Small Molecule Inhibitors of the Histone Lysine Demethylase JARID1B/PLU1

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ABSTRACT

To identify small molecule inhibitors of JARID1B, we performed a high throughput screen using the Amplified Luminescent Proximity Homogenous Assay (ALPHA) platform (**Figure 1**). JARID1B was screened against a diverse library set consisting of 15,600 molecules. From the screen we have identified several compounds that yield low micromolar IC_{50} values for JARID1B upon dose response. Specifically, the novel small molecule known as "compound 5" and also 2,4-pyridine dicarboxylic acid (2,4-PDCA) effectively inhibit PLU1 upon *in vitro* confirmation. We are now in the process of testing of these compounds on H3K4 methylation and cell proliferation in breast cancer and melanoma cell lines. These studies reveal specific and potent inhibitors of JARID1B that can be further developed for use as therapeutic agents targeting these enzymes in cancer.

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.

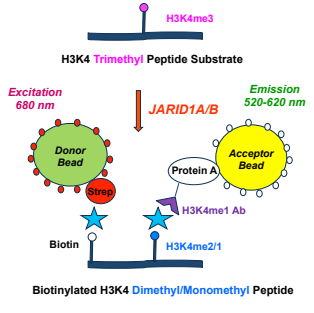


Figure 1. Schematic of the AlphaScreen assay used for high through-put screening of PLU1. PLU1 acts on a the biotin-labeled H3K4me3 peptide to form a bio-H3K4me2/1 peptide product. Upon laser excitation, energy is transferred from the bio-H3K4me2/1 peptide bound to streptavidin-coated donor beads to the H3K4me1 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-PLU1 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-PLU1 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

PLU1 Demethylase Activity

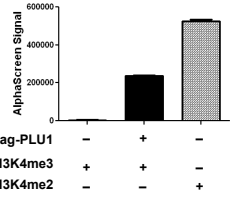


Figure 2. Demethylase activity of Flag-PLU1. AlphaScreen signal representing PLU1 demethylase activity on a biotinylated H3K4me3 peptide substrate is shown. Activity is seen only in the presence of both the PLU1 enzyme and bio-H3K4me3 peptide substrate. Bio-H3K4me2 peptide assayed in the absence of PLU1 enzyme is a positive control for Alpha-Screen detection.

Overview of the Small Molecule Inhibitor Screen Results

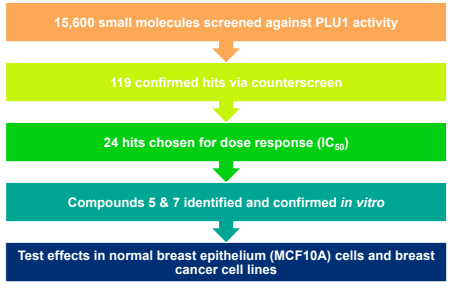


Figure 3. Overview of the high throughput screen to identify compounds that inhibit PLU1 activity, and characterization in breast cancer cells.

RESULTS

Dose-Response Curve for PLU1 with Compound 5

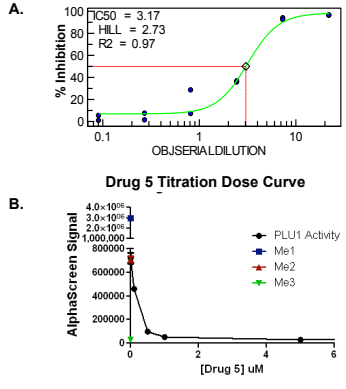
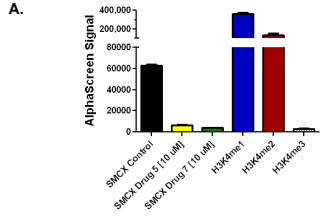


Figure 4. Inhibition of PLU1 by compound 5. **A.** Dose response curve (IC_{50}) for compound 5. Percent inhibition of PLU1 activity is illustrated as a function of increasing drug concentration. **B**. *In vitro* titration of compound 5 against Flag-PLU1 activity. The IC_{50} of compound 5 for PLU1 is about 3 μM. Increasing concentration of compound 5 leads to decreased AlphaScreen signal and thus decreased PLU1 enzymatic activity.

Effect of Drug 5 on SMCX Demethylase Activity



Effect of Drug 5 on UTX Demethylase Activity

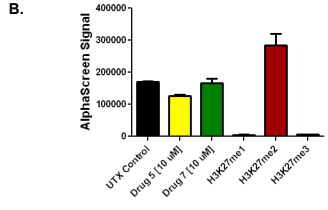


Figure 5. Specificity of compounds 5 and 7. AlphaScreen assay of the demethylase activity of Flag-SMCX (**A**) and His-Flag-UTX (**B**) in the presence of compounds 5 and 7 (10 μM). The Flag-SMCX reaction uses bio-H3K4me3 peptide substrate (64 nM), and the His-Flag-UTX reaction uses bio-H3K27me3 peptide substrate (64 nM).

Cell Proliferation (WST-1) Assay: 72 hr Exposure to Drug 5

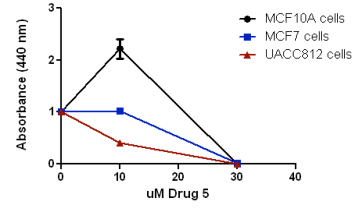


Figure 6. Cell proliferation (WST-1) assay of MCF10A, MCF7, and UACC812 cells exposed to compound 5. Cells were treated with 0.1% DMSO, 10 μM, or 30 μM compound 5 for 72 hrs. The WST-1 colormetric reagent was added for 4 hrs and the plate was read at 440 nm absorbance. Cell proliferation of UACC812 cells is compromised upon exposure to 10 μM compound 5.

CONCLUSIONS

Upon optimization of the AlphaScreen assay for JARID1 demethylase activity on biotinylated H3K4me3 peptide substrates, we now have a very sensitive assay for detecting PLU1 activity and thus its inhibition. As a result of screening over 15,000 small molecules and known medicines for inhibition of PLU1 activity, we have identified over 100 validated compounds that inhibit PLU1 activity by at least 30%. Dose response challenge of 24 of these compounds resulted in low micromolar IC_{50} values for PLU1 inhibition. One of these identified small molecules, compound 5, was further validated *in vitro*, and appears to be specific for H3K4me3 demethylases as seen by inhibition of other JARID1 family members. Compound 5 shows increased specificity for PLU1 compared to the other JARID1 family members. In contrast, compound 5 has little effect on the activity of the H3K27me3 demethylase UTX.

Upon exposure of normal and breast cancer cell lines to 10 μM compound 5, decreased cell proliferation of UACC812 cells (containing a 15-fold amplification of the HER2/Neu oncogene sequence) was quantified. In contrast, a protective effect was seen at this concentration for normal (MCF10A) cells and breast cancer MCF7 cells. This is consistent with previous data where MCF7 cells showed no change in cell proliferation upon PLU1 knockdown. It remains to be seen if the proliferation effect of compound 5 in the UACC812 cells is specifically due to inhibition of the PLU1 enzyme.

ACKNOWLEDGEMENTS

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