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Inhibition of Histone Deacetylases

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Abstract

Lysine acetylation of histones is one of the major epigenetic regulators of chromatin conformation and gene expression. The dynamic nature of histone acetylation is determined by the counterbalancing activity of histone acetyltransferase and histone deacetylase (HDAC) enzymes. Acetylation of histones is generally associated with open and transcriptionally active chromatin, whereas the activity of HDACs leads to histone deacetylation, condensation of chromatin, and inhibition of transcription. Aberrant silencing of tumor suppressors and other genes has been found in different types of cancer. Abnormal activity of HDACs has been implicated in tumorigenesis and therefore considerable effort has been put into the development of HDAC inhibitors as a means of modifying histone acetylation status and reexpressing aberrantly silenced tumor suppressor genes. This has led to the generation of a number of structurally diverse compounds that can effectively inhibit HDAC activity, thus altering chromatin structure in cancer cells. This unit discusses the methods and recent technological developments with respect to the studies of HDAC inhibition in cancer.

Keywords

Histone deacetylases; Histone acetyltransferases; HDAC inhibitors; Epigenetic gene silencing; Chromatin remodeling

1. Introduction

Epigenetic modifications refer to heritable and reversible changes in chromatin structure that are not due to alterations in primary DNA sequence (1, 2). The biochemical modifications that dictate epigenetic changes include methylation of cytosine residues in CpG dinucleotides and posttranslational modifications of the histone tails such as acetylation, methylation, phosphorylation, ubiquitination, sumoylation, and ADP-ribosylation (3, 4). Among these modifications, histone acetylation is one of the major regulators of chromatin conformation and gene expression. The histone deacetylase (HDAC) family is divided into zinc-dependent (class I, IIa, IIb, and IV of which there are 11 subtype enzymes) and zinc-independent enzymes (class III, also called sirtuins), which require NAD^+ for their catalytic activities (Table 1) (5). Acetylation by histone acetyltransferases (HATs) is generally associated with transcriptionally active chromatin (euchromatin) and activity of HDACs typically leads to chromatin condensation and inhibition of transcription (heterochromatin). Over the past decade, a number of HDAC inhibitors have been rationally designed and developed. These HDAC inhibitors have been examined for their ability to alter chromatin structure and reexpress aberrantly silenced genes which is associated with growth inhibition and apoptosis in cancer cells (6, 7). The field of HDAC inhibitors is moving rapidly into a new stage of development that has now started to produce success in the clinic, particularly in the field of cancer therapy. Based on their chemical structures, HDAC inhibitors are divided into four groups: hydroxamic acids, cyclic tetrapeptides, short-chain fatty acids, and

benzamides (Table 2). Most of the HDAC inhibitors developed so far are nonselective reagents and among the most potent inhibitors are those that have been designed to target primarily the zinc cofactor of the enzyme active site and exhibit their effects in nano- or micro-molar levels (8, 9). However, several recent studies revealed some unique features of class IIa HDAC biochemistry and demonstrated unexpected selectivity of HDAC inhibitors presumed to be nonselective (10, 11).

The efficacy of HDAC inhibitors such as TSA (trichostatin A), SAHA (vorinostat), romidepsin (FK-228), LBH589 (Panobinostat), PDX101 (Belinostat), and MS-275 (Entinostat) as antitumor agents has been demonstrated in a wide range of cancer cell lines as well as in animal models (12–15). Two pharmaceutical HDAC inhibitors, SAHA and romidepsin, have already been approved by the US-FDA for the clinical treatment of cutaneous T-cell lymphoma (CTCL). A number of other promising HDAC inhibitors are currently under evaluation in advanced clinical trials. The exact mechanisms through which HDAC inhibitors mediate anticancer activity have not been fully elucidated. One model suggests that HDAC inhibitor-induced hyperacetylation of histones activates tumor-suppressor genes and represses oncogenes, thus activating intrinsic apoptotic pathways (16, 17). For example, in ER-negative breast cancer cells, inhibition of HDAC activity by specific HDAC inhibitors reactivates aberrantly silenced estrogen receptor alpha (ERα) and progesterone receptor (PR) gene expression (18–21). Pruitt et al., demonstrated that the inhibition of class III HDAC SIRT1 using a pharmacologic inhibitor, splitomicin, or siRNA reactivates epigenetically silenced *SFRP1, SFRP2, E-cadherin*, and *CRBP1* genes in human breast and colon cancer cells despite full retention of DNA hypermethylation at promoters of reactivated genes (22). A recent study demonstrated that HDAC inhibitors induce cellular senescence through downregulation of polycomb group genes, suggesting that HDAC activity is important for self-renewal of human multipotent stem cells (MSCs) (23). In addition, a growing field of mass spectrometry-based proteomic techniques have identified several nonhistone proteins whose lysine acetylation is directly regulated by HDACs (24–26). These studies suggest that HDAC inhibitors can also affect diverse pathways in the cell and have recently been used to predict lysine acetylation motifs (27). A broader discussion of mass spectrometry techniques used in epigenetic research can be found in volume 593, Chapter 13 of this series (28).

Studies investigating the effects of HDAC inhibitors on chromatin and gene transcription generally involve measuring the alterations of histone acetylation levels or expression of genes and gene products associated with acetylated histones induced by drug (Fig. 1). Although histones are often enriched prior to analysis using a classical acid extraction protocol (29–31), we find that nuclear extraction using a kit-based method described here is faster and typically sufficient. Additionally, more efficient methods for histone isolation which preserve more labile modifications such as phosphorylation have recently been developed (32).

As part of an effort to define the “histone code” of variable histone tail modifications, a significant area of research focuses on the detection of specific acetylated lysines of histones. Immunological detection (Western blots or immunochemistry) has become the method of choice to determine histone acetylation in cancer cells as a result of the growing availability of site-specific, histone family-specific, or pan-acetylation antibodies. While recent developments in mass spectrometry enable accurate quantification of isoform-specific histone modifications (33), the existing array of specific antibodies remains indispensable for the analysis of gene expression in concert with histone acetylation.

A range of methods is now available for assessing gene regulation relevant to histone acetylation in a gene-specific or genome-wide manner. The relationship between a gene of

interest and site-specific histone acetylation can be analyzed by chromatin immunoprecipitation (ChIP) using antibodies (preferably monoclonal) to identify specifically modified histones bound to DNA. To globally assess genes correlated with specific histone acetylation sites, the ChIP-on-chip method (ChIP in combination with microarray) is used. This strategy has been extensively described in other recent editions of this collection (34–36). Quantitative-PCR has been widely used to quantify specific gene expression changes, and microarray expression analysis has been successfully used in our laboratory to measure the global gene expression after HDAC inhibitor treatment (37). The methods and protocols for the analysis of the cellular effects of histone deacetylase inhibition in human breast cancer cells are described below.

2. Materials

2.1. Cell Culture, HDAC Inhibitors

1. Phosphate-buffered saline (PBS), pH 7.2.
2. Dulbecco's modified Eagle's medium (DMEM, Mediatech) containing 5% fetal bovine serum (Mediatech) used for culture of MDA-MB-231 human breast cancer cells.
3. Trypsin/EDTA solution: 0.05% trypsin/0.53 mM EDTA (Mediatech).
4. HDAC inhibitors: SAHA (suberoylanilide hydroxamic acid, vorinostat, Cayman); TSA (7-[4-(dimethylamino) phenyl]-*N*-hydroxy-4,6-dimethyl-7-oxo-2,4-heptadienamide; Sigma); MS275 (Selleck Chemicals); belinostat (PXD101, Selleck Chemicals); and panobinostat (LBH589, Selleck Chemicals). These HDAC inhibitors are dissolved in 100% DMSO and stored at −20°C (see Note 1).

2.2. Histone Isolation

1. PBS, pH 7.2.
2. Lysis buffer A: 10 mM Tris–HCl, 1 mM $MgCl_2$, 5 mM butyrate, 1% Triton X-100.
3. Buffer B: 0.25 M sucrose, 3 mM $CaCl_2$, 10 mM Tris–HCl, and 5 mM butyrate.
4. Sulfuric acid.
5. Acetone.

2.3. Nuclear Protein Extraction

1. NE-PER® nuclear and cytoplasmic extraction reagents (Thermo Scientific/Pierce).
2. Protease inhibitors: benzamidine 250 mg/ml; aprotinin 2 mg/ml; leupeptin 2 mg/ml; PMSF (phenlymethlysulfonyl flouride) 0.2 M.

2.4. Western Blots and Antibodies

2.4.1. Chemiluminescence Detection

1. Tris–HCl SDS-PAGE precast gels, gel running and protein transfer apparatus, and PVDF membrane.
2. ECL plus Western blotting detection system (GE Healthcare).

[1]HDAC inhibitors should be dissolved in DMSO first for maximum solubility and then diluted in aqueous buffer of choice. Since some HDAC inhibitors, such as vorinostat, are unstable in aqueous media, we replace the drug-containing medium every day if the length of treatment time is more than 24 h.

2.4.2. Odyssey Quantitative Fluorescence Detection

1. Infrared dye 800CW goat anti-mouse secondary antibody and infrared dye 680 goat anti-rabbit secondary antibody (Li-COR).
2. Odyssey blocking buffer (Li-COR).
3. Tween 20, PBS buffer, methanol, and SDS.
4. The Odyssey® infrared imaging system (Li-COR).

2.4.3. Histone Antibodies—Rabbit anti-acetyl-histone H3 polyclonal IgG (Millipore), Rabbit anti-acetyl-histone H4 polyclonal IgG (Millipore), Rabbit anti-acetyl-histone H3 (Lys 9) polyclonal IgG (Millipore), Rabbit anti-acetyl-histone H3 (Lys 27) polyclonal IgG (Millipore), Rabbit anti-histone H3 polyclonal IgG (Abcam), Goat anti-rabbit immunoglobulin/HRP (DAKO).

2.5. In vitro HDAC Activity Assay

1. HDAC assay buffer: 50 mM Tris–Cl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$.
2. HDAC substrate (Calbiochem/EMD).
3. HDAC developer concentrate (20×) (Calbiochem/EMD).
4. Deacetylated standard (Calbiochem/EMD).
5. 96-Well microplates.
6. Fluorimeter.

2.6. RNA Extraction, cDNA Synthesis, PCR

1. RNA extraction: TRIzol® reagent (Invitrogen).
2. First-strand cDNA synthesis: $Oligo(dT)_{12–18}$, dNTP Mix (dATP, dGTP, dCTP, and dTTP), 5× first-strand buffer, DTT, RNaseOUT™, M-MLV reverse transcriptase (Invitrogen).
3. PCR reagent: JumpStart™ Taq ready mix (Sigma).
4. Real-Time PCR reagents: SYBR green or Taqman® (Applied Biosystems).
5. Applied Biosystems Real-Time PCR system.

2.7. Chromatin Immunoprecipitation

1. 37% Formaldehyde.
2. SDS lysis buffer: 1% SDS, 10 mM EDTA pH 8.0, 50 mM Tris–HCl pH 8.1.
3. ChIP diluent buffer: 0.01% SDS, 1.1% Triton-X 100, 1.2 mM EDTA pH 8.0, 16.7 mM Tris–HCl pH 8.1, 167 mM NaCl.
4. Low salt buffer: 0.1% SDS, 1.0% Triton X-100, 2 mM EDTA pH 8.0, 20 mM Tris–HCl pH 8.1, 150 mM NaCl.
5. High salt buffer: 0.1% SDS, 1.0% Triton X-100, 2 mM EDTA pH 8.0, 20 mM Tris–HCl pH 8.1, 500 mM NaCl.
6. LiCl immune complex: 0.25 M LiCl, 1% NP40, 1% deoxycholate, 1% SDS, 1 mM EDTA pH 8.0, 10 mM Tris–HCl pH 8.1.

7. Elution buffer: 1% SDS, 0.1 M $NaHCO_3$.
8. Protein A agarose slurry/Salmon Sperm DNA (Millipore).
9. Protein A agarose beads (Millipore).
10. Magnetic separator.
11. Sonifier (Branson).

3. Methods

3.1. Histone Extraction and Isolation

Histone proteins from HDAC inhibitor-treated human breast cancer cells are isolated according to previously published method (29).

1. Treat human breast cancer cells with HDAC inhibitors.
2. Harvest cells by scraping or trypsinization and centrifugation at 500 × *g* for 5 min and wash cells three times in ice-cold PBS.
3. Remove the supernatant and resuspend in 1 ml lysis buffer A with protease inhibitor and transfer to a 1.5-ml eppendorf tube on ice for 30 min.
4. Centrifuge for 15 min at 16,000 × *g* at 4°C.
5. Resuspend the pellet in 250 μl buffer B with protease inhibitor.
6. Add 11 μl 3.8N H_2SO_4 to make final concentration of 0.4N and leave the tube at 4°C overnight.
7. Centrifuge at 16,000 × *g* for 15 min at 4°C and transfer the supernatant to a new tube and precipitate with 10× cold acetone at −20°C.
8. Centrifuge 16,000 × *g* 15 min at 4°C and wash the pellet with cold acetone containing 0.2% H_2SO_4.
9. Dry pellet and dissolve pellet in ddH_2O.

3.2. Nuclear Protein Extraction (See Note 2)

This protocol is derived from the published protocol from manufacturer (Thermo Scientific/ Pierce).

1. Isolate approximately 20 μl packed cell volume of breast cancer cells treated with HDAC inhibitors or vehicle control by centrifugation at 500 × *g* for 2–3 min.
2. Add 200 μl of ice-cold CER I reagent with 1× protease inhibitor mixture.
3. Vortex vigorously for 15 s to fully resuspend the cell pellet and incubate the samples on ice for 10 min.
4. Add 11 μl of ice-cold CER II.
5. Vortex the tube for 5 s on the highest setting. Incubate tube on ice for 1 min.
6. Vortex the tube for 5 s and centrifuge the tube for 5 min at 16,000 × *g*.

[2]This method has been successfully used to extract nuclear proteins and examine the expression level of nuclear histone proteins (39). The CER I reagent from cytoplasmic/nuclear protein extraction kit induces swelling of the cell leading to stress on the cellular membrane and the CER II reagent lyses the cell membrane, allowing cytoplasmic proteins to be collected. The NER reagent is then used to extract nuclear proteins from the intact nucleus. EDTA-free protease inhibitors can be used for the extraction agents. However, protease inhibitors that contain alcohols should be avoided.

7. Immediately transfer the supernatant (cytoplasmic extract) fraction to a clean prechilled tube and resuspend the insoluble pellet fraction containing nuclei in 100 μl of ice-cold NER with 1× protease inhibitor mixture.
8. Vortex for 15 s every 10 min, for a total of 40 min.
9. Centrifuge the tube at 16,000 × *g* for 10 min and transfer the supernatant (nuclear extract) fraction to a clean prechilled tube.
10. Store all extracts at −80°C until use.

3.3. Western Blot Analysis of Histone Acetylation

3.3.1. Chemiluminescence Western Blot

1. Treat cells with HDAC inhibitors.
2. Extract nuclear proteins or histone proteins as described above in Subheadings 3.1 and 3.2.
3. Dilute equal amounts of proteins in 2× SDS loading buffer, and boil at 95°C for 5 min.
4. Separate nuclear extract or histones on SDS-PAGE gels and transfer them onto PVDF membrane according to appropriate protocols.
5. Block blot in TBST with 5% nonfat dry milk for 2 h at room temperature or 4°C overnight.
6. Add the primary antibodies against acetylated histone proteins at a dilution of 1:2,000, and incubate the membranes with the diluted antibody at room temperature for 2 h.
7. Wash the membrane with TBST on a shaker with the revolution at 40–50 rpm for 3 × 10 min.
8. Incubate blot with secondary antibody (rabbit) at a concentration of 1:3,000 in TBST containing 5% nonfat dry milk at room temperature for 1.5 h.
9. Wash the blot three times for 10 min in TBST and once for 5 min in 1× TBS, and rinse with water.
10. Visualize the acetylated histones with the ECL kit.

3.3.2. Odyssey Western Blot (See Note 3)

1. Protein sample preparation, gel running and PVDF membrane transfer should be performed using standard blotting procedures as described in Subheading 3.3.1.
2. Place membrane in Odyssey blocking buffer (without Tween 20) for at least 1 h with gentle shaking at room temperature.
3. Incubate blot with primary antibodies in Odyssey blocking buffer with 0.1% Tween 20 for 2 h at room temperature or overnight at 4°C.
4. Rinse membrane with 1× PBST (0.2% Tween 20).

[3]By use of fluorescence-labeled antibodies rather than enzyme labels, Odyssey infrared image system quantitatively detects protein expression on the Western blot with wide linear dynamic range that cannot be achieved by conventional chemiluminescence. With two detection channels, multiple separate targets can be probed in the same experiment. Therefore, quantification accuracy is improved when the second channel is used for loading normalization. This method has been used in our recent study to quantify drug-induced changes in global histone marks (39).

5. Incubate blot with infrared dye secondary antibody at a concentration of 1:5,000 in Odyssey blocking buffer with 0.1% Tween 20 and 0.01% SDS at room temperature for 60 min. Protect membrane from light during incubation.
6. Rinse membrane with 1× PBST (0.1% Tween 20). The membrane can be scanned wet or dry.

3.4. HDAC Activity Assay

In vitro HDAC activity assays are performed using the Calbiochem® HDAC activity assay kit according to the manufacturer's instructions. This method is an assay system to measure HDAC activity in whole cell or nuclear extracts, immunoprecipitates, or purified recombinant human HDACs. In this unit, we describe the method of in vitro measurement of nuclear HDAC activity using the peptide substrate comprising an acetylated side chain lysine residue and a bound fluorescent group (Calbiochem/EMD Chemical).

1. Treat cells with HDAC inhibitors.
2. Extract nuclear proteins using the methods as described in Subheading 3.2.
3. Prepare the standard curve of deacetylation. Optimize the concentration ranges of deaceylated standard.
4. Add HDAC assay buffer to appropriate wells of the 96-well plate.
5. Add diluted cell nuclear extract or other HDAC containing samples to designated wells in triplicate.
6. Add HDAC substrate to each well containing nuclear extract or "no enzyme control."
7. Mix thoroughly and incubate the plate at room temperature (~25°C) for 10–15 min.
8. Read samples in a fluorimeter at an excitation wavelength of 350–380 nm and an emission wavelength of 440–460 nm (see Note 4).

3.5. RT-PCR to Detect the Reexpression of Epigenetically Silenced Genes by HDAC Inhibitors in Human Breast Cancer Cells

Previous studies in our laboratory showed that pharmacological inhibition of histone deacetylation resulted in the expression of functional ERα mRNA and protein in ER negative human breast cancer cells (18–20). Here, we describe the method to detect the reactivation of ERα mRNA by SAHA treatment using RT-PCR in ER negative MDA-MB-231 cells. The RT-PCR primers and conditions for some other genes reactivated by HDAC inhibitors in human breast cancer cells are summarized in Table 3.

1. Treat ER negative human breast cancer cells (MDA-MB-231) with 1–10 μM SAHA for 24 h.
2. Rinse cells with ice-cold PBS and lyse cells directly by adding 1 ml of TRIZOL reagent (Invitrogen) per 10-cm^2 dish and scraping with cell scraper.
3. Add 0.2 ml of chloroform per 1 ml of TRIzol reagent. Vortex samples for 15 s and incubate them at room temperature for 2–3 min.

[4]This method has been successfully used with preparations of all class I and II HDACs and class III HDAC SIRT1. It is necessary to use a potent HDAC inhibitor, such as TSA, as a positive control in experiments. The exact concentration range of the deaceylated standard, substrate and inhibitors should be carefully optimized and determined.

4. Centrifuge the samples at 12,000 × *g* for 15 min at 4°C and transfer upper aqueous phase containing RNA into nuclease-free tubes.
5. Add 0.5 ml of isopropyl alcohol per 1 ml of TRIZOL reagent to precipitate the RNA and centrifuge at 12,000 × *g* for 10 min at 4°C.
6. Wash the RNA pellet with 75% ethanol, centrifuge, and dissolve RNA in 100 μl DEPC-treated water. Measure the RNA concentrations.
7. First strand cDNA is synthesized by mixing 3 μg total RNA with 1 μl oligo $(dT)_{12–18}$ (500 μg/ml), and 1 μl of 10 mM dNTP mix (Invitrogen), then adding sterile ddH_2O to 12 μl.
8. Heat mixture to 65°C for 5 min and quickly chill on ice.
9. Add 4 μl 5× first strand buffer, 2 μl 0.1 M DTT and 1 μl RNaseOUT™ Recombinant Ribonuclease Inhibitor (40 U/μl) (Invitrogen), mix contents of the tube gently and incubate at 37°C for 2 min.
10. Add 1 μl (200 U) of M-MLV reverse transcriptase (Invitrogen) and incubate 50 min at 37°C followed by heating at 70°C for 15 min to inactivate the reaction.
11. Conventional PCR was performed in cDNA samples as previously described (20) using the following primers: ERα S: GCACCCTGAAGTCTCTGGAA; AS: TGGCTAAAGTGGTGCATGAT; Actin S: ACCATGGATGATGATATCGC; AS: ACATGGCTGGGGTGTTGAAG. Amplified products are analyzed on 1.5% agarose gels.

3.6. Chromatin Immunoprecipitation to Analyze Changes in Regulatory Chromatin Marks by HDAC Inhibitors at the Specific Gene Promoters

ChIP is a powerful tool to study protein–DNA interaction in HDAC inhibitor treated cells. This technique can map minute-byminute changes of histone acetylation at a single promoter, or over the entire genome by using advanced ChIP on DNA microarray technology (ChIP-on-chip) (38). The protocol and reagents for ChIP used in our laboratory are described below as recommended by the manufacturer's instructions (Millipore/Upstate).

1. Treat 2×10^6 human breast cancer cells with 1–10 μM HDAC inhibitors for 24 h.
2. Crosslink DNA and proteins by adding 37% formaldehyde directly to growth media to a final concentration of 1%, and gently shake dishes to mix, and incubate at room temperature for 10 min.
3. Add glycine to a final concentration of 0.125 M to quench crosslinking reactions.
4. Wash cells with cold PBS containing 1× protease inhibitor and scrape cells from each dish into a microcentrifuge tube.
5. Spin at 500 × *g* at 4°C for 2–5 min to pellet cells.
6. Resuspend cell pellet in 200 μl of SDS lysis buffer containing 1× protease inhibitor.
7. Sonicate cell lysate to shear DNA to ~200–1,000 bp in length (see Note 5). In between pulses, let samples sit on ice for at least 2 min.

[5]It is important to optimize conditions for shearing crosslinked DNA to 200–1,000 bp in length. These conditions vary with different cell types, cell density, and the specific sonication equipment setting including the power output, duty cycle, and number of pulses. During the sonication, keep all the samples on ice to avoid the occurrence of protein denaturation.

8. Centrifuge samples at 14,000 × *g* at 4°C for 10 min and transfer 200 μl of the sonicated cell supernatant to a new microcentrifuge tube.
9. Dilute the sonicated cell supernatant tenfold in 1.8 ml ChIP dilution buffer with 1× protease inhibitor.
10. Preclear the 2-ml diluted cell supernatant with 80 μl of salmon sperm DNA/Protein A Agarose beads (50% slurry) for 30 min at 4°C with agitation.
11. Separate beads by brief centrifugation and transfer the supernatant to a fresh tube (if using magnetic beads, beads are separated on magnetic rack).
12. Remove 10 μl (1%) of the supernatant as input and save at 4°C.
13. Collect the supernatant by aliquoting 1 ml into fresh microfuge tubes.
14. Add the immunoprecipitating antibodies against acetylated H3, H4, or specific lysine residues on histone tail to the supernatant fraction and incubate overnight at 4°C with rotation. Rabbit immunoglobulin G (IgG) and H3 antibodies are used for negative and quantitative controls, respectively (see Note 6).
15. Add 60 μl of Protein A Agarose beads (Millipore) and mix for 1 h at 4°C with rotation.
16. Separate beads by brief centrifugation or magnetic rack and remove the supernatant fraction.
17. Wash the beads once in 1 ml of low salt immune complex wash buffer (Millipore), once in high salt immune complex wash buffer (Millipore), once in LiCl Immune complex wash buffer (Millipore), and twice in TE buffer.
18. Add 100 μl of elution buffer (20% SDS, 1 M $NaHCO_3$) to each tube containing the antibody/bead complex. Mix and incubate at room temperature for 15 min. Transfer the eluate to fresh tube and wash the beads with 250 μl ChIP elution buffer. Repeat the wash and pool the eluates.
19. Add 20 μl 5 M NaCl to the pooled eluates and reverse cross-links at least 4 h at 65°C.
20. Add 1 μl of RNase A and incubate for 30 min at 37°C. Add 4 μl 0.5 M EDTA, 8 μl 1 M Tris–HCl and 1 μl Proteinase K, and incubate at 45°C for 1 h.
21. Extract the samples with phenol/chloroform (1:1), ethanol-precipitate DNA in the presence of 20 μg of glycogen, wash with 70% ethanol, and dissolve in 50 μl TE.
22. IP DNA can be further analyzed by quantitative Real-Time PCR to quantify alteration in acetylated histone marks at the promoter region of gene of interest (see Note 7). DNA immunoprecipitated by H3 antibody is used for normalization.

[6]It is possible that an anti-acetylated histone antibody used in ChIP will not recognize the epitope of the antigen in fixed chromatin. In such case, choose an antibody with higher affinity that has been validated as suitable for ChIP. It is important to use a negative control in every ChIP experiment (such as IgG) to detect nonspecific binding. If polyclonal antibodies are used, a control using unimmunized sera from the same species should be included.

[7]ChIP Primers for silenced genes reactivated with HDAC inhibition in human breast cancer cells: ERα, forward: TGAACCGTCCGCAGCTCAAGATC and reverse: GTCTGACCGTAGACCTGCGCGTTG (19); SFRP1, forward: AGCCGCGTCTGGTTCTAGT and reverse: GGAGGCTGCAGGGCTG; E-cadherin, forward: TAGAGGGTCACCGCGTCTATG and reverse: GGGTGCGTGGCTGCAGCCAGG (22).

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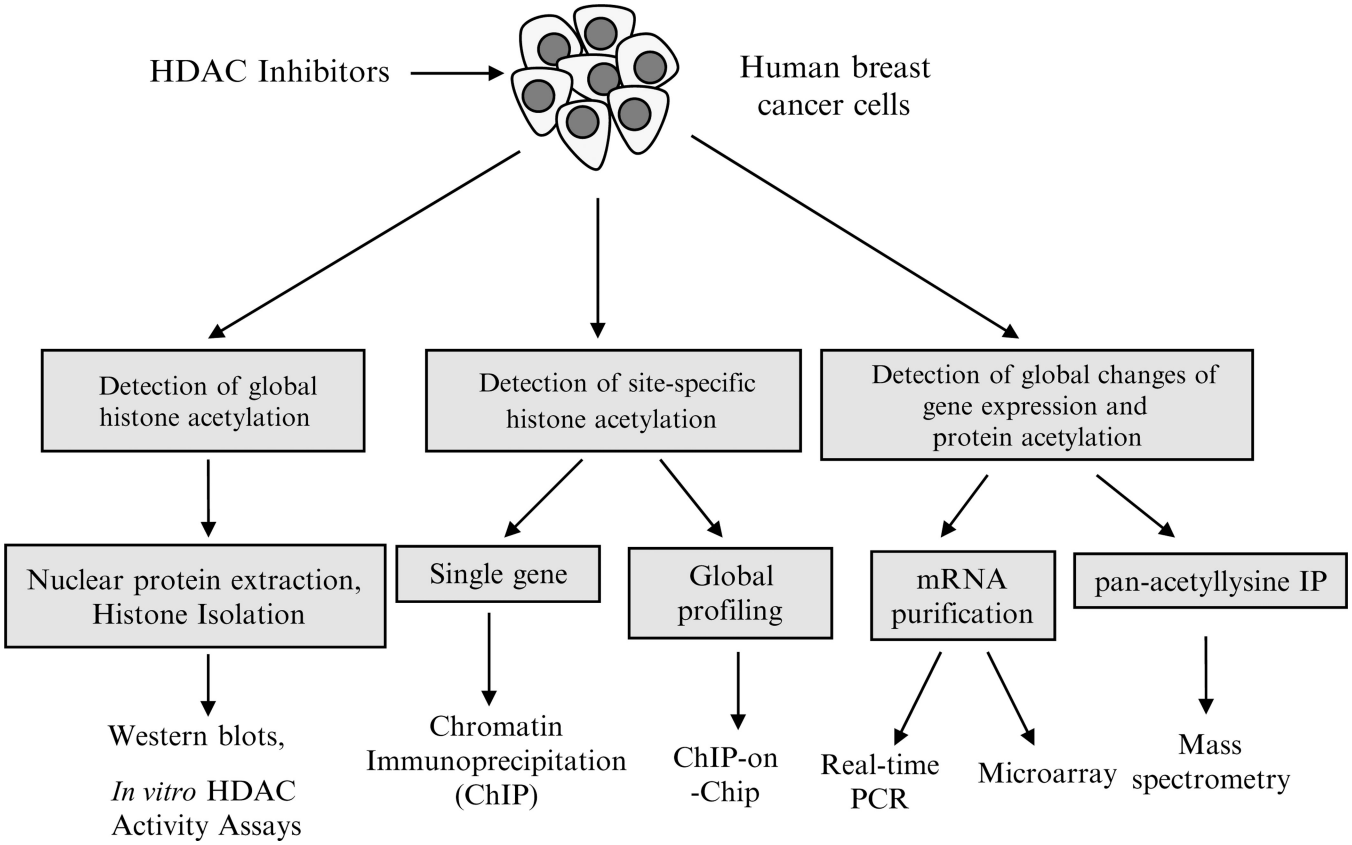

Fig. 1.

Techniques for studying HDAC inhibition in human breast cancer. After human breast cancer cells are treated with HDAC inhibitors, immunological detection methods such as Western blot or immunochemistry can be used to determine the level of histone acetylation using specific antibodies against histone H3 or H4 or specific histone lysine residues such as AcH3K9, AcH3K27, and AcH4K20. Chromatin immunoprecipitation (ChIP) is used to determine the interaction of site-specific acetylated histones with promoters of genes of interest in breast cancer cells after HDAC inhibitor treatment. DNA sequences bound to a particular acetylated histone or nonhistone protein can be isolated by ChIP and these fragments can subsequently be hybridized to a DNA microarray (such as a tiling array). This so-called ChIP-on-chip technology allows the determination of acetylated histone binding occupancy throughout the genome of the cancer cell. Quantitative-PCR is able to precisely measure the specific gene expression changes in the presence of HDAC inhibitor treatment. Microarray-based gene expression profiling can be used to identify genes whose expression is altered by HDAC inhibitor treatment. Several mass spectrometry (MS)-based proteomic methods exist to quantitatively analyze proteins that are hyperacetylated after treatment with HDAC inhibitors as well as determine isoform specific occupancy of histone modifications.

Table 1

Human histone deacetylase subunits

Class	Subunit	Zinc-dependent	NAD+dependent
I	HDAC 1, 2, 3, 8	Yes	No
IIa	HDAC 4, 5, 7, 9	Yes	No
IIb	HDAC 6, 10	Yes	No
III	SIRT 1, 2, 3, 4, 5, 6, 7	No	Yes
IV	HDAC 11	Yes	No

HDAC histone deacetylase, *SIRT* sirtuin

Table 2

Characteristics of some HDAC inhibitors in clinical trials

Class	Compound	Targeted HDACs	Clinical trial stage
Hydroxamic acid	SAHA (Vorinostat)	Class I, II, IV	USFDA approved for CTCL
	LBH-589 (Panobinostat)	Class I, II, IV	Phase III
	PXD-101 (Belinostat)	Class I, II, IV	Phase II
	ITF2357	Class I, II	Phase I
Cyclic peptide	Romidepsin (FK/228)	Class I, II	USFDA approved for CTCL
Short-chain fatty acid	Valproic acid	Class I HDAC 1	Phase II
	Phenylbutyrate	Class I	Phase I, II
Benzamide	MS-275 (Entinostat)	Class I HDAC 1, 2, 3	Phase II
	MGC0103	Class I, 11	Phase II

CTCL cutaneous T-cell lymphoma, *HDAC* histone deacetylase, *SAHA* suberoylanilide hydroxamic acid, *USFDA* US Food and Drug Administration

Table 3

RT-PCR primers and conditions for genes reactivated by HDAC inhibitors in human breast cancer cells

Gene	Primers	Annealing temperature (°C)	HDAC inhibitor	Reference
ERα	S: GCACCCTGAAGTCTCTGGAA AS: TGGCTAAAGTGGTGCATGAT	55	TSA Scriptaid LBH SAHA	(18–21)
PR	S: TCATTACCTCAGAAGATTTGTTTAATC AS: TGATCTATGCAGGACTAGACAA	60	TSA Scriptaid	(18–21)
SFRP1	S: GGCCCATCTACCCGTGTCG AS: GATGGCCTCAGATTTCAACTCGT	60	Splitomicin	(22)
SFRP2	S: AAGCCTGCAAAAATAAAAATGATG AS: TGTAAATGGTCTTGCTCTTGGTCT	53	Splitomicin	(22)
E-cadherin	S: CCGCCGGCGTCTGTAGGAA AS: AGGGCTCTTTGACCACCGCTCTC	57	Splitomicin	(22)
CRBP1	S: CATCCGCACGCTGAGCACTTTTAG AS: CACGCCCCTCCTTCTCACCCTTCT	58	Splitomicin	(22)

ERα estrogen receptor alpha, *PR* progesterone receptor, *SFRP* secreted frizzled-related protein, *CRPB1* cellular retinol binding protein 1