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Histone deacetylase inhibition by suberoylanilide hydroxamic acid: a therapeutic approach to treat human uterine leiomyoma

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Objective: To evaluate the effect of inhibition of histone deacetylases (HDACs) by suberoylanilide hydroxamic acid (SAHA) treatment of human uterine leiomyoma primary (HULP) cells in vitro on cell proliferation, cell cycle, extracellular matrix (ECM) formation, and transforming growth factor $\beta 3$ (TGF-$\beta 3$) signaling.
Design: Prospective study comparing uterine leiomyoma (UL) vs. adjacent myometrium (MM) tissue and cells with or without SAHA treatment.
Setting: Hospital and university laboratories.
Patient(s): Women with UL without any hormone treatment.
Intervention(s): Myomectomy or hysterectomy surgery in women for leiomyoma disease.
Main Outcome Measure(s): HDAC activity was assessed by enzyme-linked immunosorbent assay, and gene expression was assessed by quantitative real-time polymerase chain reaction. Effects of SAHA on HULP cells were analyzed by CellTiter (Promega, Madison, Wisconsin), Western blot, and quantitative real-time polymerase chain reaction.
Result(s): The expression of HDAC genes (*HDAC1*, fold change [FC] = 1.65; *HDAC3*, FC = 2.08; *HDAC6*, FC = 2.42) and activity (0.56 vs. 0.10 optical density [OD]/h/mg) was significantly increased in UL vs. MM tissue. SAHA decreased HDAC activity in HULP cells but not in MM cells. Cell viability significantly decreased in HULP cells (81.68% at 5 μM SAHA, 73.46% at 10 μM SAHA), but not in MM cells. Proliferating cell nuclear antigen expression was significantly inhibited in SAHA-treated HULP cells (5 μM SAHA, FC = 0.556; 10 μM SAHA, FC = 0.622). Cell cycle markers, including *C-MYC* (5 μM SAHA, FC = 0.828) and *CCND1* (5 μM SAHA, FC = 0.583; 10 μM SAHA, FC = 0.482), were significantly down-regulated after SAHA treatment. SAHA significantly inhibited ECM protein expression, including FIBRONECTIN (5 μM SAHA, FC = 0.815; 10 μM SAHA, FC = 0.673) and COLLAGEN I (5 μM SAHA, FC = 0.599; 10 μM SAHA, FC = 0.635), in HULP cells. *TGFβ3* and *MMP9* gene expression was also significantly down-regulated by 10 μM SAHA (*TGFβ3*, FC = 0.596; *MMP9*, FC = 0.677).
Conclusion(s): SAHA treatment inhibits cell proliferation, cell cycle, ECM formation, and TGF-$\beta 3$ signaling in HULP cells, suggesting that histone deacetylation may be useful for treatment of UL. (Fertil Steril® 2022;117:433-43. ©2021 by American Society for Reproductive Medicine.)
El resumen está disponible en Español al final del artículo.

Key Words: Cell proliferation, extracellular matrix, SAHA, ULS-$\beta 3$ pathway, uterine leiomyoma

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Uterine leiomyomas (ULs) or fibroids are benign neoplasms of the smooth muscle cells of the uterine myometrium (MM) (1). They are the most common tumor in premenopausal women, affecting 20% to 50% of women over the age of 30 years (2–4). The incidence increases with age, and UL development occurs earlier in Black women than in White women (5, 6). ULs are associated with several symptoms, including pelvic pain, abnormal uterine bleeding, and infertility, leading to recurrent abortion and preterm labor (7, 8). Currently, hysterectomy and myomectomy are the chosen treatments for UL (9). Other, less invasive, treatments based on hormone therapy are available but cause side effects, such as premenopausal symptoms or hepatic damage, and myomas recover their initial size once treatment is stopped (10). Thus, there is no effective and noninvasive treatment for UL. Although the molecular mechanisms involved in UL development remain unclear, many factors are proposed to contribute to UL, including steroid hormones (11, 12), growth factors (13), the Wnt/β-catenin pathway (14), and genetic and epigenetic mutations (15). Epigenetic mutations can be reversed by chemical agents. Therefore, targeting epigenetic modifications could be a viable therapeutic strategy to treat UL.

Histone modification is a key component in the epigenetic regulation of genes. These modifications occur at the N-terminal tail or the globular domains of core histones and include acetylation, methylation, phosphorylation, ubiquitination, and SUMOylation (16). Histone acetylation consists of the transfer of an acetyl group from acetyl coenzyme A to the lysine e-amino group on the N-terminal tails of histones. Several studies have described altered acetylation of H3K27ac histone marks in ULs compared with the adjacent MM, suggesting that acetylation plays a significant role in the development of ULs (17–19). Generally, histone acetylation has been correlated with gene expression, and deacetylation leads to transcriptional repression (20). Histone acetyltransferases add acetyl groups to histones, whereas histone deacetylases (HDACs) erase these marks (20). There are four different classes of HDACs based on their sequence similarities: class I proteins (HDAC1, HDAC2, HDAC3, and HDAC8), class II proteins (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10), class III proteins or sirtuins (SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, and SIRT7), and class IV protein (HDAC11) (21). HDACs play an important role in multiple biologic processes, such as cell cycle progression, cell differentiation, and cell survival, and are involved in, for instance, the development of different tumors (22), such as breast cancer and ovarian cancer (23, 24). Notably, HDAC activity is higher in ULs than in the adjacent MM, suggesting that the transcription of genes implicated in the normal function of MM may be repressed because of a decrease in histone acetylation, resulting in an advantage for the growth and maintenance of ULs (16, 25). Suberoylanilide hydroxamic acid (SAHA), or vorinostat, is a competitive inhibitor of class I and class II HDAC (26) that is used as an anticancer drug to treat different types of tumors (27, 28). One of the well-characterized biochemical effects of SAHA is increased accumulation of acetylated core histones caused by inhibition of HDAC activity (29, 30), which blocks cell proliferation and tumor growth in hepatoid adenocarcinoma (31), myeloid leukemia (32), and prostate cancer (33).

Therefore, if HDACs are involved in UL development through epigenetic modification of histones, the use of inhibitors such as SAHA could reduce UL size to provide a novel therapeutic option. For this purpose, we evaluated the effect of SAHA in human uterine leiomyoma primary (HULP) cells on cell proliferation, cell cycle progression, apoptosis, extracellular matrix (ECM) formation, and the transforming growth factor $\beta 3$ (TGF-$\beta 3$) signaling pathway—which is widely implicated in UL development—to test its potential as a new therapeutic option to treat leiomyoma.

MATERIALS AND METHODS

Human Tissue Collection

Samples of human UL and adjacent MM were gathered from premenopausal Caucasian women aged 31 to 48 years who were undergoing myomectomy or hysterectomy as treatment for symptomatic UL pathology (n = 15) and who had not received any hormonal treatment in the previous 3 months (Supplemental Table 1, available online). A body mass index >35 kg/m^2 and current smoking were exclusion criteria. This study was approved by the Clinical Ethics Committee at the Hospital Universitario y Politécnico La Fe (Spain) (approval no. 2018/0097), and all participants provided informed consent. The tissue samples were divided into three fragments for assessment of HDAC activity, measurement of HDAC gene expression, and isolation of single cells for in vitro study. The UL and adjacent MM cells were cultured in vitro as primary cultures from the individual patients.

Human Uterine Leiomyoma Primary Cell Isolation

The UL and MM fragments were mechanically and enzymatically processed to isolated UL and MM cells from each individual patient. The tissue was treated with 2 mg/mL of type II collagenase (Labclinics, Madrid, Spain) and 1 mg/mL of DNase I (Sigma-Aldrich, St. Louis, Missouri) to obtain single-cell suspensions. The cells were incubated in vitro at 37°C and 5% CO_2 in culture medium (Dulbecco's modified Eagle medium/F-12; GIBCO, Waltham, Massachusetts) with 10% fetal bovine serum (Fisher Scientific, Waltham, Massachusetts) and antibiotic–antimycotic solution (Fisher Scientific, Waltham, Massachusetts) to study HDAC activity and the effects of SAHA treatment.

Assessment of HDAC Activity in Tissue and HULP and MM cells

HDAC activity was evaluated in UL vs. adjacent MM tissues (n = 6) as well as in HULP and MM cells from the same individual patient (n = 5) treated with and without SAHA inhibitor. Nuclear protein extracts of tissues and cells were isolated with an EpiQuik nuclear extraction kit (Epigentek, Brooklyn, New York). HDAC activity was determined with an Epigenase HDAC activity/inhibition direct assay kit (Epigentek) and a microplate reader (Synergy HT, Bio-Tek). HDAC activity is

proportional to the amount of deacetylated products colorimetrically measured at 450 nm and was calculated according to the formula provided by the manufacturer:

$$\text{HDAC activity}(\text{optical density[OD]} / \text{min} / \text{mg}) = \frac{(\text{Sample OD} - \text{Blank OD})}{(\text{Protein amount}(\mu g) \times \text{min})} \times 1,000$$

SAHA Treatment

The effects of SAHA on HULP and MM cells were assessed in vitro by culture of isolated cells from individual patients. The cells were incubated in culture medium until they achieved a confluence of 70%. The cells (n = 15) were then starved in serum-free medium overnight and were treated with different doses of SAHA (Abcam, Cambridge, UK) dissolved in dimethyl sulfoxide (DMSO). The treatment groups included 0 μM SAHA (control), 0.01% DMSO (vehicle), 5 μM SAHA, and 10 μM SAHA for 48 hours.

Cell Viability Assay

To determine cell viability after treatment with SAHA, HULP cells and MM cells from the same individual patient (n = 10) were incubated with culture medium in a 96-well plate. After the cells reached 70% confluence, they were starved in serum-free medium overnight and treated with 0 μM SAHA, 0.01% DMSO, 5 μM SAHA, or 10 μM SAHA for 48 hours. A CellTiter 96 AQueous One Solution cell proliferation assay (Promega, Madison, Wisconsin) was used to measure the quantity of proliferating viable cells, and absorbance was measured on a microplate reader (Synergy HT, Bio-Tek) at 490 nm.

Protein Extraction and Western Blotting

A radioimmunoprecipitation assay buffer containing protease inhibitors was used to extract protein from SAHA-treated and -untreated HULP cells (n = 10). Subsequently, 20 μg of each sample was analyzed by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Western blotting was performed to determine the expression of ECM proteins, such as collagen I (COL-I; 70R-CR007X, 1:1,000; Fitzgerald, Acton, Massachusetts) and fibronectin (F3648, 1:2,000, Sigma-Aldrich), proliferation proteins, such as proliferating cell nuclear antigen (PCNA; sc-56, 1:200, Santa Cruz Biotechnology, Santa Cruz, California), and apoptosis proteins, such as B-cell lymphoma-2 (BCL2; sc-7382, 1:200, Santa Cruz Biotechnology) and BCL2-associated X (BAX; sc-20067, 1:200, Santa Cruz Biotechnology). Antigen–antibody complex was detected with a chemiluminescence detection system (Thermo Fisher), and specific protein bands were visualized by chemiluminescence imaging with an LAS-3000 Imaging System (Fujifilm). Band intensity was quantified by ImageJ software and was normalized to β-actin (1:1,000; sc-47778, Santa Cruz Biotechnology).

Analysis of Gene Expression

Total RNA was extracted from UL and MM tissues with TRIzol reagent (Fisher Scientific, Waltham, Massachusetts) and from HULP cells with an RNeasy Mini kit (Qiagen, Hilden, Germany). Quantitative real-time polymerase chain reaction analysis was conducted using a StepOnePlus system (Applied Biosystems, Foster City, California) and PowerUp SYBR Green (Fisher Scientific). *HDAC1*, *HDAC3*, and *HDAC6* gene expression was evaluated in UL and MM tissues (n = 10). *CCND1*, *MMP9*, *c-MYC*, and *TGFβ3* gene expression was assessed in SAHA-treated and -untreated HULP cells (n = 10). Primers were designed with the Primer Quest tool (DNA Integrated Technologies, Coralville, Iowa) (Supplemental Table 2). Data were normalized to the expression of *GAPDH*. The $\Delta\Delta C_t$ method was used to calculate fold change.

Statistical Analysis

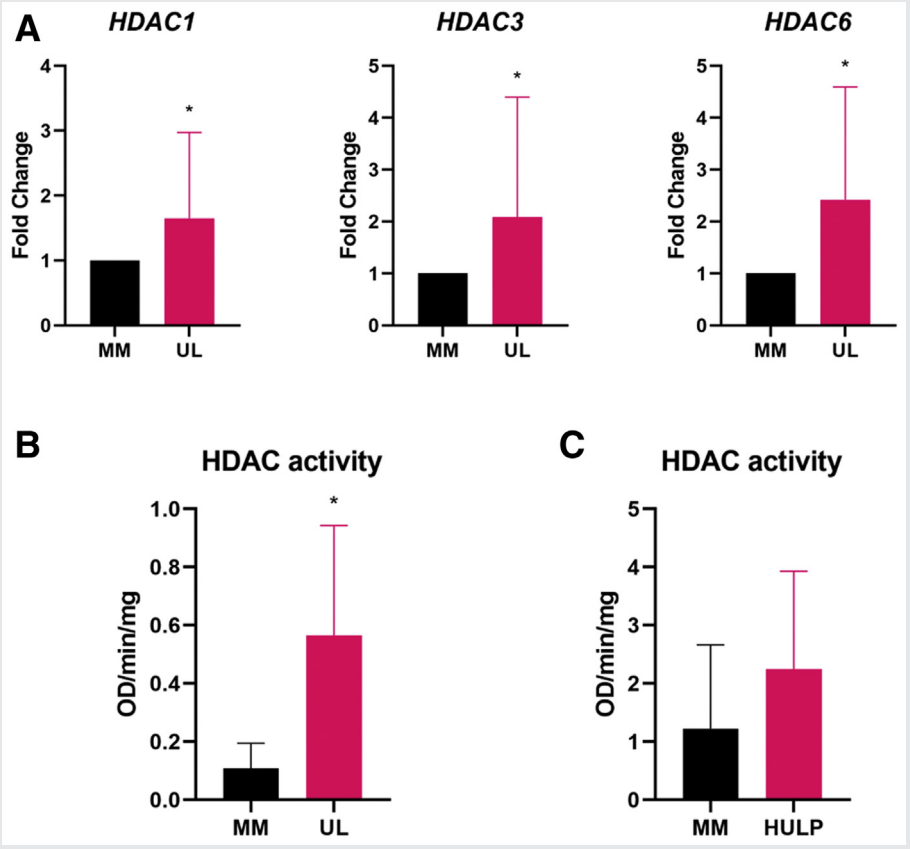
GraphPad Prism 8.0 (San Diego, California) was used for statistical analyses and generation of figures. A paired *t* test was performed to compare HDAC activity in UL vs. MM tissue and in HULP vs. MM cells. A Wilcoxon test for paired samples was performed to analyze *HDAC1*, *HDAC3*, and *HDAC6* gene expression in UL compared with adjacent MM tissues. A repeated-measures one-way analysis of variance test with a Geisser–Greenhouse correction was performed to assess cell viability and HDAC activity in HULP and MM cells after SAHA treatment, as well as *TGFβ3* and *MMP9* gene expression and PCNA, fibronectin, and collagen I protein expression. A Friedmann test was used to analyze the BAX/BCL2 ratio and *MMP9* gene expression. All SAHA treatment results were normalized and compared with control or vehicle (untreated HULP cells) groups. Data are presented as mean ± SD. A *P* value <.05 was considered to indicate statistical significance.

RESULTS

HDAC Gene Expression and HDAC Activity in UL Compared with MM Tissues

To assess whether ULs have higher expression of HDAC enzymes than adjacent MM, *HDAC1*, *HDAC3*, and *HDAC6* gene expression was compared between UL and. MM tissue samples from the same patient. UL samples displayed a statistically significant increase in *HDAC1*, *HDAC3*, and *HDAC6* gene expression with MM tissue (fold change [FC] = 1.65, *P*=.048; FC = 2.08, *P*=.042; FC = 2.42, *P*=.0413, respectively) (Fig. 1A). Global HDAC activity was measured to determine if increased gene expression of HDACs is correlated with higher activity. UL tissue exhibited significantly higher HDAC activity than MM tissue (0.56 vs. 0.10 OD/h/mg, *P*=.034) (Fig. 1B).

FIGURE 1



Histone acetylation status in UL compared with adjacent MM. (**A**) Gene expression of *HDAC1*, *HDAC3*, and *HDAC6* represented as fold change (n = 10), and (**B**) HDAC activity (OD/min/mg) in UL compared with MM tissue (n = 6). (**C**) HDAC activity (OD/min/mg) in HULP cells compared with MM cells after 7 days of cell culture (n = 5). Data are presented as the mean value ± SEM. *Significantly different between the pairs, $P<.05$. HDAC = histone deacetylase; HULP = human uterine leiomyoma primary; MM = myometrium; OD = optical density; UL = uterine leiomyoma.

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HDAC Activity of HULP and MM Cells In Vitro

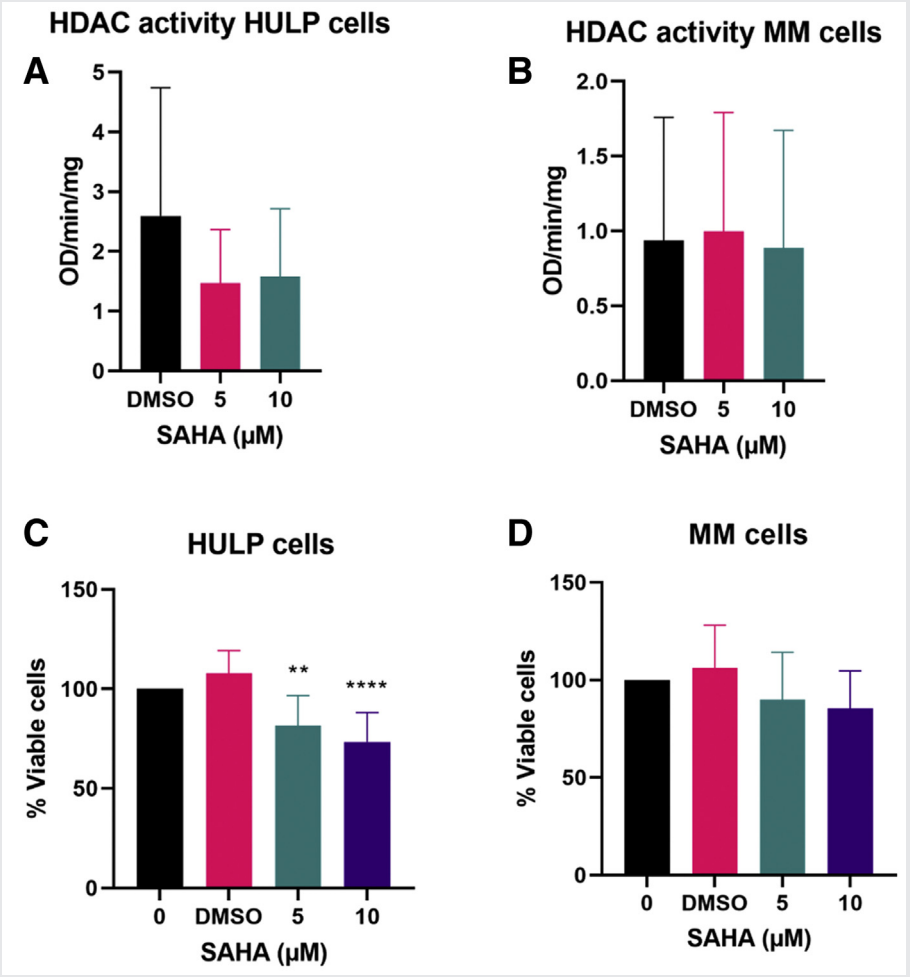
Because of the strong interaction between the environment and epigenetics, we analyzed HDAC activity in HULP compared with MM cells from the same patient after 7 days of in vitro culture to confirm that HDAC activity is not modified by the in vitro environment. The results showed that HDAC activity tended to be increased in HULP cells compared with MM cells (2.24 vs. 1.22 OD/min/mg, $P=.365$) (Fig. 1C). These results suggested that the increased HDAC activity observed in UL compared with MM tissue could not be modified in vitro by cell culture conditions, and, therefore, in vitro studies could provide a reliable framework to investigate HDACs in ULs.

In addition, we evaluated the effect of SAHA treatment on HDAC activity in vitro in HULP and MM cells from the same patient. Although HDAC activity tended to decrease in HULP cells after SAHA treatment (2.594 OD/min/mg with DMSO vs. 1.472 OD/min/mg with 5 μM SAHA [$P=.200$] and 1.575 OD/min/mg with 10 μM SAHA [$P=.422$] [Fig. 2A]), this tendency to decreased HDAC activity was not observed in MM cells treated with SAHA (0.93 OD/min/mg with DMSO vs. 0.99 OD/min/mg with 5 μM SAHA [$P=.512$] and 0.88 OD/min/mg with 10 μM SAHA [$P=.951$] [Fig. 2B]).

In Vitro Effects of SAHA on Cell Viability in HULP and MM Cells

The number of proliferating cells was determined to investigate the possible effects of SAHA on proliferation in HULP and MM cells from the same patient. Treatment with SAHA significantly decreased the percentage of viable HULP cells in a dose-dependent manner (81.68% with 5 μM SAHA [$P=.001$]; 73.46% with 10 μM SAHA [$P=.0001$] [Fig. 2C]). In contrast, the number of proliferating MM cells was not altered after SAHA treatment (89.90% with 5 μM SAHA [$P=.760$]; 85.44% with 10 μM SAHA [$P=.429$] [Fig. 2D]), suggesting that SAHA treatment has a greater effect on HULP cells than on MM cells. In addition, no effect of the treatment vehicle (DMSO) was observed in HULP and MM cells compared with untreated cells with cell culture medium only.

FIGURE 2



Effect of histone acetylation inhibition by SAHA on HDACs activity and cell viability in HULP and MM cells. HDACs activity (OD/min/mg) after SAHA treatment of HULP cells (**A**) and MM cells (**B**). Percentage of viable cells after 48 hours of treatment with 0 μM SAHA, DMSO, 5 μM SAHA, or 10 μM SAHA in HULP cells (**C**) (n = 14) and MM cells (**D**). Significantly different, ***P*<.01, ****P*<.001. DMSO = dimethyl sulfoxide; HDAC = histone deacetylase; HULP = human uterine leiomyoma primary; MM = myometrium; OD = optical density; SAHA = suberoylanilide hydroxamic acid.

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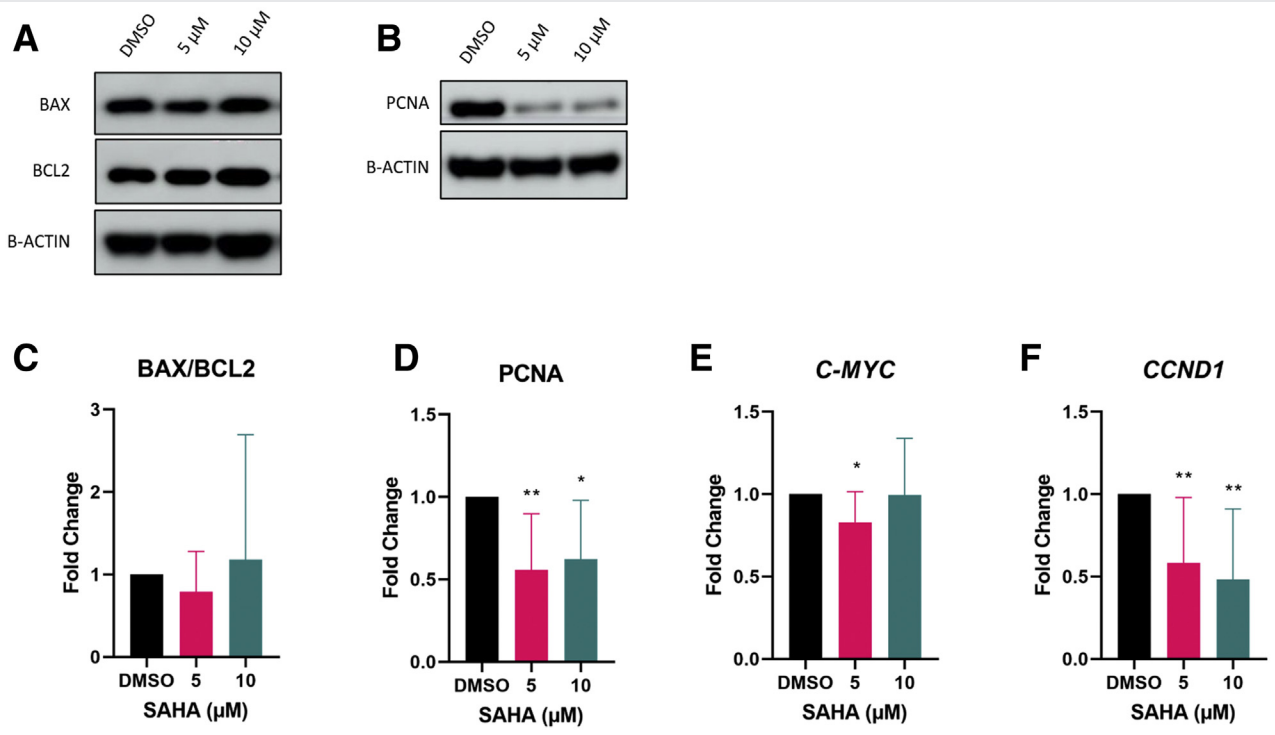
In Vitro Effects of SAHA on Apoptosis and Cell Proliferation in HULP Cells

To describe the effect of SAHA treatment on cell proliferation and apoptosis in HULP cells, we analyzed protein markers of apoptosis and proliferation by Western blot. We did not observe significant changes in BAX (proapoptotic) and BCL2 (antiapoptotic) protein expression in HULP cells treated with 5 or 10 μM SAHA compared with untreated cells (Fig. 3A), suggesting no alterations in apoptosis. Consequently, no significant differences were observed in the BAX/BCL2 ratio (5 μM SAHA, FC = 0.78 and *P*=.147; 10 μM SAHA, FC = 1.18 and *P*=.235) (Fig. 3C). We analyzed protein expression of the gold-standard proliferation marker PCNA by Western blot in SAHA-treated compared with untreated HULP cells (Fig. 3B). SAHA treatment decreased PCNA protein expression in HULP cells in a dose-dependent manner, and this decrease was statistically significant at both doses (5 μM SAHA, FC = 0.556 and *P*=.004; 10 μM SAHA, FC = 0.622 and *P*=.015) (Fig. 3D). To confirm the antiproliferative effect of SAHA treatment in HULP cells, gene expression of the cell cycle markers *C-MYC* and *CCND1* was studied by quantitative real-time polymerase chain reaction. *C-MYC* expression was significantly down-regulated in HULP cells after treatment with 5 μM SAHA (FC = 0.828 and *P*=.032) (Fig. 3E). *CCND1* expression was significantly down-regulated in HULP cells after treatment with 5 μM SAHA (FC = 0.583 and *P*=.003) and 10 μM SAHA (FC = 0.482 and *P*=.001) (Fig. 3F). Thus, SAHA inhibits gene expression of cell cycle markers in HULP cells in vitro in a dose-dependent manner.

FIGURE 3



Effect of histone acetylation inhibition by SAHA on apoptosis, cell proliferation, and cell cycle progression in HULP cells. Representative images of protein expression levels for (**A**) BAX (26 kDa), BCL2 (23 kDa), and (**B**) PCNA (36 kDa) in HULP cells (n = 10) after 48 hours of treatment with SAHA at 5 μM or 10 μM compared with cells treated with vehicle (DMSO). Quantification of normalized protein expression of the BAX/BCL2 ratio (**C**) and PCNA (**D**) represented as fold change. Gene expression levels of *C-MYC* (**E**) and *CCND1* (**F**) in HULP cells treated for 48 hours with SAHA at 5 μM or 10 μM compared with untreated HULP cells (DMSO vehicle) are represented as fold change. Significantly different, **P*<.05, ***P*<.01. DMSO = dimethyl sulfoxide; HDAC = histone deacetylase; HULP = human uterine leiomyoma primary; MM = myometrium; OD = optical density; PCNA = proliferating cell nuclear antigen; SAHA = suberoylanilide hydroxamic acid.

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In Vitro Effects of SAHA Treatment on the ECM in HULP Cells

To determine ECM status in HULP cells after SAHA treatment, we analyzed the expression of the main proteins involved in ECM formation, fibronectin and collagen I, by Western blot (Figs. 4A and B, respectively). Fibronectin expression was decreased in a dose-dependent manner, and this decrease was statistically significant at both doses (5 μM SAHA, FC = 0.815 and *P*=.010; 10 μM SAHA, FC = 0.673 and *P*=.0006) (Fig. 4C). Collagen I expression showed a statistically significant decrease in expression in a dose-dependent manner (5 μM SAHA, FC = 0.599 and *P*=.0015; 10 μM SAHA, FC = 0.635 and *P*=.008) (Fig. 4D). These results show that SAHA inhibits ECM protein expression in HULP cells in vitro in a dose-dependent manner.

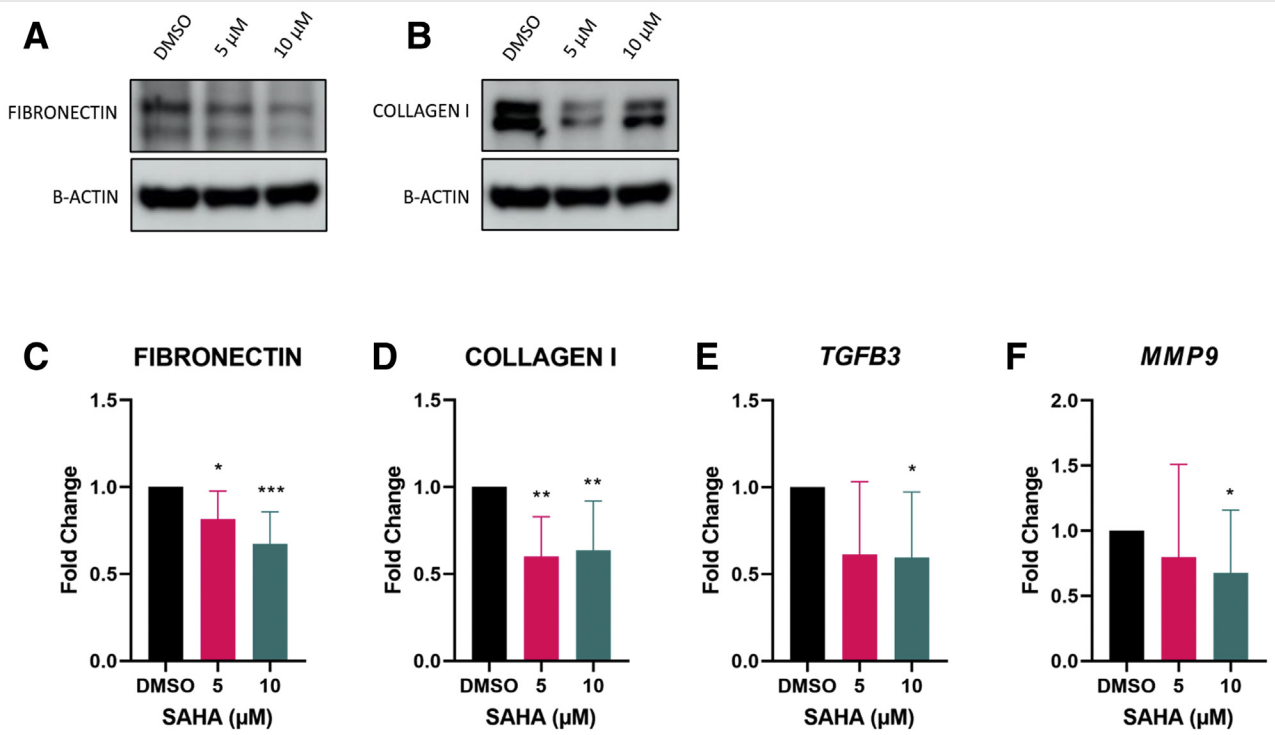
In Vitro Effects of SAHA on the TGF-β3 Signaling Pathway in HULP Cells

To evaluate the effect of SAHA treatment on the TGF-β3 signaling pathway in HULP cells, we assessed the expression of genes involved in this pathway, such as *TGFβ3* and *MMP9*, by quantitative real-time polymerase chain reaction. *TGFβ3* gene expression decreased in HULP cells after SAHA treatment (5 μM SAHA, FC = 0.611 and *P*=.060; 10 μM SAHA, FC = 0.596 and *P*=.033) (Fig. 4E). Accordingly, *MMP9* gene expression was down-regulated in treated HULP cells (5 μM SAHA, FC = 0.795 and *P*=.085; 10 μM SAHA, FC = 0.677 and *P*=.028) (Fig. 4F). These results suggest that SAHA treatment decreases the TGF-β3 signaling pathway in HULP cells.

DISCUSSION

Our study showed that SAHA inhibits cell proliferation, the cell cycle, ECM protein expression, and the TGF-β3 signaling pathway in HULP cells, which are involved in leiomyoma development, suggesting that HDACs may be a viable therapeutic target for treatment of UL. The molecular mechanisms involved in UL development remain unclear, and there is no effective and noninvasive treatment (10–14). Therefore, defining new molecular mechanisms involved in UL pathogenesis would allow us to describe new therapeutic

FIGURE 4



Effect of histone acetylation inhibition by SAHA on ECM formation in HULP cells. Representative images of protein expression levels for fibronectin (220 kDa) (**A**) and collagen I (140 kDa) (**B**) and quantitation of normalized protein expression of fibronectin (220 kDa) (**C**) and collagen I (**D**) in HULP cells treated with SAHA at 5 μM or 10 μM for 48 hours compared with control vehicle-treated HULP cells (DMSO) (n = 10). Gene expression analysis of *TGFβ3* (**E**) and *MMP9* (**F**) after SAHA treatment at 5 μM or 10 μM in HULP cells represented as fold change. Data are represented as mean and standard deviation of fold change. Significantly different, *$P<.05$, **$P<.01$, ***$P<.001$. DMSO = dimethyl sulfoxide; ECM = extracellular matrix; HULP = human uterine leiomyoma primary; SAHA = suberoylanilide hydroxamic acid.

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targets for UL. Alterations in the expression of genes that regulate epigenetic processes are frequently found as cancer drivers and may cause widespread alterations of histone posttranslational modifications, which are involved in abnormal patterns of gene expression (15, 16). Because of the inherent reversibility of epigenetic changes, inhibitors targeting these processes are promising anticancer strategies (34). By removing acetyl groups, HDACs reverse chromatin acetylation and alter the transcription of oncogenes and tumor suppressor genes (35, 36). HDAC inhibitors, such as SAHA (vorinostat, MK0683), have been emerging as promising therapies that control cancer growth and metastasis (37). On the basis of these findings, we focused on inhibition of HDACs by SAHA as a new therapeutic strategy for UL through treatment of HULP cells with those doses of SAHA tested in cells isolated from hepatocellular carcinoma (38, 39), lung cancer (28), and endometriosis (27).

For this purpose, we first studied the expression and activity of HDACs on ULs and adjacent MM tissue. It has been reported that SAHA inhibits class I and class II HDACs (40, 41). We studied HDAC1 and HDAC3 as class I HDACs and HDAC6 as a class II HDAC, because they have been proved to be important in the pathogenesis of UL (42). We confirmed that there is higher *HDAC1*, *HDAC3*, and *HDAC6* gene expression as well as higher HDAC activity in UL tissue than in adjacent MM tissue. Increased expression and activity of HDACs were reported in other studies in both breast cancer and UL (16, 25, 42), suggesting that transcription of genes involved in normal MM function may be repressed, promoting tumor growth and maintenance. On the basis of these findings, the use of HDAC inhibitors to reverse histone deacetylation could be a good therapeutic approach to treat UL. Subsequently, because of the interaction between the environment and epigenetics, we studied HDAC activity in cell culture to confirm the maintenance of this epigenetic feature in an in vitro model. HDAC activity tended to be increased in HULP cells compared with MM cells, suggesting that the in vitro environment does not modify HDAC status observed in the tissue. These results allow the study of epigenetic modifications such as inhibition of HDACs by SAHA under in vitro conditions.

To assess the effectiveness of SAHA treatment on HULP and MM cells, we studied HDAC activity after SAHA treatment in vitro. We found that HDAC activity tended to be decreased in HULP cells after SAHA treatment but not in MM cells. Accordingly, analysis of the number of

proliferating cells showed that treatment with SAHA significantly decreased the percentage of viable HULP cells but not of MM cells. These findings suggest, for the first time to our knowledge, that SAHA treatment is more effective in HULP cells than in MM cells, proving its potential as a UL treatment in which the adjacent MM will likely not be affected.

It is widely known that increased proliferation of UL cells may be involved in UL development and growth (14, 43). HDACs are an important epigenetic feature in UL development, and cell proliferation is one of the key biologic processes involved in UL development. Therefore, the reversal of histone hypoacetylation by SAHA could reduce UL size by decreasing cell proliferation. We found that treatment of HULP cells with SAHA decreased cell proliferation, as demonstrated by decreased PCNA protein expression. These findings suggest an antiproliferative effect of SAHA treatment on UL, as was previously described in other tumors (31–33). Additionally, the cell cycle regulatory genes *CCND1* and *C-MYC* were down-regulated after SAHA treatment. *CCND1* is overexpressed in UL compared with MM (44, 45), as well as in other tumor types, thus making it a negative prognostic marker of UL (46, 47). It has been reported that other HDAC inhibitors decrease UL cell growth through cell cycle arrest (44). In addition, induction of cell cycle arrest by SAHA is reported in cancer (37). Apoptosis did not increase after SAHA treatment, demonstrating the absence of SAHA toxicity in HULP cells. These results collectively indicate that HDACs are critical in the regulation of cell proliferation, and SAHA could reduce the growth of ULs by decreased cell proliferation and cell cycle arrest.

Uterine leiomyomas are characterized by excessive deposition of ECM, specifically collagen, fibronectin, and sulfated proteoglycans, causing expansion of the UL (48). ECM should be considered as a crucial target for UL therapeutics (49). Our results showed that SAHA treatment significantly decreased the expression of ECM-associated proteins, such as fibronectin and collagen I, suggesting an important role of histone acetylation in the regulation of ECM formation. Other studies have demonstrated the success of treatments targeting ECM formation to reduce UL growth, such as treatment with vitamin D (50–52). In addition, the role of histone acetylation in ECM regulation has been shown in renal tubulointerstitial fibrosis (53), in which HDAC inhibitors had an antifibrotic effect.

The TGF-β3 signaling pathway is overexpressed in UL compared with the adjacent MM and is directly responsible for the development of the UL fibrotic phenotype by increasing ECM deposition and cell proliferation (54–56). Elevated levels of matrix metalloproteinase 9 (MMP-9) and total MMPs, which are targets of the TGF-β3 signaling pathway, are evident in leiomyoma (57). In previous studies of cancer, acetylation has been linked to TGF-β3 signaling (58), and TGF-β3-induced genes that regulate matrix turnover were regulated by HDACs (59). Considering that TGF-β3 has a principal role in ECM overproduction and is related to HDACs, SAHA treatment would decrease the expression of these proteins, reducing ECM deposition. Our study showed that SAHA treatment down-regulated *TGFβ3* and *MMP9* gene expression. The positive antifibrotic effect of TGF-β3 inhibition in UL treatment has been shown with the use of ulipristal acetate (55) and vitamin D (56). In addition, vitamin D effectively reduces the expression and activities of MMP-9 in cultured human uterine fibroid cells (52), as does the HDAC inhibitor trichostatin A in trophoblast cells (60). On the basis of these findings, we suggest that the TGF-β3 signaling pathway is regulated by epigenetic deacetylation and that inhibition of HDACs by SAHA treatment alters the functionality of this pathway, leading to a reduction in ECM deposition.

CONCLUSION

We suggest that the reduction of ECM deposition and the decrease in cell cycle progression and cell proliferation generated by the reversal of histone deacetylation with SAHA treatment induces inhibition of UL growth. These findings highlight that inhibition pf HDACs may be a viable therapeutic target to treat UL. Further studies are necessary to assess the potential effects of HDAC inhibition on the TGF-β3 signaling pathway as well as the in vivo effect of SAHA treatment on UL.

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Inhibición de las histonas deacetilasas mediante el ácido hidroxámico suberoilanilida: una aproximación terapéutica para tratar los miomas uterinos humanos.

Objetivo: Evaluar el efecto de la inhibición de las histonas deacetilasas (HDACs) mediante el tratamiento con ácido hidroxámico suberoilanilida (SAHA) en células primarias de mioma humano (HULP) *in vitro* sobre la proliferación celular, ciclo celular, formación de matriz extraceluar (ECM), y señalización del factor de crecimiento transformante $\beta 3$ (TGF-$\beta 3$).

Entorno: Estudio prospectivo comparando tejido de miomas uterinos (UL) versus miometrio adyacente (MM) y células con o sin tratamiento con SAHA.

Paciente(s): Hospital y laboratorios universitarios.

Intervención(es): Mujeres con UL sin ningún tratamiento hormonal.

Principales medidas de resultado(s): La actividad de HDAC se evaluó mediante un ensayo inmunoabsorbente ligado a enzimas, y la expresión génica se evaluó mediante la reacción cuantitativa en cadena de la polimerasa a tiempo real. Los efectos de SAHA sobre las células HULP se analizaron mediante CellTiter (Promega, Madison, Wisconsin), Western Blot y reacción cuantitativa en cadena de la polimerasa a tiempo real.

Resultado(s): La expresión de los genes HDAC (*HDAC1,* tasa de cambio [FC] = 1,65; *HDAC3,* FC=2,08; *HDAC6,* FC=2,42) y actividad (0,56 vs. 0,10 densidad óptica [OD]/h/mg) fueron significativamente aumentadas en tejido de UL vs. MM. SAHA disminuyó la actividad de HDAC en las células HULP, pero no en las células de MM. La viabilidad celular disminuyó significativamente en las células HULP (81,68% a 5 μM de SAHA; 73,46% a 10 μM de SAHA), pero no en las células de MM. La expresión del antígeno nuclear de proliferación celular fue significativamente inhibida en las células HULP tratadas con SAHA (5 μM de SAHA, FC= 0,556; 10 μM de SAHA, FC= 0,622). Los marcadores de ciclo celular, incluyendo *C-MYC (*5 μM de SAHA, FC= 0,828) y *CCND1* (5 μM de SAHA, FC=0,583; 10 μM de SAHA, FC= 0,482, fueron significativamente regulados a la baja después del tratamiento con SAHA. SAHA significativamente inhibió la expresión de proteínas de ECM, incluyendo FIBRONECTINA (5 μM de SAHA, FC= 0,815; 10 μM de SAHA, FC= 0,673) y COLAGENO I (5 μM de SAHA, FC= 0,599; 10 μM de SAHA, FC= 0,635) en las células HULP. La expresión génica de *TGF-$\beta 3$* y *MMP9* fue también significativamente regulada a la baja tras el tratamiento con SAHA a 10 μM (*TGF-$\beta 3$,* FC= 0,596; *MMP9*, FC= 0,677).

Conclusión(s): El tratamiento con SAHA inhibe la proliferación celular, el ciclo celular, la formación de ECM y la señalización TGF-$\beta 3$ en las células HULP, sugiriendo que a deacetilación de histonas pueden ser útiles para tratar los miomas uterinos.