Identification of Small Molecule Inhibitors of Jumonji AT-rich Interactive Domain 1B (JARID1B) Histone Demethylase by a Sensitive High Throughput Screen

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Background: JARID1B is an H3K4 histone demethylase and an attractive target for cancer therapy.

Results: High throughput screen identified novel compounds that can inhibit JARID1B demethylase activity.

Conclusion: Drug-like small molecules can be identified to inhibit JARID1B.

Significance: The identified JARID1B inhibitors are lead compounds that can be developed into anti-cancer epigenetic drugs.

JARID1B (also known as KDM5B or PLU1) is a member of the JARID1 family of histone lysine demethylases responsible for the demethylation of trimethylated lysine 27 in histone H3 (H3K4me3), a mark for actively transcribed genes. JARID1B is overexpressed in several cancers, including breast cancer, prostate cancer, and lung cancer. In addition, JARID1B is required for mammary tumor formation in syngeneic or xenograft mouse models. JARID1B-expressing melanoma cells are associated with increased self-renewal character. Therefore, JARID1B represents an attractive target for cancer therapy. Here we characterized JARID1B using a homogeneous luminescence-based demethylase assay. We then conducted a high throughput screen of over 15,000 small molecules to identify inhibitors of JARID1B. From this screen, we identified several known JmjC histone demethylase inhibitors, including 2,4-pyridinedicarboxylic acid and catechols. More importantly, we identified several novel inhibitors, including 2-(4-methylphenyl)-1,2-benzisothiazol-3(2H)-one (PBIT), which inhibits JARID1B with an IC50 of about 3 μM in vitro. Consistent with this, PBIT treatment inhibited removal of H3K4me3 by JARID1B in cells. Furthermore, this compound inhibited proliferation of cells expressing higher levels of JARID1B. These results suggest that this novel small molecule inhibitor is a lead compound that can be further optimized for cancer therapy.

Covalent posttranslational modification of histones on lysine tails is essential for gene regulation and DNA repair (1). Histone lysine methylations are now widely accepted modifications for activating or silencing gene transcription, depending on the site and degree of methylation (2). For example, trimethylated lysine 4 in histone H3 (H3K4me3) is associated with active transcription, whereas trimethylated lysine 27 in histone H3 (H3K27me3) is associated with gene silencing.

The enzymes responsible for the demethylation of H3K4me3 are the Jumonji AT-rich interactive domain 1 (JARID1)² or lysine demethylase 5 (KDM5) family of lysine demethylases (3–8). This family consists of JARID1A (also known as KDM5A or RBP2), JARID1B (also known as KDM5B or PLU1), JARID1C (also known as KDM5C or SMCY), and JARID1D (also known as KDM5D or SMCY) in mammals (2). Similar to other Jumonji C (JmjC) domain-containing demethylases, the JARID1 enzymes catalyze the demethylation of histones in an iron(II) and α-ketoglutarate (α-KG)-dependent reaction (9). In this reaction, the oxidative decarboxylation of α-KG results in a hydroxylated methyl-lysine intermediate, which is thermodynamically unstable. The release of the hydroxyl and methyl groups as formaldehyde from this intermediate results in a demethylated lysine residue. Although all the JmjC domain histone demethylases catalyze the reaction via a similar mechanism, they clearly demonstrate specificity toward particular lysine residue(s) (10).

The JARID1 demethylases have been linked to human diseases such as cancer and X-linked mental retardation (2). Both JARID1A and JARID1B are potential oncoproteins, and both are overexpressed in a variety of cancers (2). Increased expression of JARID1A promotes a more stem-like phenotype and enhanced resistance to anticancer agents (11).

The abbreviations used are: JARID1, Jumonji AT-rich interactive domain 1; KDM5, lysine demethylase 5; JmjC, Jumonji; 2,4-PDCA, 2,4-pyridinedicarboxylic acid monohydrate; α-KG, α-ketoglutarate; bio-, biotinylated; DMSO, dimethyl sulfoxide; HER2*, HER2-positive; PBIT, 2-(4-methylphenyl)-1,2-benzisothiazol-3(2H)-one; UTX, ubiquitously transcribed tetratricopeptide repeat protein X-linked; JMD3, JmjC domain-containing protein 3.

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Moreover, loss of JARID1A inhibits tumorigenesis in two genetically engineered mouse cancer models (12). JARID1B is highly expressed in human mammary tumors and breast cancer cell lines, but not in normal adult breast tissue (13). Knockdown of JARID1B leads to up-regulation of tumor suppressor genes including BRCA1 (8). Down-regulation of JARID1B in breast cancer cells decreased tumor formation potential of these cells in mouse syngeneic or xenograft models (8, 14). JARID1B is also up-regulated in advanced and metastatic prostate tumors (15) and is required for continuous growth of melanoma cells (16). Taken together, both JARID1A and JARID1B enzymes are very attractive targets for cancer therapy (2). Even so, no specific inhibitor of these two epigenetic regulators is currently available, and the development of small molecule inhibitors is in demand.

Until now, no high throughput screen has been reported for the JARID1 family of histone lysine demethylases. Small molecule inhibitor screens of other JmJc domain-containing demethylases employed methods including detection of the reaction byproduct formaldehyde (17, 18), mass spectrometry (19), AlphaScreen (20), and LANCE Ultra and AlphaLisa assays (21). In these studies, α-KG analogues were reported to inhibit the JmJc demethylases (22). One such analogue, 2,4-pyridinedicarboxylic acid (2,4-PDCA), has been shown to inhibit the catalytic core of JARID1B (23). However, the specificity is likely compromised as these analogues may inhibit other Fe(II)- and α-KG-dependent enzymes, such as prolyl hydroxylases (22).

Here we describe a high throughput screen to identify small molecule inhibitors of full-length JARID1B using the AlphaScreen platform. By implementing AlphaScreen technology, we developed a very sensitive assay for detecting demethylation of a biotinylated (bio-) H3K4me3 peptide in vitro. We screened JARID1B against a diverse library consisting of 15,134 molecules and identified several compounds that yielded low μM IC_{50} values. One novel inhibitor, PBIT, inhibits JARID1B up to 95%, with an IC_{50} value of about 3 μM. This compound can also inhibit other members of the JARID1 family. It, however, did not inhibit the H3K27me3 demethylases ubiquitously transcribed tetratricopeptide repeat protein, X-linked (UTX) or JmjC domain-containing protein 3 (JMJD3), indicating that PBIT is specific for the JARID1 enzymes. Furthermore, this small molecule is able to modulate H3K4me3 levels in cells and attenuate proliferation of UACC-812 breast cancer cells. Taken together, these studies reveal the identification of novel inhibitors of JARID1B in vitro with therapeutic implications for breast cancer.

**EXPERIMENTAL PROCEDURES**

**Histone Peptides and Antibodies**—C-terminal biotinylated peptides used in assays were as follows: H3K4me3 (ART-K(Me3)-GTARKSTGGKAPRQKLAA-GGK(biotin)), H3K4me2 (ART-K(Me2)-GTARKSTGGKAPRQKLAA-GGK(biotin)), H3K4me1 (ART-K(Me)-GTARKSTGGKAPRQKLAA-GGK(biotin)), H3K27me3 (ATKAAR-K(Me3)-SAPATGGVKKPHRYPG-GK(biotin)), H3K27me2 (ATKAAR-K(Me2)-SAPATGGVKKPHRYPG-GK(biotin)), and H3K27me1 (ATKAAR-K(Me1)-SAPATGGVKKPHRYPG-GK(biotin)). These were obtained from AnaSpec. Anti-H3K4me3 polyclonal antibody (ab8580), anti-H3K4me2 polyclonal antibody (ab7766), anti-H3K4me1 polyclonal antibody (ab8895), and anti-H3 polyclonal antibody (ab1791) were purchased from Abcam, and anti-H3K27me2 polyclonal antibody (ab7766) was obtained from EMD Millipore. Anti-JARID1A monoclonal antibody (3876S) was purchased from Cell Signaling, anti-JARID1B polyclonal antibody (A301-813A) and anti-JARID1C polyclonal antibody (A301-035A) were obtained from Bethyl Laboratories, anti-UTX antibody (M30076) was from Abmart, and anti-HA antibody (MMS-101P) was from Covance.

**Cell Lines**—SF21 insect cells were cultured in Grace’s medium with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. MCF7 and UACC-812 cells were cultured in RPMI 1640 with 10% FBS and 1% penicillin/streptomycin. MCF10A cells were cultured in Dulbecco’s modified Eagle’s medium: Ham’s F12 medium (1:1), 5% horse serum, 0.1 μg/ml cholera toxin, 10 ng/ml insulin, 0.5 μg/ml hydrocortisone, 20 ng/ml epidermal growth factor, and 1% penicillin/streptomycin.

**Enzyme Production**—SF21 cells infected with baculoviruses expressing FLAG-JARID1A (3), FLAG-JARID1B (8), FLAG-JARID1C (6), or His-FLAG-UTX (24) were cultured at 27 °C for 3 days, and the FLAG-tagged enzymes were purified via anti-FLAG M2 beads (Sigma). Purification of these histone demethylases was confirmed by Coomassie Brilliant Blue staining and Western blot analysis using the specific antibodies against these enzymes.

**Histone Demethylase Assay**—Histone demethylase assays were performed in 384-well white plates (Corning 3574). Demethylase buffer conditions for FLAG-JARID1B were as follows: 10 μM α-KG, 100 μM ascorbate, 50 μM (NH₄)₂Fe(SO₄)₂, 50 mM Hepes (pH 7.5), 0.01% (v/v) Tween 20, and 0.1% (w/v) bovine serum albumin. The demethylase reactions included 64 nM bio-H3K4me3 peptide alone or in the presence of 4 nM FLAG-JARID1B enzyme in a 10-μl reaction at 25 °C for 30 min. As a positive control, 64 nM bio-H3K4me3 peptide was assayed in the absence of enzyme. Assay conditions for FLAG-JARID1C were the same as for FLAG-JARID1B except that 20 nM enzyme was used. For FLAG-JARID1A, the demethylase buffer was similar to FLAG-JARID1B except that 125 μM α-KG and 13 nM FLAG-JARID1A enzyme were used. The His-FLAG-UTX and FLAG-JMJD3 demethylase assays also employed the same buffer conditions as for FLAG-JARID1B, with 64 nM bio-H3K27me3 peptide assayed with or without 25 nM His-FLAG-UTX enzyme or 50 nM FLAG-JMJD3 (BPS Bioscience, 50115) and 64 nM bio-H3K27me2 peptide as a positive control. JARID1A, JARID1C, UTX, and JMJD3 histone demethylase assays proceeded at 37 °C for 1 h.

**AlphaScreen Assay**—The AlphaScreen general IgG (protein A) detection kit was obtained from PerkinElmer Life Sciences. Demethylated H3K4 products were detected using AlphaScreen antibody/bead mix containing 7.5 mM EDTA and 0.15 μg/ml anti-H3K4me1 antibody in a final volume of 20 μl. For detection of demethylated H3K27 products, the AlphaScreen antibody/bead mix containing 7.5 mM EDTA and 0.15 μg/ml anti-H3K27me2 antibody in a final volume of 20 μl was used. The luminescence signal was measured using the
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Environ (PerkinElmer Life Sciences) or Pherastar (BMG Labtech) plate readers.

**Drug Screening Libraries and Conditions**—FLAG-JARID1B was screened against 15,134 compounds. These compound libraries were from the Yale Center for Molecular Discovery. The libraries screened included the MicroSource Gen-Plus, MicroSource Pure Natural Products, National Institutes of Health Clinical Collection, Enzo Epigenetics, Yale Compound, and ChemBridge MW-Set libraries, plus selected plates from the Maybridge Diversity, ChemBridge MicroFormats, DIVER-Set, and ChemDiv libraries containing 8-hydroxyquinoline analogues. The first five libraries were screened twice, once with the standard demethylase assay condition and once with similar conditions except that 1 mM α-KG was used. Compounds dissolved in dimethyl sulfoxide (DMSO) were added into a 384-well plate containing bio-H3K4me3 peptide in demethylase buffer to a final concentration of 20 μM. The reactions were initiated by the addition of 4 nM FLAG-JARID1B and detected as described above. To eliminate the false positive hits, a counter-screen was performed against bio-H3K4me2 in the absence of enzyme. IC_{50} values were generated from dose-response curves using 0.1–11 μM of compound and 15 or 50 μM Fe(II).

**Chemicals**—PBIT (PH009215) and 2,4-PDCA (P63395) were purchased from Sigma-Aldrich. DMSO (9224-01) was purchased from J.T. Baker.

**Immunostaining**—pcDNA3.1(−)−3×HA-JARID1B construct was generated by inserting 3×HA-JARID1B between BamHI and XbaI of pcDNA3.1(−)− vector. MCF7 cells were plated on 12-mm-diameter coverslips in 24-well plates and transfected with pcDNA3.1(−)−3×HA-JARID1B in the presence of 0, 10, or 30 μM PBIT. After incubation for 24 h, the cells were fixed, permeabilized, and stained with antibodies against HA and H3K4me3 for 2 h. The coverslips were then incubated with anti-mouse Alexa Fluor-546 (Invitrogen, A-11003) and goat anti-rabbit Envision (Dako, K4002) for 1 h. Cy5-Tyramide (PerkinElmer Life Sciences, NEL775001KT) and 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Biotium, GW1-0001) were used to visualize 3×HA-tagged JARID1B and nuclei, respectively. The slides were sealed and analyzed under an Olympus fluorescence microscope.

**Histone Extraction and Western Blot**—MCF7 cells treated with PBIT (10 μM) or DMSO (0.1%) for 72 h were harvested and lysed with PBS containing 0.5% Triton X-100. Nuclei were spun down by centrifugation at 6,500 g for 10 min, and the pellets were resuspended in 0.2 N HCl. The histones were extracted overnight, and cellular debris was removed by centrifugation. The samples were loaded onto 16% SDS-PAGE gels and probed with antibodies against H3K4me3, H3K4me2, H3K4me1, and H3.

**Cell Proliferation Assay**—The colorimetric assay (WST-1 reagent) from Roche Applied Science (11644807001) was performed in 96-well white clear bottom plates (Costar, 3610). 1,000 cells were seeded per well (in quadruplicate) overnight, and PBIT was added to the cells to the indicated concentration for 72 h. 0.01% DMSO was included as the control. The WST-1 reagent was added (5 μl/well) for 4 h, and absorbance at 440 nm (which reflects the number of viable cells) was measured with the BioTek Synergy Mx plate reader.

**Generation of JARID1B Knockdown Cell Lines**—Stable knockdowns of JARID1B in UACC-812, MCF7, and MCF10A cells were performed as described previously (25) using two lentiviral shRNAs, pLKO.1-JARID1B sh1 (targeting CGAGATGGAATTAACAGTCTT) and pLKO.1-JARID1B sh2 (targeting AGGGAGATGCACCTCTGATATA). pLKO.1-shScr (scramble shRNA control) was described previously (25, 26). pLKO.1-shGFP control shRNA was a gift from William Hahn (Dana-Farber Cancer Institute, Boston, MA). Knockdown cells were selected and maintained in medium containing 2 μg/ml puromycin.

**Real Time Reverse-Transcription (RT) PCR**—Real-time RT-PCR experiments were performed as described (12). Values were normalized to the level of GAPDH or ACTB. Primers specific for JARID1B were hPLU1F2 (CCATAGCCGAGGATCGAAGTGG) and hPLU1R2 (GGATACGTTGGGTAAATGAAGT). Primers specific for GAPDH were hGAPDH (CCGATCCCCCTCAGAACCAAAATGAA) and hGAPDH (GTCTTCTGGTGTCAGTGAT). Primers specific for ACTB were described previously (27).

**RESULTS**

**AlphaScreen Assay Setup**—To identify small molecule inhibitors of the JARID1B enzyme, AlphaScreen technology was employed to monitor JARID1B activity (Fig. 1A) (20). In our demethylase assays, a biotinylated H3K4me3 peptide substrate underwent demethylation by JARID1B. The demethylated products (bio-H3K4me2/1) were detected by interaction with both streptavidin-coated donor beads (via biotin label) and protein A-coated acceptor beads (via interaction with the H3K4me2/1 antibody). Laser excitation leads to a luminescence signal that corresponds to the amount of bio-H3K4me2/1 and thus demethylase activity. Antibody optimization for the AlphaScreen assay in the absence of enzyme was performed using various antibodies against H3K4me2 and H3K4me1. Among these antibodies, the H3K4me1 antibody can generate homogeneous luminescence signals for both the bio-H3K4me2/1 and the bio-H3K4me1 peptides (Fig. 1B). More importantly, the signal for the bio-H3K4me1 peptide is about twice that of the bio-H3K4me2 peptide. Therefore, the AlphaScreen signal can also indicate the degree of demethylation.

**Characterization of JARID1B**—The FLAG-tagged full-length JARID1B enzyme was expressed in Sf21 insect cells using FLAG-JARID1B baculoviruses and affinity-purified using anti-FLAG antibody. FLAG-JARID1B was analyzed by SDS-PAGE for purity (Fig. 2A), and by Western blot for JARID1B expression (Fig. 2B). To assess the activity of FLAG-JARID1B, demethylase assays were performed in triplicate using AlphaScreen platform in the presence and absence of JARID1B (Fig. 3A). AlphaScreen signal is detected in demethylase assays performed in the presence of both the bio-H3K4me3 peptide and FLAG-JARID1B. Assays performed using only the bio-H3K4me2 peptide served as a positive control.

To optimize screening conditions, FLAG-JARID1B activity was further investigated in a time course and enzyme
titration experiment (Fig. 3B). Robust AlphaScreen signal was observed using only 5 nM FLAG-JARID1B, and the demethylase reaction was essentially complete after 30 min at room temperature. Further optimization of the FLAG-JARID1B demethylase reaction included titration of the bio-H3K4me3 peptide (Fig. 3C), α-KG (Fig. 3D), and Fe(II) and ascorbate (data not shown). These results showed that the $K_m$ for bio-H3K4me3 is $\sim 15$ nM and the $K_m$ for α-KG is $\sim 5$ μM. Final screening conditions for JARID1B were 4 nM enzyme, 64 nM bio-H3K4me3 peptide, 50 μM Fe(II), 10 μM α-KG, and 100 μM ascorbate, and demethylase reactions proceeded for 30 min at room temperature.

**High Throughput Screening for JARID1B Inhibitors**—FLAG-JARID1B was screened against 15,134 compounds from several small molecule libraries. At a threshold of inhibition more than three standard deviations (about 30–40% inhibition), we identified 298 hits (Fig. 1C and supplemental Table 2). Of these confirmed hits, 24 compounds were selected based on their inhibition efficiency and structure for further dose-response analysis. As iron chelators tend to inhibit more efficiently at lower iron concentrations, dose-response analysis was performed in the presence of 15 and 50 μM Fe(II) to eliminate potential iron chelators. Many of these 24 compounds yielded low micromolar IC$_{50}$ values (Table 1 and supplemental Table 3), including several known demethylase inhibitors, such as 2,4-PDCA and catechols. As 2,4-PDCA was recently shown to inhibit the JARID1B catalytic core (23), these results validated our screening method. Consistent with this previous study, 2,4-PDCA inhibited JARID1B with an IC$_{50}$ value of about 5 μM (supplemental Table 3).

Among the top inhibitors, ChemBridge compounds 7812482 and 6339039 have very similar structures, but are likely reactive and were not further pursued. Caffeic acid and esculetin are catechols, which are potential iron chelators and reactive (17, 28). Consistent with this, lower IC$_{50}$ values for these catechols were observed in the presence of 15 μM Fe(II) than in the presence of 50 μM Fe(II). Furthermore, caffeic acid was identified as an inhibitor of JMJD2C/KDM4C and UTX/KDM6A (29).

A novel demethylase inhibitor, PBIT, was also identified as a potent inhibitor of JARID1B, with an IC$_{50}$ value of about 3 μM at both 15 μM and 50 μM Fe(II) (supplemental Table 3). To address the inhibitory specificity of PBIT and 2,4-PDCA against other JARID1 demethylases, these two compounds were tested against JARID1B (Fig. 4A), JARID1A (Fig. 4B), and JARID1C...
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FIGURE 3. Characterization of JARID1B. A, enzymatic activity of FLAG-JARID1B (4 nM) as monitored by AlphaScreen signal in the presence and absence of bio-H3K4me3 peptide substrate (64 nM). Bio-H3K4me2 peptide (64 nM) in the absence of enzyme serves as a positive control for the AlphaScreen assay. 
B, titration and time course of the FLAG-JARID1B. All assays were carried out in triplicate using 64 nM bio-H3K4me3 peptide and 2, 5, or 7.5 nM FLAG-JARID1B. Reactions were quenched with EDTA at various time points. No signal is seen for bio-H3K4me3 peptide assayed in the absence of FLAG-JARID1B, and bio-H3K4me2 (64 nM) assayed in the absence of enzyme represents a positive control for the AlphaScreen assay. C, demethylase activity of FLAG-JARID1B upon titration of the bio-H3K4me3 peptide for Kd determination. D, demethylase activity of FLAG-JARID1B on the bio-H3K4me3 peptide substrate upon titration of \( \alpha \)-ketoglutarate for Kd determination. Error bars in panels A–D indicate S.E.

TABLE 1
List of selected active compounds that inhibit the demethylase activity of JARID1B

The compounds are listed by structure, supplier ID or name (if available), and IC50 value from dose-response curves performed at 50 \( \mu \)M (high) and 15 \( \mu \)M (low) Fe(II).

| Compound structure | Supplier ID/name | IC50 (\( \mu \)M) high/low Fe(II) |
|--------------------|-----------------|-------------------------------|
| ![Compound structure 1](image1.png) | ChemBridge 7812182 | 1.15/1.06 |
| ![Compound structure 2](image2.png) | ChemBridge 6339039 | 1.31/1.00 |
| ![Compound structure 3](image3.png) | 2-[(4-methylphenyl)-1,2-benzisothiazol-3(2H)-one (PILOT) | 2.78/3.17 |
| ![Compound structure 4](image4.png) | Caffeic acid | 2.88/1.71 |
| ![Compound structure 5](image5.png) | 2,4-pyridinedicarboxylic acid (2,4-PDCA) | 4.47/4.07 |
| ![Compound structure 6](image6.png) | Esculetin | 4.60/2.57 |
| ![Compound structure 7](image7.png) | Ebselen | 5.17/7.63 |

(Fig. 4C). 10 \( \mu \)M PBIT inhibited the activities of all the JARID1 enzymes tested (Fig. 4, A–C). Dose-response analysis showed that PBIT is also a potent inhibitor of JARID1A and JARID1C, with IC50 values of 6 and 4.9 \( \mu \)M, respectively (supplemental Fig. 1A and 1B). Similarly, 2,4-PDCA inhibited all the JARID1 proteins tested, with an IC50 of 4.3 \( \mu \)M for JARID1B and 4.1 \( \mu \)M for JARID1A (Fig. 4, A–C, supplemental Fig. 1C, and supplemental Table 3). Next, we examined the specificity of PBIT and 2,4-PDCA for other JmjC domain-containing histone lysine demethylases. After initial optimization of the AlphaScreen assay for antibody specificity in the absence of enzyme (supplemental Fig. 2), analysis of the H3K27 demethylases UTX/KDM6A and JMJD3/KDM6B revealed that PBIT did not inhibit the activity of UTX or JMJD3 at 10 \( \mu \)M (Fig. 4, D and E). Likewise, 2,4-PDCA did not inhibit UTX at 10 \( \mu \)M (Fig. 4D). These results suggest that PBIT is a specific inhibitor of the JARID1 enzymes. 

In Vivo Validation of Inhibitors—To determine whether JARID1B could be inhibited by PBIT in cells, HeLa cells over-expressing full-length JARID1B were treated with 10 or 30 \( \mu \)M PBIT. As expected, in JARID1B-transfected cells, the levels of H3K4me3 decreased dramatically when compared with untransfected cells (Fig. 5). In contrast, in PBIT-treated cells, this decrease is blocked (Fig. 5).

To determine whether PBIT affects H3K4 methylation globally in vivo, H3K4 methylation levels were analyzed in histone extracts prepared from MCF7 cells after exposure to PBIT. Treatment of MCF7 cells with 10 \( \mu \)M PBIT for 72 h led to a dramatic increase of H3K4me3 levels, whereas H3K4me2 and H3K4me1 levels did not change significantly (supplemental Fig. 3). Similar results were obtained from MCF10A cells and 1445
mouse melanoma cells (data not shown), indicating that PBIT acts to inhibit the JARID1 H3K4 demethylases in vivo.

**PBIT Inhibits Cell Proliferation in a JARID1B Level-dependent Manner**—As JARID1B is overexpressed in human breast tumors (13), we asked whether inhibition of JARID1B activity has any growth inhibitory effect on breast cancer cells. To this end, we first analyzed the expression levels of JARID1B in immortalized human mammary epithelial cells (MCF10A) and human breast cancer cell lines (MCF7 and UACC-812). UACC-812 cells expressed a higher level of JARID1B than MCF7 or MCF10A cells (Fig. 6A). We then treated these cells with PBIT and analyzed for cell proliferation. Consistent with the higher expression levels of JARID1B in UACC-812 cells, exposure to 10 μM PBIT killed most of the UACC-812 cells (Fig. 6B), but showed minimal toxicity to MCF7 cells (Fig. 6D) and MCF10A cells (Fig. 6D). Similar results were obtained when we downregulated JARID1B by shRNA (Fig. 6E), where JARID1B shRNA inhibited proliferation of UACC-812 cells (Fig. 6F), but not MCF7 cells (Fig. 6G) or MCF10A cells (Fig. 6H).

**DISCUSSION**

The current study is the first report of a high throughput screen for inhibitors of the JARID1 family of demethylases. In this study, we first characterized full-length JARID1B. We showed that the apparent $K_m$ for bio-H3K4me3 is 15 nM. This is much lower than the reported $K_m$ for the JARID1B catalytic core (23), suggesting that other domains of JARID1B contribute to folding of the protein or substrate recognition and can be targeted for inhibition. We developed a very robust high throughput screen using the AlphaScreen platform to search for novel small molecule inhibitors of the histone lysine demethylase JARID1B. The signal-to-noise ratio was high (~17), even with only 4 μM enzyme, producing a Z’ factor of ~0.8 (supplemental Table 4). This allows for usage of small amounts...
of enzymes and for the identification of inhibitors with very low IC_{50} values. After screening over 15,000 small molecules, we identified over 90 validated compounds that inhibit JARID1B activity (supplemental Table 2), many of which have IC_{50} values in the low micromolar range.

Several types of JmjC demethylase inhibitors have been identified previously, including α-KG analogues, methyl-lysine analogues, 2,4-PDCA, 8-hydroxyquinoline, catechols, Ni(II), bipyridine, NCDM-32, disulfiram analogues, and hydroxamic acids (22). In this screen, we also identified many of these known JmjC demethylase inhibitors. For example, several of the hits here were identified in the miniaturized screen for inhibitors of the H3K9 demethylase JMJD2E with similar IC_{50} values (Table 1) (17), suggesting that these are nonspecific demethylase inhibitors. Some of these structures contain catechols, which are likely iron chelators and thus nonspecific inhibitors (28). Another potent hit, 2,4-PDCA, was identified as an inhibitor for multiple demethylases (18, 30, 31) and was recently shown to inhibit the JARID1B catalytic domain (23). Here we showed that 2,4-PDCA can also efficiently inhibit the JARID1 proteins, suggesting that it is a nonspecific demethylase inhibitor.

In addition to the known inhibitors, we also identified several novel inhibitors. One such hit, named PBIT, inhibited JARID1B with a low micromolar IC_{50} value. PBIT is unlikely to be an iron chelator as similar IC_{50} values were obtained from experiments performed at both 15 μM and 50 μM Fe(II). True iron chelators are more effective at lower iron concentrations by scavenging much of the available iron. PBIT potently inhibits JARID1A/B/C, suggesting that it can act as a pan-JARID1 inhibitor. 10 μM PBIT has a minimal effect on the H3K27 demethylases UTX and JMJD3 (Fig. 4, D and E). In addition, the IC_{50} value of PBIT for JMJD2E is 28 μM (18). Although we cannot exclude the possibility that PBIT also inhibits other JmjC demethylases and hydroxylases, our results suggest that PBIT is specific for the JARID1 enzymes.

PBIT is a derivative of benzisothiazolinone, a widely used microbicide and fungicide used in many home cleaning products (32). PBIT and its analogues were previously identified as inhibitors of salicylate synthase from Mycobacterium tuberculosis (33). The derivatives of benzisothiazolinone were explored as potential antiviral drugs by inhibiting enzymes such as macrophage migration inhibitory factor (34). Therefore, many PBIT analogues are available and may inhibit the JARID1 enzymes. In fact, the PBIT analogue ebselen exhibited an IC_{50} of ~6 μM against JARID1B (Table 1). Further studies optimizing the PBIT lead compound are currently ongoing. No crystal structure of the catalytic domains of the JARID1 enzymes has been published. It was shown recently that structure-guided virtual screen was essential to identify potent UTX inhibitors (35). Structural studies of the JARID1B enzyme with its inhibitors will be necessary to decipher their inhibitory mechanisms and to derive more potent inhibitors.

PBIT treatment prevented the JARID1B overexpression-induced decrease of H3K4me3 in HeLa cells (Fig. 5). In addition, treatment of MCF7 cells with PBIT increased global levels of H3K4me3 (supplemental Fig. 3), suggesting that this compound is capable of entering the nucleus and inhibiting JARID1 H3K4 demethylases. Our cell-based assays showed that PBIT inhibited cell growth in a JARID1B level-dependent manner (Fig. 6, A–D). Consistent with these experiments, we showed that JARID1B knockdown decreased the proliferation of UACC-812 cells, but not MCF7 or MCF10A cells (Fig. 6, E–H). The effect of JARID1B knockdown on UACC-812 cells is not as dramatic as PBIT treatment, suggesting that either incomplete knockdown of JARID1B or functional compensation of JARID1A contributes to proliferation and survival of HER2-positive (HER2+) UACC-812 cells. JARID1B is overexpressed in HER2+ cells and human tumors, suggesting that PBIT could be used to treat the HER2+ subtype of breast cancer. JARID1B knockdown was reported to decrease growth of MCF7 cells (8). In contrast, similar to our studies, JARID1B knockdown did not affect the proliferation of MCF7 cells (36). This discrepancy is likely due to different culture media used in these studies. Interestingly, PBIT treatment increased H3K4me3 level in MCF7 cells, but did not inhibit growth of these cells, suggesting that additional non-histone substrates of the JARID1 enzymes play critical roles in cell growth.

JARID1A and JARID1B knock-out mice are viable (2, 3, 37), suggesting that inhibition of JARID1A or JARID1B has minimal effects on normal cells in vivo. JARID1A loss inhibits tumorigenesis in two mouse endocrine cancer models (12), suggesting that a JARID1A inhibitor could be used to treat these cancers. In addition, the tumors formed in the JARID1A knock-out mice showed increased JARID1B expression, implying that inhibitors that block both JARID1A and JARID1B enzymes are more effective.

3 J. Cao and Q. Yan, unpublished data.
effective in preventing tumor formation (12). The importance of JARID1 inhibitors will need to be confirmed in mouse models in which the endogenous JARID1 genes were replaced with the genes encoding catalytic inactive enzymes. Although we showed that the apparent JARID1 inhibitor PBIT has selective inhibitory activity on a HER2⁺ breast cancer cell line, the effi-

**FIGURE 6. PBIT inhibits cell proliferation in a JARID1B level-dependent manner.** A, Western blot analysis of UACC-812, MCF7, and MCF10A cells with the indicated antibodies. B–D, WST-1 cell proliferation assays of UACC-812 (B), MCF7 (C), and MCF10A (D) cells in the presence of PBIT at the indicated concentrations. Shown are the ratio of absorbance at 440 nm of day 3/day 0 (D3/D0) with S.E. E, real time RT-PCR analysis of JARID1B mRNA in stable cell lines with the indicated shRNA hairpins. Shown are mean values with S.E. F–H, WST-1 cell proliferation assays of UACC-812 (F), MCF7 (G), and MCF10A (H) cells with control or JARID1B shRNA hairpins. Shown are the ratio of absorbance at 440 nm of day 3 or 4/day 0 (D3 or D4/D0) with S.E.
cacy of PBIT and its derivatives on breast cancer needs to be further investigated with additional cell lines and in xenograft or genetically engineered mouse cancer models. As the JARID1 enzymes contribute strongly to tumorigenesis and drug resistance in multiple cancer types (2, 38), these inhibitors may also be effective for cancer therapy in those settings.

Acknowledgments—We thank Dr. Yi Zhang for providing FLAG-JARID1A and FLAG-JARID1B baculoviral constructs, Dr. Yang Shi for providing FLAG-JARID1C baculoviral construct, Drs. Kristian Helin and Stuart Orkin for providing His-FLAG-UTX baculoviral construct, and Dr. William Hahn for providing PLKO.1-shGFP construct. We thank Drs. Jon Morrow and Tian Xu for sharing their construct, and Dr. William Hahn for providing PLKO.1-shGFP construct. We thank members of the Rosenberg, Nguyen, Stern, and Yan laboratories for their kind help and valuable discussions. We thank Laura Abriola for technical help on hit validation and Amber Anders for help on generating the pCDNA3.1(−)-3×HA-JARID1B plasmid.

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