Histone deacetylase inhibitors induce growth arrest, apoptosis, and differentiation in clear cell sarcoma models

Shuzhen Liu, Hongwei Cheng, Wanda Kwan, Joanna M. Lubieniecka, and Torsten O. Nielsen

Genetic Pathology Evaluation Centre, University of British Columbia, Vancouver, British Columbia, Canada

Abstract
Clear cell sarcoma is an aggressive malignancy occurring most commonly in the distal extremities of young adults, characterized by t(12;22)(q13;q12) creating the chimeric fusion oncogene EWS-ATF1. We assessed growth inhibition and differentiation effects of histone deacetylase inhibitors MS-275 and romidepsin (depsipeptide, FK228) on clear cell sarcoma cells and evaluated drug sensitivity among related translocation-associated sarcomas and other cell models. Three clear cell sarcoma cell lines, seven other sarcomas, six nonsarcoma malignant cell lines, and two nonneoplastic mesenchymal cell models were treated with MS-275 or romidepsin. Growth inhibition was assayed by monolayer 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Induction of cell cycle arrest and apoptosis were assessed by propidium iodide/Annexin V flow cytometry in monolayer and spheroid cultures and by immunoblotting analysis. Expression levels of key genes involved in mesenchymal differentiation and of EWS-ATF1 were measured by quantitative real-time PCR in clear cell sarcoma cells treated with histone deacetylase inhibitors. MS-275 and romidepsin inhibited growth in clear cell sarcoma cells by inducing cell cycle arrest and apoptosis in a time- and dose-dependent manner. Sarcomas showed greater sensitivity than other tumor types, with clear cell sarcomas most sensitive of all, whereas nonmalignant mesenchymal cells were highly resistant. MS-275 at 1 μmol/L and romidepsin at 1 nmol/L induced histone H3 acetylation, cell cycle arrest, apoptosis, and differentiation in clear cell sarcoma cells within 24 hours. Histone deacetylase inhibitors increased expression of SOX9, MYOD1, and PPARγ and decreased EWS-ATF1 expression in clear cell sarcoma cells. Histone deacetylase inhibitors show promising preclinical activity in multiple clear cell sarcoma models. [Mol Cancer Ther 2008;7(6):1751–61]

Introduction
Clear cell sarcoma typically presents in the distal extremities of young adult patients and is characterized by t(12;22)(q13;q12) (ref. 1). This translocation fuses the 5’ portion of the Ewing sarcoma (EWSR1) oncogene on chromosome 22q with the 3’ portion of the activating transcription factor 1 (ATF1) oncogene on chromosome 12q, resulting in the EWS-ATF1 chimeric fusion oncprotein. Treatment for clear cell sarcoma is wide excision of the tumor as soon as the diagnosis is established, with neoadjuvant or adjuvant radiation therapy to improve the rate of local control. Nevertheless, more than half of patients develop metastases and 5-year disease-specific survival is <50%. No targeted chemotherapies are available and conventional agents offer little benefit (2).

Clear cell sarcoma, formerly termed “malignant melanoma of soft parts,” is now recognized to fall into a category also including Ewing sarcoma, synovial sarcoma, desmoplastic round cell tumor, myxoid liposarcoma, and several other malignancies, which can be grouped together as the “fusion oncogene sarcomas of young adults.” The defining translocations in many of these sarcomas have a common structure: the non–DNA-binding EWSR1 protein or a homologue (FUS, TAF2N) forms the NH2 terminus, and a DNA binding transcription factor forms the COOH terminus. In doing so, EWSR1 loses its RNA binding domain, and the transcription factor its regulatory domain, resulting in the EWSR1 trans-regulatory domain being aberrantly fused to the DNA binding domain of an otherwise unrelated transcription factor (3). The retained NH2-terminal SYQG domain of EWSR1 is thought to function as a transcriptional activator (4), the functions of which could involve changes in histone acetylation status. EWS-FLI1 expression in myoblasts blocks mesenchymal differentiation and induces neural crest genes and Wnt signaling pathway members (5). In myxoid liposarcoma and rare cases of Ewing sarcoma, FUS substitutes for its homologue EWSR1 (6), and the SYT-SSX oncprotein in synovial sarcoma may also invoke similar effects by binding an EWS homologue called SIP/CoAA (4). The features of these translocation-associated sarcomas, including clear cell sarcoma, have many similarities to the translocation-associated leukemias, for which related translocations have been implicated in transforming committed hematopoietic progenitors back to a stem cell state.
(7), and a model has been described linking fusion oncoproteins to aberrant chromatin remodeling and impaired differentiation (8).

Gene transcription is regulated by coactivator and corepressor complexes. Whereas acetylation of histones is associated with chromatin relaxation and transcriptional activation, histone deacetylases repress transcription by condensing chromatin. Thus, histone deacetylases are enzymatic effectors of transcriptional regulatory complexes performing epigenetic histone modifications, repressing key genes, many of which control growth and induce differentiation (9). Histone deacetylases have been shown to associate with promoters and repress transcription of neuronal, intestinal, and muscle differentiation genes; such epigenetic alterations may represent very early events in the development of cancer (10). Overexpression of histone deacetylases can induce cell proliferation (11), and deregulation of histone deacetylase recruitment seems to play an important role in tumorigenesis.

For these reasons, inhibition of histone deacetylases is considered a promising antineoplastic targeted intervention in many cancer models and several such drugs are in active development. Histone deacetylase inhibitors are effective in leukemias, including those bearing fusion oncogenes, where they induce cell growth arrest and differentiation (12). Histone deacetylase inhibitors induce markers of differentiation in myeloid leukemia, bone, and chondrosarcoma models (13–15). MS-275, a recently developed class I histone deacetylase inhibitor, represses growth of human malignancies such as leukemias and solid tumors in preclinical models (16, 17). Another histone deacetylase inhibitor, romidepsin (depsipeptide, FK228), inhibits proliferation, reverses EWS-FLI1 mediated histone deacetylation, inhibits the level of EWS-FLI1 mRNA and protein in Ewing sarcoma cells (18), and induces differentiation in chondrosarcoma cells (15). Phase I/II studies have shown that both MS-275 and romidepsin can be used in humans (19, 20).

For most soft tissue sarcomas, no adjuvant chemotherapy offers proven benefit to overall or even progression-free survival, and metastatic disease is generally fatal (21). Finding new approaches to targeted therapy is a priority for improving patient outcomes. Recent efforts focusing on Ewing sarcoma (18), chondrosarcoma, and synovial sarcoma (22) indicate that the histone deacetylase inhibitor romidepsin inhibits growth of those tumor cells in vitro and in xenograft models. To date, there have been no studies testing the effect of histone deacetylase inhibitors against clear cell sarcomas. We conducted this study to investigate the growth inhibition and differentiation activities of the histone deacetylase inhibitors MS-275 and romidepsin on clear cell sarcoma cell line models. We compare the sensitivity translocation-associated sarcomas and other cell line models to these histone deacetylase inhibitors, and evaluate the effect of MS-275 and romidepsin on the expression of genes associated with mesenchymal cell growth and differentiation.

### Materials and Methods

#### Drugs and Cell Lines

MS-275 and romidepsin (depsipeptide, FK228) were generously provided by Berlex Pharmaceuticals (Montville, NJ) and the Developmental Therapeutics Program of the National Cancer Institute (Bethesda, MD). Doxorubicin was purchased from Sigma and was used as an experimental

| Table 1. Cell lines and IC50 of MS-275 and romidepsin at 72 h of treatment |
|-----------------------------|-------------------------------|--------------------------|-------------------------------|--------------------------|
| Cell line                  | Provider                      | Tumor type               | Fusion gene                  | IC50 of MS-275 (μmol/L)   | IC50 of romidepsin (nmol/L) |
| DTC1                       | Dr. KL. Schaefer              | Clear cell sarcoma       | EWS-ATF1                     | 0.28                      | 0.51                        |
| KAO                        | Dr. KL. Schaefer              | Clear cell sarcoma       | EWS-ATF1                     | 0.38                      | 0.91                        |
| SU-CCS-1                   | Dr. AL. Epstein               | Clear cell sarcoma       | EWS-ATF1                     | 0.63                      | 0.89                        |
| SYO-1                      | Dr. A. Kawai                  | Synovial sarcoma         | SYT-SSX2                     | 0.44                      | 0.67                        |
| FUJI                       | Dr. K. Nagashima              | Synovial sarcoma         | SYT-SSX2                     | 2.51                      | 1.31                        |
| SKNMC                      | ATCC                          | Ewing sarcoma            | EWS-FLI1                     | 0.79                      | 1.17                        |
| 4102-91                    | Dr. P. Aman                   | Myxoid liposarcoma       | FUS-DDIT3                    | 1.23                      | 1.26                        |
| 1765-92                    | Dr. P. Aman                   | Myxoid liposarcoma       | FUS-DDIT3                    | 1.25                      | 1.77                        |
| JN-DSRCT-1                 | Dr. H. Iwasaki                | DSRCT                    | EWS-WT1                      | 1.12                      | 1.25                        |
| NMS-2PC                    | Dr. A. Ogose                  | MPNST                    |                                | 1.20                      | 0.81                        |
| HL60                       | ATCC                          | Myeloid leukemia         |                                | 0.75                      | 1.86                        |
| A549                       | ATCC                          | Lung carcinoma           |                                | 3.55                      | 3.24                        |
| SW480                      | ATCC                          | Colon carcinoma          |                                | 4.67                      | 2.69                        |
| MCF7                       | ATCC                          | Breast carcinoma         |                                | 4.79                      | 3.55                        |
| PC-3                       | ATCC                          | Prostate cancer          |                                | 1.27                      | 2.51                        |
| MMRU                       | Dr. R. Byers                  | Melanoma                 |                                | 4.37                      | 2.57                        |
| Hs68                       | ATCC                          | Human fibroblasts        |                                | >10                       | >10                          |
| hMSC-001F                  | Stem Cell Technologies        | hMSC                     |                                | >10                       | >10                          |

Abbreviations: ATCC, American Type Culture Collection; DSRCT, desmoplastic small round cell tumor; MPNST, malignant peripheral nerve sheath tumor; hMSC, bone marrow–derived human mesenchymal stem cells.
positive control. MS-275, romidepsin, and doxorubicin were dissolved to appropriate 1,000× stock concentrations in DMSO and distilled water, respectively. The study was approved by the Clinical Research Ethics Board of the British Columbia Cancer Agency.

To assess relative sensitivity to histone deacetylase inhibitors, we obtained different cancer cell lines that could be categorized into three groups: soft tissue sarcomas, hematopoietic/epithelial malignancies, and nonmalignant mesenchymal cells (listed in Table 1). Three clear cell sarcoma cell lines, DTC1 (23), KAO (24), and SU-CCS-1 (25), were included, and human fibroblasts and bone marrow–derived mesenchymal stem cells were used as negative control cells. RPMI 1640 supplemented with 10% fetal bovine serum was used to culture all cell lines with the following exceptions: SW-480, MCF7, PC3, MMU, and Hs68 used DMEM supplemented with 10% fetal bovine serum; JN-DSRCT-1 used MEM/F12 with 10% fetal bovine serum; A549 used F12K Nutrition Medium with 10% fetal bovine serum; and hMSC required Mesencult basal medium with mesenchymal stem cell stimulatory supplements (Stem Cell Technologies, Inc.). All cells were cultured under standard incubation conditions (37°C, 95% humidity, 5% CO2).

Table 2. Primer sequences for quantitative real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EWS-ATF1</td>
<td>GAAGAGGGGGATTTGATCGT</td>
<td>TTTCTGCCCCGTTGTA1CTT</td>
<td>113</td>
</tr>
<tr>
<td>SOX9</td>
<td>ATTTCCTCGTGGCTTGCTT</td>
<td>ATTTGGCTGCTGAAACATTCC</td>
<td>99</td>
</tr>
<tr>
<td>MYOD1</td>
<td>CGCCAGGATATGGAGCTACT</td>
<td>CCGGTCGTCAATAGAACCTGCT</td>
<td>108</td>
</tr>
<tr>
<td>PPARG</td>
<td>ATCAAAGTGAGGGCTGCAATC</td>
<td>CGCATAACCATGCTGAGGAG</td>
<td>104</td>
</tr>
<tr>
<td>MITF</td>
<td>GAGGCTTCAAAAACAGGCA</td>
<td>AGGAAGCAGTTCGGAAT</td>
<td>105</td>
</tr>
<tr>
<td>CDKN1A</td>
<td>TTACGACGGAACACGAGGAGT</td>
<td>TCAACGTGATGCACAGGAAA</td>
<td>120</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CTGACTCAACAGCGACACC</td>
<td>CCAAGCTCGTGAACAAAT</td>
<td>120</td>
</tr>
</tbody>
</table>

Cell Growth Assays and Cell Cycle Analysis
The growth of cells was assessed as previously described (26) by measuring the absorbance of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Monolayer cultured cells were subcultured in 24-well plates and treated in triplicate wells with media containing final concentrations of MS-275, romidepsin, doxorubicin, or vehicle control (DMSO). Photographic images of cells were captured with an inverted light microscope (Hund Wetzlar) using a consumer digital camera. MTT formazan absorbance was measured at 24, 48, and 72 h. HL60 cells, growing in suspension, were subcultured and seeded at 1 × 10^5 per well in 96-well plates. After incubating with MTT at treatment time points, 100 μL of lysis buffer (20% SDS, 50% N,N-dimethylformamide, 0.4% glacial acetic acid, pH 4.8) were added and incubated overnight. Assay equivalency was validated by comparing the readings between monolayer- and suspension-cultured A549 cells, which showed no significant differences. IC_{50} values were determined with the formula log (fraction killed/fraction survived) = mlog (dose) – mlog (IC_{50}) (ref. 27).

For cell cycle analysis, cells treated with MS-275, romidepsin, or vehicle were collected and fixed with 70% ethanol on ice for 30 min, then washed with PBSTB (PBS containing 0.1% Tween 20 and 0.1% bovine serum albumin). After being resuspended in PBSTB with 50 μg/mL RNase and 50 μg/mL propidium iodide, cell cycle distributions were analyzed using an EPICS MXL flow cytometer (Beckman Coulter). The percentages of cell populations in each cell cycle phase (sub-G1, G1, S, G2-M) were calculated from DNA content histograms using ESP032 Software.

Detection of Apoptosis in Three-Dimensional Spheroid Culture
Spheroid cell cultures were produced as previously described (26) and treated with MS-275, romidepsin, doxorubicin, or vehicle. The proportion of apoptotic cells within treated spheroids was measured by Annexin V/propidium iodide flow cytometry.

Immunoblotting Analyses
Primary antibodies anti–histone H3 (rabbit polyclonal), anti–caspase-3 (rabbit monoclonal), and anti-p21 (mouse monoclonal) were purchased from Cell Signaling Technologies. Anti–acetyl-histone H3 (rabbit polyclonal) was purchased from Upstate Biotechnology and anti–glycer-aldehyde-3-phosphate dehydrogenase (GAPDH; mouse monoclonal) from Santa Cruz Biotechnology. Antimouse and antirabbit fluorescently labeled secondary antibodies were purchased from LI-COR Biosciences. Total cellular protein lysates were harvested, quantified, and immunoblotted as previously described (26). Western blot analysis was done using an Odyssey infrared imaging system.

Quantitative Real Time PCR
Total RNA was isolated using TRIzol and treated with DNase I according to the manufacturer’s instructions (Invitrogen). Two micrograms of total RNA from each sample were reverse transcribed using the SuperScript first-strand synthesis system (Invitrogen) as per manufacturer’s instructions. The primer sets used for quantitative real-time PCR were designed to amplify the fusion gene in clear cell sarcoma (EWS-ATF1), genes associated with mesenchymal (SOX9, MYOD1, and PPARG) or melanocytic (MITF) differentiation, CDKN1A (encoding the p21 cell cycle suppressor, a gene associated with cell cycle control and response to histone deacetylase inhibitors), and GAPDH as an internal control (Table 2). Two microliters
of cDNA template were added in triplicate to a MicroAmp Optical 96-well Reaction Plate (Applied Biosystems). Each 25-μL reaction mixture contained 12.5 μL of SYBR Green PCR Master Mix (Applied Biosystems) and 10 pmol each of forward and reverse primers. Quantitative real-time PCR was conducted using a 7900HT Fast Real-time PCR System (Applied Biosystems). Following 2 min at 50°C and 10 min at 95°C, the amplification was carried out with 40 cycles of 15 s at 95°C and 1 min at 60°C. The threshold cycle (Ct) value represents the cycle number at which the sample fluorescence is statistically higher than background. The relative amount of gene expression was analyzed by the 2^[-ΔΔCt] method (28). In all experiments, GAPDH mRNA was used as an internal standard to normalize the mRNA levels, and mRNA from vehicle-treated cells was used as a baseline standard for the analysis of relative mRNA levels of assayed genes in drug-treated cells. The specificity of primers was tested using dissociation curves of real-time PCR and agarose gel analysis of PCR products.

siRNA Transfection

siRNAs (On-TARGETplus SMARTpool) directed against SOX9, MOYD1, and PPARG, and nontarget siRNA were synthesized by Dharmacon. siRNA transfections were done in 24-well plates in triplicate wells using the HiPerfect siRNA transfection reagent (Qiagen) according to the manufacturer’s instructions. After optimizing transfection parameters for each cell line, 1 d after seeding cells onto 24-well plates (8 × 10^4 per well), each well was transfected with 10 nmol/L of siRNA. Twenty-four hours posttransfection, cells were treated with 5 μmol/L MS-275, 5 nmol/L romidepsin, or vehicle. Cell growth was measured by MTT assay after 24 h.

Results

**MS-275 and Romidepsin Inhibit Cell Growth in Clear Cell Sarcoma and Other Translocation-Associated Sarcomas**

Three clear cell sarcoma cell lines were treated with MS-275 (0.1, 1, and 10 μmol/L), romidepsin (0.1, 1, and 10 nmol/L), doxorubicin (1 μmol/L), or vehicle (0.1% DMSO). MS-275 and romidepsin significantly inhibited cell growth in a dose- and time-dependent manner in all three clear cell sarcoma lines (Fig. 1). Effects of inhibition were observed at 24 hours with 1 μmol/L MS-275 or 1 nmol/L romidepsin. By comparison, the doxorubicin-positive control was also effective but had much greater toxicity.
to nonneoplastic fibroblasts (Hs68) and human bone marrow–derived mesenchymal multipotential stem cells. IC_{50} values for these and 12 other cell lines (including translocation-associated sarcomas and other human malignancies) are presented in Table 1. These experiments show that MS-275 and romidepsin inhibit cell growth in all cancer cells in a dose- and time-dependent manner, but with varying degrees of sensitivity in different cancer cell types. The IC_{50} values of MS-275 at 72 hours are much lower in most of the tested sarcomas than those in other cancer cell types. The results from romidepsin showed a similar pattern of sensitivity. By comparison, human fibroblast and mesenchymal stem cells are highly resistant to MS-275 and romidepsin, with IC_{50} values greater than 10 μmol/L and 1 nmol/L, respectively, at 72 hours. No significant inhibition of cell growth was observed in these nonneoplastic cells after treatment with 1 μmol/L MS-275 and 1 nmol/L romidepsin. Propidium iodide flow cytometry for DNA content confirmed that in the clear cell sarcoma lines, 1 μmol/L MS-275 and 1 nmol/L romidepsin have similar effects (Fig. 2A). At 24 hours, DTC1 clear cell sarcoma cells display a greatly increased sub-G_1 fraction, suggestive of apoptosis; SU-CCS-1 cells show prominent S-phase depletion; and KAO clear cell sarcoma cells display both types of change. Western blot analysis after 24-hour treatment with either of the tested histone deacetylase inhibitors shows the expected accumulation of acetylated histone H3 as well as a dose-dependent induction of p21^{waft/cip1} (a marker of cell cycle arrest) and cleaved caspase-3 (a marker of apoptosis) in all three cell lines (Fig. 2B).

MS-275 and Romidepsin Induce Apoptosis in Clear Cell Sarcoma Spheroids

Three-dimensional spheroid cultures are more resistant to chemotherapy and are thought to be a more representative preclinical model than monolayer cultures, yet can be assayed for growth and apoptosis more readily than xenografts. We cultured SU-CCS-1, DTC1, and KAO clear cell sarcoma cells as spheroids to assess the induction of apoptosis by histone deacetylase inhibitors by flow cytometric analysis of Annexin V/propidium iodide binding. Both MS-275 and romidepsin induced apoptosis in all three clear cell sarcoma spheroid models in a dose- and time-dependent manner (Table 3). We also tested the effect of MS-275 on synovial sarcoma cell line SYO-1 spheroids and detected significant apoptotic induction by the histone deacetylase inhibitor in a similar time- and dose-dependent manner (data not shown).

Histone Deacetylase Inhibitors Induce Morphologic Change and Transcriptional Evidence of Differentiation in Clear Cell Sarcoma Cells

We observed morphologic changes in all three clear cell sarcoma cell lines on treatment with relatively low concentrations of MS-275 (1 μmol/L) and romidepsin (1 nmol/L). Starting from 24 hours of treatment, tumor cells converted from their baseline ovoid appearance to a fibroblastic morphology, with progressive changes continuing to 48 hours (Supplementary Fig. S1). To investigate the gene transcriptional expression associated with possible induction of cartilaginous, skeletal muscle, adipose, and melanocytic differentiation, we performed quantitative real-time PCR analysis of the mRNA levels of SOX9, MYOD1, PPARG, and MITF. CDKN1A, known to be induced by histone deacetylase inhibitor treatment in other systems, was used as a positive control. We observed minimal induction of differentiation-associated gene expression after 4-hour exposure to histone deacetylase inhibitors, but consistent induction of SOX9, MYOD1, and PPARG expression in all three clear cell sarcoma cell lines at 24 and 48 hours of treatment (Fig. 3A–C). The induction of expression of these genes is slightly cell line dependent, with an earlier occurrence in DTC1 cells than in SU-CCS-1 and KAO cells. The results also showed that MS-275 and romidepsin induce CDKN1A in the clear cell sarcoma cells, but to a considerably lesser extent than is seen for SOX9, MYOD1, and PPARG. Because clear cell sarcoma was previously defined as malignant melanoma of soft parts, we examined the induction of MITF expression as a marker of melanocytic differentiation and saw no significant induction after histone deacetylase inhibitor treatment. To determine if the observed induction of SOX9, MYOD1, or PPARG might be causative of cell growth inhibition, we sequentially transfected each cell line with siRNA against each of these genes, then treated the clear cell sarcoma cells with each histone deacetylase inhibitor and measured cell growth. No significant differences in cell growth were found between siRNA-transfected and nontransfected histone deacetylase inhibitor–treated cells in any of the three tested cell lines (Supplementary Fig. S2).

MS-275 and Romidepsin Decrease mRNA Levels of EWS-ATFI in Clear Cell Sarcoma Cells

To examine whether histone deacetylase inhibitors alter expression of the fusion oncogene, we designed primers flanking the break point of EWS-ATFI and compared its transcriptional expression level with the vehicle-treated cells for all three clear cell sarcoma cell lines. The results of quantitative real-time PCR showed that 1 μmol/L MS-275 and 1 nmol/L romidepsin decreased EWS-ATFI transcriptional expression in DTC1, KAO, and SU-CCS-1 cells after as little as 4 hours of treatment, and the suppression was maintained through 24 and 48 hours of treatment (Fig. 3D).

Discussion

Cancer cells display multiple changes in gene expression, leading to decreased expression of cell cycle checkpoint, apoptosis, and differentiation genes. Many such genes are subject to epigenetic transcriptional repression, and therefore drugs targeting epigenetic regulation could be more effective than those targeting a single gene. Histone

1 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
Figure 2. Cell cycle effects of histone deacetylase inhibitors on clear cell sarcoma models. A, propidium iodide flow cytometry for cell lines treated with 1 μmol/L MS-275, 1 nmol/L romidepsin, or vehicle only (0.1% DMSO) for 24 h. B, immunoblotting analysis of protein expression in clear cell sarcoma cell lines treated with two doses of MS-275 or romidepsin. Antibodies were used to measure direct drug effects (acetylated histone H3), cell cycle arrest (p21), and apoptosis (cleaved caspase-3), in comparison with GAPDH, total H3, and intact caspase-3 controls.
histone deacetylase inhibitors are an emerging class of antineoplastic agents that are thought to act by net acetylation of core histones, leading in turn to the uncoiling of chromatin and activation of a variety of genes implicated in the regulation of cell survival, growth, differentiation, and apoptosis. MS-275 and romidepsin are histone deacetylase inhibitors currently undergoing clinical evaluation in solid tumors and leukemia. We conducted this study to assess the effect of these two clinically applicable histone deacetylase inhibitors with known pharmacologic properties on clear cell sarcoma, a soft tissue malignancy affecting young adults for which current systemic therapy is almost impossible to reverse; epigenetic aberrations may well be reversible; targeting such alterations is an emerging approach to cancer chemotherapy and chemoprevention (31). The hallmarks of the malignant phenotype include loss of differentiation. We observed morphologic changes consistent with differentiation in all three clear cell sarcoma cell lines after treatment with relatively low concentrations of MS-275 and romidepsin. To further characterize this apparent differentiation, we tested the transcriptional expression levels of specific genes associated with mesenchymal differentiation, including SOX9, MYOD1, and PPARG. Sox9 is a transcription factor playing a key role in early chondrogenic and neural cell fate and differentiation (32). Sox9 activates transcription by interacting with chromatin and recruiting chromatin modifiers (33). The multifunctional coactivator p300 is a member of the Sox9-related transcriptional apparatus and has histone acetyltransferase activity, activating Sox-dependent transcription during chondrogenesis. Histone deacetylase inhibitors induce the expression of Sox9-regulated cartilage matrix genes and induce histone acetylation around the enhancer region of the collagen α1 gene in chondrocytes. The most extensively studied model of differentiation in which histone deacetylases play an important role is that of myoblast differentiation (34). MyoD1 is induced at an early stage of myogenic differentiation. HDAC1 acts to silence MyoD1-dependent transcription of muscle-specific genes in skeletal muscle cells (35), and transcriptional activity of MYOD reporters is up-regulated by histone deacetylase inhibitors (34). Peroxisome proliferator–activated receptor γ (encoded by PPARG) belongs to the family of nuclear hormone receptors and is a ligand-activated transcription factor. Activation of peroxisome proliferator–activated receptor γ by natural or pharmacologic ligands leads to inhibition of cell growth and/or induction of apoptosis or terminal differentiation, and may represent a promising novel therapeutic approach for certain human malignancies (36). PPARG induces terminal differentiation in many cancer cells, including human primary liposarcoma (37) and osteosarcoma (38) cells. PPARG activity is repressed by histone deacetylases (39), and down-regulation of histone deacetylases is an important process during adipocyte differentiation (40). Our study shows that both MS-275 and romidepsin induce significant transcriptional expression of SOX9, MYOD1, and PPARG in all three CCS cell lines, suggesting that in these cells histone deacetylase inhibitors induce early changes also seen in chondrogenic, myogenic,
Figure 3. mRNA levels of SOX9, MYOD1, PPARG, MITF, and CDKN1A determined by quantitative real-time PCR in clear cell sarcoma cell lines DTC1 (A), KAO (B), and SU-CCS-1 (C) after 24 and 48 h of treatment with histone deacetylase inhibitors. mRNA levels of the fusion oncogene EWS-ATF1 were also determined in the three clear cell sarcoma lines at 4, 24, and 48 h of treatment (D). Cells were treated with 0.1% DMSO (vehicle), 1 μmol/L MS-275, or 1 nmol/L romidepsin. Results are shown in a log scale relative to untreated (vehicle) control cells, where a value of zero means no induction, +1 equates to 10-fold relative increase, and −1 indicates 10-fold decrease. Bars, 95% confidence interval of the mean of three replicates.
and adipogenic differentiation. siRNA knockdowns of these genes did not preclude growth inhibition by histone deacetylase inhibitors, suggesting that the induction of these key markers of mesenchymal differentiation is a parallel epiphenomenon of treatment rather than a required mechanism for cell death. Histone deacetylase inhibitors did not activate the expression of the microphthalmia-associated transcription factor (MITF), a major inducer of melanocytic differentiation.

The development of treatment strategies identifying tumor-specific biological mechanisms is at the front line of cancer therapy. For the translocation-associated sarcomas, it is important to understand the mechanisms through which the fusion oncogenes possess transforming activity, which may lead to the identification of novel and highly effective therapeutic approaches. Fusion oncogenes carried in certain soft tissue sarcomas may invoke histone deacetylase activity, thereby, for example, inhibiting p21 expression by antagonizing the histone acetyltransferase activity of p300 in Ewing family tumors (41), where histone deacetylase inhibitors suppress EWS-FLI1 expression (18). A murine mesenchymal multipotent cell line transduced with EWS-FLI1 acquired dramatic morphologic changes and conferred oncogenic properties such as an increased rate of proliferation, repression of adipogenic differentiation programs, and an aberrant neural phenotype (42). Others have shown that EWS-FLI1 inhibits adipogenic, osteogenic, and myogenic differentiation (43, 44), and inhibition of EWS-FLI1 could lead Ewing sarcoma cells to recover the features of their mesenchymal stem cell progenitor. Ito et al. showed that synovial sarcoma cells carrying the SYT-SSX fusion gene are highly sensitive to romidepsin (results we were able to confirm in our spheroid model), and that introduction of SYT-SSX into HEK293 cells confers sensitivity to this agent (22). Our results in clear cell sarcoma are consistent with these studies. We found that the histone deacetylase inhibitors MS-275 and romidepsin suppress the transcriptional expression of the fusion gene in all three clear cell sarcoma cell lines, suggesting that histone deacetylase inhibitors may directly affect the transforming activity of EWS-ATF1. Although there are no previous reports of histone deacetylase inhibitor actions on clear cell sarcoma, our results are consistent with two published reports on Ewing sarcoma showing growth inhibition and apoptosis in monolayer cell models and xenografts, suppression of EWS-FLI1, and reversal of EWS-FLI1–mediated histone deacetylation (18, 45).

In acute myelogenous leukemia, the translocation oncprotein AML1-ETO regulates a number of target genes critical to normal hematopoiesis via its interaction with the N-CoR/mSin3/HDAC1 complex (46). The AML1-ETO fusion protein promotes leukemogenesis by recruiting histone deacetylases and silencing AML1 target genes important for hematopoietic differentiation. Histone deacetylase inhibitors partially reverse ETO-mediated transcriptional repression and induce differentiation of AML cells carrying the translocation. In leukemias, the AML1-ETO and TEL-AML1 fusions convert the AML1 transcription factor from an activator to a repressor through altered recruitment of histone deacetylases (13, 47). The features of the translocation-associated soft tissue sarcomas, including clear cell sarcoma, are similar to leukemias in which related translocations switch committed hematopoietic progenitors back to a stem cell state (7). A recently described model links fusion oncoproteins to aberrant chromatin remodeling and impaired differentiation (8). In this model, level 1 proteins (e.g., FLI1 and ATF1) recognize and bind specific DNA sequences, but whether they activate or repress transcription is dependent on recruited cofactors. Level 2 proteins (e.g., EWS and SYT-SSX) confer cell type–specific coordinated regulation of banks of transcription factors by acting as adaptors linking a restricted set of level 1 proteins to effector activities. Level 3 proteins (histone deacetylases, p300/CBP binding protein, hSWI/SNF, DNA methyltransferases, and histone methyltransferases) are expressed more constitutively and carry enzymatic activities altering chromatin structure to change transcriptional status. Level 1 or 2 proteins might make the most specific drug targets, but level 3 proteins, such as enzymes, are more readily targetable by current drugs.

MS-275 is currently in phase I/II clinical trials to evaluate its efficacy and tolerability. Results obtained thus far in patients with refractory solid tumors or hematologic malignancies suggest that MS-275 is well tolerated and shows potential antitumor activity (48, 49). MS-275 is rapidly absorbed and distributed into deep tissue compartments with slow redistribution into the systemic circulation, resulting in a long half life (39–80 hours) and tolerable dose-limiting toxicities (49). Studies with romidepsin in patients with advanced or refractory neoplasms have shown some partial or complete tumor responses, especially in T-cell lymphomas, with increased histone acetylation in patient-derived peripheral blood mononuclear cells; occasional arrhythmias have been observed (19, 50).

The recent Food and Drug Administration approval of the histone deacetylase inhibitor suberoylanilide hydroxamic acid for T-cell lymphoma is a major step forward and marks the transition of histone deacetylase inhibitors from purely experimental agents to an approved cancer therapy (20). This approval is expected to stimulate additional clinical trials with histone deacetylase inhibitors.

Our study provides evidence that histone deacetylase inhibitors have selective antitumor effects against clear cell sarcoma, at concentrations where no significant cytotoxicity is observed in human nonneoplastic cells. By comparing the cytotoxicity of two histone deacetylase inhibitors across different cancer cell lines, we found that soft tissue sarcoma cells bearing fusion oncogenes were more sensitive than most other cancer cells lacking known translocations, and that clear cell sarcomas seem to be particularly sensitive to these agents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
Acknowledgments

We thank Drs. AL. Epstein, KL. Schaefer, A. Kawai, K. Nagashima, P. Aman, H. Iwasaki, A. Ogose, and R. Byers for kindly providing us with soft tissue sarcoma and melanoma cell lines; Berlex Pharmaceuticals and the Developmental Therapeutics Program of the National Cancer Institute for supplying MS-275 and romidepsin; and Guangming Han and Forrest Hsu for cell culture assistance with preliminary experiments.

References


Downloaded from mct.aacrjournals.org on June 21, 2017. © 2008 American Association for Cancer Research.


Histone deacetylase inhibitors induce growth arrest, apoptosis, and differentiation in clear cell sarcoma models
