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Use of Polyamine Derivatives as Selective Histone Deacetylase Inhibitors

Patrick M. Woster

Abstract

Histone acetylation and deacetylation, mediated by histone acetyltransferase and the 11 isoforms of histone deacetylase, play an important role in gene expression. Histone deacetylase inhibitors have found utility in the treatment of cancer by promoting the reexpression of aberrantly silenced genes that code for tumor suppressor factors. It is unclear which of the 11 histone deacetylase isoforms are important in human cancer. We have designed a series of polyaminohydroxamic acid (PAHA) and polyaminobenzamide (PABA) histone deacetylase inhibitors that exhibit selectivity among four histone deacetylase isoforms. Although all of the active inhibitors promote reexpression of tumor suppressor factors, they produce variable cellular effects ranging from stimulation of growth to cytostasis and cytotoxicity. This chapter describes the procedures used to quantify the global and isoform-specific inhibition caused by these inhibitors, and techniques used to measure cellular effects such as reexpression of tumor suppressor proteins and hyperacetylation of histones H3 and H4. Procedures are also described to examine the ability of PAHAs and PABAs to utilize the polyamine transport system and to induce overexpression of the early apoptotic factor annexin A1.

Keywords

Histone deacetylase; Polyaminohydroxamic acid; Polyaminobenzamide; Annexin A1; Polyamine transport; Breast cancer

1. Introduction

Chromatin architecture, which is strongly influenced by post-translational modifications of histones, is a key factor in the regulation of gene expression (1–3). Several posttranslational modifications of histones (4, 5) mediate epigenetic chromatin remodeling, and histone acetylation/deacetylation are the best-characterized processes (1, 6). The acetylation status of histones is controlled by a balance between two enzymes, histone acetyltransferase (HAT), which promotes histone hyperacetylation, and the histone deacetylases (HDACs), which catalyze acetyl group cleavage (6, 7). Normal mammalian cells exhibit an exquisite level of control of chromatin architecture by maintaining a balance between these two enzymes (8). There are 11 known zinc-dependent HDAC isoforms (9–11) that belong to four structural classes: class I (isoforms 1–3 and 8) (12), class IIa (isoforms 4, 5, 7, and 9) (9), class IIb (isoforms 6 and 10) (9), and class IV (HDAC 11) (9).

In some tumor cells, hypoacetylation of histones results in aberrant gene silencing leading to the underexpression of growth-regulatory proteins such as the cyclin-dependent kinase inhibitor p21^{Waf1}, thus contributing to tumorigenesis (1, 6). Despite extensive study, it is still uncertain which HDAC isoforms play the most important roles in human cancer.

Histone hyperacetylation caused by HDAC inhibitors such as trichostatin A (TSA, **1**, Fig. 1), *N*-(2-aminophenyl)-4-[*N*-(pyridin-3-ylmethoxycarbonyl)-aminomethyl]benzamide (MS-275, **2**, Fig. 1), and suberoylanilide hydroxamic acid (SAHA, **3**, Fig. 1) can cause growth arrest in a wide range of transformed cells, and can inhibit the growth of human tumor xenografts (6, 7, 12–14), especially when used in combination with DNA methyltransferase (DNMT) inhibitors (15–18). Although several HDAC inhibitors are in clinical trials and one agent has been marketed, dose-limiting toxicity remains a problem (19). Most existing HDAC inhibitors do not discriminate between the 11 isoforms of HDAC. Isoform-specific HDAC inhibitors would be of great value in elucidating the roles of HDAC isoforms in both normal and cancer cells, but very few isoform selective inhibitors have been synthesized.

HDAC inhibitors such as **1–3** possess three structural features that are required for optimal activity: an aromatic cap group, an aliphatic chain, and a metal binding functional group (Fig. 2). We reported a series of polyaminohydroxamic acid (PAHA) derivatives that incorporate structural features of alkylpolyamines with antitumor activity and the metal binding moiety, linker, and cap groups found in molecules such as **2** and **3**. It was postulated that these compounds could enter cells using the polyamine cellular transporter, and be selectively directed to DNA and the associated HDACs by the positively charged polyamine portion of the structure. An additional advantage to this approach is that it may ultimately be possible to produce isoform-specific inhibitors for individual HDACs by altering the polyamine chain composition and the terminal alkyl group on that chain, taking advantage of the fact that HDACs differ in primary sequence at specific residues in the rim region of the HDAC active site (12).

An initial series of 16 PAHA analogues were synthesized and screened for inhibitory activity against a mixture of HDACs derived from HeLa cell lysates (20). Two of these compounds, **4** and **5** (Fig. 2), inhibited HDAC in this assay by 75% at a 1.0 μ M concentration. Compound **5** at 1.0 μ M produced significantly greater induction of acetylated histone H3 (AcH3) and acetylated histone H4 (AcH4) than a like concentration of **2**, and was markedly more effective at reexpressing the cyclin-dependent kinase inhibitor p21^{Waf1} in the ML-1 mouse leukemia cell line. Compound **6** at 1.0 μ M (Fig. 2) inhibited the HDAC mixture by 51.5%, but caused a 253-fold induction of acetylated α -tubulin while only causing a minimal increase in AcH3, AcH4, and p21^{Waf1} (21), suggesting that **6** shows a marked selectivity toward HDAC 6, which is known to deacetylate α -tubulin (22). Compound **6** was later found to inhibit HDAC 6 by 99.7% at 1.0 μ M.

Using the same design strategy, a series of polyaminobenzamides (PABAs) and their homologues were synthesized and evaluated as inhibitors of HDAC (21). Compound **7** (Fig. 3), one of the first entries in the series, exhibits an IC₅₀ value against global HDAC from the HeLa cell lysate of 4.9 μ M, which is comparable to the reported IC₅₀ value for **2** (4.8 μ M) (23). Analogues in the PABA series were evaluated against global HDAC and against 4 HDAC isoforms representing Class I (HDAC 1, 3, and 8) and Class II (HDAC 6). Isoform selectivity among the four HDACs evaluated varied significantly, demonstrating that global percent HDAC inhibition is a composite of strong and weak inhibition at different isoforms. The observed HDAC selectivity with PAHAs and PABAs is in part due to structural variations in their polyamine side chains (24). PAHA and PABA HDAC inhibitors were also evaluated for their ability to utilize the polyamine transport system (25). The hydroxamic acid-containing PAHAs, with pK_a values of approximately 9, are not substrates for the mammalian transporter, while PABAs such as **7** are effectively imported using the polyamine transport system (21).

The most active global HDAC inhibitors in the PABA series were evaluated against MCF7 wild-type tumor cells in vitro, and structure-dependent variations in growth inhibitory

activity were observed, as exemplified by PABA compounds **8–10** (Fig. 3). All these three analogues inhibited global HDAC between 30 and 40%. However, compounds **8** and **10** showed a marked selectivity for HDAC 1, while compound **9** has negligible activity against this isoform. Over a range of concentrations between 0.3 and 30 μM , benzamide **8** appeared to stimulate tumor cell growth, while **9**, which features a shorter linker chain region and an α -ketoamide metal-binding moiety, was cytostatic. However, benzamide **10**, featuring a shorter polyamine substituent, initiated apoptotic cell death in the MCF7 cell line, with an IC_{50} of 0.9 μM . In the MCF10A normal breast epithelial cell line, **10** exhibited an IC_{50} of 24 μM , and thus demonstrated significant selectivity for tumor cells. Subsequent experiments showed that **10**, but not **8**, promoted the induction of annexin A1, an early intermediate in the apoptosis pathway (26). HDAC inhibitors have been shown to promote apoptosis through induction of annexin A1, and recent studies suggest that breast tumors expressing high levels of annexin I are more likely to respond to neoadjuvant chemotherapy than cells with low levels of the protein (27).

Aberrant HDAC activity plays a role in other diseases where an HDAC inhibitor with no inherent cytotoxicity would be of great value. Recent studies have suggested that histone acetylation status plays a role in the development of diabetes mellitus (28, 29) and that HDAC inhibitors may serve as a novel class of agents for the treatment of diabetes and its complications (30, 31). We thus evaluated PABA HDAC inhibitors for their cyto-protective effects against IL-1 β -induced damage to isolated β -cells. Three PABAs, compounds **11–13** (Fig. 3), significantly inhibited HDAC activity and increased the acetylation of histone H4 in isolated β -cells. These compounds further exerted no toxic effects on metabolic cell viability in these cells. PABA **11** protected against IL-1 β -mediated loss in β -cell viability (32). Remarkably, compound **11** differs from **13**, which does not protect against this loss in β -cell viability, by only a single carbon in the linker chain region. Compound **11** was also able to attenuate IL-1 β -induced iNOS expression and subsequent NO release. These data suggest that the cytoprotective properties of **11** against IL-1 β -mediated effects may, in part, be due to inhibition of IL-1 β -induced transactivation of NF κ B in these cells. A novel HDAC inhibitor such as **11** that can prevent IL-1 β -mediated effects on isolated β -cells supports the development of nontoxic PABAs to prevent deleterious effects of cytokines and the onset of autoimmune diabetes.

This chapter describes the procedures used to quantify inhibition caused by these inhibitors and to measure cellular effects such as reexpression of tumor suppressor proteins and hyperacetylation of histones H3 and H4. Procedures are also described to examine their ability to utilize the polyamine transport system and to induce overexpression of the early apoptotic factor annexin A1. A full description of the synthesis of PAHA and PABA HDAC inhibitors is beyond the scope of this manuscript. A full description of these syntheses, including step-by-step procedures and reaction conditions, can be found in refs. (20) and (21).

2. Materials

2.1. HDAC Inhibition Assay

1. Fluor de Lys[®] HDAC Assay Kit AK500 (BIOMOL International, LP, Plymouth Meeting, CA).
2. 96-Well microplates (BD Falcon Microtest 96-well Assay Optilux, Black/Clear Bottom, TC) Surface (BD Biosciences, Franklin Lakes, NJ).
3. Assay buffer: 50 mM Tris-HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl_2 .
4. SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA).

2.2. Isoform Selectivity Studies

1. Fluorogenic HDAC Assay Kit (BPS Bioscience, San Diego, CA).
2. HDAC 1 (C-Flag) (BPS Bioscience, San Diego, CA).
3. HDAC 3 (NcoR2) (BPS Bioscience, San Diego, CA).
4. HDAC 6 (BPS Bioscience, San Diego, CA).
5. HDAC 8 (BPS Bioscience, San Diego, CA).
6. Fluorogenic HDAC Substrate 1 (BPS Bioscience, San Diego, CA).
7. 96-Well microplates (BD Falcon Microtest 96-well Assay Optilux, Black/Clear Bottom, TC) Surface (BD Biosciences).
8. Assay buffer: 50 mM Tris-HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂.
9. SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA).

2.3. HDAC Cellular Assay

1. MCF7 Human breast carcinoma cells (ATCC, Manassas, VA).
2. MCF10A Human breast carcinoma cells (ATCC, Manassas, VA).
3. MCF7 culture medium: RPMI 1640 (ATCC, Manassas, VA) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA), 10 µg/mL insulin (Sigma/ Aldrich, St. Louis, MO), 100 IU/100 µg/mL penicillin/ streptomycin solution (Mediatech, Manassas, VA).
4. MCF10A culture medium: DMEM/Ham's F12:50/50 (Mediatech, Manassas, VA) supplemented with 5% horse serum (Atlanta Biologicals, Lawrenceville, GA), 20 ng/mL epidermal growth factor, 100 ng/mL cholera toxin, 10 µg/ mL insulin, 500 ng/mL hydrocortisone (Sigma/Aldrich, St. Louis, MO), 100 IU/100 µg/mL penicillin/streptomycin solution (Mediatech).
5. Cell detaching solution: TrypLE Express (Gibco, Bethesda, MD).
6. HDAC Fluorometric Cellular Assay Kit AK503 (BIOMOL International, LP, Plymouth Meeting, CA).
7. 96-Well microplates (BD Falcon Microtest 96-well Assay Optilux, Black/Clear Bottom, TC Surface (BD Biosciences, Franklin Lakes, NJ).
8. SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA).

2.4. Cell Culture

1. MCF7 Human breast carcinoma cells (ATCC).
2. MCF10A Human breast carcinoma cells (ATCC).
3. MCF7 culture medium: RPMI 1640 (ATCC) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals), 10 µg/mL insulin (Sigma/Aldrich), 100 IU/100 µg/mL penicillin/streptomycin solution (Mediatech).
4. MCF10A culture medium: DMEM/Ham's F12:50/50 (Mediatech) supplemented with 5% horse serum (Atlanta Biologicals), 20 ng/mL epidermal growth factor, 100 ng/mL cholera toxin, 10 µg/mL insulin, 500 ng/mL hydrocortisone (Sigma/ Aldrich), 100 IU/100 µg/mL penicillin/streptomycin solution (Mediatech).

- INS 832/13 culture medium: RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum supplemented with 100 IU/mL penicillin and 100 IU/mL streptomycin, 1.0 mM sodium pyruvate, 50 μ M 2-mercaptoethanol, and 10 mM HEPES (pH 7.4).

2.5. Determination of Cell Viability for MCF-7 and MCF 10A Cells In Vitro

- MCF7 Human breast carcinoma cells (ATCC).
- MCF10A Human breast carcinoma cells (ATCC).
- MCF7 culture medium: RPMI 1640 (ATCC) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals), 10 μ g/mL insulin (Sigma/Aldrich), 100 IU/100 μ g/mL penicillin/streptomycin solution (Mediatech).
- MCF10A culture medium: DMEM/Ham's F12:50/50 (Mediatech) supplemented with 5% horse serum (Atlanta Biologicals), 20 ng/mL epidermal growth factor, 100 ng/mL cholera toxin, 10 μ g/mL insulin, 500 ng/mL hydrocortisone (Sigma/Aldrich), 100 IU/100 μ g/mL penicillin/ streptomycin solution (Mediatech).
- Cell detaching solution: TrypLE Express (Gibco).
- CellTiter 96^R AQueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI).
- CellTiter 96^R AQueous One Solution Reagent; MTS (3-[4,5-dimethylthiazo-2-yl]-2,5-diphenyltetrazolium bromide) (Promega, Madison, WI).
- 96-Well flat bottom plates (Corning, Corning, NY).
- Microplate reader (E max precision microplate reader) (Molecular Devices, Sunnyvale, CA).

2.6. Western Blotting

- 125 mM Tris-HCl, pH 6.8.
- BCATM Protein Assay Reagent Kit (PIERCE, Rockford, IL).
- Millipore Immobilon Western Chemiluminescent HRP Substrate.
- Science Lab Image Gauge software (FujiFilm, USA).

2.7. Determination of Histone Acetylation by Western Blotting

2.7.1. Determination of Acetylated Histones H3 and H4 in ML-1 Human Acute Myeloid Leukemia Cells

- Buffer A: 25 mM HEPES, pH 7.9, 150 mM NaCl, 0.5 mM EDTA, 0.1% Triton X, 10% Glycerol, 0.1% mg/mL BSA.
- Lysis Buffer B: 10 mM Tris-HCl pH 7.6, 0.25 M sucrose, 3 mM calcium chloride, and 5 mM butyric acid.
- Primary antibodies against acetyl-histone H3 (diluted 1:1,000), acetyl-histone H4 (diluted 1:500), and histone – H2A (diluted 1:1,000) (Upstate Biotechnologies, Lake Placid, NY).
- Odyssey blocking buffer #927-40000 (Li-Cor Bioscience, Lincoln, NE).
- Infrared labeled antibodies: anti-mouse IRdye 800, anti-rabbit AlexaFluor 680 (Li-Cor Bioscience, Lincoln, NE).

6. PBS-T (phosphate-buffered saline (PBS) containing 0.1% Tween 20.

2.7.2. Determination of Acetylated Histones H3 and H4 in Insulin-Secreting Rat INS-832/31 Cells

1. 1× Cell lysis buffer: 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1.0 mM EDTA, 1.0 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1.0 mM β -glycerophosphate, 1.0 mM Na_3VO_4 , 1.0 $\mu\text{g/mL}$ Leupeptin, 1.0 mM PMSF (see Note 1).
2. 3× Sample loading buffer: 2.4 mL of 1.0 M Tris-HCl pH 6.8 stock, 3 mL of 20% SDS stock, 3 mL of 100% Glycerol, 1.6 mL β -mercaptoethanol, 6.0 mg bromophenol blue, diluted to 10 mL with dH_2O .
3. Antibodies (Cell Signaling Technologies, Danvers, MA): Histone H4- 2592- Rabbit, AcH4- 2594 S- Rabbit, Histone H3- 9715- Rabbit, AcH3- 9649 S- Rabbit.
4. Insulin-secreting rat INS-832/31 cells (33).

2.8. Determination of Annex 1A expression

1. MCF7 Human breast carcinoma cells (ATCC).
2. MCF7-AnxA1 stable cells, transfected with annexin A1 with GFP (provided by Dr. F. Hirata at Wayne State University).
3. MCF10A Human breast carcinoma cells (ATCC).
4. MCF7 culture medium: RPMI 1640 (ATCC) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals), 10 $\mu\text{g/mL}$ insulin (Sigma/Aldrich), 100 IU/100 $\mu\text{g/mL}$ penicillin/streptomycin solution (Mediatech).
5. MCF7-AnxA1 culture medium: Minimum essential medium, Eagle with Earle's balanced salt solution (ATCC, Manassas, VA) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals), 10 $\mu\text{g/mL}$ insulin (Sigma/Aldrich), 100IU/100 $\mu\text{g/mL}$ penicillin/streptomycin solution (Mediatech).
6. MCF10A culture medium: DMEM/Ham's F12:50/50 (Mediatech) supplemented with 5% horse serum (Atlanta Biologicals), 20 ng/mL epidermal growth factor, 100 ng/ mL cholera toxin, 10 $\mu\text{g/mL}$ insulin, 500 ng/mL hydrocortisone (Sigma/Aldrich), 100 IU/100 $\mu\text{g/mL}$ penicillin/ streptomycin solution (Mediatech).
7. Cell detaching solution: TrypLE Express (Gibco).
8. Cell lysis buffer: 10 mM Tris-HCl, pH 7.4, 1% SDS, 1 mM EDTA, 1 mM EGTA, 2 mM PMSF, 3 mM-activated Na_3VO_4 , protease inhibitor cocktail.
9. BCATM Protein Assay Reagent Kit (PIERCE).
10. Polyacrylamide gel: 10% Bis-Tris precast (Bio-Rad Labs, Hercules, CA).
11. Gel running buffer: XT MOPS running buffer (Bio-Rad Labs, Hercules, CA).
12. Precision Plus protein standards (Bio-Rad Labs, Hercules, CA).
13. Sample buffer: XT Sample Buffer (Bio-Rad Labs, Hercules, CA).
14. Blotting buffer: PVDF Westran clear signal membrane (Whatman, Sanford, ME).

¹Utilization of this lysis buffer is important to prevent the loss of acetyl groups. If you are not probing for acetylated histones, then other lysis buffers may be substituted.

15. Transfer buffer: Towbin's Bis-Tris/Glycin/20% methanol without SDS.
16. Antibodies: Anti-Annexin A1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti- β -actin antibody (Sigma-Aldrich, St. Louis, MO), anti-mouse IgG horseradish peroxidase-conjugated antibody (Bio-Rad Labs, Hercules, CA).
17. Immunodetection reagents: ECL advance western blotting detection reagent (Amersham, Piscataway, PA).
18. 6-Well flat bottom plates (Corning).
19. PBS: Mediatech, Manassas, VA).
20. Tris-buffered saline (TBS): 20 mM Tris-HCl, pH 7.5, 137 mM NaCl.
21. Chemiluminescence detection and analyzer, Fuji LAS-1000 Plus and Science Lab Image Gauge (Fuji Film, USA).

2.9. Polyamine Uptake Assay

1. MCF7 Human breast carcinoma cells (ATCC).
2. MCF10A Human breast carcinoma cells (ATCC).
3. MCF7 culture medium: RPMI 1640 (ATCC) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals), 10 μ g/mL insulin (Sigma/Aldrich), 100 IU/100 μ g/mL penicillin/streptomycin solution (Mediatech).
4. MCF10A culture medium: DMEM/Ham's F12:50/50 (Mediatech) supplemented with 5% horse serum (Atlanta Biologicals), 20 ng/mL epidermal growth factor, 100 ng/mL cholera toxin, 10 μ g/mL insulin, 500 ng/mL hydrocortisone (Sigma/Aldrich), 100 IU/100 μ g/mL penicillin/ streptomycin solution (Mediatech).
5. Cell detaching solution: TrypLE Express (Gibco).
6. [3 H]-spermidine trihydrochloride (Perkin-Elmer, Boston, MA) (see Note 2).
7. Spermidine trihydrochloride (Acros Organics, Morris Plains, NJ).
8. PBS-T (PBS containing 0.1% Tween 20).
9. BCATM Protein Assay Reagent Kit (PIERCE).
10. 24-Well plates, flat bottom (Corning).

3. Methods

3.1. HDAC Inhibition Assay

1. HDAC inhibition activity was evaluated in vitro by using HDAC fluorimetric assay/drug discovery kit AK500. The assay was performed in a 96-well plate. 30-fold diluted HeLa extract was used as source of HDAC activity.
2. The Fluor de LysTM substrate containing acetylated lysine side chains was normalized by plotting a standard curve at various substrate concentrations.
3. A 10 μ L portion of the desired inhibitor molecule (5 μ M final concentration) in assay buffer, 25 μ L of Fluor de LysTM substrate (250 μ M), 0.5 μ L of HeLa cell

²Use of tritiated substances requires institutional training and approval by the office of radiation safety. All procedures should be carried out in areas within laboratories that have been approved for use of radioisotopes. Radioactive waste should be disposed of in approved containers.

extract (6–9 mg/mL), and 14.5 μ L assay buffer were incubated at 37°C in an appropriate well of the microtiter plate for 30 min (see Note 3).

4. The reaction was stopped by adding 50 μ L Fluor de Lys™ developer cocktail, and the plate was incubated at room temperature for 15 min.
5. Fluorescence was measured using a SpectraMax M5 plate reader (excitation wave length 360 nm, emission wave length 450 nm).

3.2. Isoform Selectivity Studies

1. Inhibition of individual HDAC isoform activity was evaluated using a Fluorogenic HDAC Assay Kit (BPS Bioscience) performed in a 96-well plate. Human recombinant HDAC immunoprecipitates were used as the source of HDAC activity.
2. A 10 μ L portion of the desired inhibitor molecule (5 μ M final concentration) in assay buffer, 5 μ L of substrate (200 μ M), 5.0 μ L HDAC 6 (0.2 μ g/ μ L), HDAC 3 (0.2 μ g/ μ L), HDAC 1 (0.04 μ g/ μ L) or HDAC 8 (0.6 μ g/ μ L), and 35.0 μ L assay buffer were incubated at 37°C in an appropriate well of the microtiter plate for 30 min.
3. The reaction was stopped by adding 50 μ L of the developer cocktail.
4. The plate was incubated at room temperature for 15 min.
5. Fluorescence was measured using a SpectraMax M5 plate reader (excitation wave length 360 nm, emission wave length 450 nm).

3.3. HDAC Cellular Assay

1. Cells were seeded in 200 μ L of the appropriate medium at 7,000 cells per well without phenol red, and were cultured at 37°C in 5% CO₂ for 2.5 days (80% confluences in a 96-well microplate).
2. Cells were exposed to test compounds in 100 μ L of medium for 6 days. The medium containing the compound was changed every 3 days.
3. Medium was replaced with 50 μ L/well of media containing 200 μ M of the Fluor de Lys™ substrate. The plates were incubated at 37°C for 3 h and 40 min.
4. The reaction was stopped by adding 50 μ L Fluor de Lys™ developer cocktail containing 1 $\times$ developer in cold lysis buffer and 2 mM trichostatin A.
5. The plate was incubated at 37°C for 15 min.
6. Fluorescence was measured using a SpectraMax M5 plate reader (excitation wave length 360 nm, emission wave length 450 nm).

3.4. Cell Culture

1. Human breast carcinoma MCF-7 (HTB-22) was maintained in the logarithmic phase at 37°C in a 5% CO₂ humidified atmosphere in the medium described above.
2. Human mammary epithelial cells (MCF 10A) were obtained from Karmanos Cancer Institute, Detroit, MI, and grown in the logarithmic phase at 37°C in a 5% CO₂ humidified atmosphere in the DMEM described above.

³All aqueous solutions should be prepared in water that has a resistivity of 18.2 M Ω -cm and total organic content of less than five parts per billion.

3. INS 832/13 cells were cultured in the medium described above at 37°C in a 5% CO₂ humidified atmosphere. The medium was changed twice weekly and cells were trypsinized and subcloned weekly.

3.5. Determination of Cell Viability for MCF-7 and MCF 10A Cells In Vitro

1. 7,500 Cells/well in 200 µL medium were seeded in a 96-well plate, and the cells were allowed to attach at 37°C in 5% CO₂ for 2.5 days.
2. The medium was aspirated, and cells were treated with 200 µL fresh medium containing gradually increasing concentrations of the test compound. The cells were incubated for 6 days at 37°C in 5% CO₂. Medium containing compounds was replaced every 3 days.
3. After 6 days, the medium was removed and replaced with a mixture of 100 µL phenol red free medium and 20 µL of the MTS reagent solution. The cells were incubated for another 4 h at 37°C under 5% CO₂ environment.
4. Absorbance was measured at 490 nm on a microplate reader equipped with SOFTmax PRO 4.0 software to determine the cell viability.

3.6. Western Blotting

1. Protein extracts were prepared in 125 mM Tris-HCl (pH 6.8), denatured using 4% SDS.
2. Protein content was quantitated using the BCATM Protein Assay Reagent Kit.
3. Extracts were subjected to polyacrylamide gel electrophoresis (PAGE) and electroblotted to nitrocellulose.
4. Blots were probed with antibodies and developed with Millipore Immobilon Western Chemiluminescent HRP Substrate.
5. Chemiluminescence was detected by Luminescence Image Analyzer, Fuji LAS-1000 Plus or Odyssey Infrared Imaging system.
6. Bands were analyzed by the Science Lab Image Gauge software (Fuji Photo Film).

3.7. Determination of Histone Acetylation

3.7.1. Determination of Acetylated Histones H3 and H4 in ML-1 Human Acute Myeloid Leukemia Cells

1. ML-1 human acute myeloid leukemia cells were seeded at the rate of 2×10^6 cells/ T-25 flask in 5.0 mL McCoy's 5A medium containing 9% fetal bovine serum and allowed to attach overnight.
2. Cells were treated with appropriate concentration of PABAs and PAHAs in the presence of 1.0 mM aminoguanidine and incubated for 24 h at 37°C with 5% CO₂.
3. At the end of 24 h, cells were harvested with trypsin and were converted to pellets, which were washed with 1× PBS and repelleted.
4. The pellet was resuspended in buffer A and protease inhibitors (~200 µL per T-25 flask).
5. The samples were transferred to chilled 1.5 mL tubes and were incubated on ice for 20 min. Nuclei were collected by centrifugation at 14,000 rpm for 15 min.
6. The pellets were resuspended in 250 µL ice cold lysis buffer B. 0.4 N Sulfuric acid was added to the samples, and they were incubated at 4°C overnight.

7. Debris was pelleted by centrifugation and the supernatant was collected. Histones were precipitated by addition of 10 volumes of acetone and incubated at -20°C overnight.
8. Pellets were collected by centrifugation, briefly dried under vacuum and resuspended in ddH₂O.
9. Proteins (10–20 μg per lane) are separated on 15% SDS-PAGE and visualized by Western blot analysis (see Subheading 3.6) using primary antibodies against AcH3, H4, and H2A.
10. The immunoreactive proteins were quantified using Odyssey Infrared Imaging System.

Protein was transferred to PVDF, and the membrane was blocked for 1 h with Odyssey blocking buffer. Primary antibodies in the same dilution as mentioned above were added to the blocking buffer along with 0.1% Tween 20. The membrane was washed 5 times with PBS-T for 5 min. Infrared labeled antibodies anti-mouse IRdye 800 and anti-rabbit AlexaFluor 680 were diluted in blocking buffer with 0.1% Tween 20 to 1:4,000. The antibody solution was added to the membrane and it was rocked for 1 h at room temperature. It was washed again for 5 min, and the membrane is scanned on Odyssey Infrared Imaging system.

3.7.2. Determination of Acetylated Histones H3 and H4 in Insulin-Secreting Rat INS-832/31 Cells

1. INS-832/13 (33) cells were cultured for 2 days in a 24-well plate in RPMI media.
2. The media was aspirated and the cells were rinsed twice with ice cold PBS.
3. 50 μL of ice cold $1\times$ cell lysis buffer was pipetted into each well and immediately scraped and collected into microcentrifuge tubes such that 3 wells were combined per tube.
4. The cells were then lysed by sonication.
5. Cell lysate protein concentration was then quantitated using Bio-Rad's Bradford reagent standardized with BSA.
6. 10 μg of protein was then taken from each sample and combined with a 1:3 ratio of $3\times$ sample loading buffer.
7. The proteins were then separated on a 12% SDS-PAGE gel.
8. The proteins were then transferred, overnight, to a nitrocellulose membrane by wet transfer.
9. The membrane was then blocked with 5% BSA in TBST for 1 h with gentle agitation.
10. The 1° antibody was diluted 1:1,000 in 5% BSA in TBST and applied over night to the membrane at 4°C with gentle agitation. Acetylated histones must be probed first because stripping will remove the acetyl groups.
11. The membrane was then washed 3×10 min in TBST with gentle agitation.
12. The 2° antibody was then applied to the membrane with a dilution of 1:1,000 in 5% BSA in TBST for 1 h. The 2° anti-rabbit antibody was purchased from GE Health Sciences #NA931V.
13. The membrane was then washed 3×10 min in TBST.

14. The membrane was then developed with ECL Plus (GE Health Science).
15. The blot was then imaged using a Kodak Image Station 4000R Pro.

3.8. Determination of Annex 1A Expression

1. Cells were seeded at the rate of 1.0×10^6 cells/well in 6-well plate in 2 mL of the indicated medium and allowed to grow to 90% confluency.
2. Cells were treated with an appropriate concentration of inhibitor and incubated for 2.5 days at 37°C with 5% CO₂.
3. Cells were detached using the cell detaching solution, and washed twice with PBS.
4. A 120 mM portion of lysis buffer was added, and the cells were incubated for 5 min on ice.
5. The lysed cells were collected with a scraper, samples were transferred to chilled 1.5 mL tubes, and nuclei were collected by centrifugation at 15,000 rpm for 5 min at 4°C.
6. The pellets were resuspended in 80 µL ice cold lysis buffer. Samples were sonicated 3 times for 10 s followed by 10 s break in between them and boiled for 5 min at 95°C.
7. Protein content was quantitated using BCA™ Protein Assay Reagent Kit.
8. Proteins (10 or 3 µg per lane) were separated by SDS-PAGE.
9. Protein was transferred to PVDF membrane and blocked overnight at 4°C with 1% ECL blocking buffer.
10. Blots were probed with primary antibodies against annexin A1 and β-actin diluted in blocking buffer, then washed 4 times with 1% TBS for 10 min on a shaker.
11. Blots were then treated with anti-m-IgG-HRP diluted in blocking buffer with 0.1% Tween 20.
12. The membrane was washed 4 times with 1% TBS with 0.1% Tween 20 for 10 min on a shaker.
13. The membrane was developed with ECL advance blotting reagent.
14. Chemiluminescence signals were detected with the Fuji LAS-1000 Plus, and bands were analyzed by the Science Lab Image Gauge software.

3.9. Polyamine Uptake Assay

1. MCF7 cells were seeded at the rate of 5×10^5 cells/well or MCF10A cells were seeded at 3×10^5 cells/well in the appropriate medium and cultured for 2.5 days at 37°C in 5% CO₂ in 24-well plates. Cells were allowed to exponential growth at 37°C for 2.5 days.
2. The medium was removed by aspiration and replaced with 0.5 mL serum-free medium to each well and allowed to incubate for 1 h at 37°C in 5% CO₂.
3. A mixture of ³H spermidine trihydrochloride (34) and unlabeled spermidine (5 µL, 96 µM) was added to each well with increasing concentrations of PAHA or PABA inhibitor from 0.3–30 µM and incubated at 37°C for 30 min.
4. Medium containing residual ³H spermidine was aspirated, and cells were placed on ice and rinsed 3 times with 1 mL cold phosphate-buffer saline (PBS).

5. Cells were digested for 1 h at room temperature in 400 μ L 1 N NaOH.
6. Prior to determination of cell associated radioactivity, 400 μ L of the cell digest was neutralized with 420 μ L of 1 N acetic acid.
7. The 720 μ L of neutralized digest was added to 4 mL of scintillation fluid, and the radioactivity was counted using a β -rack liquid scintillation counter (Perkin Elmer, MA).

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Fig. 1. Histone deacetylase inhibitors trichostatin A (1), MS-275 (2) and SAHA (3).

Fig. 2.
A model for the design of polyaminohydroxamic acid (PAHA) and polyaminobenzamide (PABA) HDAC inhibitors, and PAHAs 4-6.

Fig. 3.
Polyaminobenzamide (PABA) HDAC inhibitors **7–13**.