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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ABSTRACT

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 4 in histone H3 (H3K4me3), a mark for actively transcribed genes. JARID1B is over-expressed in several cancers, including breast cancer, prostate cancer and lung cancer. 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-PDCA and catechols. More importantly, we identified several novel inhibitors, including N-phenyl-benzisothiazolinone (PBIT), which inhibits JARID1B with an IC_{50} of about 3 μM *in vitro*. PBIT treatment inhibited removal of H3K4me3 by JARID1B in cells. Furthermore, this compound inhibited proliferation of cells expressing higher level of JARID1B. These results suggest that this novel small molecule inhibitor is a lead compound that can be further optimized for cancer therapy.

BACKGROUND

Methylation at histone H3 lysine 4 (H3K4) is associated with active transcription, while demethylation at this site typically results in gene silencing. The demethylases responsible for the demethylation of tri- and di- methylated H3K4 are known as the JARID1(KDM5) family of enzymes. This family consists of JARID1A (RBP2/KDM5A), JARID1B (PLU1/ KDM5B), JARID1C (SMCX/ KDM5C), and JARID1D (SMCY/ KDM5D). The demethylase reaction for JARID1 enzymes, like that of other JmjC domain containing demethylases, is that of an Fe (II) – alpha-ketoglutarate (α-KG) dependent catalytic mechanism.

The JARID1 family of demethylases have been linked to diseases such as X-linked mental retardation and cancer. Specifically, JARID1A is over-expressed in gastric and lung cancers, and forms a fusion protein with the nuclear pore complex protein (NUP98) in acute myeloid leukemia patients (van Zutven et al., 2006). JARID1B is over-expressed in breast, ovarian, prostate, bladder, and lung cancers, and acts to repress the transcription of the tumor suppressor gene BRCA1 (Blair et al., 2011). JARID1B is also required for the slow-cycling growth of a subpopulation of melanoma cells (Roesch et al., 2010). These studies suggest that JARID1A and JARID1B are potential oncogenes, and therefore represent attractive targets for anti-cancer therapies.

Detection of JARID1B demethylase activity

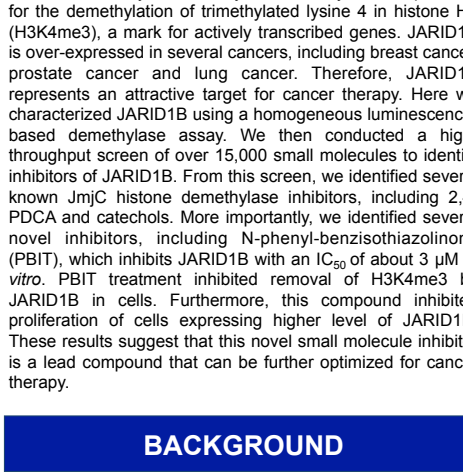

Figure 1. Schematic of the AlphaScreen assay used for high through-put screening of JARID1B. JARID1B acts on a the biotin-labeled H3K4me3 peptide to form a bio-H3K4me2/1 peptide product. Upon laser excitation, energy is transferred from the bio-H3K4me2/1 peptide bound to streptavidin-coated donor beads to the H3K4me2/1 antibody bound protein A coated acceptor beads. Enzymatic activity is seen as a signal at 520-620 nm.

METHODS

Histone Demethylase Assay- Histone demethylase assays were performed in 384 well white plates (Corning 3574). Demethylase buffer conditions were as follows: 10 μM α -KG, 100 μM ascorbate, 50 μM $(NH_4)_2Fe(SO_4)_2$, 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 biotinylated H3K4me3 peptide and 4 nM Flag-JARID1B enzyme in a 10 μl reaction at 25 ℃ for 30 min. Demethylated H3K4 products were detected using AlphaScreen antibody/ bead mix containing 7.5 mM ethylenediaminetetraacetic acid (EDTA) and 0.15 μg/ml H3K4me1 antibody in a 20 μl final volume.

Drug Screening Libraries and Conditions-Flag-JARID1B was screened against 15,600 compounds. These compounds were derived from the Yale Small Molecule Discovery Center (YSMDC) pilot collection (MicroSource Gen-Plus, MicroSource Pure Natural Products, NIH Clinical Collection), the Enzo Epigenetics Library, and the ChemBridge mw-set library.

RESULTS

Overview of HTS results

Figure 2. Overview of the high throughput screen to identify compounds that inhibit JARID1B activity, and characterization in breast cancer cells.

2-(4-methylphenyl)-1,2-benzisothiazol-3(2H)-one (PBIT)

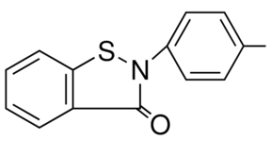

Figure 3. Structure of 2-(4-methylphenyl)-1,2-benzisothiazol-3(2H)-one (PBIT).

Purification and expression of FLAG-JARID1B

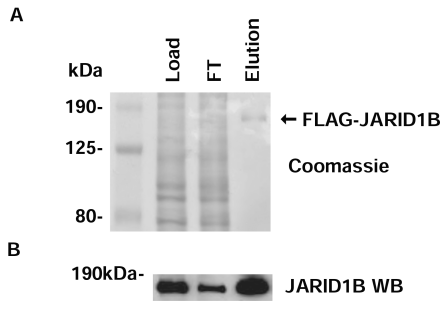

Figure 4. Analysis of recombinant FLAG-JARID1B by coomassie staining **(A)** and western blot analysis **(B)**. FT, flow-through. FLAG-JARID1B appears as a ~170 kDa band.

PBIT is selective for the JARID1 enzymes

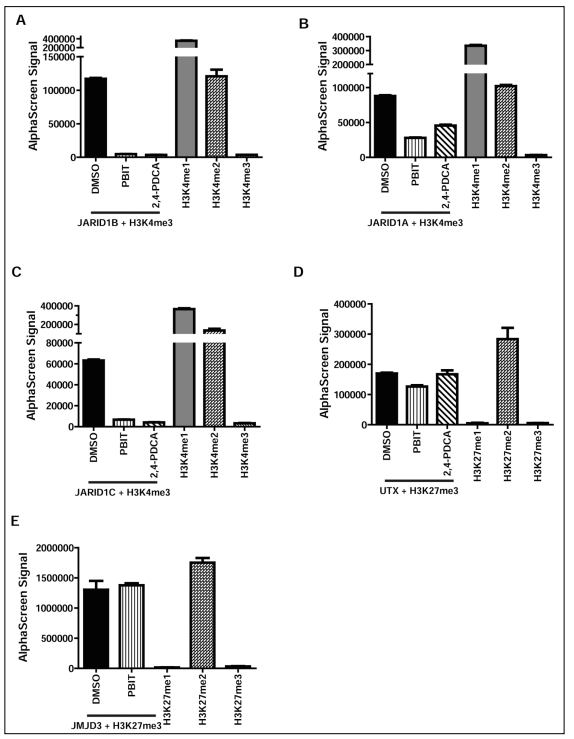

Figure 5. JARID1B **(A)**, JARID1A **(B)**, and JARID1C **(C)** were assayed with 64 nM bio-H3K4me3 peptide and PBIT or 2,4-PDCA (10 μM). UTX **(D)** and JMJD3 **(E)** were assayed with 64 nM bio-H3K27me3 peptide and PBIT or 2,4-PDCA (10 μM), and demethylase activity was detected using anti-H3K27me2 antibody.

PBIT inhibits H3K4me3 demethylaton *in vivo*

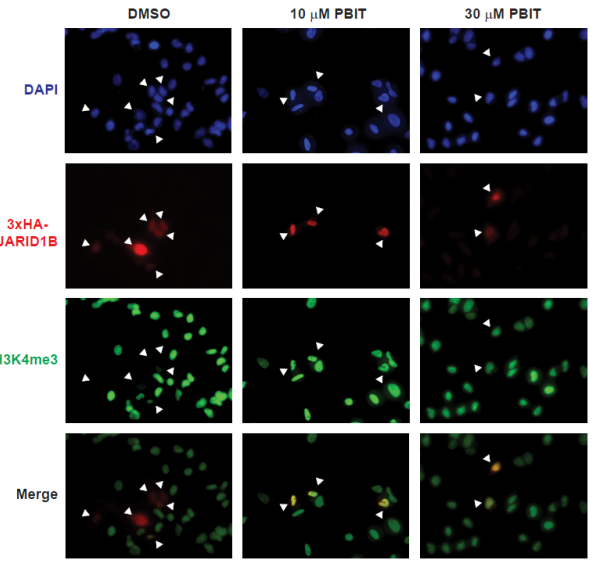

Figure 6. 3xHA-JARID1B was expressed in HeLa cells, and cells were incubated with 0.1% DMSO, or 10 μM or 30 μM PBIT for 24 hours. Cell nuclei were identified by DAPI staining (top panel, blue). 3xHA-JARID1B was identified by HA-immunofluorescence (second panel, red), and H3K4me3 was visualized by H3K4me3 immunofluorescence (third panel, green). The merged images of HA and H3K4me3 immunofluorescence are shown in the bottom panel. Triangles indicate transfected cells.

PBIT inhibits cell proliferation in a JARID1 dependent manner

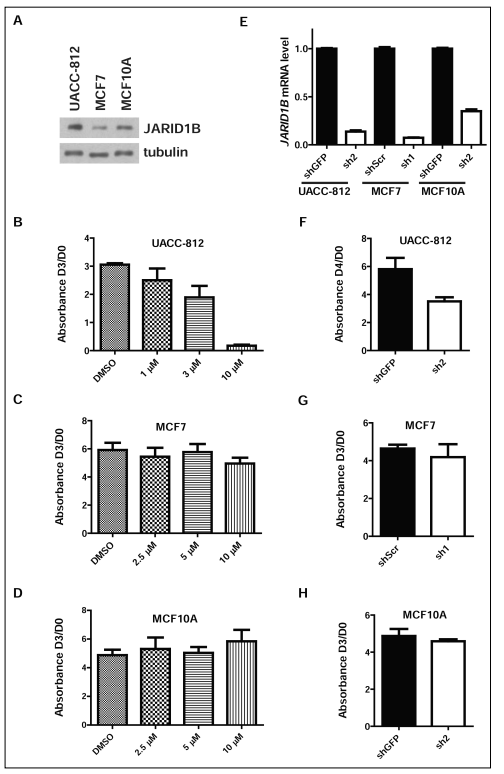

Figure 7. (A) Western blot analysis of UACC-812, MCF7 and MCF10A cells with the indicated antibodies. **(B-D)** WST-1 cell proliferation assays of UACC-812 **(B)**, MCF7 **(C)** and MCF10A **(D)** cells in the presence of PBIT at the indicated concentrations. Shown are the ratio of absorbance at 440 nm of day 4/day 0 (D4/D0) with SEM. **(E)** Real time RT-PCR analysis of *JARID1B* mRNA in stable cell lines with the indicated shRNA hairpins. Shown are mean values with SEM. **(F-H)** WST-1 cell proliferation assays of UACC-812 **(F)**, MCF7 **(G)** and MCF10A **(H)** cells with control or JARID1B shRNA hairpins.

CONCLUSIONS

- We identified over 100 validated compounds that inhibit JARID1B activity by at least 30%. Dose response challenge of 24 of these compounds resulted in low micromolar IC_{50} values for JARID1B inhibition.
- One hit, named PBIT, was further validated *in vitro*, and appears to be specific for H3K4me3 demethylases.
- PBIT treatment prevented the JARID1B overexpression-induced decrease of H3K4me3 in HeLa cells, suggesting this compound is capable of entering the nucleus and inhibiting JARID1 H3K4 demethylases.
- PBIT inhibits cell proliferation in a JARID1 dependent manner. PBIT decreased cell proliferation of UACC-812 cells. This finding was confirmed by analysis of JARID1B knockdown in UACC812 cells, and could be explained by increased JARID1B expression. In contrast, no effect was seen at this concentration for immortalized (MCF10A) cells or breast cancer MCF7 cells.

ACKNOWLEDGEMENTS

We would like to acknowledge the following funding sources for this work, including the Yale Small Molecule Discovery Center (YSMDC) Pilot Grant, and the Yale Center for Clinical Investigation (YCCI) Junior Faculty Scholar Award.