD3 for H3K4me3 Peptide

<table>
<thead>
<tr>
<th>clone</th>
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<th>$K_d$ (μM)</th>
<th>error (μM)</th>
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<tr>
<td>WT</td>
<td>H3K4</td>
<td>9.35</td>
<td>1.57</td>
<td>0.954</td>
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<tr>
<td></td>
<td>H3K4me3</td>
<td>0.294</td>
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<tr>
<td>D1624A</td>
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<td>H3K4me3</td>
<td>1.15</td>
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<tr>
<td>D1624N</td>
<td>H3K4</td>
<td>NM*</td>
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<tr>
<td></td>
<td>H3K4me3</td>
<td>0.634</td>
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<td>0.986</td>
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<td>W1625A</td>
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<td>H3K4me3</td>
<td>4.05</td>
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<tr>
<td>D1629A</td>
<td>H3K4</td>
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<td>3.81</td>
<td>0.551</td>
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*NM: Not measurable within the concentration range utilized in the fluorescence polarization assay.

a model where D1629 provides a major contribution to binding of amiodarone analogues and tegaserod to JARID1A PHD3. We predict that these interactions likely occur through electrostatic contacts between inhibitor amine groups and the carboxyl group of the aspartate side chain, as the D1629N mutation did not increase the dose response. Because D1624 forms part of the binding site for di-N-desethylamiodarone and tegaserod in modeling predictions, it is unsurprising that mutating this residue could impair inhibitor interactions with JARID1A PHD3, albeit to a lesser extent than D1629. In fact, the hydroxyl group extending from the indole ring of tegaserod may form a hydrogen bond with D1624. Weaker interactions with this site on JARID1A PHD3 may account for the reduced dose response in D1624 mutants relative to wild-type protein.

**DISCUSSION**

Utilizing a newly developed HaloTag assay, a small molecule screen identified disulfiram, amiodarone, and tegaserod as inhibitors of the interaction between JARID1A PHD3 and H3K4me3. Compounds were validated biochemically via dose dependent-binding inhibition using the HaloTag, affinity pull down, and fluorescence polarization assays. Structural destabilization and chemical reactivity toward JARID1A PHD3 were monitored by zinc release studies. The potency of amiodarone was improved by chemical modification, following fragment-based dose dependence studies that suggested that the alkylated amine chain played an important role in binding inhibition. Following molecular modeling and site-directed mutagenesis, we determined that aspartate 1629 in JARID1A PHD3 makes a significant contribution to amiodarone-derivatives and tegaserod binding to this PHD finger.

Our studies demonstrate the value of developing new assays for small molecule screening. Many fluorescence-based binding assays are prone to compound interference; the HaloTag assay described here appears to be very resilient to such effects and provided greater stringency for inhibitors during primary screening. It is important to note that even though the HaloTag assay incorporated several washing and incubation steps, low affinity peptide binding is readily detectable due to the high sensitivity of the HaloTag assay format. High sensitivity SPOT peptide and CADOR (chromatin associated domain) array formats have been used for detection of weak peptide and histone binding domains.37,38 Our ability to readily adapt the HaloTag assay to different histone reader domains demonstrated the flexibility and utility of the HaloTag assay for low to midrange micromolar affinity interactions. Given the ease with which HaloTag fusion proteins and biotinylated ligands can be prepared, we anticipate that the assay would function well for a number of protein–ligand interactions.

Disulfiram inhibits JARID1A PHD3 and other PHD fingers not by acting as a ligand, but through ejection of structural zinc, thus revealing a general susceptibility specific to PHD fingers as a histone reader domain. Disulfiram is most well-known for its use in the treatment of alcoholism, where it inhibits acetaldehyde dehydrogenase via modification of active site cysteine residues and subsequent zinc ejection.31 In addition to acetaldehyde dehydrogenase, disulfiram inhibits a number of proteins with diverse functions, including dopamine beta-hydroxylase, viral nucleocapsin protein, DNA methyltransferase I, and histone demethylases.32,33,35 It is interesting to note that disulfiram exhibits antitumor activity, demonstrating its potential for a broad range of therapeutic indications.42

Amiodarone was initially identified from screening as a weak inhibitor of JARID1A PHD3. We hypothesized that the alkylated amine chain inhibited histone binding by acting as a competitive ligand for JARID1A PHD3. By preparing a series of derivatives that probed the role of the amine chain, we were able to identify inhibitors that were approximately 10-fold more potent than amiodarone. Our data suggest that amiodarone inhibits JARID1A PHD3 independently of the aromatic cage, as the unmethylated and trimethylated forms of the amine chain were equally potent inhibitors against histone binding.

Informed by molecular modeling studies, we identified two probable sites where amiodarone analogues appeared to bind JARID1A PHD3 and one site for tegaserod. Sites for both inhibitors contain aspartate residues that contribute to histone binding. To test our modeling predictions, we performed site-directed mutagenesis on JARID1A PHD3. Dose ratio analyses with D1624 and D1629 mutants support a model where amiodarone analogues and tegaserod bind near aspartate 1629, which interacts with arginine 2 on histone H3.

Amiodarone was developed for the treatment of arrhythmias for patients with atrial fibrillations.43 Like disulfiram, amiodarone may be a candidate for drug repositioning, since a recent preclinical mouse model study demonstrated that amiodarone could treat leukemia.44 Another study performed in Saccharomyces cerevisiae found that amiodarone significantly down-regulated the transcription of genes involved in cell cycle and DNA processing.45 To date, the mechanisms of action underlying these activities are unclear, but suggest amiodarone could influence a number of cellular processes. Tegaserod was developed as a partial agonist for 5-HT4 receptors in the treatment of irritable bowel syndrome.46,47 Our studies demonstrate novel activities for amiodarone derivatives and tegaserod through inhibition of histone reader domains.

In conclusion, identifying inhibitors of JARID1A PHD3 adds to the growing body of studies that target histone readers as a strategy for drug development efforts. The development of JQ1 as a small molecule inhibitor of the bromodomain of BRD4 shows great promise in mouse cancer models, and others have developed in vitro inhibitors of MBT methyl-lysine readers.14,48 PHD fingers and other histone reader domains play critical roles in gene regulation by recruiting protein complexes to specific regions of the genome.49 By disrupting histone reader proteins from chromatin via small molecule inhibitors, it may be possible to alter transcriptional programs that are
misregulated in cancer cells and thereby develop novel therapeutics.

ASSOCIATED CONTENT

Supporting Information

Additional data for assay optimization, inhibitor validation, and molecular modeling, as well as compound synthesis methods. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Address: University of Wisconsin-Madison School of Medicine and Public Health, 2178 Wisconsin Institutes for Discovery, 330 N. Orchard St., Madison, WI 53715. E-mail: jmdenu@wisc.edu. Phone: 608-316-4342. Fax: 608-316-4602.
Biochemistry

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Notes
The authors declare no competing financial interest.

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ABBREVIATIONS USED:
PHD finger, plant homedomain finger; JARID1A, Jumonji A/T rich interaction domain; NUP98, nucleoporin 98; AML, acute myeloid leukemia; ING, inhibitor of growth; DTD, double tudor domain; TTD, tandem tudor domain; H3K4me3, histone H3 trimethylated at lysine 4; H3K9me3, histone H3 trimethylated at lysine 9; HRP, horseradish peroxidase; DMSO, dimethyl sulfoxide; TMR, tetramethylrhodamine; SDS-PAGE, sodium dodecyl sulfate polyacrylamide electrophoresis; GST, glutathione S-transferase; IC50, half-maximal inhibitory concentration; PAR, 4-(2-pyridylazo) resorcinol; MMTS, methyl methanethiosulfonate; AMI, amiodarone; desethyl-AMI, desethylamiodarone; di-MMTS, methyl methanethiosulfonate; TCEP, tris(2-carboxyethyl)phosphine; LOD, limit of detection; HPLC, high performance liquid chromatography; Mel, methyl iodide; rt, room temperature

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