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Evaluation of entinostat alone and in combination with standard-of-care cytotoxic agents against rhabdomyosarcoma xenograft models

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Abstract

Background: Entinostat, a selective class I histone deacetylase inhibitor, has been reported to enhance the activity of cytotoxic agents and suppress expression of PAX3-FOXO1 in alveolar rhabdomyosarcoma (ARMS).

Procedures: Entinostat was tested against three rhabdomyosarcoma cell lines using 96-hour drug exposure. Entinostat alone or in binary combination with vincristine, actinomycin D or cyclophosphamide was tested in ARMS and two embryonal rhabdomyosarcoma (ERMS) xenograft models. Tumor growth was measured at weekly intervals. Drug-induced changes in acetylated histone H3(K9) and entinostat pharmacokinetics were determined.

Results: In vitro, the IC₅₀ concentration of entinostat ranged from 280 to 1300 nM. In vivo, entinostat significantly inhibited the growth of only Rh10 xenografts. For most studies, entinostat did not potentiate the activity of the cytotoxic agent. Exceptions included the vincristine and entinostat combination for Rh10 and the entinostat and actinomycin D combination for Rh10 and Rh18, although the effects were modest. For Rh18, the combination of entinostat with vincristine showed evidence of an antagonistic interaction compared with single-agent vincristine. Pharmacokinetic studies showed the average C_{max} was 569.4 ng/mL (1.51 μ M) with T_{max} at 15 minutes, and total exposure (AUC_{0-12 h}) was 435.6 h $\times$ ng/mL. Entinostat treatment increased acetylated histone H3.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CONFLICTS OF INTEREST

The authors have declared that no conflict of interest exists.

Conclusions: Entinostat demonstrated modest antitumor activity in only one of four models at dose and schedule that gave drug exposures relevant to human treatment. The addition of entinostat to standard-of-care cytotoxic agents was in most instances no more effective than the cytotoxic agents used alone. Entinostat demonstrated target inhibition with increased histone 2A acetylation.

Keywords

chemotherapy; pediatric oncology; pharmacokinetics; pharmacology; rhabdomyosarcoma

1| INTRODUCTION

There is increasing evidence that the cause of progression of many childhood cancers may have a strong epigenetic basis. For tumors such as malignant rhabdoid tumor, loss of *SMARCB1*, a component of the SWI/SNF chromatin remodeling complex, is one of the only genetic changes.¹ Similarly, in synovial sarcoma, mutations that lead to functional dysregulation of the BAF complex are tumorigenic.² Mutations in H3.1K27 and H3.3K27 or H3K36 inhibit their methyltransferases in glioblastoma and sarcoma, respectively.^{3,4} In glioblastoma, these dominant-negative histone mutations result in global reprogramming of histone marks. Thus, epigenetic modulation has become a major target for drug development with an armamentarium of drugs that target histone “writers” (DNA methyltransferases), “readers” (bromodomain proteins recruited to sites of active transcription), and “erasers” (histone deacetylases).⁵

Transcription is regulated by many posttranslational processes, including methylation and acetylation of histones. These processes are mediated by histone lysine methyltransferases (KMTs) and demethylases (KDMs) and histone acetyltransferases (HATs) and deacetylases (HDACs) that balance access of transcriptional machinery required to initiate and elongate mRNA molecules leading to efficient transcription.⁶ In simplistic terms, the state of acetylation of histones dictates the open chromatin formation that is transcriptionally active.⁷ However, the function of specific histone marks that are associated with promoters of actively transcribed genes (histone H3 lysine 4 trimethylation, and histone H3 and H4 acetylation) may be context specific and have higher levels of trimethylation of both H3K36 and H3K79 in the gene body. H3K27ac and H3K4me1, but lower relative levels of H3K4me3, seem to mark active cis-regulatory enhancer elements. In contrast, H3K9 methylation and trimethylation of H3K27 and H4K20 mark repressed genes. H3K9ac is associated with active gene promoters in association with H3K4me3, and may be involved in the recruitment of bromodomain protein 4 (BRD4) and to mediate POLII pause release from promoter proximal regions, allowing mRNA elongation.^{6,8–10}

HDACs remove acetyl marks from histones and other proteins, such as transcription factors (TP53, NF- κ B, GATA-1, and Bcl-6) Ku70, Hsp90, and α -tubulin and in general lead to suppression of gene expression.^{6,7} Eighteen HDACs have been identified and are classified based on their structural conservation. Zinc-dependent HDACs comprise classes I, II, and IV, whereas nicotinamide adenine dinucleotide (NAD⁺)-dependent HDACs comprise class III enzymes. Class I HDACs are predominantly nuclear (HDACs 1, 2, 3, and 8) and are ubiquitously expressed in tissues. These enzymes have an N-terminal deacetylase domain,

with greatest variation in the C-terminus. Class 2 enzymes may be both nuclear and cytoplasmic and, with the exception of HDAC6, have a C-terminal deacetylase domain.¹¹ HDAC6 is unique in having two deacetylase domains.

Class I HDAC enzymes, HDAC2 and HDAC3, are strongly expressed in subgroups of breast cancer with aggressive characteristics,¹² and in sarcoma HDACs 1,4,6,7, and 8 were associated with advanced disease and lower disease-free survival.¹³ Other reports link upregulation of HDAC4 to chordoma pathogenesis.^{14,15}

Several HDAC inhibitors have been approved by the US Food and Drug Administration for the treatment of cutaneous T-cell lymphoma.^{7,16} Entinostat (MS-275, SNDX-275) has received breakthrough therapy status in estrogen-positive breast cancer.¹⁷ Vorinostat is an inhibitor of class I and II enzymes, whereas entinostat inhibits only class I enzymes.¹⁸ Although the single-agent activity of HDAC inhibitors is relatively restricted, combination of entinostat potentiated the activity of the aromatase inhibitor exemestane,¹⁹ and it is speculated that epigenetic modifiers such as HDAC or DNA methyltransferase inhibitors may resensitize tumor cells with acquired resistance to cytotoxic agents^{20,21} and radiation.^{22,23} Entinostat is also reported to have preclinical activity against pediatric solid tumors.²⁴ Recently, Abraham et al. reported that entinostat treatment downregulated the PAX3:FOXO1 fusion oncogene in preclinical models of ARMS through direct transcriptional repression.²⁵ Further, limited in vivo data suggested that entinostat could enhance the activity of actinomycin D. We were therefore interested in determining the antitumor activity of entinostat alone or in combination with standard-of-care agents in xenograft models of RMS.

2 | MATERIALS AND METHODS

2.1 | Cell lines and xenografts

All RMS cell lines and xenografts were developed in this laboratory, and authenticity was confirmed against a reference short tandem repeat (STR) profile established on early-passage lines. Rh10, Rh41, and Rh65 t(2;13) translocation-positive alveolar rhabdomyosarcomas (ARMS) are patient-derived xenografts (PDX) obtained at autopsy (Rh10) or relapse on conventional multimodality therapy. In addition, we evaluated the sensitivity of two embryonal rhabdomyosarcoma (ERMS): Rh18 (established as a PDX at diagnosis) and Rh36 (established as a PDX at relapse). Rh30, Rh41, and Rh18 continuous cell lines were derived from their respective xenografts, and were grown in RPMI-1640 with 10% FBS without antibiotics. Cell lines and xenografts were authenticated by STR profiling and compared with profiles archived from early-passage tumor tissue.

2.2 | In vitro growth inhibition studies

Sarcoma cells were cultured in RPMI medium and were seeded on 6-well plates at a density of approximately 1000 cells/well and allowed to attach overnight. Entinostat was dissolved in DMSO, and used at final concentrations of <0.1% DMSO in medium. Cells were treated with various concentrations of entinostat one day after seeding. After four days, Calcein AB (Life Technologies) was added directly into culture media at a final concentration of 10%,

and the plates were incubated at 37°C. Optical density was measured spectrophotometrically at 540 and 630 nm after adding Calcein AB for 3 to 4 hours. As a negative control, Calcein AB was added to the medium without cells.²⁶ The IC₅₀ concentration was calculated by interpolation (Prism Software).

2.3 | Pharmacokinetics studies

The pharmacokinetics (PK) of entinostat were determined in non-tumor-bearing C.B.17SC *scid*^{-/-} female mice (Taconic Farms, Germantown, NY). Mice received twice-daily (b.i.d.) oral dosing at 2.5 mg/kg for 7 doses (two doses on days 1, 2, and 3; one dose on day 4). Blood for PK was collected via cardiac puncture on anesthetized mice at predose of the day 4 dose, and 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours, and 12 hours after dose on day 4. Each time point was collected in triplicate (21 mice total). The blood was collected into EDTA-treated tubes, centrifuged to obtain plasma, transferred to cryovials, and stored at -80°C until analysis. Mice were euthanized under anesthesia after blood collection.

Entinostat mouse plasma concentrations were measured using a validated LC/MS assay that was adapted from a human plasma assay.²⁷ Briefly, 100 μ L of mouse plasma was aliquoted and mixed with 1 mL of methyl t-butyl ether containing 100 ng/mL of the internal standard benzanilide. After vortex and centrifugation, the organic supernatant was transferred to a 96-well plate, evaporated, and reconstituted in (65/35) 10 mM NH₄COH, pH 2.9/methanol directly in the plate. The assay calibration range was 1 to 250 ng/mL, with a validated dilution up to 1000 ng/mL.

The maximum plasma concentration ($C_{\max}$) was recorded as the time point ($T_{\max}$) with the highest average ($n = 3$) concentration. The area under the curve to the last time point (AUC_{0-12}) was calculated using the linear trapezoidal rule with adjustments for destructive sampling²⁸ and the Bailer's method for estimating variance around the estimated AUC.²⁹ The elimination rate (K_{EL}) was the slope of the line through the terminal natural log-transformed average plasma concentrations; half-life ($T_{1/2}$) was calculated as natural log 2/ K_{EL} . Clearance (CL) was calculated as dose/AUC; volume of distribution was calculated as CL/ K_{EL} .

2.4 | Pharmacodynamic studies

In vitro, cells were exposed to entinostat at over a concentration range (1–2000 nM) that covered its physiological $C_{\max}$ (~120 nM³⁰) for 24 or 48 hours. Levels of PAX3, FOXO1, and acetylated histone H3(K9) were determined by immunoblotting.²⁶ For Rh10 and Rh65 xenografts, mice were treated as in the therapeutic protocol (2.5 mg/kg twice daily for 4 days) for up to three consecutive weeks. Tumors ($n = 3$) were harvested at 4 hours after dose 7 (week 1), dose 15 (week 2), or dose 23 (week 3), and lysates prepared for immunoblotting as previously described.³¹ Antibodies used to detect PAX3 (#sc-81351 mouse monoclonal, Santa Cruz Biotechnology, Dallas), acetyl-histone H3(K9) rabbit monoclonal (#9649, Cell Signaling Technology, Danvers, MA), and FOXO1 (#2880 rabbit monoclonal; Cell Signaling) were used at 1:100, 1:2,000, and 1:1,000 dilutions, respectively.

Secondary antibodies were rabbit IgG, HRP linked (#7074), mouse IgG, HRP linked (7076) (Cell Signaling) and used at a dilution of 1:5000.

2.5 | In vivo testing

C.B.17SC *scid*^{-/-} (C.B-*Igh-1^b/IcrTac-Prkdc^{scid}*) female mice were used to propagate subcutaneous xenografts. All mice were maintained under barrier conditions, and experiments were conducted using protocols and conditions approved by the institutional animal care and use committee at UTHSCSA. Ten mice were used in each control or treatment group. Tumor volumes (cm³) were determined, and responses were determined using activity measures similar to those previously described.³² Details of the statistical analytic methods are provided in Appendix 1.

2.6 | Drugs and formulation

Entinostat was provided to the Pediatric Preclinical Testing Consortium by Syndax Pharmaceuticals Inc., through the Cancer Therapy Evaluation Program (NCI). Entinostat was formulated as a 0.25 mg/mL suspension in 0.5% methylcellulose in sterile water with brief sonication to disperse the drug. Solutions were stored for up to 7 days at 4°C, with sonication before dosing if necessary. Entinostat was administered twice daily (b.i.d.) by oral gavage (0.1 mL/10 g body weight) for four consecutive days for three consecutive weeks (days 1–4, 7–11, and 14–18). Entinostat was provided in coded vials for blinded testing. Cyclophosphamide (150 mg/kg), vincristine (1 mg/kg) were administered i.p. every 7 days for three consecutive weeks, with or without entinostat. Actinomycin D (0.325 mg/kg), or at reduced dose (0.275 mg/kg) when combined with entinostat, due to toxicity, was administered on day 1 of the first cycle. Irinotecan was administered i.p. at 2.5 mg/kg days 1–5.

2.7 | Statistical methods

The Peto and Peto modification of the Gehan-Wilcoxon test was used to compare event-free survival distributions between treatment and control groups. To control experiment-wise Type I error at 5%, statistical tests are evaluated at the Bonferroni-corrected significance level $\alpha = 0.0125$, due to the four possible comparisons being made (tumor (entinostat vs control, cytotoxic agent vs control, combination vs control, and combination vs cytotoxic agent).

3. | RESULTS

3.1 | In vitro testing

The sensitivity of the Rh30, Rh41 (ARMS), and Rh18 (ERMS) cell lines was assessed by Calcein AB staining after 4 days of exposure to increasing concentrations of entinostat. IC₅₀ concentrations from duplicate experiments were 265 nM (Rh41), 840 nM (Rh18), and 1110 nM (Rh30) (Figure 1A). The levels of acetylated histone H3(K9), PAX3, FOXO1, and the PAX3-FOXO1 fusion protein were determined at 24 and 48 Hours for cells exposed to different concentrations of entinostat (Figure 1B). Increased acetylated histone H3 was detected at concentrations of 500 nM or above in both ARMS cell lines, although Rh18 cells appeared slightly less sensitive with increases detected at 1 μ M or above. PAX3-FOXO1 was

decreased in Rh30 cells at concentrations of entinostat above 500 nM and in Rh41 cells at 1 or above. Rh18 is a fusion-negative ERMS, hence does not express the PAX3-FOXO1 band. Entinostat had only slight effects on endogenous PAX3 (Rh30, Rh41) and inconsistent effect on endogenous FOXO1.

3.2 | Pharmacokinetics study

Entinostat plasma concentrations were measured for 21 mice, and the average ($n = 3$) value at each time point was plotted (Figure 1C). The average observed $C_{\max}$ was 569 ng/mL (1.51 μM) that occurred at the first sampling point after dose ($T_{\max} = 15$ minutes). The total exposure (AUC_{0-12}) was 436 h $\times$ ng/mL with a rapid early distribution phase, followed by a slower terminal elimination phase with a $T_{1/2}$ of 14.7 hours. The distribution volume (116 mL) was much larger than blood volume, consistent with its hydrophobicity ($\text{LogD} = 1.79$). Entinostat demonstrated a plasma clearance rate of 143 mL/h. The predose plasma levels (i.e., 16 hours after dose 6 to mice and 12 hours after dose number 7) were 0.8 ng/mL and 3.8 ng/mL, respectively

3.3 | Single-agent testing

The activity of entinostat as single-agent and of standard-of-care agents (vincristine, actinomycin D, cyclophosphamide, and irinotecan) are presented in Table 1. Entinostat inhibited the growth of Rh10 tumors, relative to controls, by 22 days ($P < 0.001$; PD2) (Figure 2) and Rh36 xenografts by 2 days ($P = 0.004$; PD1) (Figure 3). Growth of neither of the other two models (Rh18 and Rh41) was significantly inhibited. No tumor regressions were observed. In contrast, vincristine induced significant growth delays for all models and regressions in 3 of 4 lines. Actinomycin D was less active, but significantly inhibited the growth of 3 PDX models, although delays in time to event were small (5.5–7.3 days) and no tumor regressions were noted. Cyclophosphamide induced regressions in all four PDX models with delays in time to event from 37 to >87 days, and irinotecan induced PRorCR in three of four models with significant delays in time to event for all four models (14–45 days).

3.4 | Combination testing

Entinostat was combined with full doses of vincristine, cyclophosphamide, and irinotecan, but the dose of actinomycin D had to be reduced from 0.325 mg/kg to 0.275 mg/kg in combination. Results for combination testing are presented in Table 1 and are shown in Figure 2 for Rh10 and Rh18 and Figure 3 for Rh36 and Rh41. Kaplan-Meier event-free survival (EFS) plots for all treatments are shown in Figure 4.

The entinostat plus vincristine combination was significantly superior to single-agent vincristine in prolonging time to event only for Rh10, the line for which entinostat most significantly prolonged time to event as a single agent. The minimum RTV was also significantly smaller for the combination for Rh10. There was a trend toward antagonism for the vincristine plus entinostat combination for Rh18, as the combination showed reduced time to event compared with single-agent vincristine, and the minimum RTV for the combination was significantly greater than that observed for single-agent vincristine.

For actinomycin D, time to event was significantly prolonged and minimum RTV was significantly reduced for its combination with entinostat compared with single-agent actinomycin D for Rh 10 and Rh18, although the combination induced only PD2 and PD1 responses, respectively, in these models. For cyclophosphamide, the combination compared with the single-agent produced no significant changes in either time to event or minimum RTV, and similarly for irinotecan the combination was no more effective than single-agent irinotecan.

3.5 | Pharmacodynamic studies

For the determination of target inhibition, mice bearing Rh10 and Rh65 (ARMS) were treated with entinostat (2.5 mg/kg b.i.d. × 4 days) for 1, 2, or 3 consecutive weeks. Tumors were harvested 4 hours after the seventh dose each week, and rapidly frozen in liquid N₂ and stored at −80°C until assayed. Levels of PAX3-FOXO1, PAX3, and acetyl-histone H3 were assayed by immunoblotting. There was a progressive increase in acetylated histone H3 in both tumor models, indicating target inhibition by entinostat (Figure 5). PAX3 and FOXO1 were not consistently changed by drug treatment, whereas PAX3-FOXO1 was decreased after 1 and 2 weeks of treatment in Rh10 tumors, but not after 3 weeks of treatment. There was a slight decrease in PAX3-FOXO1 in Rh65 xenografts.

4 | DISCUSSION

Entinostat inhibited rhabdomyosarcoma cells in vitro with IC₅₀ concentrations between 240 and 1300 nM. Obvious increases in acetylated histone H3 were observed at entinostat concentrations of 500 nM and above. In agreement with the studies from Abraham et al., there was a modest decrease in PAX3-FOXO1 protein in both Rh30 and Rh41 cells,²⁵ although this did not correspond to sensitivity to entinostat of these cell lines.

Dosing of entinostat for preclinical studies in mice is inherently challenging because of the dramatic differences in pharmacokinetic behavior of entinostat between mice and humans. In humans, entinostat has a terminal half-life that approaches or exceeds 50 hours.³³ When given on a weekly schedule, entinostat produces $C_{\max}$ concentrations in the 30–60 ng/mL range (80–160 nM) with concentrations decreasing to less than 5 ng/mL (~13 nM) within 24 hours and then lower thereafter for six days for doses of 5–6 mg/m².^{34–36} In mice, by contrast, entinostat has a much shorter half-life, and hence to maintain trough levels in the 2–5 ng/mL range, it is necessary to dose more frequently with resultant higher peak concentrations during the dosing period and higher weekly systemic exposure compared with humans. The 2.5 mg/kg twice daily for four days dosing schedule that we used to model the weekly dosing schedule used in humans maintained predose trough levels near the 2–5 ng/mL range (5–13 nM). The twice-daily peak drug levels (569 ng/mL) for the entinostat dose/schedule that we used are much higher than $C_{\max}$ values observed in humans, as is the systemic drug exposure in mice per one week treatment course of approximately 3500 h × ng/mL. Within the limitations imposed by the markedly different pharmacokinetic profiles of entinostat in mice and humans, we believe that the dose and schedule utilized provides a sufficient approximation to human exposure so as to produce

results with relevance to the clinical setting with low likelihood for underpredicting the true activity of entinostat for rhabdomyosarcoma.

Our in vivo results can be compared with those of Abraham et al., who described testing for the entinostat and actinomycin D for a single mouse model of ARMS,²⁵ and to those of Bharathy et al., who studied entinostat in combination with vincristine for PDX models of ARMS.³⁷

Abraham et al. used a higher dose of entinostat (10 mg/kg with reduction to 5 mg/kg for toxicity) and a duration of treatment (9 days) than we used. Toxicity limited their ability to compare treatment arms beyond 7 days from treatment initiation. In their experiment, singleagent entinostat slowed tumor growth and the combination with actinomycin D appeared to slow tumor growth more than either of the single agents. Bharathy et al. used entinostat administered daily for 21 days at doses of 4 or 25 mg/kg, producing daily exposure to high entinostat levels in contrast to the exposure profile observed in the clinical setting.³⁷ The impact of the addition of entinostat to the activity of vincristine for the PDX models tested was modest and did not result in statistically significant differences in tumor volume. We confirm that entinostat can cause the slowing of tumor growth for ARMS models, as illustrated by the PD2 response against Rh10. However, entinostat as a single agent had little or no antitumor effect against the other ARMS model (Rh41) or two ERMS models. With regard to the activity for entinostat combined with cytotoxic agents, enhanced activity for the combination compared with the single chemotherapy agent was observed in only 3 of 16 experiments (Rh10 for actinomycin D and vincristine and Rh18 for actinomycin D). By contrast, entinostat showed a trend toward antagonism of the vincristine treatment effect for Rh18. Our results indicate that entinostat does not have a broad effect on enhancing the activity of standard chemotherapy agents in rhabdomyosarcoma preclinical models.

In summary, we have evaluated entinostat at a dose and schedule of administration that designed to simulate entinostat exposure in patients, within the limitations imposed by mouse versus human differences in entinostat pharmacokinetics. At the dose and schedule used, there was pharmacodynamic evidence of HDAC inhibition at 4 hours after dosing in the ARMS models studied. However, the in vivo preclinical activity of entinostat as a single agent was modest, and in most cases its combination with cytotoxic agents did not result in significantly increased antitumor activity beyond that of the cytotoxic agents themselves.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Funding information

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Abbreviations:

ARMS	alveolar rhabdomyosarcoma
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AUC_{0–12}	area under the concentration x time curve 0–12 h
C_{max}	maximum drug concentration
ERMS	embryonal rhabdomyosarcoma
PK	pharmacokinetic
STR	short tandem repeat
T_{max}	time to C _{max}

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FIGURE 1.

A, Sensitivity of rhabdomyosarcoma cell lines to entinostat. Cells were exposed to increasing concentrations of drug for 4 days, and viability assessed by Calcein Alamar Blue staining. Each concentration was done in triplicate ($\pm$ SD), and duplicate experiments are presented (e.g., Rh18–1 and Rh18–2 etc.). The IC₅₀ concentrations were Rh18 (840/840 nM), Rh30 (1300/920 nM), and Rh41 (280/250 nM), respectively, for duplicate experiments. **B**, Cells were exposed to concentrations of entinostat shown for 24 or 48 hours, and immunoblots stained for PAX3, FOXO1 acetylated histone H3(K9). Rh30 and Rh41 ARMS

cells express the PAX3-FOXO1 fusion oncoprotein, whereas Rh18 is fusion negative. **C**, Plasma pharmacokinetics for entinostat in non-tumor-bearing SCID female mice. Mice received entinostat (2.5 mg/kg b.i.d., p.o.) for 7 administrations and blood sampled from 15 minutes to 12 hours ($N = 3$ /time point). Mean $\pm$ SD ($n = 3$)

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FIGURE 2.

A, Sensitivity of Rh10 ARMS xenografts treated with (top left) control (vehicle), (top right) entinostat (2.5 mg/kg twice daily $\times$ 4 days PO for 3 consecutive weeks), (bottom left) vincristine (1 mg/kg q 7 days $\times$ 3 i.p.), or (bottom right) the combination of entinostat and vincristine. Each curve shows the growth of an individual tumor. **B**, Sensitivity of Rh18 embryonal rhabdomyosarcoma xenografts treated with (top left) control (vehicle), (top right) entinostat (2.5 mg/kg twice daily $\times$ 4 days PO for 3 consecutive weeks), (bottom left)

vincristine (1 mg/kg q 7 days x 3 i.p.), or (bottom right) the combination of entinostat and vincristine. Each curve shows the growth of an individual tumor

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FIGURE 3.

A. Sensitivity of Rh36 embryonal rhabdomyosarcoma xenografts treated with (top left) control (vehicle), (top right) entinostat (2.5 mg/kg twice daily $\times$ 4 days p.o. for 3 consecutive weeks), (bottom left) vincristine (1 mg/kg q 7 days $\times$ 3 i.p.), or (bottom right) the combination of entinostat and vincristine. Each curve shows the growth of an individual tumor. **B.** Sensitivity of Rh41 embryonal rhabdomyosarcoma xenografts treated with (top left) control (vehicle), (top right) entinostat (2.5 mg/kg twice daily $\times$ 4 days p.o. for 3 consecutive weeks), (bottom left) vincristine (1 mg/kg q 7 days $\times$ 3 i.p.), or (bottom right)

the combination of entinostat and vincristine. Each curve shows the growth of an individual tumor

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FIGURE 4.

EFS for each single-agent treatment and combination treatments. The time for a tumor to “event” (defined as 400% of the volume on the first day of treatment) is plotted against time (days after treatment initiation)

FIGURE 5.

Entinostat-induced changes in histone H3 acetylation and in expression of PAX3, FOXO1, and the PAX3-FOXO1 oncogenic fusion protein in ARMS xenografts ($n = 3$). Mice bearing Rh10 or Rh65 ARMS received entinostat (2.5 mg/kg b.i.d.) for 7 doses (week 1), 15 doses (week 2), or 23 doses (week 3). Tumors were harvested 4 hours after the last dose of entinostat and processed for immunoblotting as described in Materials and Methods

TABLE 1

Summary of in vivo single-agent and combination testing

Testing groups	Line	KM med	EFST-C	EFST/C	P value ^a	P value ^b	minRTV mean $\pm$ SD	minRTV P value ^c	Median response
Vincristine									
Entinostat	Rh10	35.1	22.4	2.77	$P < 0.001$		1.222 $\pm$ 0.220		PD2
Vincristine	Rh10	29.5	16.9	2.34	$P < 0.001$		1.441 $\pm$ 0.829		PD2
Combination	Rh10	45.2	32.6	3.58	$P < 0.001$	$P < 0.001$	0.641 $\pm$ 0.263	$P < 0.001$	PD2
Entinostat	Rh18	13.3	-0.6	0.95	$P = 0.707$		1.897 $\pm$ 0.351		PD1
Vincristine	Rh18	84.4	70.5	6.07	$P < 0.001$		0.903 $\pm$ 1.169		PR
Combination	Rh18	59.1	45.2	4.25	$P < 0.001$	$P = 0.123$	1.591 $\pm$ 0.936	$P < 0.001$	PD2
Entinostat	Rh36	19.5	2.1	1.12	$P = 0.002$		1.506 $\pm$ 0.270		PD1
Vincristine	Rh36	> 105	> 87.6	> 6.03	$P < 0.001$		0.000 $\pm$ 0.000		MCR
Combination	Rh36	> 105	> 87.6	> 6.03	$P < 0.001$	$P = 0.608$	0.000 $\pm$ 0.000	$P = 0.400$	MCR
Entinostat	Rh41	13.3	1.0	1.08	$P = 0.328$		2.042 $\pm$ 0.659		PD1
Vincristine	Rh41	64.8	52.5	5.25	$P < 0.001$		0.159 $\pm$ 0.255		MCR
Combination	Rh41	60.9	48.5	4.93	$P < 0.001$	$P = 0.498$	0.019 $\pm$ 0.041	$P = 0.105$	MCR
Actinomycin D									
Entinostat	Rh10	35.1	22.4	2.77	$P < 0.001$		1.222 $\pm$ 0.220		PD2
Actinomycin	Rh10	18.2	5.5	1.44	$P = 0.019$		2.331 $\pm$ 0.996		PD1
Combination	Rh10	32.4	19.7	2.56	$P < 0.001$	$P < 0.001$	0.727 $\pm$ 0.419	$P < 0.001$	PD2
Entinostat	Rh18	13.3	-0.6	0.95	$P = 0.707$		1.897 $\pm$ 0.351		PD1
Actinomycin	Rh18	10.7	-3.2	0.77	$P = 0.003$		2.072 $\pm$ 0.488		PD1
Combination	Rh18	28.5	14.6	2.05	$P < 0.001$	$P < 0.001$	1.279 $\pm$ 0.253	$P < 0.001$	PD1
Entinostat	Rh36	19.5	2.1	1.12	$P = 0.002$		1.506 $\pm$ 0.270		PD1
Actinomycin	Rh36	24.7	7.3	1.42	$P < 0.001$		1.048 $\pm$ 0.251		PD1
Combination	Rh36	22.9	5.5	1.32	$P = 0.001$	$P = 0.251$	1.232 $\pm$ 0.328	$P = 0.243$	PD1
Entinostat	Rh41	13.3	1.0	1.08	$P = 0.328$		2.042 $\pm$ 0.659		PD1
Actinomycin	Rh41	18.0	5.6	1.45	$P < 0.001$		1.791 $\pm$ 0.450		PD1
Combination	Rh41	18.7	6.4	1.52	$P < 0.001$	$P = 0.516$	1.653 $\pm$ 0.415	$P = 0.497$	PD1
Cyclophosphamide									
Entinostat	Rh10	35.1	22.4	2.77	$P < 0.001$		1.222 $\pm$ 0.220		PD2

Testing groups	Line	KM med	EFST-C	EFST/C	P value ^a	P value ^b	minRTV mean ± SD	minRTV P value ^c	Median response
Cyclophosphamide	Rh10	84.0	71.4	6.65	$P < 0.001$		0.000 ± 0.000		MCR
	Rh10	93.8	81.2	7.43	$P < 0.001$	$P = 0.428$	0.000 ± 0.000	$P = 0.825$	MCR
	Rh18	13.3	-0.6	0.95	$P = 0.707$		1.897 ± 0.351		PD1
	Rh18	73.3	59.4	5.27	$P < 0.001$		0.277 ± 0.450		PR
Combination	Rh18	79.1	65.2	5.69	$P < 0.001$	$P = 0.258$	0.037 ± 0.059	$P = 0.123$	MCR
	Rh36	19.5	2.1	1.12	$P = 0.002$		1.506 ± 0.270		PD1
Combination	Rh36	> 105	> 87.6	> 6.03	$P < 0.001$		0.000 ± 0.000		MCR
	Rh36	102.8	85.4	5.91	$P < 0.001$	$P = 0.075$	0.000 ± 0.000	$P = 0.190$	MCR
Combination	Rh41	13.3	1.0	1.08	$P = 0.328$		2.042 ± 0.659		PD1
	Rh41	50.0	37.7	4.05	$P < 0.001$		0.187 ± 0.219		PR
Combination	Rh41	51.1	38.8	4.14	$P < 0.001$	$P = 0.687$	0.079 ± 0.131	$P = 0.400$	CR
Irinotecan									
Combination	Rh10	35.1	22.4	2.77	$P < 0.001$		1.222 ± 0.220		PD2
	Rh10	58.1	45.4	4.59	$P < 0.001$		0.000 ± 0.000		CR
Combination	Rh10	61.2	48.6	4.85	$P < 0.001$	$P = 0.194$	0.000 ± 0.000	$P = 0.278$	CR
	Rh18	13.3	-0.6	0.95	$P = 0.707$		1.897 ± 0.351		PD1
Combination	Rh18	32.3	18.4	2.33	$P = 0.001$		0.812 ± 1.289		SD
	Rh18	32.7	18.8	2.35	$P < 0.001$	$P = 0.940$	0.930 ± 0.244	$P = 0.005$	PD2
Combination	Rh36	19.5	2.1	1.12	$P = 0.002$		1.506 ± 0.270		PD1
	Rh36	31.4	14.0	1.80	$P < 0.001$		0.333 ± 0.316		PR
Combination	Rh36	27.9	10.5	1.60	$P < 0.001$	$P = 0.597$	0.167 ± 0.099	$P = 0.529$	PR
	Rh41	13.3	1.0	1.08	$P = 0.328$		2.042 ± 0.659		PD1
Combination	Rh41	35.6	23.2	2.88	$P < 0.001$		0.373 ± 0.164		PR
	Rh41	31.6	19.3	2.56	$P < 0.001$	$P = 0.164$	0.367 ± 0.234	$P = 0.912$	PR

^a P value for EFS distribution for treatment group versus control.

^b P value for EFS distribution for combination versus single-agent treatment groups.

^c P value for comparison of minimum RTV for combination versus the single chemotherapy agent treatment groups.