HDAC Inhibition for the Treatment of Epithelioid Sarcoma: Novel Cross Talk Between Epigenetic Components

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Abstract

Epithelioid sarcoma is a rare neoplasm uniquely comprised of cells exhibiting both mesenchymal and epithelial features. Having propensity for local and distant recurrence, it poses a diagnostic dilemma secondary to pathologic complexity. Patients have dismal prognosis due to lack of effective therapy. HDAC inhibitors (HDACi) exhibit marked antitumor effects in various malignancies. The studies here demonstrate that pan-HDAC inhibitors constitute novel therapeutic strategies versus epithelioid sarcoma. Human ES cells (VAESBJ, HS-ES, Epi-544) were studied in preclinical models to evaluate HDACi effects. Immunoblot and RT-PCR were used to evaluate expression of acetylated tubulin, histones H3/H4, EZH2 upon HDACi. MTS and clonogenic assays were used to assess the impact of HDACi on cell growth. Cell culture assays were used to evaluate the impact of HDACi and EZH2-specific siRNA inhibition on cell-cycle progression and survival. Unbiased gene array analysis was used to identify the impact of HDACi on epithelioid sarcoma gene expression. Xenografts were used to evaluate epithelioid sarcoma tumor growth in response to HDACi. HDACi inhibition increased target protein acetylation and abrogated cell growth and colony formation in epithelioid sarcoma cells. HDACi induced G2 cell-cycle arrest and marked apoptosis, and reduced tumor growth in xenograft models. HDACi induced widespread gene expression changes, and EZH2 was significantly downregulated. EZH2 knockdown resulted in abrogated cell growth in vitro.

Implications: The current study suggests a clinical role for HDACi in human epithelioid sarcoma, which, when combined with EZH2 inhibitors, could serve as a novel therapeutic strategy for epithelioid sarcoma patients. Future investigations targeting specific HDAC isoforms along with EZH2 may potentially maximize treatment efficacy. Mol Cancer Res; 14(1): 1–9. ©2015 AACR.

Introduction

Epithelioid sarcoma is a rare soft tissue malignancy of unknown origin, exhibiting unique histologic characteristics reminiscent of both mesenchymal and epithelial differentiation (1, 2). This rare subtype of soft tissue sarcoma (STS) comprises <1% of cases and exhibits marked propensity for lymphatic spread (3). Typically affecting young adults, it is classified into two distinct histopathologic subtypes: conventional classic distal type, comprised of epithelioid tumor cells in nodular morphology surrounding an area of central necrosis, and the more recently identified aggressive proximal or axial type, typically exhibiting an epithelioid or rhabdoid morphology (1, 4, 5). To date, complete surgical resection of localized tumors remains the primary therapy; locally advanced and metastatic epithelioid sarcoma are usually unresectable, chemoresistant, and fatal (6). Emerging evidence suggests that epigenetic alterations in the integrity of the nucleosomal structure can lead to various oncogenic events, including tumor progression. Studies have shown correlation between several molecular targets and pathways linked to the aggressiveness and metastatic potential of epithelioid sarcoma [notably EGFR/mTOR deregulation; (7), and the loss of INI-1 observed in approximately 95% of epithelioid sarcoma (2)]. The SMARC1/INI1 gene encodes for a subunit of SWItch/sucrose non-fermentable (SWI/SNF) chromatin remodeling complex; it is reported to function as a tumor suppressor gene in infantile malignant rhabdoid tumors (8, 9).

Histone deacetylases (HDAC) are a family of enzymes that deacetylate lysines on core histone and nonhistone proteins (10). HDACs play a significant epigenetic role by inducing changes in chromatin structure or functioning as a “cog” in a protein complex.
Eleven mammalian HDAC isoforms have been identified and categorized into four classes based on sequence identity and domain organization (10). Many broad-spectrum compounds have been developed that target numerous HDAC isoforms, and are currently being evaluated for treatment of various malignancies (11). HDAC inhibitors (HDACi) display an array of anticaner effects including tumor cell growth inhibition, cell death, antiangiogenesis in vitro and in vivo (12). Previously, our group demonstrated remarkable anti-SIS effects of HDAC inhibition alone and in combination with chemotherapy (13); effects of HDAC inhibition alone in NF1-associated malignant peripheral nerve sheath tumors (MPNST) as well as combined HDACi and autophagy inhibition in vitro and in vivo (14).

In this study, we explored HDAC-inhibitory drugs in epithelioid sarcoma, demonstrating that their mechanisms of action lead to abrogated cell growth, proliferation, and increased apoptosis in both in vitro and in vivo preclinical contexts.

**Materials and Methods**

**Cell lines and reagents**

Human epithelioid sarcoma cell lines used for this study: VAESBI, HS-Es, Epi-544. VAESBI was obtained from the ATCC; HS-Es was obtained from Hiroshi Sonobe (Kochi Medical School, Nankoku, Japan); Epi-544 was established in our laboratory (2); all three are p53 wild-type as sequenced in our laboratory. All epithelioid sarcoma cell lines were subjected to short tandem repeat (STR) analysis. STR DNA fingerprinting was done by the Cancer Center support grant-funded characterized cell line core (University of Texas MD Anderson Cancer Center, Houston, TX, NCI #CA0116672. Cell lines received from collaborators and ATCC were tested upon arrival in our laboratory. Stock of STR confirmed cell lines were frozen at large quantities to have an original stock of cells. Cell lines were passed no more than 4 to 50 passages, after which researchers utilized cells from original stocks. ES cells were cultured in DMEM 1× supplemented with 10% FBS (Life Technologies). Pan-HDAC inhibitor abexinostat was obtained from Pharmacysts; suberylanilide hydroxamic acid (SAHA), and MS-275 were acquired from Dr. David McConkey, MD Anderson Cancer Center (initially obtained from Syndax Pharmaceuticals, Inc.) and dissolved in DMSO to create stock solutions. All additional dilutions were completed using the respective cell culture medium for each cell line. Commercial antibodies were used for Western blot analysis or immunohistochemical detection: acetylated H3, acetylated H4 (Millipore); acetylated tubulin (Sigma); EZH2, caspase-3 (Cell Signaling Technology); β-actin (Santa Cruz Biotechnology); PARP, Survivin, HDAC1, HDAC2, HDAC3 (Abcam); Ki67 (MIB-1). Secondary antibodies included horseradish peroxidase–conjugated [Western blot analysis: anti-rabbit, anti-mouse (Santa Cruz Biotechnology); Immunohistochemistry: Universal kit HRP, Biocare Medical]. Hoechst 33342 (Polysciences) was used for immunofluorescence experiments.

**Growth-related studies: MTS, clonogenicity, cell cycle, apoptosis**

MTS, clonogenicity, cell cycle, and Annexin V assays were performed as previously described (13). Apoptosis was measured using the Apoptosis Detection kit I (BD Biosciences) as per manufacturer's recommendations. Further information is available as Supplementary Data.

**Western blot analyses**

Western blot analyses were performed by standard methods (13).

**Quantitative reverse transcription PCR**

These assays were conducted as previously described (13). EZH2 and actin PCR primers for quantitative reverse transcription PCR (qRTP-PCR) were purchased from Sigma.

**Gene arrays**

Gene expression profiling was conducted using an Illumina gene array. Target preparation of the samples were conducted using Illumina TotalPrep RNA kit (Ambion, Cat.# AM11791). Two hundred nanograms total RNA was used in each amplification reaction. The TotalPrep RNA Amplification Kit is based on the RNA amplification protocol developed in the laboratory of James Eberwine (15). The procedure consists of reverse transcription with an oligo(dT) primer bearing a T7 promoter using Ambion's proprietary ArrayScript, a reverse transcriptase (RT) engineered to produce higher yields of first strand cDNA than wild-type enzymes. ArrayScript catalyzes the synthesis of virtually full-length cDNA, which is the best way to ensure production of reproducible microarray samples. The cDNA then undergoes second strand synthesis and clean-up to become a template for in vitro transcription with T7 RNA polymerase. To maximize cRNA yield, Ambion proprietary MEGAscript in vitro transcription (IVT) technology, along with biotin UTP (provided in the kit), is used to generate hundreds to thousands of biotinylated, antisense RNA copies of each mRNA in a sample. Hybridization was carried out following Illumina's direct hyb gene expression protocol. We hybridized 750 ng of amplified biotinylated cRNA onto Illumina's HumanHT12v4 gene expression arrays. The arrays were washed according to manufacturer's specifications and imaged using Illumina iScan Scanner. Raw feature intensity files (.iadt) were generated using Illumina’s GenomeStudio software. Quantile normalized array values were log-transformed. Array data were deposited in the Gene Expression Omnibus (accession GSE66800). SigTerms (16) software was used to search The Molecular Signatures Database (MsigDB, version 3.0) for significantly enriched pathway associations.

**siRNA knockdown**

siRNAs (20 nmol/L pools targeting EZH2, HDAC1, HDAC2, HDAC3, and control nontargeting constructs; Thermo Scientific) were introduced into cells using X-treme Gene as per manufacturer’s instructions (Roche). Briefly, 2 × 10^5 cells were plated in each well of a six-well plate and incubated overnight. A mixture of siRNA (20 nmol/L) and X-treme Gene (6 µL) diluted in 100 µL Dulbecco modified Eagle medium (DMEM) was added for 24 hours, followed by incubation in regular medium. Cells were harvested at indicated time points for specific experiments.

**In vivo therapeutic experiments**

All animal procedures and care were approved by the MD Anderson Cancer Center Institutional Animal Care and Usage Committee. Animals received humane care as per the Animal...
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Welfare Act and the NIH "Guide for the Care and Use of Laboratory Animals." For experiments evaluating effect of abexinostat monotherapy on local tumor growth, Trypan blue staining confirmed viable VAESBJ cells. Cell suspensions (2 × 10⁶) were injected subcutaneously into the flank of 6- to 7-week-old female hairless SCID mice and growth was measured twice weekly. After establishment of palpable lesions (average diameter ~4–5 mm), mice were randomly assigned to receive either vehicle control or abexinostat (25 mg/kg twice daily). Abexinostat was solubilized in 50 mmol/L sodium lactate, pH 4.2, and administered intraperitoneally twice daily, 5 days/week. Treatment continued until mice in control group mandated euthanasia. Tumors were resected, weighed, and fixed in formalin and paraffin-embedded for immunohistochemical studies. Immunohistochemistry on paraffinized, xenograft-derived specimen was conducted as previously described (7).

Statistical analyses
Cell culture–based assays were repeated at least three times; mean and SD was calculated and shown in the figures. Cell lines were examined separately. For outcomes that were measured at a single time point, two-sample t tests were used to assess the differences for independent data. Differences in xenograft growth in vivo were analyzed with linear mixed effects models to take account of the correlations among observations from the same animal as the tumor volume was measured over time. Tumor weight between groups was compared with ANOVA P value < 0.05 was considered as significant.

Results

HDAC inhibitors abrogate epithelioid sarcoma cell growth in vitro

Pan-HDACi abexinostat was utilized in all experiments; pan-HDACIs SAHA and MS-275 were used as comparative compounds to show that abexinostat effects were not unique to a single HDACi agent. Previously, we demonstrated antitumor efficacy of abexinostat in numerous STS histologies, and dose and administration regimen for this study were based on previously published data (13, 14). As anticipated, all three compounds induced acetylation of target proteins in all epithelioid sarcoma cell lines tested (Fig. 1A). The effects of HDACi inhibition on epithelioid sarcoma cell growth and colony formation were evaluated. Abexinostat (96 hrs) induced a time- and dose-dependent decrease in VAESBJ, HS-ES, and Epi-544 cell growth (Fig. 1B). All epithelioid sarcoma cell lines tested also exhibited growth inhibition in response to SAHA and MS-275 treatment. Similarly, HDACi inhibition decreased epithelioid sarcoma cell colony formation compared with DMSO-treated controls (Fig. 1C and Supplementary Fig. S1).

HDACi induces G₂ cell-cycle arrest and apoptosis in epithelioid sarcoma cells

We evaluated whether HDACi-induced epithelioid sarcoma growth inhibition could be due to effects on epithelioid sarcoma cell-cycle progression and/or induction of apoptosis. Epithelioid sarcoma cell lines were treated with increasing doses (0.5, 1 µmol/L) of abexinostat for 48 hours; samples were then analyzed using propidium iodide staining/FACS analysis. Significant dose-dependent increases (P < 0.05) in G₂ cell-cycle arrest was observed in abexinostat-treated cells compared with DMSO-treated controls (Fig. 2A). Cell-cycle analysis also revealed an increase in the sub-G₁ fraction in abexinostat-treated cells, suggesting HDACi-induced apoptosis. To identify potential HDACi-induced apoptosis, the effects of abexinostat on phosphatidylserine exposure was tested by Annexin-V staining and FACS analysis. A significant increase in HDACi-induced apoptosis was observed in all three cell lines (P < 0.05; Fig. 2B). Abexinostat-induced increases in apoptosis markers, cleaved PARP, and cleaved caspase-3 were observed in Western blot analysis (Fig. 2C). Together, these data demonstrate the in vitro monotherapeutic efficacy of pan-HDACi in epithelioid sarcoma cells.

HDACi abrogates epithelioid sarcoma xenograft growth

We next assessed the in vivo effect of HDACi on epithelioid sarcoma using human VAESBJ xenograft tumors. Mice were injected with viable VAESBJ cells subcutaneously (2 × 10⁶); therapy was administered once tumors grew to a mean diameter of 0.5 cm. No apparent side effects were noticed in mice receiving vehicle or abexinostat during the course of therapy. Results (Fig. 3A) demonstrate abrogation of tumor growth in mice treated with low dose abexinostat (25 mg/kg twice daily) with significantly reduced tumor volume over time compared with vehicle control (P < 0.0001). Moreover, abexinostat induced a significant decrease in tumor weight compared with vehicle-treated mice as well (P < 0.0001). Immunohistochemical analysis of the tumor samples exhibit a significant decrease in proliferation (Ki67, P < 0.05) and increase in apoptosis (cleaved caspase-3, P < 0.05; Fig. 3B). Together, in vitro and in vivo data suggests that HDACi inhibition is potentially effective in epithelioid sarcoma.

HDACi induces gene expression signature in epithelioid sarcoma

To identify possible HDACi-induced gene changes in epithelioid sarcoma cells, epithelioid sarcoma cells were treated with abexinostat (0.5 µmol/L, 24 hours) and gene expression profiles of each cell line (DMSO- and abexinostat-treated samples) were conducted using an Illumina gene array. Array analysis identified an increase in 966 genes in abexinostat-treated samples versus DMSO control and a decrease in 696 genes in abexinostat-treated samples versus DMSO control (Fig. 4A, P < 0.01, t test, fold changes > 1.5). Among the differentially expressed genes, BIRC5/survivin protein expression was assessed to confirm the array (Fig. 4A); this protein being of particular interest due to its well-established role in apoptosis (17).

To carry out pathway analysis to identify global association, our gene array results were run against The Molecular Signatures Database (MSigDB), from which a significant number of genes were found to be commonly up- and downregulated in our gene sets and in the NUYTTEN_EZH2_TARGETS gene sets (ref. 18; Supplementary Table S1). In following up these bioinformatics results, abexinostat reduced EZH2 expression in a time- and dose-dependent manner in epithelioid sarcoma cells (Fig. 4B and Supplementary Fig. S2).

To identify if EZH2 plays a role in epithelioid sarcoma cell growth, mock (transfection reagent alone), nontarget control siRNA (NT), and EZH2 siRNA transfection was used. Confirmation of EZH2 by siRNA knockdown was demonstrated by Western blot analysis which show decrease in EZH2 protein expression.

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EZH2 knockdown induced epithelioid sarcoma cell growth inhibition: counted cell numbers were significantly lower in siRNA treated cells versus mock or nontarget siRNA controls; knockdown of EZH2 in VAESBJ also induced an increase in apoptosis (Fig. 4C). These data demonstrate a potential role for EZH2 in epithelioid sarcoma, and suggests that HDACi-induced EZH2 regulation may possibly be relevant in HDACi-induced anti-epithelioid sarcoma effects.
Figure 2. HDACi induces G2 cell-cycle arrest and apoptosis in epithelioid sarcoma cells in vitro. A, propidium iodide–FACS analysis abexinostat (0.5, 1 μmol/L/48 hours) induced a significant increase in the G2–M phase (48 hours) in tested epithelioid sarcoma cells (P < 0.05). VAESBJ exhibited the strongest G2–M phase shift after abexinostat treatment compared with HS-ES and Epi-544. B, Annexin V/PI-FACS analysis demonstrated a significant increase in abexinostat-induced (1 μmol/L/48 hours) apoptosis was observed in all three epithelioid sarcoma cell lines. C, Western blot analysis confirmed Annexin V data where abexinostat (1 μmol/L/48 hours) induced an increase in apoptosis markers cleaved PARP and cleaved caspase-3.
Conclusions

Our current study highlights a unique epithelioid sarcoma–associated epigenetic crosstalk between HDACs and EZH2 that is of potential translational and clinical importance. HDAC inhibitors have gained greater focus in anticancer therapy because their deacetylation inhibition of both histones and nonhistone cellular proteins facilitates important transcriptional changes in target genes, especially those involved in differentiation, cellular proliferation, and apoptosis (12). Various HDACis cause numerous effects in different malignancies, including induction of differentiation, cell death, cell-cycle arrest, altered cell migration, and apoptosis (11–14). Our findings suggest an important oncogenic suppression: upon treatment with pan-HDAC inhibitors, abrogation of colony formation and reduced cell growth was apparent in three epithelioid sarcoma cell lines tested (VAESBJ, HS-ES, Epis44). We evaluated the effects of pan-HDACis on epithelioid sarcoma growth in relation to cell-cycle progression and induction of apoptosis, demonstrating significant dose-dependent increases in G2–M cell-cycle arrest and increased apoptosis. Similar anti-epithelioid sarcoma effects in response to HDAC inhibition were also seen in vivo.

EZH2 is a histone methyltransferase that catalyzes methylation of histone H3 lysine 27; along with SUZ12, EED, and RbAp46/48 it forms the Polycomb repressive complex 2 (PRC2; ref. 19). EZH2 is overexpressed in many tumors, including high-grade and advanced-stage breast, prostate, and lung cancers (20). It mainly serves to maintain the transcriptional repressive state of genes over successive cell generations (19). In our study, we saw a significant reduction in cell growth and proliferation when we targeted EZH2 via siRNA. EZH2 knockdown also showed an increase in apoptosis in VAESBJ. Previous studies have shown Polycomb-group proteins (PcG), particularly PRC2, mediating repression of gene activity by their involvement with HDAC proteins (21, 22). Moreover, we found that some HDAC isoforms have a more profound role than others regarding the regulation of EZH2 (Supplementary Figure 3).
Fig. S3); specifically, we observed that HDAC1/2 have greater effects on the protein level expression of EZH2. When we knocked down HDAC1, we saw an induced reduction in EZH2, as well as similar results in HDAC2 knockdown experiments. When we induced knockdown of HDAC3; however, we did not see changes in levels of EZH2 protein. Although a more inclusive study examining molecular interactions between HDACs and EZH2 is needed, it is likely an important function of the HDAC activity. Whether the mechanism by which HDACs deplete EZH2 is at the transcriptional or post-transcriptional level, or perhaps via increased protein degradation remains to be elucidated.
It will be clinically relevant to explore rational targeted therapeutic combinations to achieve more potent antitumor effects while overcoming initial epithelioid sarcoma therapeutic resistance (7). Identifying additional epithelioid sarcoma–associated targetable molecular deregulations in combination with HDAC inhibitors would be of great therapeutic utility. Tumors frequently exhibit alterations in the expression and composition of proteins that regulate cellular proliferation and death. Yamaguchi and colleagues demonstrated significant synergistic inhibition of gallbladder carcinoma proliferation via HDAC inhibition and repression of EZH2 (23). Fiskus and colleagues observed similar downregulation of EZH2 upon HDAC inhibition in human acute leukemia cells (24).

Taken together, EZH2 and its cooperation with HDACs may be related to the tumorigenesis of epithelioid sarcoma, as has been reported in other types of cancers (25–27). Although the limitation of having only a small cohort of epithelioid sarcoma cell lines available for testing suggests caution, these findings support further investigation of targeted therapeutics versus HDAC isoforms with combined EZH2 inhibitors, a novel strategy which could ultimately contribute to meaningful clinical options for patients burdened by epithelioid sarcoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Authors’ Contributions

Conception and design: G. Lopez
Development of methodology: G. Lopez, Y. Song
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Song, R. Lam, D. Ruder
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Song, R. Lam, C.J. Creighton, H.K. Bid, X. Zhang, R.E. Pollock
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Lopez, K.L. Bill, S. Bolshakov, R.E. Pollock
Other (made contributions in acquisition of scientific data, and analysis and interpretation of data): S. Bolshakov

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