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TITLE:

Assays for Validating Histone Acetyltransferase Inhibitors

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SUMMARY:

Inhibitors of histone acetyltransferases (HATs, also known as lysine acetyltransferases), such as CBP/p300, are potential therapeutics for treating cancer. However, rigorous methods for validating these inhibitors are needed. Three in vitro methods for validation include HAT assays with recombinant acetyltransferases, immunoblotting for histone acetylation in cell culture, and ChIP-qPCR.

ABSTRACT:

Lysine acetyltransferases (KATs) catalyze acetylation of lysine residues on histones and other proteins to regulate chromatin dynamics and gene expression. KATs, such as CBP/p300, are under intense investigation as therapeutic targets due to their critical role in tumorigenesis of diverse cancers. The development of novel small molecule inhibitors targeting the histone acetyltransferase (HAT) function of KATs is challenging and requires robust assays that can validate the specificity and potency of potential inhibitors.

This article outlines a pipeline of three methods that provide rigorous in vitro validation for novel HAT inhibitors (HATi). These methods include a test tube HAT assay, Chromatin Hyperacetylation Inhibition (ChHAI) assay, and Chromatin Immunoprecipitation-quantitative PCR (ChIP-qPCR). In the HAT assay, recombinant HATs are incubated with histones in a test tube reaction, allowing for acetylation of specific lysine residues on the histone tails. This reaction can be blocked by a HATi and the relative levels of site-specific histone acetylation can be measured via immunoblotting. Inhibitors identified in the HAT assay need to be confirmed in the cellular environment.

The ChHAI assay uses immunoblotting to screen for novel HATi that attenuate the robust hyperacetylation of histones induced by a histone deacetylase inhibitor (HDACi). The addition of an HDACi is helpful because basal levels of histone acetylation can be difficult to detect via immunoblotting.

The HAT and ChHAI assays measure global changes in histone acetylation, but do not provide information regarding acetylation at specific genomic regions. Therefore, ChIP-qPCR is used to investigate the effects of HATi on histone acetylation levels at gene regulatory elements. This is accomplished through selective immunoprecipitation of histone-DNA complexes and analysis of the purified DNA through qPCR. Together, these three assays allow for the careful validation of the specificity, potency, and mechanism of action of novel HATi.

INTRODUCTION:

Lysine acetyltransferases (KATs) catalyze the acetylation of lysine residues on both histone and non-histone proteins[1–4]. Recent research reveals that KATs and their acetyltransferase function can promote solid tumor growth[4–9]. For example, CREB-binding protein (CBP)/p300 are two paralogous KATs that regulate numerous signaling pathways in cancer[2,3]. CBP/p300 have a well characterized histone acetyltransferase (HAT) function and catalyze Histone 3 Lysine 27 acetylation (H3K27ac)[2,4,5,10,11], an important marker for active enhancers, promoter regions and active gene transcription[12–14]. CBP/p300 serve as critical co-activators for pro-growth signaling pathways in solid tumors by activating transcription of oncogenes through acetylation of histones and other transcription factors[4,9,15–18]. Due to their role in tumor progression, CBP/p300 and other KATs are under investigation for the development of novel inhibitors that block their oncogenic function[4–9,18–20]. A-485 and GNE-049 represent two successful attempts to develop potent and specific inhibitors for CBP/p300[4,9]. Additional inhibitors are currently under investigation for CBP/p300 and other KATs.

The quality of previously described KAT inhibitors (KATi) is being called into question, with many inhibitors showing off target effects and poor characterization[21]. Therefore, rigorous characterization and validation of novel drug candidates is essential for the development of high-quality chemical probes. Outlined here are three protocols that form a pipeline for screening and rigorously validating the potency and specificity of novel KATi, with a specific focus on inhibiting the HAT function (HATi) of KATs. CBP/p300 and their inhibitors are used as examples, but these protocols can be adapted for other KATs that have a HAT function[7].

The first protocol is an in vitro histone acetyltransferase (HAT) assay that utilizes purified recombinant p300 and histones in a controlled test tube reaction. This assay is simple to perform, is cost-effective, can be used to screen compounds in a low throughput setting, and does not require radioactive materials. In this protocol, recombinant p300 catalyzes lysine acetylation on histone tails during a brief incubation period and the levels of histone acetylation are measured using standard immunoblotting procedures. The enzymatic reaction can be performed in the presence or absence of CBP/p300 inhibitors to screen for compounds that reduce histone acetylation. Additionally, the HAT assay can be used to verify whether novel compounds are selective for CBP/p300 by assessing their activity against other purified KATs,

such as PCAF. The HAT assay is an excellent starting point for investigating novel inhibitors due to its simplicity, low cost, and the ability to determine the potency/selectivity of an inhibitor. Indeed, this protocol is often used in the literature as an in vitro screen[5,10]. However, inhibitors identified in the HAT assay are not always effective in cell culture because a test tube reaction is much simpler than a living cell system. Therefore, it is essential to further characterize inhibitors in cell culture experiments[22,23].

The second protocol in the pipeline is the Chromatin Hyperacetylation Inhibition (ChHAI) assay. This cell based assay utilizes histone deacetylase inhibitors (HDACi) as a tool to hyperacetylate histones in chromatin before co-incubation with a HATi[24]. Basal histone acetylation can be low in cell culture, making it difficult to probe for via immunoblotting without the addition of an HDACi to increase acetylation. The purpose of the ChHAI assay is to identify novel HATi that can attenuate the increase in histone acetylation caused by HDAC inhibition. The advantages of this assay include its low cost, relative ease to perform, and the use of cells in culture, which provides more physiological relevance than the test tube HAT assay. Similar to the HAT assay, this protocol uses standard immunoblotting for data collection.

The HAT and ChHAI assays provide data about the potency of novel compounds for inhibiting global histone acetylation, but do not provide insight into how these compounds affect modifications at specific genomic regions. Therefore, the final protocol, Chromatin Immunoprecipitation-quantitative Polymerase Chain Reaction (ChIP-qPCR) is a cell culture experiment that investigates DNA-protein interactions at specific regions of the genome. In the ChIP protocol, chromatin is crosslinked to preserve DNA-protein interactions. The chromatin is then extracted from cells and the DNA-protein complex undergoes selective immunoprecipitation for the protein of interest (e.g., using an antibody specific for H3K27ac). The DNA is then purified and analyzed using qPCR. For example, ChIP-qPCR can be used to determine if a novel HATi downregulates histone acetylation at individual oncogenes, such as Cyclin D1[25]. While ChIP-qPCR is a common technique used in the field, it can be difficult to optimize[4,10,26]. This protocol provides tips for avoiding potential pitfalls that can occur while performing the ChIP-qPCR procedure and, also includes several quality control checks that should be performed on the data.

When used together, these three protocols allow for the rigorous characterization and validation of novel HATi. Additionally, these methods offer many advantages because they are easy to perform, relatively cheap and provide data on global as well as regional histone acetylation.

PROTOCOL:

1. In vitro HAT assay

1.1. Buffer preparation

NOTE: See **Table 1** for buffer recipes.

1.1.1. Prepare 5x assay buffer and 6x Sodium Dodecyl Sulfate (SDS) and store at -20 ˚C. Aliquot SDS in 1 mL aliquots.

1.1.2. Prepare 10x SDS gel running buffer and 10x TBST and store at room temperature.

1.1.3. Prepare 1x transfer buffer and store at 4 ˚C.

CAUTION: Check safety data sheet for all chemicals used in this protocol. SDS, DTT, and bromophenol blue are toxic and should not be ingested, inhaled, or exposed to the skin or eyes. Please see safety data sheet for proper handling procedures. Please use a chemical fume hood for handling dangerous chemicals.

1.2. HAT reaction

NOTE: Anacardic acid is a known p300 inhibitor[3] and is used as an example to demonstrate how the HAT assay can identify novel p300 inhibitors. See **Supplementary Protocol** (**Schematic 1**) for a schematic of step 1.2.1.

1.2.1. Prepare the following enzymatic reaction in a 0.2 mL PCR tube: 2 µL of 5x assay buffer, 1 µL of purified p300 (0.19 µg/µL), 1 µL of anacardic acid (HATi) or DMSO control diluted in 1x assay buffer and 2 µL of autoclaved ddH_2O. Pre-incubate this mixture for 10 min at room temperature. Then add 3 µL of 100 µM Acetyl-CoA and 1 µL of purified H3.1 (0.2 µg/µL) to the reaction.

1.2.2. Incubate the complete reaction mixture at 30 ˚C for 1 h in a PCR thermal cycler.

1.2.3. Add 2-mercaptoethanol at a 1:10 ratio to the 6x SDS sample buffer.

1.2.4. Remove samples from the PCR thermal cycler and add 2 µL of 6x SDS (with 2-mercaptoethanol added) to the reaction mix.

CAUTION: 2-mercaptoethanol is toxic and should be used inside a chemical fume hood. Please see safety data sheet for proper handling.

1.2.5. Heat samples at 95 ˚C for 5 min on a heat block and cool on ice. Store the samples at -20 or -80 ˚C or perform gel electrophoresis and immunoblotting as detailed below.

1.3. Gel electrophoresis and immunoblotting

NOTE: If unfamiliar with gel electrophoresis and immunoblotting, see this standard procedure[27] for additional details on how to perform steps 1.3.1-1.3.17. Additional information can be found here[28–33].

1.3.1. Pipette 10 μL of samples (from step 1.2.5.) into the wells of a 4-20% gradient polyacrylamide gel. Pipette 5 μL of protein ladder into one of the wells as a molecular weight reference. Run the gel at 120 V for 90 min using a gel tank.

1.3.2. Transfer the gel to a polyvinylidene difluoride (PVDF) membrane at 100 V for 70 min using a transfer tank.

1.3.3. Remove the membrane from the transfer apparatus and place it in a plastic container. Block the membrane by adding 1x TBST (containing 5% milk) to the container and gently shake for 1 h at room temperature.

1.3.4. Remove the 1x TBST from step 1.3.3. Incubate the membrane overnight with selected site-specific acetyl antibodies (e.g., H3K18ac or H3K27ac primary antibodies at a 1:5,000 dilution in 1x TBST containing 5% milk) at 4 ˚C with gentle shaking.

1.3.5. Remove the primary antibody solution. Wash the membrane 2x with 1x TBST (no milk) at room temperature with gentle shaking for 15 min each wash.

1.3.6. Dilute the secondary antibody at 1:20,000 in 1x TBST (containing 5% milk) and incubate the membrane for 1 h at room temperature with gentle shaking.

1.3.7. Remove the secondary antibody solution. Wash the membrane 2x with 1x TBST (no milk) at room temperature with gentle shaking for 15 min each wash.

1.3.8. Drain the 1x TBST from the membrane. Mix HRP substrate peroxide solution and HRP substrate luminol solution in a 1:1 ratio (1 mL of each) and pipette 2 mL of the combined solution to the membrane surface.

1.3.9. Incubate the solution with the membrane for 5 min at room temperature.

1.3.10. Drain excess chemiluminescent substrate from the membrane onto a paper towel and place the membrane in plastic wrap inside an x-ray cassette holder.

1.3.11. Move to a dark room dedicated to x-ray film processing. Expose the membrane to an x-ray film by placing the film on top of the membrane and closing the cassette for 30 s.

NOTE: Time of contact between the film and membrane must be determined experimentally. Strong signals will need short exposures (seconds) and weaker signals may need longer exposures.

1.3.12. Remove the x-ray film from the cassette and process the film by running it through an x-ray film processor. See the manufacturer manuals for specific instructions on how to process the x-ray film.

1.3.13. Remove the membrane from the plastic wrap and wash it with ddH_2O for 5 min at room temperature with gentle shaking.

1.3.14. Incubate the membrane with 0.2 M NaOH for 5 min at room temperature with gentle shaking.

1.3.15. Wash the membrane with ddH_2O for 5 min at room temperature with gentle shaking.

1.3.16. Block the membrane by adding 1x TBST (containing 5% milk) to the container and gently shake for 1 h at room temperature.

1.3.17. Add the next primary antibody dilution (e.g., probe for H3K27ac if first antibody used was H3K18ac) and shake overnight at 4 ˚C. Repeat steps 1.3.4-1.3.17 until all antibody probes are completed.

2. ChHAI assay

2.1. In vitro drug treatments and analysis of acetylated histones

NOTE: A-485 is a potent and well characterized p300 HATi[2,4] . This inhibitor will be utilized in the remaining assays due to its efficacy and specificity in cell culture. MS-275 (Entinostat)[24] is an HDACi that markedly increases histone acetylation levels and is used to facilitate easier detection of acetylation probes with standard immunoblotting. See **Supplementary Protocol** (**Schematic 2**) for a schematic of the drug dilutions used in step 2.1.

2.1.1. Seed 100,000 MCF-7 cells in a 12 well plate and allow cells to grow to 80-90% confluency in 1 mL of cell culture medium. Mark the wells for the following experimental design: well 1: DMSO control (reference point); well 2: A-485 (3 μM); well 3: A-485 (10 μM); well 4: MS-275 (3 μM); well 5: MS-275 (3 μM) + A-485 (3 μM); well 6: MS-275 (3 μM) + A-485 (10 μM).

NOTE: For culturing MCF-7 cells, use complete DMEM media and allow cells to grow at 37 ˚C with 5% CO_2. See **Table 1** for complete DMEM recipe.

2.1.2. At 24 h after seeding, pipette 4 mL of complete DMEM media to a sterile 15 mL conical tube. Pipette 2 μL of MS-275 (6 mM in DMSO) to the 4 mL of medium for a final concentration of 3 μM MS-275.

2.1.3. Pipette 2 μL of DMSO to 4 mL of medium in a separate sterile 15 mL conical tube.

CAUTION: Please check safety data sheet for proper handling of DMSO. Some glove types are not rated for handling DMSO.

2.1.4. Aspirate the cell culture medium from wells 4-6 and pipette 1 mL of 3 μM MS-275 in medium (step 2.1.2) to each well. Discard unused diluted MS-275.

2.1.5. Aspirate the cell culture medium from wells 1-3 and pipette 1 mL of diluted DMSO (step 2.1.3) to each well. Discard unused diluted DMSO.

2.1.6. Return the cells to the incubator and incubate for 4 h to allow accumulation of acetylated histones in cells exposed to MS-275 (wells 4-6).

NOTE: MS-275 is an HDACi and will cause histone hyperacetylation[24]. This 4 h pre-incubation is necessary to allow MS-275 to induce hyperacetylation before the addition of A-485, which reduces histone acetylation[2,4].

2.1.7. After 4 h of incubation with MS-275, prepare the following dilutions in separate sterile 1.5 mL tubes by pipetting: 1.0 μL of DMSO to 1 mL of DMEM media; 0.5 μL of DMSO and 0.5 μL A-485 (6 mM) to 1 mL of DMEM media; 0.5 μL of DMSO and 0.5 μL of A-485 (20 mM) to 1 mL of DMEM media; 0.5 μL of DMSO and 0.5 μL of MS-275 (6 mM) to 1 mL of DMEM media; 0.5 μL of A-485 (6 mM) and 0.5 μL of MS-275 (6 mM) to 1 mL of DMEM media; 0.5 μL of A-485 (20 mM) and 0.5 μL of MS-275 (6 mM) to 1 mL of DMEM media.

2.1.8. Aspirate the cell culture medium from wells 1-6 and pipette 1 mL of dilution 1 to well 1, dilution 2 to well 2, dilution 3 to well 3, dilution 4 to well 4, dilution 5 to well 5, and dilution 6 to well 6.

NOTE: A general rule is to balance DMSO (solvent) content between experimental groups and not to exceed 0.1% DMSO content in cell culture to avoid cellular toxicity and changes in proliferation.

2.1.9. Return the cells to the incubator and culture for 20 h.

2.1.10. After 20 h, aspirate the cell culture medium from wells 1-6.

2.1.11. Wash the cells by pipetting 1 mL of PBS to wells 1-6. Aspirate the PBS.

2.1.12. Add 100 μL of 1x passive lysis buffer (see **Table 1**) to wells 1-6. Store cell culture plate (with samples in passive lysis buffer) at −80 °C overnight for freeze-thaw and lysis of cells.

CAUTION: Please check safety data sheet for all chemicals before making buffers. CDTA can cause serious eye damage and irritation.

2.1.13. Thaw samples at room temperature with gentle shaking for 10 min. Transfer samples to separate 1.5 mL tubes and immediately place on ice.

2.1.14. Measure protein concentration of each sample. Protein concentration can be determined using several well-established protocols[34].

2.1.15. Equilibrate protein concentration between samples 1-6 (in an equal volume) using 1x passive lysis buffer to dilute, as necessary.

2.1.16. Add 2-mercaptoethanol at a 1:10 ratio to 6x SDS sample buffer.

2.1.17. Add 6x SDS sample buffer with 2-mercaptoethanol to samples 1-6 to a final concentration of 1x SDS sample buffer.

2.1.18. Heat samples at 95 ˚C for 5 min on a heat block and cool on ice. Samples can be stored at -20 ˚C or -80 ˚C until step 2.1.19.

2.1.19. Pipette a volume containing 30 μg of protein for samples 1-6 to the wells in a 4-20% gradient polyacrylamide gel. Perform immunoblotting procedure according to protocol described in Protocol 1.

3. ChIP-qPCR

NOTE: The protocol below is described for inhibitors of p300 as an example.

3.1. Buffer preparation

NOTE: See **Table 1** for buffer recipes. The general steps of the ChIP protocol (e.g. buffer recipes, wash times and centrifugation times) below are modified and adapted from the manufacturer's recommendations of a commercially available kit (see **Table of Materials**) and from the literature[35,36].

3.1.1. Prepare ChIP dilution buffer, nuclei swelling buffer, low salt wash buffer, high salt wash buffer, LiCl wash buffer and TE buffer. Store at 4 ˚C.

3.1.2. Prepare SDS lysis buffer, 10x glycine buffer and ChIP elution buffer. Store at room temperature.

CAUTION: Please check safety data sheet for all chemicals before making buffers to ensure proper handling.

3.2. Drug treatment

NOTE: See **Supplementary Protocol** (**Schematic 3**) for a schematic of the drug dilutions used in step 3.2.

3.2.1. Seed MCF-7 cells in two 15 cm culture dishes and grow cells to 90% confluency in 12 mL of the complete DMEM medium. Mark the dishes for the following experimental design: Dish 1: DMSO control (reference point); Dish 2: A-485 (3 μM).

NOTE: For culturing MCF-7 cells, use complete DMEM media and grow at 37 ˚C with 5% CO_2. See **Table 1** for complete DMEM recipe.

3.2.2. In a sterile 15 mL conical tube, pipette 12 mL of DMEM media and pipette 6 μL of DMSO. Mix well.

3.2.3. In a separate sterile 15 mL conical tube, pipette 12 mL of DMEM media and pipette 6 μL of A-485 (6 mM in DMSO) to get a final concentration of 3 μM A-485. Mix well.

3.2.4. Aspirate the media from Dish 1 and 2.

3.2.5. Add the 12 mL of diluted DMSO in DMEM (step 3.2.2.) to Dish 1.

3.2.6. Add the 12 mL of 3 μM A-485 in DMEM (step 3.2.3.) to Dish 2.

3.2.7. Return the cell culture dishes to the incubator and incubate for 24 h.

3.3. Cell fixation

3.3.1. Pipette 330 μL (27.5 μL per mL) of 37% formaldehyde to the complete media and gently swirl the plate to mix.

CAUTION: Formaldehyde is toxic. Please see safety data sheet for proper handling procedures.

3.3.2. Incubate for 10 min at room temperature.

3.3.3. Pipette 2 mL of 10x glycine to the plate and swirl to mix.

3.3.4. Incubate for 5 min at room temperature.

3.3.5. After incubation, place dishes on ice and thaw an aliquot of protease inhibitor cocktail.

3.3.6. Prepare the following solutions for both the DMSO and A-485 samples using the buffers prepared in step 3.1.

3.3.6.1. 2 mL of PBS with a 1:1,000 dilution of the protease inhibitor cocktail

3.3.6.2. 1 mL of nuclei swelling buffer with a 1:1,000 dilution of the protease inhibitor cocktail

3.3.6.3. 0.5 mL of SDS lysis buffer with a 1:1,000 dilution of the protease inhibitor cocktail

3.3.7. Aspirate the media from the cell culture dishes and wash the cells twice with 15 mL of cold PBS.

3.3.8. Pipette 2 mL of PBS with the protease inhibitor cocktail (step 3.3.6.1) to the cell culture dishes. Lift the cells into solution using a cell scraper.

3.3.9. Transfer the cell suspension to a microcentrifuge tube. Collect remaining cells with additional PBS if necessary.

3.3.10. Spin tubes at 800 *x g* at 4 ˚C for 5 min to pellet the cells.

3.3.11. Aspirate the supernatant and pipette 1 mL of nuclei swelling buffer with the protease inhibitor cocktail (step 3.3.6.2) to the pellet. Resuspend the pellet and incubate on ice for 10 min.

3.3.12. Centrifuge the tubes at 2,700 *x g* at 4 ˚C for 5 min to pellet nuclei.

3.3.13. Aspirate the supernatant and pipette 0.5 mL of SDS lysis buffer with the protease inhibitor cocktail (step 3.3.6.3) to the pellet. Resuspend the pellet and incubate on ice for 10 min.

3.3.14. Store the chromatin samples at -80 ˚C until step 3.4.1 or proceed immediately to step 3.4.

3.4. DNA sonication

3.4.1. Transfer 130 µL of chromatin from DMSO sample (step 3.3.14) to two DNA sonication tubes using a pipette (130 µL each).

3.4.2. Transfer 130 µL of chromatin from A-485 sample (step 3.3.14) to two DNA sonication tubes using a pipette (130 µL each).

3.4.3. Sonicate the DNA to roughly 150-200 base pair fragments using the following sonicator settings: Peak Incident Power (W) of 175, Duty Factor of 10%, 200 Cycles per Burst and 430 s treatment time.

NOTE: Sonication settings may differ between models and settings may need to be adjusted to achieve appropriate fragment size for different cell lines.

3.4.4. Keep samples on ice after sonication.

3.4.5. Transfer the sonicated chromatin to a 1.5 mL tube using a pipette and centrifuge at 10,000 *x g* at 4 ˚C for 10 min to pellet debris.

3.4.6. Pipette the supernatant (contains sonicated chromatin) to a new tube and discard the debris. Sonicated chromatin can be stored at -80 ˚C.

3.5. Chromatin immunoprecipitation (ChIP)

NOTE: See **Supplementary Protocol** (**Schematic 3**) for a schematic of the IP groups in Step 3.5.

3.5.1. Measure the protein content of the sonicated chromatin for the DMSO and A-485 samples from Step 3.4.6. Protein content can be measured using well-established protocols[34].

NOTE: For simplicity, this protocol will assume protein content is equal and 100 µL of sonicated chromatin will be used. Otherwise, protein content will need to be equilibrated between all samples in an equal volume.

3.5.2. Pipette 100 µL of DMSO sonicated chromatin to two 1.5 mL tubes (100 µL each). Pipette 400 µL of ChIP dilution buffer (containing a 1:1,000 dilution of the protease inhibitor cocktail) to each tube to bring total volume up to 500 µL. Remove 5 µL of the solution from one of the tubes and store at -20 ˚C as DMSO Input.

3.5.3. Pipette 100 µL of A-485 sonicated chromatin to two 1.5 mL tubes (100 µL each). Pipette 400 µL of ChIP dilution buffer (containing a 1:1000 dilution of the protease inhibitor cocktail) to each tube to bring total volume up to 500 µL. Remove 5 µL of the solution from one of the tubes and store at -20 ˚C as A-485 Input.

3.5.4. Using a pipette, add the immunoprecipitation (IP) antibody (e.g. non-specific IgG control or H3K27ac specific antibody) to the corresponding tubes for the DMSO and A-485 samples: IP #1 DMSO chromatin with IgG antibody (5-10 µg); IP #2 DMSO chromatin with H3K27ac antibody (5-10 µg); IP #3 A-485 chromatin with IgG antibody (5-10 µg); IP #4 A-485 chromatin with H3K27ac antibody (5-10 µg).

3.5.5. Add 20 µL of protein A magnetic beads to each tube. Make sure the beads are well resuspended.

3.5.6. Rotate the samples overnight at 4 ˚C.

3.5.7. Pellet the protein A magnetic beads using a magnetic separator and remove the supernatant. Do not disturb the beads.

3.5.8. Wash the beads with 500 µL to 1 mL of the low salt wash buffer and rotate for 5 min at 4 ˚C. Perform a quick spin down, pellet the beads using a magnetic separator, and remove the supernatant.

3.5.9. Wash the beads with 500 µL to 1 mL of the high salt wash buffer and rotate for 5 min at 4 ˚C. Perform a quick spin down, pellet the beads using a magnetic separator, and remove the supernatant.

3.5.10. Wash the beads with 500 µL to 1 mL of the LiCl wash buffer and rotate for 5 min at 4 ˚C. Perform a quick spin down, pellet the beads using a magnetic separator, and remove the supernatant.

3.5.11. Wash the beads with 500 µL to 1 mL of TE buffer and rotate for 5 min at 4 ˚C. Perform a quick spin down. Keep the beads in the TE buffer until step 3.5.14.

3.5.12. Remove Input samples (from step 3.5.2 and 3.5.3) from freezer and keep on ice.

3.5.13. Thaw an aliquot of Proteinase K.

3.5.14. Pellet the beads using a magnetic separator and remove the TE buffer from the beads (from step 3.5.11).

3.5.15. Add 100 µL ChIP elution buffer + 1 µL of Proteinase K to every sample, including the Input samples. Incubate samples with shaking at 62 ˚C for 2 h using a thermocycler.

3.5.16. After 2 h, heat samples to 95 ˚C for 10 min using a thermocycler.

3.5.17. Cool the samples to room temperature.

3.5.18. Pellet magnetic beads using a magnetic separator and transfer supernatant (contains the DNA of interest) to a new 1.5 mL tube.

3.5.19. Purify the DNA using a standard PCR cleanup kit.

3.5.20. The purified DNA can be stored at -20 ˚C and can be used as templates in standard qPCR protocols. Follow manufacturer protocols for running qPCR.

3.6. ChIP-qPCR data analysis

NOTE: Two common methods to analyze ChIP-qPCR results are fold enrichment over the IgG antibody and the 1% Input method. An excellent template for both analysis methods is provided by a commercial source can be used to quickly calculate fold enrichment for each IP antibody/target of interest[37].

3.6.1. To calculate fold enrichment and % Input, copy and paste the ΔCt values from the qPCR data obtained for each antibody (non-specific IgG, the IP H3K27ac antibody, and the 1% Input) into the corresponding region in the analysis template and the Fold Enrichment and Yield % Input will automatically populate.

REPRESENTATIVE RESULTS:

The in vitro histone acetyltransferase (HAT) assay can be used to probe for compounds that inhibit p300 HAT activity towards a histone substrate. **Figure 1A** provides an experimental schematic for the HAT assay. Anacardic acid, a known HATi[3,38], was utilized in this assay in a concentration range from 12.5-100 µM. At 100 µM, anacardic acid downregulates p300 catalyzed histone acetylation at Histone 3, Lysines 9 and 18 versus the control DMSO treatment (**Figure 1B**, lane 5 versus lane 1). A concentration range was utilized in this assay because lower drug dosages may not greatly inhibit p300 HAT activity (**Figure 1B**, lanes 2-4 versus lane 1). In Lane 6, no Acetyl-CoA was added to the reaction and serves as a negative control for p300 catalysis and for basal levels of histone acetylation on recombinant H3.1 (**Figure 1B**). p300 and H3.1 protein levels were utilized as loading controls (**Figure 1B**). These immunoblot results were quantified using ImageJ[39] (**Figure 1C**). Fold changes were calculated by comparing the band intensity of each sample to the band intensity of the DMSO control for each acetylation probe. Quantification for anacardic acid at 100 µM shows potent reduction in H3K18ac and H3K9ac versus the DMSO control, confirming the visual results in **Figure 1B**.

In the Chromatin Hyperacetylation Inhibition (ChHAI) assay, HDACi is used as a tool to hyperacetylate histones in chromatin before co-incubation with a HATi[24], such as p300 inhibitor A-485[2,4]. The purpose of this assay is to determine the efficacy of HATi for attenuating histone hyperacetylation induced by HDACi. **Figure 2A** provides an experimental schematic for the ChHAI assay. In this assay, treatment of MCF-7 cells with HDACi MS-275 strongly upregulated acetylation on Histone 3, on several lysine residues (**Figure 2B**, lane 4 versus lane 1). The basal levels of H3K18ac and H3K27ac were low, showing the benefits of adding an HDACi in the ChHAI assay (**Figure 2B**, lanes 1-3). The addition of A-485 with MS-275 attenuates the increased histone acetylation at H3K18 and H3K27, but not H3K9 (**Figure 2B**, lanes 4-6). Importantly, H3K9ac is not regulated by p300 in cell culture[2], showing the specificity of A-485 in this experiment. These immunoblot results were quantified in **Figure 2C**. Fold changes were calculated by comparing the band intensity of each sample to the band intensity of MS-275 alone (Lane 4) for each acetylation probe. Lanes 1-3 were not quantified because H3K18ac and H3K27ac basal levels were not detected.

Chromatin Immunoprecipitation-quantitative Polymerase Chain Reaction (ChIP-qPCR) is a cell culture experiment that investigates DNA-protein interactions at specific regions of the genome. It can be used to investigate the effects of HATi at gene regulatory elements that control oncogene expression[25]. **Figure 3A** provides an experimental schematic for the ChIP-qPCR protocol. MCF-7 cells treated with 3 µM A-485 for 24 hours were subjected to ChIP-qPCR through immunoprecipitation of histone-DNA complexes enriched in H3K27ac (**Figure 3**). The purified DNA was analyzed for the Cyclin D1 promoter sequence. ChIP-qPCR primers are designed against a specific DNA sequence in the genome and are used to detect the relative amount of precipitated DNA. The amount of precipitated DNA reflects the abundance of the protein of interest at the genomic region under investigation. Indeed, in the DMSO sample, the DNA precipitated by the IgG control antibody produces a higher Ct value than the H3K27ac antibody in the qPCR reaction for the Cyclin D1 promoter (**Figure 3B**). This indicates that the

non-specific IgG control precipitated less DNA-protein complexes than the H3K27ac specific antibody at the Cyclin D1 promoter. This translates to a 632.73 fold enrichment of H3K27ac over the non-specific IgG control (**Figure 3B**).

This fold enrichment provides evidence that the H3K27ac specific antibody successfully immunoprecipitated acetylated histones and that H3K27ac is enriched at the Cyclin D1 promoter. After validating the quality of the H3K27ac antibody, a comparison can be made between the DMSO and A-485 treated groups. As shown in **Figure 3C**, A-485 reduces H3K27ac enrichment at the Cyclin D1 promoter versus the DMSO control using the %Input method (representative result of n=2). Importantly, A-485 is known to significantly reduce H3K27ac in cell culture[2,4].

ChIP-qPCR raw %Input values can be highly variable between independent biological replicates, despite the experimental trend being reproducible. Therefore, it may be useful to present the data as a normalized percent of DMSO control to show the reproducible ratio between control and drug treatment[10]. For example, in **Figure 3D**, A-485 significantly downregulates H3K27ac occupancy at the Cyclin D1 promoter (n=2). Statistical analysis was based on the Student's t-test (*$P < 0.05$).

FIGURE AND TABLE LEGENDS:

Figure 1: Anacardic acid inhibits p300 enzymatic activity in a HAT assay. (**A**) A schematic diagram of the HAT assay, depicting the enzymatic reaction. (**B**) Anacardic acid potently inhibited p300 enzymatic activity and downregulated histone acetylation at H3K18 and H3K9 at 100 µM (lane 5) versus the DMSO control treatment (lane 1). Lane 6 lacks Acetyl-CoA in the reaction and served as a negative control for histone acetylation. (**C**) The immunoblot results in (B) were quantified. Fold changes were calculated by comparing the band intensity of each sample to the band intensity of the DMSO control for each acetylation probe.

Figure 2: p300 inhibitor A-485 potently attenuates histone hyperacetylation in the ChHAI assay. (**A**) A schematic diagram of the ChHAI assay. (**B**) In MCF-7 cells, HDAC inhibitor MS-275 potently upregulated histone acetylation at H3K18, K27 and K9 (lane 4) versus the DMSO control (lane 1). The addition of A-485, a known p300 HAT inhibitor, with MS-275 attenuated the increase in histone acetylation at H3K18 and K27, but not H3K9 (lanes 5-6 versus lane 4). (**C**) The immunoblot results in (B) were quantified. Fold changes were calculated by comparing the band intensity of each sample to the band intensity of MS-275 alone (Lane 4) for each acetylation probe. Lanes 1-3 were not quantified because H3K18ac and H3K27ac basal levels were not detected.

Figure 3: p300 inhibitor A-485 decreases H3K27ac levels at the Cyclin D1 promoter as measured by ChIP-qPCR. (**A**) A schematic diagram of the ChIP-qPCR protocol. (**B**) Representative qPCR Ct values for the Cyclin D1 promoter for the IgG and H3K27ac immunoprecipitations. The IgG control had a higher Ct value, indicating that the H3K27ac antibody successfully enriched for H3K27ac over the non-specific IgG antibody. (**C**) A-485 (3 µM)

treatment for 24 h downregulated H3K27ac at the Cyclin D1 promoter in comparison to the DMSO control treatment in MCF-7 cells (representative result of n=2). (**D**) The %Input from two independent ChIP experiments were normalized to the DMSO control as a percentage of the DMSO control. Treatment with A-485 in MCF-7 cells significantly downregulates H3K27ac occupancy at the Cyclin D1 promoter. Statistical analysis was based on the Student's t-test (*P < 0.05).

Table 1: Recipes of the buffers and solution used.

Supplementary files: Supplementary experimental schematics for Protocols 1-3.

DISCUSSION:

Lysine acetyltransferases (KATs) acetylate several lysine residues on histone tails and transcription factors to regulate gene transcription[2,3]. Work in the last two decades has revealed that KATs, such as CBP/p300, PCAF and GCN5, interact with oncogenic transcription factors and help drive tumor growth in several solid tumor types[4,5,9,15–18]. Due to their emerging role in promoting tumor growth, KATs are being investigated as novel targets in cancer treatment. Novel KAT inhibitors (KATi) need to be carefully and rigorously tested for potency, selectivity, and safety before moving to use in the clinic. Recent evidence has shown that previously described KATi compounds exhibit off target effects and were poorly characterized before being widely used in the scientific literature as chemical probes[21]. Therefore, rigorous methods are needed for KATi characterization. Described here are three protocols that can be used together to characterize and validate novel inhibitors targeting the histone acetyltransferase (HAT) function of KATs: an in vitro HAT assay, the ChHAI assay, and ChIP-qPCR. These protocols use CBP/p300 and their inhibitors as examples, but these methods can easily be adapted for future application in investigating other KATs.

The HAT assay is simple and a cost-effective way to screen compounds for potency in inhibiting HAT function in a test tube. Purified HATs (either recombinant[4,10] or immunoprecipitated[40]) can be tested in this assay, but recombinant CBP/p300 is used as an example in this protocol. CBP/p300 have an enzymatic HAT domain that transfers an acetyl group from Acetyl-CoA to a lysine residue on a target substrate[3]. Among the lysine residues modified by CBP/p300, the most characterized targets are Histone 3 Lysine 18 and 27 (H3K18 and H3K27, respectively)[2,3,10,11]. In the HAT assay, the purified p300 HAT domain is incubated with Acetyl-CoA and Histone 3.1 as a substrate. During the incubation period, p300 will catalyze acetylation on several H3.1 residues including H3K18 and H3K27. The relative abundance of acetylation on these residues can be measured via immunoblotting. This test tube reaction can be used to screen for novel compounds that bind to p300 and inhibit its HAT activity (HATi). For example, anacardic acid, a known HATi[38], potently downregulates histone acetylation at both H3K18 and H3K9 in comparison to the DMSO control (**Figure 1B**, lane 5 versus lane 1). It is critically important to add Acetyl-CoA to the experimental reactions (**Figure 1B**, Lanes 1-5) or p300 catalysis of histone acetylation will not occur (**Figure 1B**, Lane 6). The absence of Acetyl-CoA can also be used as a negative control for p300 catalysis and for basal levels of histone acetylation on recombinant H3.1.

When performing the HAT assay, it is important to ensure that each reaction receives the same amount of p300, H3.1 and Acetyl-CoA. H3.1 and p300 levels in the immunoblot serve as loading controls for the gel. For this assay, site-specific histone acetyl antibodies or a pan-acetyl antibody can be used for immunoblotting. When optimizing to improve immunoblot quality it is crucial to use validated antibodies for immunoblotting and to initially follow the manufacturer's recommendations for antibody dilution. The antibody dilutions used in the Protocol section are for reference and the dilutions can be changed based on the results of initial experiments (e.g., if the signal is too strong the antibody can be further diluted). Due to its simplicity, the HAT assay is an excellent starting experiment for screening novel inhibitors. However, the HAT assay does have disadvantages. A major concern with the HAT assay is that compounds that are effective in a test tube may prove ineffective in a living system. This is an issue because compound efficacy in cell culture can be altered by cellular permeability issues, cellular metabolism, and compound stability. In addition, KATs, specifically CBP/p300, have many protein-protein interactions that regulate their KAT activity in cell culture[3,41,42]. Therefore, it is essential to further characterize inhibitors identified in the HAT assay in cell culture experiments[20,23].

The Chromatin Hyperacetylation Inhibition (ChHAI) assay is the second protocol in the pipeline and is useful for validating the effects of novel inhibitors in cell culture. This assay utilizes HDAC inhibitors (HDACi)[24] to induce histone hyperacetylation in cells because basal acetylation can be too low to detect on an immunoblot. The cell lines of choice are pre-incubated with an HDACi to allow for accumulation of acetylated chromatin before the addition of a HATi. After co-incubating the HDACi and HATi, the cells are lysed and subjected to standard immunoblotting procedures for specific histone acetylation sites. The purpose of this assay is to determine the efficacy of novel HATi for attenuating histone hyperacetylation induced by HDACi. Cells exposed to HDACi should have significantly higher levels of histone acetylation than cells exposed to the DMSO solvent (**Figure 2B**, lane 4 versus lane 1). Addition of the HATi along with the HDACi is expected to reduce the immunoblot signal in comparison to the HDACi treatment alone (**Figure 2B**, lanes 5-6 versus lane 4). Basal levels of histone acetylation (**Figure 2B**, Lanes 1-3) are difficult to detect and highlights the importance of adding an HDACi in this protocol. MS-275 (Entinostat) is used as an example but other HDACi can be used[24,43]. MS-275 has variable reported inhibitory concentrations versus Class I HDACs and generally inhibits HDAC1 and HDAC3 with nanomolar to low micromolar concentrations, respectively[43,44]. A wide range of MS-275 concentrations is used in the literature[45–47], but a 3 μM treatment provides a robust and reproducible increase in histone acetylation in MCF-7 cells. Therefore, it may be beneficial to perform an initial screen with a wide range of HDACi and HATi concentrations to determine the optimal concentration for the ChHAI assay when using a different cell line.

Similar to the HAT assay, the immunoblot protocol may need to be optimized to obtain quality results through the use of appropriate antibodies, optimal antibody dilutions and carefully controlled sample loading. Carefully controlled sample loading is essential for the success of this protocol and can be achieved through equilibrating the protein content of all samples and through pipetting equal volume of samples into the wells of the immunoblot gel. For this

protocol, a pan-acetyl antibody can be used for immunoblotting. However, it should be supplemented with site-specific acetyl antibodies because KATs have specificity for certain histone lysine residues and HATi does not affect all histone acetylation sites in cell culture[1,2,4,5,10,11]. Inhibitors that are potent at reducing histone acetylation in both the HAT and ChHAI assays are strong candidates for further evaluation. Importantly, the HAT and ChHAI assays have the limitation of only providing data about global changes in histone acetylation. This limitation creates the need to characterize the effects of novel HATi at specific regions of the genome.

Chromatin Immunoprecipitation-quantitative Polymerase Chain Reaction (ChIP-qPCR) is the final protocol in the pipeline and evaluates DNA-protein interactions at specific regions of the genome. In this assay, genomic regions enriched in H3K27ac are purified through immunoprecipitation (IP) and analyzed using DNA primers in qPCR. This technique provides mechanistic insight into how HATi affects histone modifications at gene promoters and enhancers. ChIP-qPCR is a robust technique and is less costly than whole-genome sequencing (e.g., ChIP-seq), but it can be difficult to optimize due to many steps that affect the outcome. The most difficult step to correctly optimize is step 3.5, the immunoprecipitation. This step is difficult to optimize because if the purified DNA in step 3.5.20 is highly dilute it can cause poor results in the qPCR reaction (e.g., no amplification of the target gene sequence and very high ΔCt values). The success of the IP step is dependent on several factors, such as the abundance of the protein target of interest and the quality of the IP antibody. It is crucial to validate the quality of the IP H3K27ac antibody versus the IgG control to verify the success of the IP step. For example, in **Figure 3B**, the H3K27ac specific antibody displays 632.73-fold enrichment over the non-specific IgG control. This indicates that the H3K27ac antibody is high quality and that H3K27ac is enriched at the Cyclin D1 promoter. After validation of the IP antibody, a comparison can be made between the DMSO and A-485 treated groups. As shown in **Figure 3C**, A-485 reduces H3K27ac enrichment at the Cyclin D1 promoter versus the DMSO control using the %Input method (representative result of n=2). If the IP antibody proves to be low quality and does not show fold enrichment in initial experiments, try increasing the cell numbers in step 3.2.1. Higher cell numbers can help compensate for poor antibody quality by increasing the total protein content of the lysate and will allow more protein to be added to the IP reaction.

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DISCLOSURES:

The authors have no conflicts of interest or disclosures to make.

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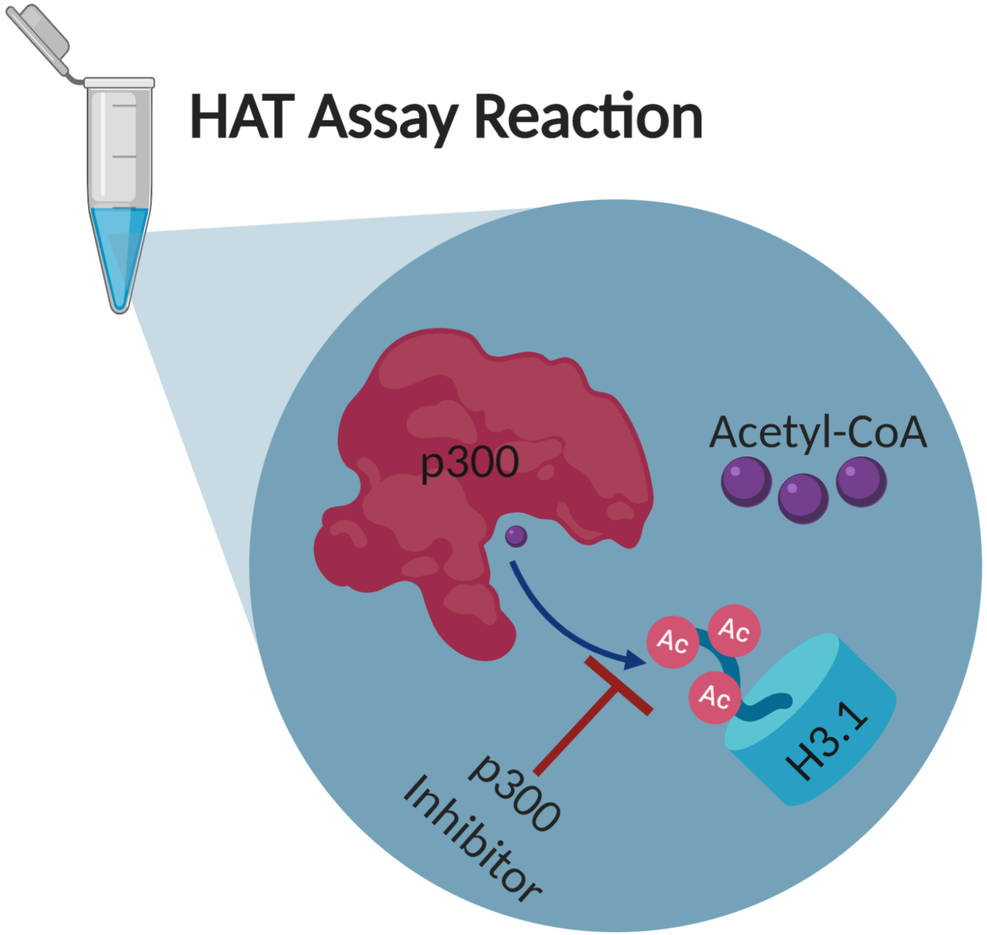
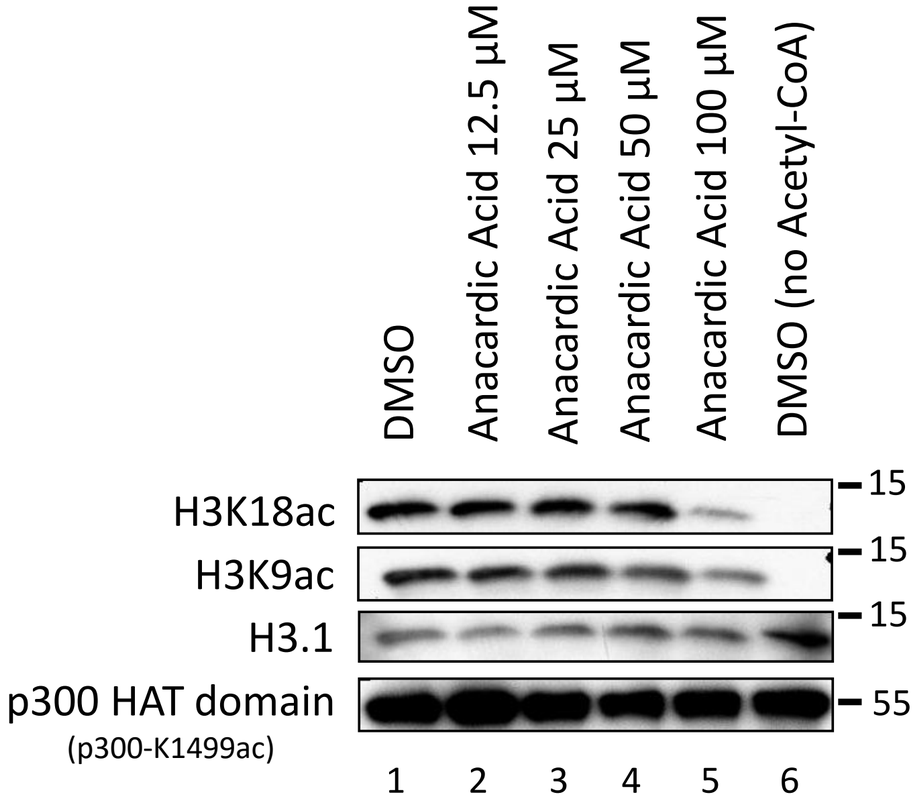
Figure 1

A



B



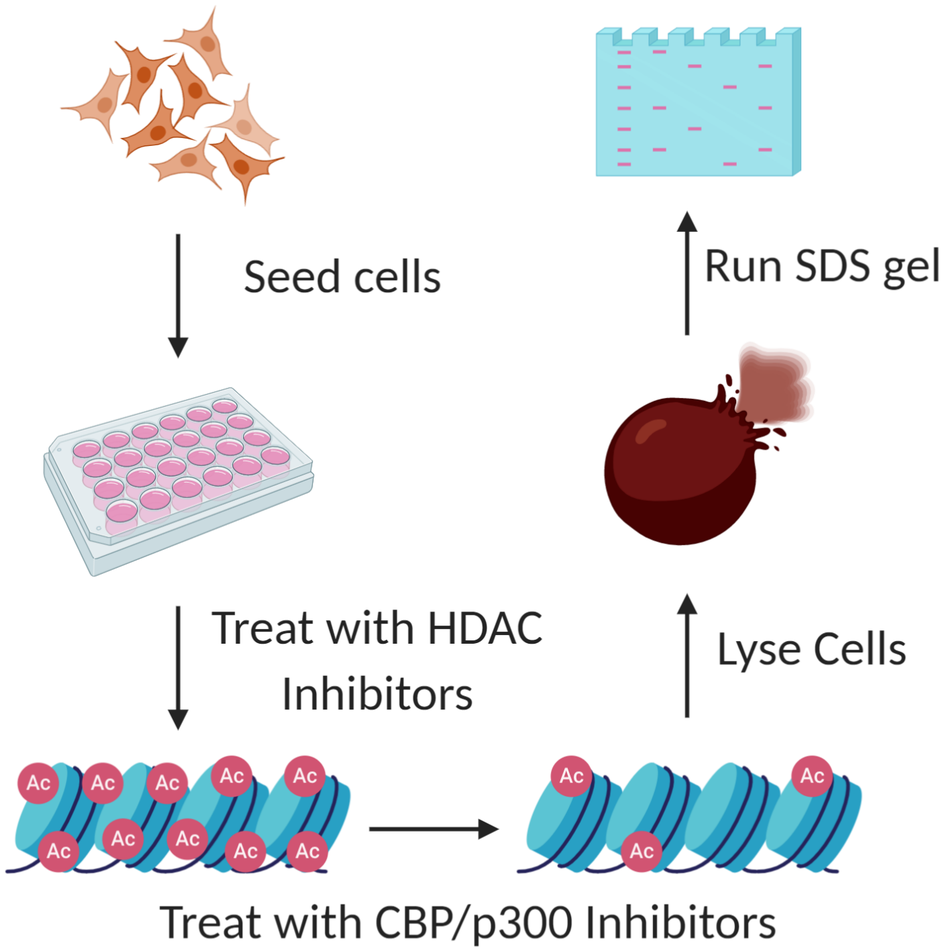
C

Quantification of HAT Assay Immunoblot Relative to DMSO Control (Fold Change)						
Histone Modification	DMSO	Anacardic Acid 12.5 µM	Anacardic Acid 25 µM	Anacardic Acid 50 µM	Anacardic Acid 100 µM	DMSO (no-acetyl-CoA)
H3K18ac	1.00	0.89	0.89	0.87	0.21	0.00
H3K9ac	1.00	0.99	1.01	0.85	0.45	0.16

Figure

Figure 2

A



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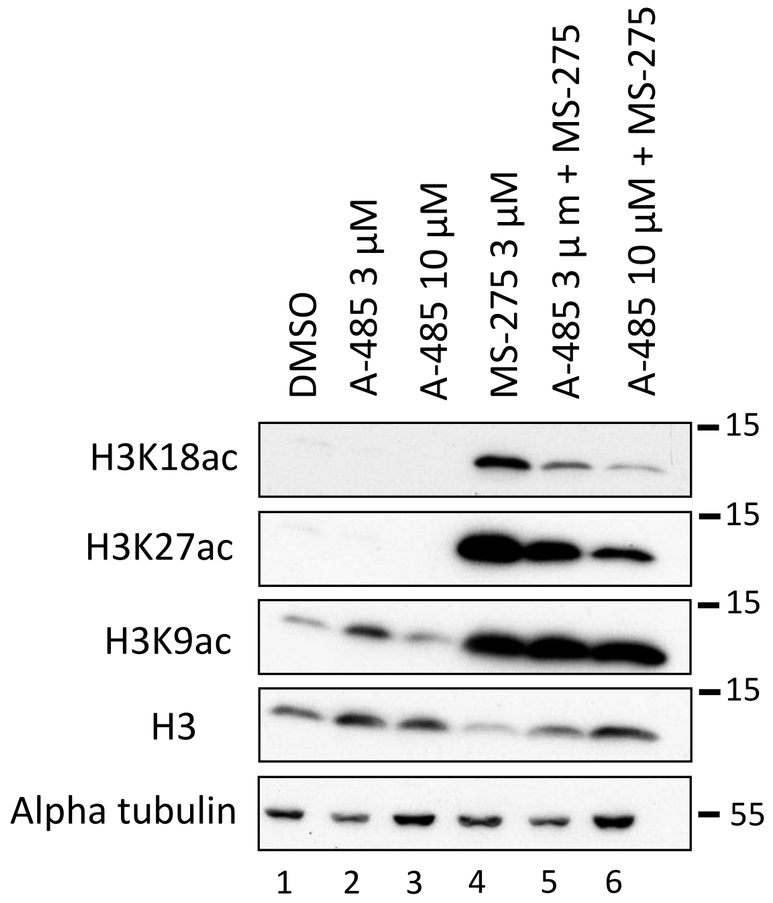
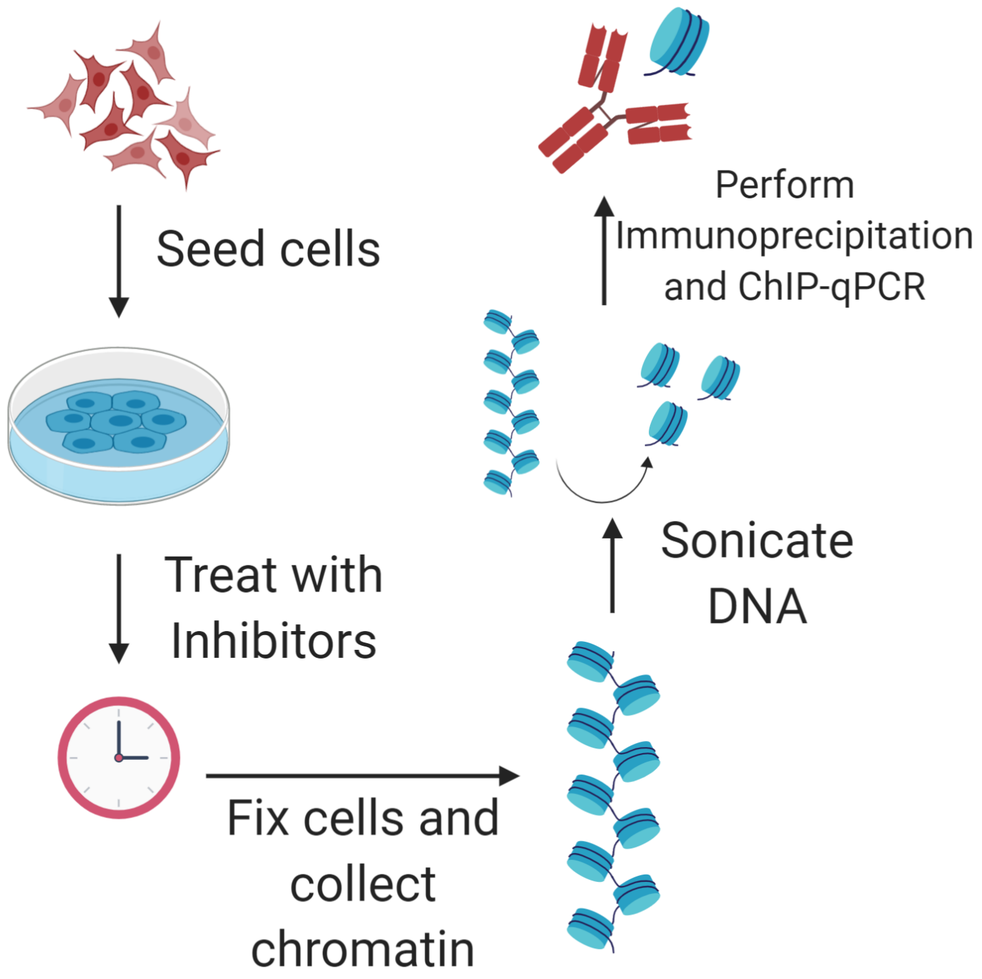


C

Quantification of Levels of Acetylated Histone H3 (fold change versus MS-275)			
Histone Modification	MS-275 3 µM	MS-275 + A-485 3 µM	MS-275 + A-485 10 µM
H3K18ac	1.00	0.32	0.11
H3K27ac	1.00	0.68	0.40
H3K9ac	1.00	1.09	0.98

Figure 3

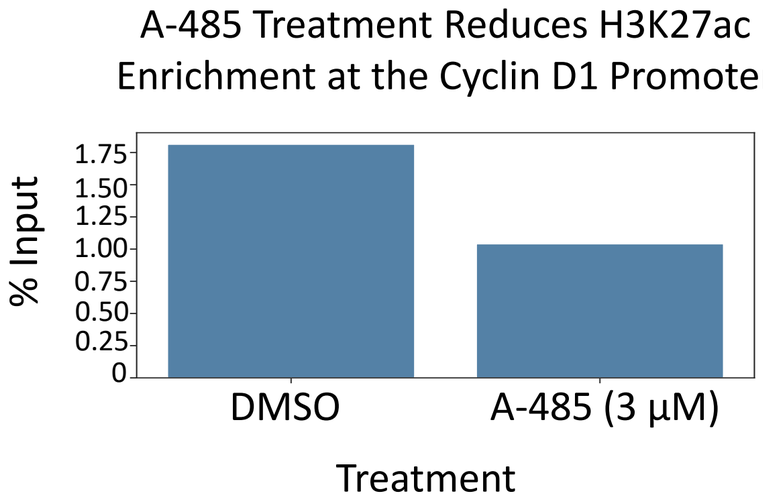
A



B

Antibody	qPCR Ct Value	Fold Enrichment
IgG	32.06990051	1
H3K27ac	22.76444435	632.73

C



D

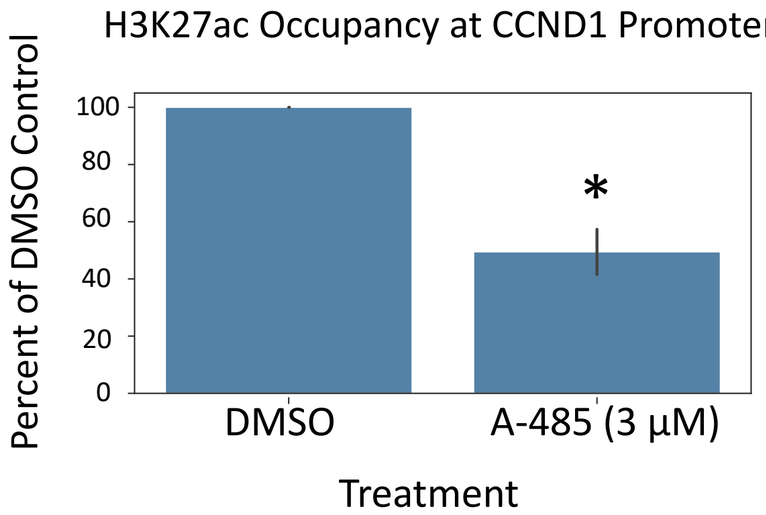


Figure 1


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Figure 2

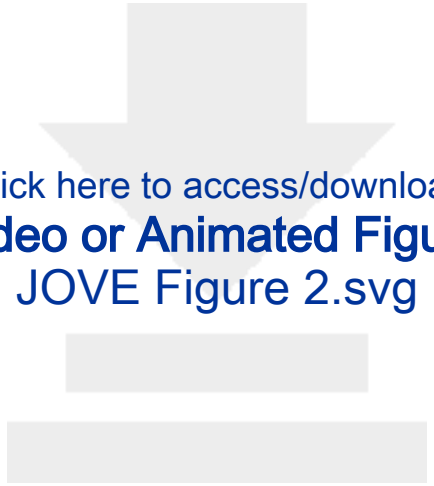
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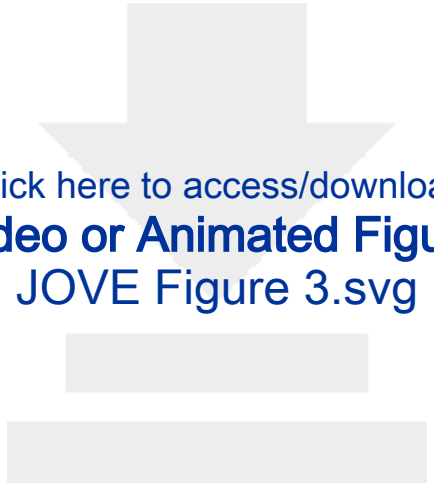
Figure 3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Buffer
10X Glycine buffer
10X Running Buffer
10X TBST
1X TBST with 5% milk
5X Assay buffer:
5X Passive lysis buffer
6X Sodium Dodecyl Sulfate (SDS)
ChIP dilution buffer
ChIP Elution Buffer
Complete DMEM for MCF-7 Cells:
High salt wash buffer
LiCl wash buffer
Low salt wash buffer
Nuclei swelling buffer
SDS lysis buffer
TE buffer
Transfer buffer

Recipe

18.74 g of glycine with PBS until dissolved and add PBS to 200 ml.
250 mM Tris, 1.9 M glycine, 1% SDS

Dissolve 30.0 g of Tris base, 144.0 g of glycine, and 10.0 g of SDS in 1000 ml of H2O. The pH of the buffer should be 8.3 and no pH adjustment is required. Store the running buffer at room temperature
2.42 g of Tris base, 8g of NaCl, 2 ml of 50% Tween 20, add ddH2O to 1 liter
5g powdered milk per approximate 100 ml of 1X TBST
500 mM HEPES, pH 7.5, 0.4 % Triton X-100
make an aqueous stock containing 125 mM Tris, pH 7.8, 10 mM 1,2-CDTA, 10 mM DTT, 5 mg/ml BSA, 5%
0.375 M Tris pH 6.8, 6 ml glycerol, 1.2 g SDS, 0.93 g 1,4-dithiothreitol (DTT), 6 mg bromophenol blue, adc
0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM Tris-HCl pH 8.0, 167 mM NaCl
1% SDS (w/v) and 0.1 M NaHCO3 in autoclaved ddH2O
Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% bovine calf serum (BCS), penicillin (
0.1% SDS, 1% Triton X-100, 2 mM EDTA, 200 mM Tris-HCl pH 8.0, 500 mM NaCl.
0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 100 mM Tris-HCl pH 8.0.
0.1% SDS, 1% Triton X-100, 2 mM EDTA, 200 mM Tris-HCl pH 8.0, 150 mM NaCl.
5 mM PIPES pH 8.0, 85 mM KCl, 0.5% NP-40
1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.0
10 mM Tris-HCl pH 8.0, 1 mM EDTA.
25 mM Tris, 190 mM glycine, 20% methanol and check the pH and adjust to pH 8.3 if necessary.

(vol/vol) Triton X-100 and 50% (vol/vol) glycerol in ddH2O.
water to 10 ml.

(10 units/ml), and streptomycin (10 mg/ml)

Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materials_Complete.xlsx

Name of Material/Equipment	Company	Catalog Number
1.5 ml tube	Fisher Scientific	05-408-129
10 cm dish	Sarstedt AG & Co.	83.3902
10 ul tips	Fisher Scientific	02-707-454
1000 ul tips	Corning	4846
10X Glycine buffer		
10X Running Buffer		
10X TBST		
12 well plate	Corning	3513
15 cm dish	Sarstedt AG & Co.	83.3903
15 ml conical tube	Santa Cruz Biotechnology	sc-200249
1X TBST with 5% milk and 0.02% Sodium Azide		
1X TBST with 5% milk		
200 ul tips	Corning	4844
2-mercaptoethanol	Sigma-Aldrich	M3148
4-20% polyacrylamide gel	Thermo Fisher: Invitrogen	XP04205BOX
5X Assay buffer		
5X Passive lysis buffer		
6X Sodium Dodecyl Sulfate (SDS)		
A-485	MedChemExpress	HY-107455
Acetyl-CBP(K1535)/p300(K1499) antibody	Cell Signaling Technology	4771
Acetyl-CoA	Sigma-Aldrich	A2056
Acetyl-Histone H3 (Lys 27) antibody (H3K27ac)	Cell Signaling Technology	CST 8173
Acetyl-Histone H3 (Lys18) antibody (H3K18ac)	Cell Signaling Technology	CST 9675
alpha tubulin antibody	Millipore Sigma	T5168
Anacardic acid	Cayman Chemical	13144
anti-mouse IgG HRP linked secondary antibody	Cell Signaling Technology	7076
anti-rabbit IgG secondary antibody	Jackson ImmunoResearch	711-035-152
Autoradiography film	MIDSCI	BX810
Belly Dancer Rotating Platform	Stovall Life Science Incorporated	not available
Bovine Calf Serum (BCS)	HyClone	SH30072.03
Bovine Serum Albumin (BSA)	Sigma-Aldrich	A2153
Bromophenol Blue	Sigma-Aldrich	B0126

CDTA	Spectrum Chemical	125572-95-4
cell scraper	Millipore Sigma	CLS3010
ChIP dilution buffer		
ChIP Elution Buffer		
Complete DMEM for MCF-7 Cells		
Covaris 130 µl microTUBE	Covaris	520045
Covaris S220 Focused-ultrasonicator	Covaris	S220
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	41639
DL-Dithiothreitol (DTT)	Sigma-Aldrich	43815
DMEM	Corning	10-013-CV
EDTA	Fisher Scientific	BP120-1
Example transfer tank and transfer apparatus	Bio-rad	1704070
EZ-Magna ChIP A/G Chromatin Immunoprecipitation Kit	Millipore Sigma	17-10086
FK228 (Romidepsin)	Cayman Chemical	128517-07-7
Formaldehyde solution	Sigma-Aldrich	F8775
glycerol	Fisher Scientific	BP229-1
glycine	Sigma-Aldrich	G7126
HEPES	Sigma-Aldrich	54457
High salt wash buffer		
IGEPAL (NP-40)	Sigma-Aldrich	I3021
Immobilon Chemiluminescent HRP Substrate	Millipore Sigma	WBKLS0500
KCl	Fisher Scientific	BP366-500
LiCl	Sigma-Aldrich	L9650
LiCl wash buffer		
Low salt wash buffer		
Magnetic Separator	Promega	Z5341
Methanol	Sigma-Aldrich	494437
Mini gel tank	Invitrogen	A25977
MS-275 (Entinostat)	Cayman Chemical	209783-80-2
NaCl	Fisher Scientific	7647-14-5
NaOH	Fisher Scientific	S318-100
Normal Rabbit IgG	Bethyl Laboratories	P120-101

Nuclei swelling buffer		
PCR Cleanup Kit	Qiagen	28104
Penicillin/Streptomycin 100X	Corning	30-002-CI
Phosphate-buffered saline (PBS)	Corning	21-040-CV
PIPES	Sigma-Aldrich	80635
powdered milk	Nestle Carnation	
Power Pac 200 for western blot transfer	Bio-rad	
Power Pac 3000 for SDS gel running	Bio-rad	
Prestained Protein Ladder	Thermo Fisher	26616
Protease Inhibitor Cocktail	Sigma-Aldrich	PI8340
Protein A Magentic Beads	New England BioLabs	S1425S
Proteinase K	New England BioLabs	P8107S
PTC-100 Programmable Thermal Controller	MJ Research Inc.	PTC-100
PVDF Transfer Membrane	Millipore Sigma	IEVH00005
Recombinant H3.1	New England BioLabs	M2503S
Recombinant p300	ENZO Life Sciences	BML-SE451-0100
SAHA (Vorinostat)	Cayman Chemical	149647-78-9
SDS lysis buffer		
Sodium Azide	Fisher Scientific	26628-22-8
Sodium Bicarbonate	Fisher Scientific	S233-500
Sodium deoxycholate	Sigma-Aldrich	D6750
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich	71725
Standard Heatblock	VWR Scientific Products	MPN: 949030
Table top centrifuge	Eppendorf	5417R
TE buffer		
Transfer buffer		
Trichostatin A	Cayman Chemical	58880-19-6
Tris	Fisher Scientific	BP152-5
Triton X-100	Sigma-Aldrich	T8787
Tween 20	Sigma-Aldrich	9005-64-5
X-ray film processor	Konica Minolta Medical & Graphic, Inc.	SRX-101A

Comments/Description
For all methods
For cell culture of MCF-7 cells
For all Methods
For all Methods
For Method 3. See Table 1 for recipe.
For Methods 1 and 2. See Table 1 for recipe.
For Methods 1 and 2. See Table 1 for recipe.
For Method 2
For Method 3
For Methods 2 and 3
For Methods 1 and 2. Can be used to dilute primary antibodies that will be used more than once. Allows for short-term storage of
For Methods 1 and 2. Used to block PVDF membrane and for antibody diltions. See Table 1 for recipe.
For all Methods
for SDS sample buffer preparation
For Methods 1 and 2
For Method 1. See Table 1 for recipe.
For Method 2. See Table 1 for recipe.
For Methods 1 and 2. See Table 1 for recipe.
CBP/p300 Inhbitor for use in Methods 2 and 3. Dissolved in DMSO.
For Method 1
for use in Method 1
antoibodies for H3K27ac for immunoblots and ChIP
antoibodies for H3K18ac for immunoblots and ChIP
For Method 2. Dilute 1:20,000
For Method 1
For Methods 1 and 2. Dilute 1:10,000
For Methods 1 and 2. Dilute 1:10,000 to 1:20,000
For Methods 1 and 2
For Methods 1 and 2
cell culture media
for buffer preparation
for SDS sample buffer preparation

For buffer preparation
For Method 3
For Method 3. See Table 1 for recipe.
For Method 3. See Table 1 for recipe.
For Methods 2 and 3. See Table 1 for recipe.
Sonication tube for use with Covaris S220 in Method 3
DNA sonicator for use in Method 3
for drug dilution and vehicle control treatment
for SDS sample buffer preparation
cell culture media
for buffer preparation
For Methods 1 and 2

For Method 3
HDAC Inhibitor for use in Method 2
for cell fixation
For buffer preparation
for buffer preparation
for buffer preparation
For Method 3
for buffer preparation
For Methods 1 and 2
for buffer preparation
For buffer preparation
For Method 3. See Table 1 for recipe.
For Method 3. See Table 1 for recipe.
For use in Method 3
For buffer preparation
For Methods 1 and 2
HDAC Inhibitor for use in Method 2. Dissolved in DMSO.
for buffer preparation
for buffer preparation in Methods 1 and 2
Control rabbit antibody for use in Method 3

For Method 3. See Table 1 for recipe.
For use in Method 3
cell culture media
For Methods 2 and 3
for buffer preparation
For Methods 1 and 2
For Methods 1 and 2
For Methods 1 and 2
For Methods 1 and 2
for use in Method 3
For use in Method 3
For use in Method 3
For Method 1
For Methods 1 and 2
for use in Method 1
for use in Method 1
HDAC Inhibitor for use in Method 2
For Method 3. See Table 1 for recipe.
For Methods 1 and 2. **CAUTION: Sodium Azide is toxic. See SDS for proper handling.**
for buffer preparation
for buffer preparation
for SDS sample buffer preparation
For Methods 1 and 2
For all methods
For Method 3. See Table 1 for recipe.
For Methods 1 and 2. See Table 1 for recipe.
HDAC Inhibitor for use in Method 2
for buffer preparation
for buffer preparation
for buffer preparation in Methods 1 and 2

For Methods 1 and 2

primary antibody dilutions. Do not use for secondary antibody diluton. **CAUTION: Sodium Azide is toxic.**

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May 5, 2020

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Dear Editors,

Thank you for your careful review of our manuscript entitled, “Assays for Validating Novel Histone Acetyltransferase Inhibitors”. With this revised manuscript, we have, to the best of our ability, addressed each of the recommendations.

We have addressed each of the points and clarified the methods as requested. With these and other edits, we believe that these revisions have improved the overall clarity of our revised manuscript. In terms of specific responses, the editorial comments are in *italics* and responses are in **bold**.

Editorial:

The editor has formatted the manuscript to match the journal's style. Please retain and use attached file for revision.

The attached file, with its corrected format, was utilized for future revisions.

Please address all specific comments marked in the manuscript.

The specific editor comments in the manuscript were addressed and all changes were tracked in the manuscript. Additional information for each specific concern can be found in the manuscript, where we have replied to each comment. Furthermore, as suggested by the editor, we have uploaded a Table of recipes and a supplementary file containing schematics of the experimental designs for each method covered in the manuscript.

There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol and is in line with the title.

2.75 pages have been highlighted in the manuscript for filming.

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No previously published figures were used in this manuscript.

Kind regards,

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University of Florida
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Supplementary Protocol

Schematic 1: HAT Assay Reaction

10 Minute Pre-incubation (do not add again in complete reaction)	**Complete HAT Assay Reaction (includes pre-incubation components)**
2 µL of 5x assay buffer	2 µL of 5x assay buffer
	3 µL of 100 µM Acetyl-CoA
1 µL of purified p300 (0.19 µg/µL)	1 µL of purified p300 (0.19 µg/µL)
	1 µL of purified H3.1 (0.2 µg/µL)
1 µL of anacardic acid (HATi) or DMSO control diluted in 1x assay buffer	1 µL of anacardic acid (HATi) or DMSO control diluted in 1x assay buffer
2 µL autoclaved ddH_2O	2 µL autoclaved ddH_2O

Schematic 2: ChHAI Assay Experimental Design

Experimental Groups	**Initial Drug Dilutions (Step 2.1.2-2.1.5)**	**Final Drug Dilutions (Step 2.1.7)**
DMSO	0.5 µL of DMSO to 1 mL of DMEM media	1.0 µL of DMSO to 1 mL of DMEM media
A-485 3 µM	0.5 µL of DMSO to 1 mL of DMEM media	0.5 µL of DMSO and 0.5 µL A-485 (6 mM) to 1 mL of DMEM media
A-485 10 µM	0.5 µL of DMSO to 1 mL of DMEM media	0.5 µL of DMSO and 0.5 µL of A-485 (20 mM) to 1 mL of DMEM media
MS-275 3 µM	0.5 µL of MS-275 (6 mM) to 1 mL of DMEM media	0.5 µL of DMSO and 0.5 µL of MS-275 (6 mM) to 1 mL of DMEM media
MS-275 3 µM + A-485 3 µM	0.5 µL of MS-275 (6 mM) to 1 mL of DMEM media	0.5 µL of A-485 (6 mM) and 0.5 µL of MS-275 (6 mM) to 1 mL of DMEM media
MS-275 3 µM + A-485 10 µM	0.5 µL of MS-275 (6 mM) to 1 mL of DMEM media	0.5 µL of A-485 (20 mM) and 0.5 µL of MS-275 (6 mM) to 1 mL of DMEM media.

Schematic 3: ChIP-qPCR Experimental Design

Experimental Groups	**Drug Dilutions (Step 3.2.2-3.2.3)**	**Immunoprecipitation Groups (Step 3.5.4)**
DMSO	6 μL of DMSO to 12 mL of DMEM media	IP #1: IgG antibody (5-10 μg)
		IP #2: H3K27ac antibody (5-10 μg)
A-485 3 μM	6 μL of A-485 (6 mM) to 12 mL of DMEM media	IP #3: IgG antibody (5-10 μg)
		IP #4: H3K27ac antibody (5-10 μg)