Combining PCI-24781, a Novel Histone Deacetylase Inhibitor, with Chemotherapy for the Treatment of Soft Tissue Sarcoma

Gonzalo Lopez\textsuperscript{1,2}, Juehui Liu\textsuperscript{2,3}, Wenhong Ren\textsuperscript{2,3}, Wei Wei\textsuperscript{6}, Suizhao Wang\textsuperscript{2,3}, Guy Lahat\textsuperscript{2,3}, Quan-Sheng Zhu\textsuperscript{2,3}, William G. Bornmann\textsuperscript{4}, David J. McConkey\textsuperscript{1,5}, Gonzalo Lopez\textsuperscript{2,3}, William G. Bornmann\textsuperscript{4}, David J. McConkey\textsuperscript{1,5}, Raphael E. Pollock\textsuperscript{2,3}, and Dina C. Lev\textsuperscript{1,2}

Abstract

Purpose: Histone deactylase inhibitors (HDACi) are a promising new class of anticancer therapeutics; however, little is known about HDACi activity in soft tissue sarcoma (STS), a heterogeneous cohort of mesenchymal origin malignancies. Consequently, we investigated the novel HDACi PCI-24781, alone/in combination with conventional chemotherapy, to determine its potential anti-STS-related effects and the underlying mechanisms involved.

Experimental Design: Immunoblotting was used to evaluate the effects of PCI-24781 on histone and nonhistone protein acetylation and expression of potential downstream targets. Cell culture–based assays were utilized to assess the effects of PCI-24781 on STS cell growth, cell cycle progression, apoptosis, and chemosensitivity. Quantitative reverse transcription-PCR, chromatin immunoprecipitation, and reporter assays helped elucidate molecular mechanisms resulting in PCI-24781–induced Rad51 repression. The effect of PCI-24781, alone or with chemotherapy, on tumor and metastatic growth was tested in vivo using human STS xenograft models.

Results: PCI-24781 exhibited significant anti-STS proliferative activity in vitro, inducing S phase depletion, G2/M cell cycle arrest, and increasing apoptosis. Superior effects were seen when combined with chemotherapy. A PCI-24781–induced reduction in Rad51, a major mediator of DNA double-strand break homologous recombination repair, was shown and may be a mechanism underlying PCI-24781 chemosensitization. We showed that PCI-24781 transcriptionally represses Rad51 through an E2F binding-site on the Rad51 proximal promoter. Although single-agent PCI-24781 had modest effects on STS growth and metastasis, marked inhibition was observed when combined with chemotherapy.

Conclusions: In light of these findings, this novel molecular-based combination may be applicable to multiple STS histologic subtypes, and potentially merits rigorous evaluation in human STS clinical trials.

Antimetastatic soft tissue sarcoma (STS) systemic therapies remain elusive, resulting in 50% 5-year overall survival rates remaining stagnant for at least 30 years (1). New approaches are urgently needed yet difficult to develop because STS are rare and typified by marked intratumor and intertumor heterogeneity (2), rendering identification of specific STS subtype molecular “addictions” and their inhibition problematic (3). Alternatively, identifying target pathways that serve as convergence points for multiple STS subtypes is appealing; histone deacetylase inhibition is one such potential therapeutic strategy not yet explored in STS (4).

Chromatin structure is affected by posttranslational core-histone modifications, including acetylation and deacetylation (5). Core-histone acetylation states, important for chromatin structure, function, and gene expression, are controlled by opposing actions of histone acetyl transferases and histone deacetylases (HDAC; ref. 6). The histone acetyl transferase enzymes:HDAC balance is crucial for normal cell growth maintenance; deregulation due to increased HDAC expression or function occurs in many malignancies, resulting in abnormal modulation of target gene transcriptional activity; prompting development of HDAC inhibitors (HDACi) as anticancer therapeutics (7, 8). Recent in vitro and in vivo studies show that HDACi treatment of hematologic and epithelial-origin malignancies results in growth inhibition, abrogated angiogenesis, and apoptosis without significant normal cell or tissue toxicities.
Translational Relevance

There is a need for new therapies to improve the outcome of patients suffering from soft tissue sarcoma (STS). Here we show that PCI-24781, a novel histone deacetylase inhibitor (HDACi), exhibits significant anti-STS effects, resulting in growth inhibition, cell cycle arrest, and apoptosis. Furthermore, synergism with low-dose conventional chemotherapy was identified in vivo. These results provide significant insight into the potential effects of HDACi in STS. Although HDACi effects alone and in combination with DNA-damaging agents were examined previously in several epithelial malignancies, there is no significant exploration of such an approach in STS, i.e. mesenchymal malignancies, which employ very different mechanisms of tumorigenesis and subsequently exhibit clinical behavior very different from carcinomas. As such, this report is an important preclinical model for the use of HDACi in sarcoma, a disease with very few effective therapeutic options.

(9). One explanation for this selective effect is that defective cell cycle checkpoint regulation in tumor cells may render them more susceptible to HDACi-induced apoptosis (10). Therapeutic strategies that exploit tumor-specific mechanisms while sparing normal cells are very appealing; several HDACi are under investigation as single agents or combined with conventional chemotherapies. The HDACi-chemotherapy combinations may merit clinical evaluation for patients burdened by STS.

Materials and Methods

Cell culture and reagents. Human HT1080 (fibrosarcoma), RD (rhabdomyosarcoma), and SKLMS1 (leiomyosarcoma) STS cells were obtained from the American Type Culture Collection. Primary normal human fibroblast cultures were acquired from Promocell GmbH. Cells were cultured in DMEM supplemented with 10% FBS (Life Technologies). The HDAC inhibitor PCI-24781 was obtained from Pharmacia, and suberoylanilide hydroxamic acid (SAHA) was synthesized at MD Anderson Cancer Center and dissolved in DMSO to create stock solutions. Doxorubicin (Ben Venue Lab) and cisplatinum (Sicor) were obtained from the American Type Culture Collection. Primary normal human fibroblast cultures were acquired from Promocell GmbH. Cells were cultured in DMEM supplemented with 10% FBS (Life Technologies). The HDAC inhibitor PCI-24781 was obtained from Pharmacia, and suberoylanilide hydroxamic acid (SAHA) was synthesized at MD Anderson Cancer Center and dissolved in DMSO to create stock solutions. Doxorubicin (Ben Venue Lab) and cisplatinum (Sicor) were obtained from the American Type Culture Collection. All additional dilutions were completed using the respective cell culture medium for each cell line.

Commercial antibodies were used for Western blot and chromatin immunoprecipitation assays, including Rad51 and β-actin (Santa Cruz Biotechnology), acetylated histones 3 and 4 (Millipore), acetylated tubulin (Sigma), RNA Polymerase II (Covance), IgG (Santa Cruz Biotechnology), and E2F1 and E2F4 (Santa Cruz Biotechnology). Immuno-histochemical antibodies included antiproliferating cell nuclear antigen (Dako Cytomation) and CD31 (BD Biosciences). Dead End Fluorometric TUNEL System (Promega) was used for terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) staining. Secondary antibodies included horseradish peroxidase–conjugated (Universal kit HRP; Biocare Medical) and fluorescent secondary antibodies (antirabbit Alexa488 and antimouse Alexa 594; Jackson Immuno Research). Other reagents included CytoQ FC Receptor blocker (Innoved Bioscience), Hoechst 33342 (Polysciences), and propyl gallate (ACROS Organics).

Western blot analysis. Western blot was done as described (14).

Measurement of cell proliferation. Cell growth assays used CellTiter96 Aqueous Non-Radioactive Cell Proliferation Assay Kit (Promega) per the manufacturer’s instructions. Growth rates were analyzed 48 h after HDACi (PCI-24781/SAHA) treatment and with doxorubicin or cisplatin, alone or combined with HDACi; several administration sequences were evaluated: 24 h pretreatment with PCI-24781 followed by the addition of chemotherapy for 24 h and conversely, pretreatment with chemotherapy followed by PCI-24781 as well as treatment with both compounds concomitantly for 24 h. Absorbance was measured at 490 nm wavelength; treated cell absorbance values are presented as a percentage of untreated cell absorbance.

Clonogenic assay. STS cells were treated in culture dishes with DMSO (control) and varying concentrations of HDACi (PCI-24781/SAHA) for 24 h. One hundred cells per well were replated, then allowed to grow in normal media for 10 d, then stained with a 6% glutaraldehyde, 0.5% crystal violet solution for 30 min. Staining solution was decanted from each well and cells washed with deionized H2O. Individual colonies retaining staining solution were counted.

Cell cycle analysis. STS cell monolayers were treated with PCI-24781/SAHA for varying periods. Propidium iodide/fluorescence-activated cell sorting (FACS) analysis was conducted as described (14).

Apoptosis assay. Apoptosis was measured using the Annexin V Apoptosis Detection Kit I (BD Biosciences) as described (14).

In vivo therapeutic studies. All animal procedures and care were approved by the MD Anderson Cancer Center Institutional Animal Care and Use Committee. Animals received humane care as per the Animal Welfare Act and the NIH “Guide for the Care and Use of Laboratory Animals.” Animal models were utilized as previously described (15). Trypsin blue staining confirmed viable STS cells (SKLMS1 or HT1080 cells × 10⁶/ml HBSS/mouse) were injected into the flank (SKLMS1 i.m., HT1080 s.c.) of 6-wk-old female SCID mice (n = 40/experiment), growth was measured twice weekly; HT1080GL cells (stably expressing green fluorescent protein/Luciferase) were tail vein injected, resulting in experimental lung metastases that could be followed by bioluminescence. When average tumor volumes reached 100 mm³, the mice were assigned to four treatment groups (7-8 mice/group): (a) control (vehicles only); (b) doxorubicin/cisplatinum (1.2 mg/kg/biweekly and 2 mg/kg/biweekly, respectively, i.p.); (c) PCI-24781 (50 mg/kg/d ×5 d/wk, i.p.); and (d) PCI-24781 plus chemotherapy (PCI-24781 initiated 24 h before chemotherapy in all cases). PCI-24781 dose and treatment schedules were determined per company recommendations. A similar experimental design was used for lung metastasis treatment, initiated when bioluminescence showed established lung metastases; the study included six arms so that PCI-24781 combined with either chemotherapy or a targeted agent could be evaluated. The mice were followed for tumor size and body weight and sacrificed when control group tumors reached 1.5 cm average largest dimension or when bioluminescence suggested control group had significant pulmonary tumor load. Tumors were

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resected, weighed, and frozen or fixed in formalin and paraffin-embedded for immunohistochemical studies. Similarly, lungs were resected, weighed, and fixed in formalin.

**Immunohistochemical analysis.** Immunohistochemistry and TUNEL assay were done as previously described (15). Staining distribution (% positive stained tumor cells) and intensity (0 = no staining, 1 = low, 2 = high) as well as CD31 counts (in 10× high-power fields) were evaluated and scored by three independent reviewers (GL, WR, and AL).

**Reverse transcription-PCR/quantitative reverse transcription-PCR.** These assays were conducted as previously described (16). Rad51 PCR primers for both assays were designed using primer 3 software. Rad51, 5′-TGGCCCAACCAACCATGCAGAC-3′ and 5′-TCATGAGCTGGTGGTGTTGAG-3′; GAPDH, 5′-GACACGATCTGAGAC-3′ and 5′-CAGCAGTTCGCAATGACC-3′. For quantitative reverse transcription-PCR (RT-PCR) Rad51 primers were 5′-GAGGTGAAGGAAAGGCCATG-3′ and 5′-GGTGTGTTGATGTCGGCATG-3′; Actin, 5′-CAGCCAATGTACGTTCACACTC-3′ and 5′-AGTCCAGAAGGGATGCAGAC-3′; and
p21, 5′-GGAAGACCATGTGGACCTGT-3′ and 5′-AATCTGTCATGCTGGTCTGC-3′.

**ChIP assay.** Chromatin immunoprecipitation assays were done using ChIP assay kits (Upstate Technology) as described (17). After appropriate treatment, STS cells were fixed in 1% formaldehyde followed by incubation in 0.125 mol/L glycine to stop cross-linking. Cells were then washed and recovered, followed by lysis in SDS buffer. Lysates were sonicated, yielding genomic DNA fragments with a bulk size of 200-1,000 bp, then centrifugation-clarified. Supernatants were diluted and preclred with salmon sperm DNA/protein G-agarose. Lysates were immunoprecipitated with 2 to 5 μg of relevant antibodies. Antibody-nucleoprotein complex mixtures were

![Fig. 2](https://www.aacrjournals.org/doi/abs/10.1158/1538-7445.CAN-08-0959)

**Fig. 2.** PCI-24781 induces S phase depletion, G2 cell cycle arrest, and apoptosis in STS cells and sensitizes them to chemotherapy. A, propidium iodide/FACS analysis showing the effect of HDACi (PCI-24781 0.5 μmol/L/48 h, SAHA 2 μmol/L/48 h) on STS cell cycle progression. A significant (*P* < 0.05) reduction in S phase cells and G2 arrest is seen. Additionally, an increase in sub G1 population is observed, suggesting the induction of apoptosis. B, PCI-24781 induces STS cell apoptosis in a time- and dose-dependent manner (Annexin V staining). C, pretreatment of STS cells with PCI-24781 (0.1 μmol/L/24 h) sensitizes STS cells to chemotherapy. A superior effect was shown when combining PCI-24781 and doxorubicin (0.5 μmol/L/24 h; *P* < 0.0001) or cisplatinum (1 μmol/L/24 h; *P* < 0.05) as compared with each agent alone. When sequencing was reversed, i.e. pretreatment with chemotherapy (24 h) followed by the addition of PCI-24781 (24 h), or when the drugs were administered concomitantly for 24 h, no such superior effect was observed (data not shown). Results represent the average of a minimum of three replications (± SD). *P*, PCI-24781.
incubated overnight and recovered by incubation with 60 μL salmon sperm DNA/protein G-agarose for 1 h at 4°C. One hundred–microliter aliquots were reserved from negative control (no antibody) samples before washes; these aliquots were processed in parallel with eluted samples and used as input DNA. Beads were washed five times, and nucleoprotein complexes were eluted from protein G-agarose beads in immunoprecipitation elution buffer. Cross-links were reversed by adding 4 μL 5 mol/L NaCl and incubating overnight at 65°C, followed by 1.5-h digestion with RNase A and proteinase K at 50°C. DNA fragments were recovered by phenol/chloroform extraction and ethanol precipitation and analyzed by PCR. Primer sequences available upon request.

Luciferase reporter assay. Rad51 promoter sequence (from –403 to +63 bp of the transcriptions start site- numbered according to Entrez GenID 5888, gi:19924132) cloned into pGL3 as well as deletion mutants were cotransfected into SKLMS1, RD, and HT1080 cells using FuGene 6 transfection reagent (Roche Diagnostics GmbH) per the manufacturer’s instructions, as described (18). The Rad51 reporter construct was further mutated at the E2F proximal binding site using Stratagene Quick Change mutagenesis kit (Stratagene) per the manufacturer’s instruction and used for transfection as above. Cells were incubated with/without PCI-24781 (0.5 μmol/L) for 24 h then harvested. Luciferase assays were done using dual-luciferase assay reagents (Promega) and assessed with a luminometer. Each experiment was repeated three times; data were normalized to renilla luciferase.

Statistical analysis. Cell culture-based assays (and Western blot analyses) were repeated at least three times; mean ± SD was calculated. Cell lines were examined separately. For outcomes that were measured at a single time point a two-sample t-test was used to assess differences; a linear mixed model was used to assess treatment effects and association between compounds. The mean, SD, and range for all in vivo experiment group variables were calculated and recorded. Tumor volume was logarithmically transformed for further statistical analysis. A linear mixed model was used to assess effects of treatment on tumor growth over time, with an AR1(1) correlation structure to examine correlations among tumor volumes of the same mice over time. A linear regression model was used to assess the effect of treatment on tumor/lung metastases weight. All pairwise comparisons between tumor weights among treatment groups were made using the Tukey-Kramer method for multiple comparison adjustment to control experimental-wise type I error rates. All tests were two-sided and P = 0.05 was considered statistically significant. Statistical analyses and plotting were done using SAS version 9 (SAS Institute) and S-Plus 7 (Insightful Inc.).

Results
HDACi abrogate human soft tissue sarcoma cell growth. Two hydroxamic acid-derived HDACi were studied, SAHA and PCI-24781; their effects on acetylated histones and acetylated tubulin expression were evaluated in different human STS subtype cell lines: HT1080 (fibrosarcoma; wt-p53), SKLMS1 (leiomyosarcoma; mut-p53), and RD (rhabdomyosarcoma; mut-p53). Time- and dose-dependent target protein acetylation increases were shown after treatment with either HDACi independent of cell p53 mutational status (Fig. 1A).

HDACi effects on STS cell growth and clonogenicity were evaluated. SAHA (48 hours) induced dose-dependent decreases in HT1080 and RD cell growth; less effects were observed in SKLMS1 (Fig. 1B). All sarcoma cell lines tested were sensitive to PCI-24781; no effect on primary cultured normal human fibroblast growth was observed after treatment with either HDACi (Fig. 1B). Similarly, HDACi induced significantly decreased STS cell colony formation compared with untreated controls (P < 0.05; Fig. 1C). These initial results suggest that HDACi abrogate growth of different histologic subtype and p53 mutational status STS cells in a reproducible manner, supporting further examination of HDACi effects in STS in vitro and in vivo.

HDACi induce STS cell S phase depletion, G2 cell cycle arrest, and enhance apoptosis. We evaluated whether HDACi STS growth inhibition could be due to effects on STS cell cycle progression and/or induction of apoptosis. PCI-24781 exhibited lower LD50 (nanomolar range) in STS cells tested as compared with SAHA; further studies therefore utilized PCI-24781. STS cell lines were treated with increasing doses of PCI-24781 for 48 hours, then underwent propidium iodide staining/FACS analysis. Significant dose-dependent S phase depletion and G2/M cell cycle arrest was observed in PCI-24781–treated cells compared with DMSO–treated controls (Fig. 2A). After PCI-24781 (0.5 μmol/L) exposure for 48 hours, the percentage of RD and HT1080 S phase cells decreased from 42 ± 6.4% to 26 ± 9.1% and from 34 ± 5% to 17 ± 5%, whereas G2/M phase cells increased from 22 ± 1.7% to 64 ± 3.6% and from 30 ± 6.4% to 49 ± 5.7% (P < 0.05). PCI-24781–induced G2/M checkpoint activation was observed in both wt-p53 and mutated p53 cells. HDACi cell cycle progression effects are suggested as potentially tumor/cell type as well as compound-specific (9). To evaluate whether these STS cell cycle changes were PCI-24781–specific, SAHA STS cell cycle effects were examined. Similar to PCI-24781, SAHA induced significant S phase depletion and G2/M arrest in drug-sensitive STS cell lines (HT1080 and RD; Fig. 2A); propidium iodide/FACS analysis showed increased cellular accumulation in the sub-G1, cell cycle fraction, suggesting drug-induced STS cell apoptosis. To confirm this observation, we assessed the effects of PCI-24781 on phospho-tyrosine exposure by Annexin V staining and FACS analysis; statistically significant dose- and time-dependent increases in STS cell apoptosis were observed (P < 0.05; Fig. 2B). Although only minimal apoptosis was seen 24 hours after exposure to PCI-24781, 74% ± 14 and 64% ± 25 Annexin V positivity was observed in HT1080 and RD cells, respectively, after 96 hours of PCI-24781 (0.5 μmol/L) treatment. In summary, these data show that single agent PCI-24871 reproducibly induces STS cell cycle alterations and apoptosis in vitro.

Combining PCI-24781 with chemotherapy results in superior anti-STS effects in vitro. In addition to monotherapy efficacy, some HDACi (including PCI-24781) have synergy with conventional chemotherapy and radiation (19–21). Consequently, we examined STS cell growth effects of PCI-24781 alone or combined with chemotherapy. Two chemotherapeutics were studied: doxorubicin, the most commonly used STS first line treatment, and cisplatinum, a DNA cross-linking agent. STS cells were treated with PCI-24781 alone for 48 hours, chemotherapy alone for 24 hours, or by the sequential combination of PCI-24781 and chemotherapy (cells pretreated with PCI-24781 for 24 hours, then chemotherapy was added for an additional 24 hours). Low-dose agents were used to evaluate this combination; cells underwent MTS assays (Fig. 2C). The combination of PCI-24781 (0.1 μmol/L) and doxorubicin (0.5 μg/mL) caused growth reductions of 77% ± 4.7, 81% ± 3.1, and 65% ± 3.5 in HT1080, RD, and SKLMS1 cells, respectively. In contrast, PCI-24781 alone resulted in 23% ± 3.2, 24% ± 5.6, and 12% ± 4.4 growth inhibition of HT1080, RD, and SKLMS1 cells, whereas doxorubicin alone caused 6% ± 9.5, 10% ± 4.5, and 9% ± 4.5 growth reduction, respectively. Statistical analysis showed a statistically significant superior effect for combination compared with each compound alone (PCI-24781 plus doxorubicin, rad51...
Interestingly, this effect was lost when drugs were administered together for 24 hours or when cells were pretreated with chemotherapy for 24 hours prior to PCI-24781 treatment (data not shown), suggesting that PCI-24781 possibly sensitizes STS cells to the effect of chemotherapy.

**Combining PCI-24781 with chemotherapy results in superior anti-STS effects in vivo.** We next evaluated the impact of PCI-24781 alone and combined with chemotherapy in vivo. SKLMS1 growing i.m. and HT1080 growing s.c. or as experimental lung metastases were used in four-armed studies comparing the effects of low-dose doxorubicin, cisplatinum, PCI-24781, or PCI-24781 combined with either chemotherapy on human STS local and metastatic growth in SCID mice. Therapy was initiated after tumor establishment (100 mm³). Similarly, in the experimental lung metastasis...
model, treatment was initiated only after established metastases were identified by bioluminescence.

A linear mixed model was used to assess tumor growth (tabulated as log-transformed tumor volume) across treatment groups over time, and a linear regression model was used to assess tumor weights across treatment groups. Treatment with low-dose doxorubicin alone did not significantly affect SKLMS1 xenograft growth (Fig. 3A); PCI-24781 alone induced significant tumor growth inhibition (slope of tumor volume versus time for PCI-24781 treated mice versus control untreated tumors; \( P = 0.001 \)). Most significantly, combined PCI-24781 and low-dose doxorubicin was markedly inhibitory compared

![Graph showing tumor growth and weight over time](image)

**Fig. 4.** Combining PCI-24781 (50 mg/kg/d) with low-dose cisplatinum (2 mg/kg/biweekly) or doxorubicin (1.2 mg/kg/biweekly) results in significant reduction of human fibrosarcoma (HT1080) local and metastatic growth in nude mice. **A,** tumor growth curves showing a significant synergy between PCI-24781 and cisplatinum in vivo \((P < 0.001)\). **B,** a highly statistical significant tumor weight reduction was observed with combined treatment compared with the other three groups \((P = 0.0003, = 0.0038, \text{and} = 0.0011 \text{ versus control, cisplatinum, and PCI-24781 groups, respectively})\). **C,** as shown above for SKLMS1 xenografts, enhanced tumor necrosis in PCI-24781–treated tumors, and most significantly in combination therapy specimens was observed in HT1080 tumors (H&E). Decreased tumor proliferation (PCNA) and increased apoptosis (TUNEL) was also seen. No significant differences in CD31 counts were shown; however, a marked decrease in the number of large, patent blood vessels was discernible in the combination group. **D,** nude mice injected with HT1080 cells \((1 \times 10^6/mouse)\) through tail vein were followed using bioluminescence. Treatment (as recorded) was initiated once bioluminescence first suggested lung metastases establishment \((\text{day 0})\). Sequential bioluminescence imaging of a representative mouse from each treatment arm show the increase in luciferase readout in control, cisplatinum–, doxorubicin–, and PCI-24781–treated groups; decreased readouts were most pronounced in combination therapy groups. H&E staining shows the reduction in lung metastatic deposits in combination-treated groups; microscopic metastasis are marked by arrow heads. \( * P, \text{PCI-24781} \).
with control, doxorubicin alone, or PCI-24781 alone treated tumor groups \( (P < 0.0001) \). Average group tumor weights at study termination were 1.62 g ± 0.47 for control, 1.34 g ± 0.43 for doxorubicin, 1.11 g ± 0.26 for PCI-24781, and 0.64 g ± 0.24 for combination (Fig. 3B). PCI-24781 alone significantly decreased tumor weight versus controls \( (P = 0.026) \). Moreover, tumor weight reduction due to combined PCI-24781-chemotherapy was markedly significant versus other treatments \( (P < 0.0001, = 0.0023, \text{ and } = 0.047 \text{ for control, doxorubicin, and PCI-24781, respectively}) \).

H&E staining of different treatment arm tumors revealed marked tumor necrosis in PCI-24781 and combination treatment groups (Fig. 3C); viable tumor sections were immunohistochemically evaluated for the impact of different therapies on STS cell proliferation (PCNA) and apoptosis (TUNEL). Average PCNA and TUNEL-positive staining nuclei revealed 88 ± 2 and 20 ± 2.4 for control, 85 ± 1.2 and 16 ± 1.4 for doxorubicin, 71 ± 2.3 and 19 ± 1.1 for PCI-24781, and 57 ± 4.6 and 61 ± 18.4 for combination, respectively, suggesting that combination therapy had the strongest antiproliferation and apoptosis-inducing effects \( (P < 0.05) \). Sections were also stained for CD31 to examine different therapy effects on tumor-associated vasculature. No significant differences in CD31 counts were seen; however, markedly decreased numbers of large, patent blood vessels was discernible in the combination group (Fig. 3C).

Similarly, the effects of PCI-24781, cisplatinum, and their combination were tested in HT1080 xenografts (Fig. 4). No significant tumor growth differences over time were identified comparing cisplatinum- or PCI-24781–treated and control mice. However, significant tumor growth reduction was observed with combined therapy \( (P < 0.001 \text{ versus the other treatment groups}; \text{ Fig. 4A}) \). Average group tumor weights at study termination were 1.23 g ± 0.15 for control, 1.01 g ± 0.39 for cisplatinum, 1.09 g ± 0.54 for PCI-24781, and 0.36 g ± 0.22 for combination (Fig. 4B). No significant tumor weight reduction occurred due to PCI-24781 or cisplatinum alone treatments, whereas significant tumor weight reduction was observed with combined treatment \( (P = 0.0003, = 0.0038, \text{ and } = 0.0011 \text{ versus control, cisplatinum, and PCI-24781 groups, respectively}) \). Immunohistochemistry results were similar to those for the SKLMS1-treated tumors described above, showing increased necrosis in PCI-24781 and combination groups (Fig. 4C). Decreased proliferation and enhanced apoptosis were most pronounced in the combination-treated group.
(average PCNA and TUNEL-positive staining nuclei were 83 ± 7.1 and 18 ± 0.3 for control, 62 ± 2.8 and 17 ± 2.5 for cisplatinum, 50 ± 2.8 and 21 ± 5.9 for PCI-24781, and 38 ± 14.1 and 33 ± 8.6 for combination, respectively; *P < 0.05). No significant difference in CD31 positivity was seen among groups, whereas a reduction in large blood vessels was observed in the combination group.

Lastly, the effect of the various therapies on STS lung metastases was evaluated. An experimental fibrosarcoma lung metastasis model was used; PCI-24781 combined with doxorubicin or cisplatinum was tested (Fig. 4D). Mice were followed by bioluminescence; representative sequential images are depicted in Fig. 4D, showing reduced bioluminescence in combination treatment mice. H&E staining revealed large metastatic deposits replacing much lung parenchyma in control- and chemotherapy-treated tumors; smaller lesions were seen in the PCI-24781 group, and small microscopic lesions were observed in combination treatment mice. Lung metastases weights were calculated by deducting the estimated average normal mouse lung weight from actual lung weight at study termination. Average lung metastases weight per group was 0.55 g ± 0.14 for control, 0.62 g ± 0.26 for cisplatinum, 0.48 g ± 0.33 for doxorubicin, 0.33 g ± 0.20 for PCI-24781 group, 0.15 g ± 0.19 for PCI-24781-cisplatinum, and 0.13 g ± 0.14 for PCI-2478-doxorubicin. A trend toward reduced metastatic load was seen in the PCI-24781–treated mice but it did not reach statistical significance, whereas PCI-24781 combined with either chemotherapy resulted in significant lung metastases weight reduction (*P < 0.05) compared with control or cisplatinum treatment groups. Taken together, these data suggest that although PCI-24781 exhibits significant anti-STS effects in vitro, it is only marginally effective as monotherapy in vivo. However, combining PCI-24781 with low-dose conventional chemotherapy results in significant STS tumor and metastasis

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**Fig. 6.** PCI-24781 induces Rad51 transcriptional repression potentially mediated via enhanced E2F1 binding to the Rad51 proximal promoter. A, chromatin immunoprecipitation assays showing decreased Polymerase II binding to a transcribed region in the proximal Rad51 gene in STS cells in response to PCI-24781 (0.5 μmol/L/24 h); in contrast increased Polymerase II binding to the p21 gene is seen. Binding of acetylated histones 3 and 4 to the Rad51 and p21 promoters is enhanced. No significant nonspecific IgG binding was observed; input DNA was used as loading control. B, PCI-24781 (0.5 μmol/L/24 h) induces suppression of Rad51 promoter activity while it enhances p21 promoter activation in STS cells (left panel); a −403 bp Rad51 luciferase reporter assay previously described (18) was utilized for these experiments. PCI-24781 induced Rad51 promoter repression is observed even when a reporter construct consisting of the proximal 50 bp of the Rad51 promoter is utilized, suggesting that the PCI-24781 response element is located within this proximal region (right panel). C, mutating the E2F binding site located within the first 50 bp of the Rad51 promoter construct resulted in decreased luciferase readout in untreated cells and most importantly abrogated the PCI-24781 (0.5 μmol/L/24 h) induced Rad51 promoter repression (left panel). Chromatin immunoprecipitation analysis showed enhanced E2F1 binding to its cis element in the proximal Rad51 promoter in response to PCI-24781 (0.5 μmol/L/24 h); a slight decrease in E2F4 binding is also observed (right panel). Results represent the average of a minimum of three replications (± SD). *P, PCI-24781.
growth inhibition in vivo, an observation of potential clinical utility.

Possible underlying mechanism for chemosensitization: PCI-24781 induces Rad51 transcriptional repression. These findings suggest that PCI-24781 is potentially a STS chemosensitizer. HDACi, including PCI-24781, can synergize with DNA-damaging agents (21, 22). One potential mechanism underlying such observations is that PCI-24781 can induce blockade of DNA double-strand break homologous recombination repair, at least in part due to down-regulation of Rad51 (23). Previously we showed that Rad51, a key regulator of homologous recombination repair, is highly overexpressed in various STS histologic subtypes (18), showing that Rad51 expression causes STS cells to acquire chemoresistance, whereas abrogating Rad51 expression sensitizes these cells to doxorubicin. In light of these findings, we evaluated whether PCI-24781 induced Rad51 down-regulation in STS cells, and considered candidate mechanisms underlying such repression. Figure 5A shows that treatment of STS cells with PCI-24781 induced a time-dependent decrease in Rad51 mRNA expression, initially apparent in all three cell lines within 4 to 10 hours of treatment. Western blot analysis showed that the reduced Rad51 transcript level is further translated into abrogated Rad51 protein expression (Fig. 5B). We next investigated whether PCI-24781 affects Rad51 mRNA stability and induces shortened Rad51 mRNA half-life in STS cells. Cells were treated with actinomycin D to block de novo mRNA transcription; PCI-24781 was added 30 minutes later to several of the cultured wells; mRNA was harvested at subsequent incremental time points and analyzed via quantitative RT-PCR. Comparable Rad51 mRNA half lives (~6-8 hours) were found when samples from PCI-24781–treated and non-treated cells were compared (data not shown), indicating that PCI-24781 does not directly enhance Rad51 mRNA transcript degradation. Next we evaluated the potential effect of PCI-24781 on Rad51 transcription by determining the recruitment of RNA polymerase II (Poly II) to the transcription initiation region of the Rad51 gene. Binding of Poly II to the p21 gene coding region was selected as control for these experiments after showing that PCI-24781 induces enhanced p21 expression in our cells (Fig. 5C). ChIP analysis using anti-Poly II antibody showed that 24 hours of PCI-24781 treatment significantly decreased the association of Poly II with the Rad51 gene whereas an increase in Poly II recruitment to the p21 gene was observed (Fig. 6A). Interestingly, ChIP analysis evaluating acetylated histones H3 and H4 binding to the Rad51 and p21 promoters showed that PCI-24781 leads to increased association of these acetylated histones with promoter nucleosomes of both targets (Fig. 6A). These data suggest that PCI-24781 induces Rad51 transcriptional repression in the presence of increased Rad51 promoter histone acetylation. To further validate the transcriptional repressive effect of PCI-24781 on Rad51 we utilized our previously described Rad51 luciferase reporter construct (containing the −403 to +63 Rad51 promoter region; 18) for transient transfection of STS cells. As shown in Fig. 6B, PCI-24781 treatment (24 hours) resulted in significant suppression of Rad51 promoter activity (∼0.05). In contrast, increased p21 promoter activity was shown after PCI-24781 treatment (Fig. 6B). To further determine which part of the Rad51 promoter is necessary for PCI-24781–induced repression, we examined the activity of serial 5’ truncated Rad51 promoter constructs after drug treatment. A significant reduction in luciferase readout in response to PCI-24781 was shown in all deleted constructs, including the −50Luc, indicating that the PCI-24781 response element is in the proximal Rad51 promoter (Fig. 6B). This region was previously identified to contain an E2F binding-site of importance in regulating the transcription of Rad51 (24). To confirm that the repressive effect of PCI-24781 is mediated through this cis element we further mutated the E2F binding-site in the −403 bp reporter construct and repeated the luciferase assays. As shown in Fig. 6C, E2F binding-site mutation resulted in decreased luciferase expression in untreated cells, suggesting that this site is a potential mediator of Rad51 transcription. Most importantly, the PCI-24781–induced decrease in luciferase readout observed after the transfection of the wild-type promoter construct was abolished with the transfection of the mutated construct (Fig. 6C). These findings support the hypothesis that PCI-24781–induced Rad51 transcriptional repression is mediated via this proximal E2F binding-site. Several E2F family members can potentially bind to the above site; E2F1 and E2F4 binding has been described (24, 25). Therefore, using a ChIP assay, the binding of E2F1 and E2F4 to the proximal Rad51 promoter in HT1080 cells in response to PCI-24781 was evaluated. Figure 6D shows increased E2F1 binding to the Rad51 promoter in response to PCI-24781; in contrast a slight reduction in E2F4 binding was seen. No significant change in E2F1 and/or E2F4 protein level in response to PCI-24781 could be observed via immunoblotting (data not shown). Taken together, these studies show that PCI-24781 transcriptionally represses the expression of Rad51. This repression is potentially modulated via increased binding of E2F1 to the proximal promoter of this gene. This observed decrease in Rad51 expression could represent one potential mechanism underlying the significant PCI-24781 chemosensitizing effects observed in STS.

Discussion

HDACi are a new class of anticancer therapeutics not yet evaluated for STS. Pleiotropic HDACi modulation of multiple pathways, genes, and biological features may be especially useful given the various oncogenic abnormalities associated with many STS subtypes. We evaluated two different hydroxamic acid-based compounds, SAHA and PCI-24781, regarding growth and survival of STS cells in vitro. SAHA has been investigated in hematologic and epithelial-origin malignancies showing antitumor effects (e.g., growth arrest, differentiation, and/or apoptosis) at micromolar concentrations (26, 27). SAHA inhibited clonogenic growth and induced apoptosis in breast cancer (28), exhibiting antiproliferative and proapoptotic effects in other cancer types (prostate, bladder carcinoma, and myeloma) independent of p53 status (29-31). Kutko et al. showed that SAHA induced significant growth-suppressive effects in rhabdomyosarcoma cells (32). Our results showed SAHA anti-STS effects, variable among cell lines, whereas PCI-24781 was growth inhibitory at nanomolar dose ranges in all STS cell lines tested and consequently further investigated. We showed that PCI-24781 induces accumulation of acetylated histones and acetylated tubulin in various histology STS cells, abrogates STS cell growth, induces significant S phase depletion and G2/M cell cycle arrest, enhances apoptosis, and possibly sensitizes STS cells to doxorubicin and cisplatinum. P53 mutations are the most common STS genetic alteration,
and p53 mutated STS are generally more chemoresistant (33). It is pertinent that no significant PCI-24781 response differences were seen in wtp53 (HT1080) versus mutated p53 gene (SKLMS1, RD) STS cells. As with other HDACi (34), minimal normal cell growth effects were seen at doses tested. HDACi cell cycle progression effects vary among tumor types and compounds studied. HDACi-induced G1 cell cycle arrest is described, often associated with p53-independent p21 induction (35, 36). HDACi-repression of cyclin D, cyclin A, CTP synthase, or thymidylate synthetase results in retinoblastoma protein hypo-phosphorylation and/or blocking S-phase progression may also underlie this G1 arrest (37–39). However, we detected more pronounced G2/M than G1 cell cycle arrest in PCI-24781–treated STS cells. HDACi-mediated G2/M arrest has been previously described, although it seems rarer than G1 arrest; the responsible HDACi-related mechanisms remain uncertain (9). PCI-24781 induced p21 expression in all STS cell lines tested; although p21 is commonly associated with the G1 checkpoint, its association with G2/M arrest is described (40). Further studies of PCI-24781 cell STS cycle effects are ongoing.

Interestingly, single drug PCI-24781 exhibited minimal anti-STS effects in vivo at doses tested whereas significant impacts were observed in combination with low-dose chemotherapy, an observation of potential clinical applicability. Synergism between several HDACi (including PCI-24781) and various anti-tumor reagents (5-fluorouracil, gemcitabine, docetaxel, as well as doxorubicin and cisplatinum) in preclinical models (breast, lung, and hematologic malignancies) has been described (11, 23, 41). This synergism generally depends on drug sequencing: HDACi pretreatment resulted in superior antitumor effects than either drug alone, whereas reversing this order showed no additional (or even antagonistic) effects (42). Our data support this finding, showing significant combinatorial effect only with STS PCI-24781 pretreatment. Although the underlying mechanisms need clarification, several potential rationales, including HDACi-induced enhanced DNA accessibility due to loosened chromatin structures, cell cycle changes conducive to chemotherapeutic cytotoxicity, defective stress signaling pathways, and/or emergence of lower apoptotic thresholds within tumor cells may be contributory (9).

An additional mechanism for HDACi synergy with DNA-damaging agents has been proposed, implicating abrogation of DNA double-strand break repair. HDACi impair homologous recombination repair, potentially via homologous recombination-related gene expression control (43, 44). One such major regulator of homologous recombination is Rad51, whose expression is decreased in PCI-24781–treated tumor cells, thereby perhaps contributing to PCI-24781–induced impaired homologous recombination (23). Previously we showed that Rad51 is highly expressed in many STS subtypes, where its overexpression potentially contributes to STS chemoresistance (18). Consequently, we evaluated PCI-24781 effects on STS Rad51 expression, showing that PCI-24781 significantly inhibits expression of STS cellular Rad51 mRNA and protein. To the best of our knowledge, mechanisms of PCI-24781–induced Rad51 repression have not been described.

Several gene expression arrays show that HDACi cancer cell treatment alters expression of cellular gene subsets (approximately 2%) in a cell- and compound-specific manner (4, 39). Of the deregulated genes, ~50% are overexpressed and ~50% are underexpressed. The mechanisms of HDACi-induced decreased gene expression remain uncertain; HDACi-induced transcriptional repression and/or decreased mRNA stability, possibly secondary to HDACi-induced increased expression of other genes, e.g., p21, may be relevant (45, 46). Furthermore, HDAC inhibitors may induce acetylation of nonhistone proteins, including several transcription factors, thereby altering function and potentially negatively affecting oncogenic target gene transcription (9). We showed that Rad51 is transcriptionally repressed by PCI-24781, and that this is possibly mediated through enhanced E2F1 binding to the Rad51 proximal promoter. This is an intriguing finding because E2F1 is primarily conceived as a positive transcriptional regulator, whereas E2F4 is considered a transcription repressor and may even specifically inhibit Rad51 transcription in response to hypoxia (25). However, a negative E2F1 regulatory role has been described for several genes including urokinase plasminogen activator, the antiapoptotic protein Mcl-1, and human telomerase reverse transcriptase (47–49). Perhaps E2F1 protein binding partners determine its activator versus repressor role, with PCI-24781 impacting on assembly of the E2F1 complex. PCI-24781 possibly induces acetylation of E2F1 which has previously been shown to enhance its binding affinity to consensus E2F binding sites (50). Studies to further elucidate the mechanism and functional effects of increased E2F1 binding in response to PCI-24781 are currently ongoing; perhaps PCI-24781–induced decreases in Rad51 may be partially responsible for its STS chemosensitizing effects.

In summary, we have shown that combined HDAC inhibition and low-dose chemotherapy induce anti-STS activity in vitro and in vivo. Our results are encouraging, supporting further investigation of this combination as a promising novel therapeutic intervention for the treatment of STS. However, it is of essence to point out the limitations of preclinical cell culture and xenograft animal experiments to consistently predict actual therapeutic responses in humans. Construction of HDA Ci clinical trials will require appropriate patient selection, drug scheduling and combination therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

4. Xu WS, Parmigiani RB, Marks PA. Histone


Correction: Combining PCI-24781, a Novel Histone Deacetylase Inhibitor, with Chemotherapy for the Treatment of Soft Tissue Sarcoma

In this article (Clin Cancer Res 2009;15:3472–83), which was published in the May 15, 2009, issue of Clinical Cancer Research (1), several actin loading control blots were inadvertently duplicated by the authors. Specifically, in Fig. 1A, the actin bands of the

![Image of Figure 1A showing actin loading control blots for HT1080, RD, and SKLMS1 cells treated with SAHA and PCI-24781]

![Image of Figure 1B showing percentage of cell viability for HT1080, RD, SKLMS1, and NHF cells treated with SAHA and PCI-24781]

![Image of Figure 1C showing colonies (avg. no.) for RD, HT1080, and SKLMS1 cells treated with DMSO, SAHA 2 μmol/L, and PCI-24781 0.5 μmol/L]
HT1080 cell line time and dose HDAC inhibitor–treated gels, as well as those of the RD cell line time and dose HDAC inhibitor–treated gels, were identical. In addition, in Fig. 5B, the actin control of HT1080 and RD cell lines appeared to be duplicate images. The authors repeated these experiments and confirmed the same results as detailed in the original investigations. The corrected versions of Figs. 1 and 5 are below. The authors regret this error.

Figure 5.

Reference

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Combining PCI-24781, a Novel Histone Deacetylase Inhibitor, with Chemotherapy for the Treatment of Soft Tissue Sarcoma

Gonzalo Lopez, Juehui Liu, Wenhong Ren, et al.


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