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Histone Deacetylase Inhibitors Inhibit Rhabdomyosarcoma by Reactive Oxygen Species-Dependent Targeting of Specificity Protein Transcription Factors

Erik Hedrick¹, Lisa Crose², Corinne M. Linardic^{2,3}, and Stephen Safe^{1,4}

¹ Department of Veterinary Physiology and Pharmacology, Texas A&M University, 4466 TAMU, College Station, TX 77843-4466 USA

² Department of Pediatrics, Duke University Medical Center, DUMC 102382, Durham, NC 27710

³ Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC 27710

⁴ Institute of Biosciences and Technology, Texas A&M Health Sciences Center, 2121 W. Holcombe Blvd., Houston, TX 77030-3303

Abstract

The two major types of rhabdomyosarcomas (RMS) are predominantly diagnosed in children, namely embryonal (ERMS) and alveolar (ARMS) RMS and patients are treated with cytotoxic drugs which results in multiple toxic side effects later in life. Therefore, development of innovative chemotherapeutic strategies is imperative and a recent genomic analysis suggested the potential efficacy of reactive oxygen species (ROS)-inducing agents. Here we demonstrate the efficacy of the potent histone deacetylase (HDAC) inhibitors, panobinostat and vorinostat, as agents that inhibit RMS tumor growth *in vivo*, induce apoptosis, and inhibit invasion of RD and Rh30 RMS cell lines. These effects are due to epigenetic repression of cMyc which leads to decreased expression of cMyc-regulated miRs-17, -20a and -27a, upregulation of ZBTB4, ZBTB10 and ZBTB34 and subsequent downregulation of Sp transcription factors. We also show that inhibition of RMS cell growth, survival and invasion and repression of Sp transcription factors by the HDAC inhibitors is independent of histone acetylation but reversible after cotreatment with the antioxidant glutathione. These results show a novel ROS-dependent mechanism of antineoplastic activity for panobinostat and vorinostat that lies outside of their canonical HDAC inhibitory activity and demonstrates the potential clinical utility for treating RMS patients with ROS-inducing agents.

Keywords

HDAC inhibitors; ROS; cMyc; ZBTBs; Sp transcription factors

Send all correspondence to: Stephen Safe, Department of Veterinary Physiology and Pharmacology, Texas A&M University, 4466 TAMU, College Station, TX 77843-4466, Tel: 979-845-5988 / Fax: 979-862-4929, ssafe@cvm.tamu.edu.

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INTRODUCTION

Rhabdomyosarcomas (RMS) is the most common soft tissue sarcoma that is primarily observed in children and adolescents and accounts for 5% of all pediatric cancers and 50% of soft tissue sarcomas in children (1-3). Embryonal RMS (ERMS) and alveolar RMS (ARMS) are the two major classes of RMS in children and adolescents and differ with respect to their histology, genetics, treatment, and prognosis (4, 5). ERMS accounts for over 60% of RMS patients and is associated with loss of heterozygosity at the 11p15 locus and mutation in Ras signaling (6, 7), whereas ARMS occurs in approximately 20% of RMS patients and is associated with translocations resulting in formation of pro-oncogenic gene products resulting from the fusion of *PAX3* or *PAX7* with the Forkhead gene *FOXO1A* (8, 9). ARMS patients have a poor diagnosis and patient survival is <20% for metastatic ARMS. Treatments include radiotherapy, surgery, and chemotherapy with cytotoxic drugs (10, 11), however, RMS patients that survive current therapies have a dramatic increased incidence of several health problems as adults (12). Thus, there is a critical need for development of new therapeutic regimens for treating childhood RMS.

Specificity protein 1 (Sp1) transcription factor is overexpressed in human RMS tumors and other Sp family members including Sp3 and Sp4 are also overexpressed in RMS cell lines (13). The importance of Sp transcription factors (TFs) in RMS is primarily due to pro-oncogenic Sp-regulated genes that are themselves drug targets for RMS and these include CXCR4, hepatocyte growth factor receptor (c-MET), insulin-like growth factor 1 receptor (IGF-1R), and platelet-derived growth-factor receptor α (PDGFR α) (14-17). Clinical studies using drugs that specifically target Sp TFs and Sp-regulated genes for treatment of RMS have not yet been reported; however, there is an open phase I/II trial (NCT01610570) evaluating the efficacy of mithramycin in solid tumors including RMS. Mithramycin acts in part by binding to GC-rich sequences and regulating chromatin accessibility, including the ability to displace Sp1 from oncogenic promoters. Thus, the therapeutic potential of Sp TF in RMS is gaining traction.

Genomic analysis of RMS from several patients indicated that “skeletal muscle (rhabdomyosarcoma) may have even higher levels of ROS than other cancer cells and may be particularly sensitive to therapeutics that induce oxidative stress” (18). This sensitivity is thought to occur because with such a high baseline burden of ROS, there is little tolerance for further oxidative stress and this was confirmed by showing that ROS inducers were highly effective inhibitors of RMS tumor growth using patient-derived xenografts in mouse models (18). Recent studies in our laboratory (19) demonstrate that ROS inducers also inhibit pancreatic cancer cell and tumor growth and this is due, in part, to a novel epigenetic pathway (20) in which ROS-mediated repression of cMyc results in downregulation of Sp TFs and pro-oncogenic Sp-regulated genes. In this study, we demonstrate that ROS-inducing histone deacetylase (HDAC) inhibitors block RMS cell and tumor growth by initially targeting cMyc, which results in downregulation of microRNAs (miRs) and induction of ZBTB transcriptional repressors, which in turn downregulate Sp TFs.

MATERIALS AND METHODS

Cell lines and antibodies

RD, Rh30 and SMS-CTR rhabdomyosarcoma cell lines were purchased from American Type Culture Collection (Manassas, VA) and cells were maintained as previously described (13, 19). Cells were authenticated in 2014 (Promega Powerplex 18D) at the Duke University DNA Analysis Laboratory (Durham, NC). Various reagents (including antibodies) are summarized in Supplemental Materials and Methods.

Cell proliferation and MTT assays

Proliferation of RD and Rh30 rhabdomyosarcoma cells (1.0×10^5 per well) in the presence or absence of transfected siRNAs and after treatment with panobinostat and vorinostat (dimethyl sulfoxide, DMSO, as empty vehicle) ($\pm$ GSH, 3 hr prior to treatment) was essentially carried out as previously described (13, 19). Primary human myoblasts (HSMM, Lonza), Rh30, or RD cells were plated in 96-well plates at a density of 10,000 cells per well. The next day, cells were treated with vehicle (DMSO) or increasing doses of panobinostat. Twenty-four hours post-treatment, cells were analyzed by the MTT assay.

Annexin V staining

RD and Rh30 rhabdomyosarcoma cell (1.0×10^5 per well) were seeded in 2-well Nunc Lab-Tek chambered B#1.0 Borosilicate coverglass slides from Thermo Scientific and were allowed to attach for 24 hr. After 24 hr or 72 hr (after Sp1 knockdown), Annexin V staining was determined as described (13, 19).

Boyden chamber assay

RD and Rh30 rhabdomyosarcoma cells (3.0×10^5 cells per well) were seeded in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and were allowed to attach for 24 hr. Cells were seeded and subsequently treated with varying concentrations of panobinostat or vorinostat for 24 hr ($\pm$ GSH, 3 hr prior to treatment) or with 100 nm of siSp1, siSp3, siSp4 for 48 hr and cells that migrated through the pores were then counted as described (19).

RT-PCR

miRNA was isolated using the mirVana miRNA isolation kit (Ambion, Austin, TX) according to the manufacturer's protocol. Quantification of miRNA (RNU6B and miR-17, -20a, and -27a) was done using the TaqMan miRNA assay kit (Life Technologies) according to the manufacturer's protocol with real-time PCR. U6 small nuclear RNA was used as a control to determine relative miRNA expression.

Chromatin immunoprecipitation

The chromatin immunoprecipitation (ChIP) assay was performed using the ChIP-IT Express Magnetic Chromatin Immunoprecipitation Kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. RMS cells (5×10^6 cells) were treated with panobinostat for 3

hr and after crosslinking, lysing and immunoprecipitation, the interactions with the cMyc and Sp1 promoters were determined as described (19).

Western blot analysis

RD and Rh30 rhabdomyosarcoma cells (3.0×10^5 per well) were seeded in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and were allowed to attach for 24 hr and after various treatments, whole cell lysates were analyzed by Western blots essentially as described (13, 19).

Small interfering RNA and ROS assays

RD and Rh30 rhabdomyosarcoma cells were seeded (1.2×10^5 cells per well) in 6-well plates in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and left to attach for 24 hr. Knockdown of NR4A1 was carried out using Lipofectamine 2000 reagent according to the manufactures protocol. Cellular ROS levels were ascertained using the cell permeable probe CM-H₂DCFDA (5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester) from Invitrogen (Grand Island, NY) (19). RNAi studies were also carried out essentially as described (19).

In vivo xenograft studies

Male SCID/beige mice were subcutaneously implanted with 5×10^6 RD cells suspended in Matrigel (BD Biosciences). At 12 days post-implantation, when tumors were palpable, mice were begun on a treatment regimen of 17.5 mg/kg panobinostat or vehicle (DMSO), dosed intraperitoneally. Mice were treated daily for four days, no treatment for two days, and then every other day for 10 days. Animals were examined every other day for tumor burden (approximated by external caliper measurements, where $[(\text{width})^2 \times \text{length}] / 2$), animal weight, and overall well-being. At study end, animals were humanely sacrificed and tumors were harvested for analysis.

Statistical analysis

Statistical significance of differences between the treatment groups was determined by student's *t* test. The results are expressed as means with error bars representing 95% confidence intervals for 3 experiments for each group unless otherwise indicated, and a *P* value less than 0.05 was considered statistically significant. All statistical tests were 2-sided.

RESULTS

1. HDAC inhibitors induce ROS which decreases cell growth, induces apoptosis, and downregulates Sp1, Sp3 and Sp4 proteins

Figure 1A confirms that both panobinostat and vorinostat induced ROS in RD and Rh30 cells as previously reported in patient-derived RMS xenografts (18) and these responses were attenuated in cells cotreated with the HDAC inhibitors plus the antioxidant glutathione (GSH). Treatment of RD and Rh30 cells with different concentrations of both HDAC inhibitors also decreased proliferation of RD and Rh30 cell lines (Fig1B). For the more potent HDAC inhibitor (panobinostat), we also observed inhibition of metabolic activity in

RD and Rh30 cells using the MTT assay, whereas in primary human skeletal muscle myoblasts (HSMs) significant inhibition was not observed at concentrations as high as 500 nM, demonstrating specificity of the panobinostat for the transformed cell lines (Fig. 1C). Finally, we also observed inhibition of RD and Rh30 cell growth by panobinostat and vorinostat was significantly attenuated after cotreatment with GSH (Fig1D), indicating that induction of ROS by the HDAC inhibitors was important for their growth inhibitory effects. However, the growth inhibitory effects of panobinostat in Rh30 cells was also ROS-independent.

Panobinostat and vorinostat also induced Annexin V staining, a marker of apoptosis in RD and Rh30 cells (Fig. 2A) and cotreatment with GSH attenuated this response. Using a similar treatment protocol, we also show that both HDAC inhibitors induced cleavage of PARP and caspase 3 (markers of apoptosis) in RD and Rh30 cells (Supplemental Figs. S1A and S1B) and these effects were attenuated after cotreatment with GSH. Panobinostat and vorinostat inhibited invasion in RD and Rh30 cells in a Boyden chamber assay (Fig. 2B) and this response was also attenuated in cells cotreated with GSH. Thus, ROS induction by both HDAC inhibitors resulted in the induction of apoptosis and inhibition of cell growth and invasion in RD and Rh30 cells. Hydrogen peroxide and other ROS-inducing anticancer agents decrease expression of Sp1, Sp3, and Sp4 transcription factors in pancreatic, colon and bladder cancer cells, and Sp1, Sp3, and Sp4 are also highly expressed in RMS and tumors (19, 21-27). Panobinostat also decreased expression of Sp1, Sp3 (high and low molecular weight forms) and Sp4 in RD and Rh30 cells and cotreatment with GSH attenuated this response (Fig. 2C). We also observed similar effects in RD and Rh30 cells treated with vorinostat alone or in combination with GSH (Figure 2D) and both HDAC inhibitors increased histone-3 acetylation in RD and Rh30 cells which was unaffected by cotreatment with GSH. This suggests that the effects of panobinostat and vorinostat in this system are due to induction of ROS and are independent of their activity as inhibitors of histone deacetylation. Supplemental Figure S1C also shows that both HDAC inhibitors downregulate Sp1, Sp3 and Sp4 expression in a third RMS cell line, SMS-CTR cells.

2. ROS decreases RMS cell growth and downregulates Sp1, Sp3 and Sp4

Figures 3A and 3B show that 75-150 μ M hydrogen peroxide and 100-200 μ M *t*-butylhydroperoxide, respectively, inhibited growth of RD and Rh30 cells and also decreased expression of Sp1, Sp3, and Sp4 proteins and cMyc (Fig. 3C and 3D). These results were consistent with the effects observed for panobinostat in RMS cells (Fig. 2C and 2D). Supplemental Figure S2 shows that after knockdown of Sp1, Sp3 and Sp4 by RNA interference there is inhibition of Rh30 and RD cell growth (Supplemental Fig. S2A), induction of Annexin V staining (Supplemental Fig. S2B) and decreased invasion in a Boyden chamber assay (Supplemental Fig. S2C), demonstrating the important role of Sp TFs in the growth, survival and invasion of RMS cells and this was consistent with previous studies on Sp TFs in RMS cells (13).

3. Mechanisms of action of HDAC inhibitor-induced ROS in RMS cells

Results illustrated in Figure 4A show that within 3 hr after treatment of RD and Rh30 cells with panobinostat, there was a significant decrease in expression of cMyc and after 6 hr Myc

protein levels were not detected for the 24 hr duration of the experiment. Interestingly, we also observed a rapid decrease in expression of Sp1 within 3-6 hr, whereas decreased expression of Sp3 and Sp4 proteins was observed only at longer time points. This suggests that like cMyc, Sp1 expression may also be reduced by rapid ROS-dependent chromatin shifts that have previously been observed in colon and pancreatic cancer cells after treatment with hydrogen peroxide and phenethylisothiocyanate (PEITC; an ROS inducer), respectively (19, 20). In addition, cMyc downregulation may be due, in part, to loss of Sp1 which also regulates cMyc as illustrated in Figure 4B which shows that Sp1 knockdown decreases cMyc expression in RD and Rh30 cells (Fig. 2B). In contrast, the rate of degradation of Sp-regulated gene products including EGFR, bcl2, and survivin (Fig. 4C) was similar to that observed for Sp3 and Sp4 proteins and different from either cMyc or Sp1. ROS-induced changes in histone marks in RD and Rh30 cells were investigated in a ChIP assay after treatment with panobinostat for 3 and 6 hr. In RD cells (Fig. 4D), there was a decrease in Pol II and the H3K4me3 activation mark on the cMyc and Sp1 promoters and an increase in H4K16Ac. In Rh30 cells (Fig. 4E), there was also a decrease in Pol II and H3K4me3 and an increase in H4K16Ac on the cMyc and Sp1 promoters as observed in RD cells; however, we also observed an increase in the H3K27me3 deactivation mark on the cMyc promoter. These results are consistent with previous studies on ROS-induced changes in histone marks (20) and with the exception of the increase in H4K16Ac (activation mark), the epigenetic changes correlate with the observed rapid decreases in cMyc and Sp1 protein expression (Fig. 4A).

Previous studies in pancreatic cancers cells show that ROS-dependent downregulation of Myc also results in decreased expression of miR-27a and miR-20a/miR-17 and upregulation of the corresponding miR-regulated Sp repressors ZBTB34/ZBTB10 and ZBTB4, respectively (19). Results in Figure 5A show that after treatment of RD and Rh30 cells with panobinostat there was a decrease in cMyc expression and this was attenuated in cells cotreated with panobinostat plus glutathione. Previous studies showed that miR-27a is expressed in RD and Rh30 cells (13), and treatment of these cell lines with 100 nM panobinostat decreased expression of miR-27a and this decrease was also attenuated in cells cotreated panobinostat plus glutathione (Fig. 5B). We also observed that miR-20a and miR-17 were expressed in both RMS cell lines and downregulation of these miRs with panobinostat was also inhibited in cells cotreated with the HDAC inhibitor plus glutathione. MiR-27a and miR20a/miR-17 are members of the miR-23a-27a-24-2 and miR-17-92 clusters, respectively, and there is evidence that both miR clusters are regulated by cMyc (19, 28-30), and this was confirmed by transfection with siMyc which decreased miR-27a and miR-20a/miR17 expression in RMS cells (Fig. 5C). There is also some evidence that Sp1 regulates these same miR clusters, and knockdown of Sp1, Sp3 and Sp4 by RNAi decreased miR-27a and miR-20/miR-17 expression and the most dramatic effects were observed for miR-27a in both RD and Rh30 cells (Fig. 5B). These results could also be due in part to downregulation of cMyc which can be regulated by Sp TFs (31).

Treatment of RD and Rh30 cells with panobinostat resulted in the induction of ZBTB10 and ZBTB34 (Fig. 6A) which are regulated by miR-27a, and ZBTB4 which is regulated by miR-20a/miR-17 (19, 22-26). In addition, transfection of RD and Rh30 cells with siMyc increased expression of ZBTB34, ZBTB10, and ZBTB4 and also decreased levels of Sp1,

Sp3, and Sp4 and antagomir-27a also induced ZBTB10 and decreased expression of Sp proteins (Fig. 6B). Thus, cMyc and miR-27a knockdown decreases expression of miRs, induces ZBTBs and decreases Sp TFs and thereby mimics the effects of ROS. *In vivo* studies with SCID mice bearing RD cell xenografts also showed that administration of panobinostat (17.5 mg/kg) decreased tumor volume and weight (Fig. 6C), and analysis of the tumors showed that panobinostat also decreased Sp1, Sp3, Sp4 and cMyc expression (Supplemental Fig. S3). These results are consistent with *in vitro* studies and demonstrate that the effectiveness of panobinostat as an anticancer agent in RMS cells is primarily due to targeting of Sp1, Sp3 and Sp4 transcription factors through an ROS-dependent mechanism involving downregulation of cMyc and Myc-regulated miRs (Fig. 6D).

DISCUSSION

Transformation of normal cells into cancer cells is a complex cell- and tissue-specific process that involves activation of oncogenes and inactivation of tumor suppressor genes (32, 33); however, many other genes that also significantly contribute to the cancer cell phenotype. These genes are typically overexpressed in tumor vs. non-tumor tissues and have been termed as non-oncogene addiction (NOA) genes and are excellent targets for mechanism-based antineoplastic agents (33). The transcription factor Sp1 plays a critical role in embryonic development but there is evidence that levels of Sp1 decrease with age in rodents and humans (34-36). In contrast, Sp1 is highly expressed in tumors from RMS patients and in many other cancers and high expression of Sp1 is a negative prognostic factor for patient survival, tumor recurrence or tumor grade (13, 37-42). Stable transduction of human skeletal muscle myoblasts with PAX3-FOXO1, telomerase and N-Myc resulted in formation of transformed cell lines similar to ARMS cells (43) and both the genetically transformed and ARMS cells expressed high levels of Sp1, Sp3 and Sp4 (13). Interestingly, transformation of the muscle myoblasts dramatically increased expression of Sp1 and Sp3 but not Sp4 proteins which were highly expressed in the non-transformed cells (13); carcinogen/oncogene-induced transformation of human fibroblasts also dramatically increased Sp1 expression but levels of Sp3 and Sp4 were not determined (44).

The pro-oncogenic functions of Sp1 have been reported in many different cell lines (reviewed in 42); however, it is also important to determine the role of Sp1, Sp3 and Sp4 since all three proteins are overexpressed in RMS and other cancer cell lines. Supplemental Figure S2 shows that after knockdown of Sp1, Sp3 and Sp4 in Rh30 and RD cells, there was a significant decreased in cell proliferation, induction of apoptosis (Annexin V staining), and inhibition of invasion indicating that all three Sp proteins are NOA genes in RMS cells. In previous studies, we demonstrated that tolifenamic acid-induced downregulation of Sp1, Sp3 and Sp4 inhibited growth, induced apoptosis and inhibited invasion in RMS cells, and this was accompanied by decreased expression of several pro-oncogenic Sp-regulated genes (13).

Based on a recent report that ERMS patient-derived xenografts were highly sensitive to drugs that induce oxidative stress (18), we hypothesized that the efficacy of the ROS-inducing agents such as panobinostat was also due, in part, to repression of Sp1, Sp3 and Sp4. This hypothesis was based on previous studies showing that several ROS-inducing

anticancer drugs including curcumin, phenethylisothiocyanate (PEITC), methyl 2-cyano-3,12-dioxooleana-1,9-dien-28-oate (CDDO-Me), GT-094 (a nitro-aspirin analog), celastrol and betulinic acid also repressed Sp1, Sp3 and Sp4 expression (ROS-dependent) in pancreatic, bladder and colon cancer cells (19, 21-26).

HDAC inhibitors typically enhance histone acetylation and this response contributes to the anticancer activities of this class of compounds; however, these inhibitors also modulate several pathways in cancer cells and this includes induction of ROS. Therefore, we used the HDAC inhibitors panobinostat and vorinostat that are also ROS inducers (45) to investigate downregulation of Sp1, Sp3 and Sp4 in RMS cells in culture and in an *in vivo* model. Both panobinostat and vorinostat induced ROS, inhibited growth, induced apoptosis and inhibited invasion of RD and Rh30 cells, and all of these responses were attenuated after cotreatment with the antioxidant GSH (Figs. 1 and 2; Supplemental Fig. S1). Moreover, panobinostat also inhibited tumor growth in SCID mice bearing RD cells as a xenograft (Fig. 6B). In parallel studies, it was observed that panobinostat and vorinostat also decreased expression of Sp1, Sp3 and Sp4 in Rh30 and RD cells (Figs. 2C and 2D) and this response was attenuated after cotreatment with GSH. Moreover, treatment with hydrogen peroxide and *t*-butylhydroperoxide also decreased expression of Sp1, Sp3 and Sp4 as previously observed in other cell lines (23, 27, 46). We also observed that panobinostat decreased cMyc, Sp1, Sp3 and Sp4 proteins levels in tumors (Fig. 6B) and this paralleled the effects observed in cell culture (Fig. 2C). Thus, both knockdown of Sp TFs by RNA (Suppl. Fig. S1) and treatment with ROS inducers results in decreased expression of Sp1, Sp3 and Sp4, decreased growth and invasion, and induction of apoptosis *in vitro* and tumor growth *in vivo*. This suggests that the ROS-mediated repression of Sp TFs plays an important role in the antineoplastic effects of these HDAC inhibitors in RMS cells and is consistent with the *in vitro* and *in vivo* efficacy of panobinostat in RMS patient-derived xenografts (18).

Previous studies show that ROS-dependent repression of Sp1, Sp3 and Sp4 was due to downregulation of miR-27a and/or miR-20a/miR-17-5 resulting in increased expression of the transcriptional repressors ZBTB10/ZBTB34 and ZBTB4, respectively (19, 21-26). The ZBTB proteins are members of the POK family of transcriptional repressors (47) and competitively bind and displace Sp TFs from GC-rich sites on Sp promoters and Sp-regulated gene promoters. Using panobinostat as a model ROS inducer, we show that this compound also decreases miR-27a and miR-20a/miR-17 in RD and Rh30 cells (ROS-dependent) (Fig. 5B) and this results in the induction of ZBTB10/ZBTB34 and ZBTB4 (Fig. 6A). These data also show that the high expression of Sp1, Sp3 and Sp4 in RMS cells is due, in part, to miR-dependent suppression of the ZBTB transcriptional repressors.

Treatment of colon cancer cells with hydrogen peroxide or pharmacological doses of ascorbate which induces hydrogen peroxide decreases expression of Sp1, Sp3 and Sp4 in colon cancer cells (46) and hydrogen peroxide also induces genomewide migration of chromatin-modifying complexes from non-GC-rich to GC-rich gene promoters and downregulates genes such as cMyc (20). Sp1, Sp3, Sp4 and cMyc all have GC-rich promoters, and treatment of RD and Rh30 cells with panobinostat rapidly decreased expression of cMyc and Sp1 proteins, whereas with the exception of the rapid decrease in expression of Sp3 (high MW band in RD cells), downregulation of Sp3 and Sp4 was

primarily observed at later time points (Fig. 4A). Results of ChIP assays confirmed that treatment with panobinostat decreased pol II and the H3K4me3 activation mark in the GC-rich cMyc and Sp1 promoters in RD and Rh30 cells, and the H3K27me3 inactivation mark was also increased on the cMyc promoter only in Rh30 cells (Figs. 4D and 4E). Surprisingly, the H4K16Ac activation mark was increased by panobinostat in RD and Rh30 cells but decreased by PEITC-induced ROS in pancreatic cancer cells (19), suggesting that the epigenetic effects of ROS inducers are cell context-dependent and this is currently being investigated.

The importance of the ROS-dependent decrease in cMyc is that there are E-box and GC-rich elements in the miR-23a~27a~24-2 and miR-17-92 gene cluster promoters (28-30) and knockdown of cMyc or Sp TFs in RMS cells significantly decreased expression of miR-27a and miR-20a/miR-27 (Figs. 5C and 5D). It was also observed for the first time that Sp1, Sp3 and Sp4 play a role in expression of miR-27a and this paralleled the highly effective downregulation of miR-27a in RMS cells treated with HDAC inhibitors (Fig. 5B).

In summary, results of this study show that HDAC inhibitors that induce ROS are highly effective inhibitors of RMS cell in culture and xenograft tumor growth in mice and the effects *in vitro* were independent of HDAC inhibition. This complements results obtained with HDAC inhibitors using tumor-derived xenografts and lineage of origin studies (18, 48), thus confirming the potential efficacy of ROS inducers for RMS chemotherapy (18). This study demonstrates that panobinostat and vorinostat decrease expression of Sp1, Sp3, Sp4 and pro-oncogenic Sp-regulated genes by ROS-dependent epigenetic downregulation of cMyc and Sp1 which in turn decrease expression of miR-27a and miR-20a/miR-17, resulting in the induction of the ZBTB transcriptional repressors (Fig. 6D). The study demonstrates why ROS inducers are highly effective for treating RMS since this pathway leads to a cascade of events leading to downregulation of Sp TFs and pro-oncogenic Sp-regulated genes. ROS inducers and other drugs targeting Sp TFs represent promising new approaches for RMS chemotherapy; moreover, since miRs that repress ZBTB repressors have been detected in serum (49, 50), this may also provide a biomarker for monitoring treatment efficacy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ARMS	alveolar rhabdomyosarcoma
ChIP	chromatin immunoprecipitation
c-MET	hepatocyte growth factor receptor

DMSO	dimethyl sulfoxide
ERMS	embryonal rhabdomyosarcoma
HDAC	histone deacetylase
HSMM	human skeletal muscle myoblast
IGFR	insulin-like growth factor receptor
NOA	non-oncogene addiction
PDGFRα	platelet-derived growth-factor receptor α
RMS	rhabdomyosarcoma
ROS	reactive oxygen species
Sp1	specificity protein 1
TFs	transcription factors

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Figure 1.

ROS-dependent inhibition of RMS cell growth by HDAC inhibitors. (A) RD and Rh30 cells were treated with DMSO, 100 nM panobinostat, or 1 μ M vorinostat alone or in combination with 5 mM GSH, and ROS was determined fluorimetrically. (B) RD and Rh 30 cells were treated with panobinostat or varinostat and after 24 hr, cell growth was determined by counting cells in a Coulter counter. (C) Metabolic activity was also determined in RD, Rh30 and HSMM cells treated with panobinostat. (D) RD and Rh30 cells were treated with DMSO, panobinostat and vorinostat alone or in combination with 5 mM GSH, and cells were counted using a Coulter counter. Results are expressed means $\pm$ SE for at least 3 replicated determinations and significant ($p < 0.05$) induction of ROS or growth inhibition (*) or reversal by GSH (**) is indicated.

Figure 2.

ROS-dependent induction of apoptosis, inhibition of invasion and downregulation of Sp proteins by panobinostat and vorinostat. RD and Rh30 cells were treated with DMSO, panobinostat or vorinostat alone or in combination with 5 mM GSH, and induction of Annexin V staining (A) or inhibition of invasion (B) were determined by fluorescence and a Boyden chamber assay, respectively. Results are expressed as means $\pm$ SE for at least 3 replicate determinations and significant ($p < 0.05$) induction of Annexin V staining or inhibition of invasion (*) and inhibition by GSH (**) are indicated. RD and Rh30 cells were treated with panobinostat (C) or vorinostat (D) alone or in combination with 5 mM GSH for 24 hr, and whole cell lysates were analyzed by western blots. The two Sp3 bands in all gels represent the full length (115 kD) and truncated (80 kD) forms.

Figure 3.

Hydrogen peroxide and *t*-butylhydroperoxide inhibit RMS cell growth and decreases Sp1, Sp3 and Sp4 in RMA cells. Rh30 and RD cells were treated with hydrogen peroxide (A) and *t*-butylhydroperoxide (B), and effects on cell proliferation were determined using a Coulter counter. Results are expressed as means $\pm$ SE for at least 3 replicate determinations and significant ($p < 0.05$) growth inhibition (*) is indicated. Rh30 (C) and RD (D) cells were treated with hydrogen peroxide and *t*-butylhydroperoxide for 24 hr, and whole cell lysates were analyzed by western blots.

Figure 4.

Panobinostat decreases Myc and Sp TFs through epigenetic pathways. (A) RD and Rh30 cells were treated with panobinostat for different times, and whole cell lysates were analyzed for cMyc and Sp TFs by western blot analysis. (B) After Knockdown of Sp1 by RNA interference (siSp1), whole cell lysates were analyzed for cMyc expression by western blots. (C) Lysates from (A) were also analyzed by western blots for selected Sp-regulated genes. RD (D) and Rh30 (E) cells were treated with DMSO or panobinostat for 3 or 6 hr and analyzed in a ChIP assay using antibodies against pol II, IgG (control), and selected histone marks.

Figure 5.

Panobinostat regulates cMyc, and cMyc and Sp TFs regulate miR-27a and miR-20/miR-17 in RMS cells. RMS cells were treated with panobinostat alone or in combination with GSH for 24 hr, and whole cell lysates were analyzed by western blots (A) or analyzed for miR expression (B) by real time PCR. RD and Rh30 cells were transfected sicMyc (C) or siSp1, siSp3 and siSp4 (D), and miR expression was determined by real time PCR. Results (B-D) are expressed as means $\pm$ SE for 3 replicate determinations and significantly ($p < 0.05$) decreased mir expression is indicated (*).

Figure 6. Panobinostat and cMyc knockdown induce ZBTB transcriptional repressors; sicMyc decreases Sp TFs in RMS cells; and panobinostat inhibits growth of RMS xenograft tumors. (A) RD and Rh30 cells were treated with panobinostat for up to 24 hr, and whole cell lysates were analyzed by western blots. (B) RD and Rh30 cells were transfected with sicMyc or antagomiR-27a (Ant27a), and whole cell lysates were analyzed for ZBTB and Sp proteins by western blots. (C) Panobinostat (17.5 mg/kg) was administered to SCID mice and tumor volumes and weights were determined. (D) Mechanism of action of ROS-inducing HDAC inhibitors in RMS cells. ROS induces downregulation of Myc and Sp1, resulting in decreased expression of Myc/Sp1-regulated miRs and induction of miR-regulated transcriptional repressors (ZBTB). The ZBTB repressors competitively bind GC-rich promoter elements to decrease expression of Sp-regulated genes.