Combined Effects of Suberoylanilide Hydroxamic Acid and Cisplatin on Radiation Sensitivity and Cancer Cell Invasion in Non-Small Cell Lung Cancer

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Abstract

Lung cancer is a leading cause of cancer-related mortality worldwide, and concurrent chemoradiotherapy has been explored as a therapeutic option. However, the chemotherapeutic agents cannot be administered for most patients at full doses safely with radical doses of thoracic radiation, and further optimization of the chemotherapy regimen to be given with radiation are needed. In this study, we examined the effects of suberoylanilide hydroxamic acid (SAHA) and cisplatin on DNA damage repairs, and determined the combination effects of SAHA and cisplatin on human non–small cell lung cancer (NSCLC) cells in response to treatment of ionizing radiation (IR), and on tumor growth of lung cancer H460 xenografts receiving radiotherapy. We also investigated the potential differentiation effect of SAHA and its consequences on cancer cell invasion. Our results showed that SAHA and cisplatin compromise distinct DNA damage repair pathways, and treatment with SAHA enhanced synergistic radiosensitization effects of cisplatin in established NSCLC cell lines in a p53-independent manner, and decreased the DNA damage repair capability in cisplatin-treated primary NSCLC tumor tissues in response to IR. SAHA combined with cisplatin also significantly increased inhibitory effect of radiotherapy on tumor growth in the mouse xenograft model. In addition, SAHA can induce differentiation in stem cell–like cancer cell population, reduce tumorigenicity, and decrease invasiveness of human lung cancer cells. In conclusion, our data suggest a potential clinical impact for SAHA as a radiosensitizer and as a part of a chemoradiotherapy regimen for NSCLC. Mol Cancer Ther; 15(5); 842–53. ©2016 AACR.

Introduction

Lung cancer is the most common cancer and the leading cause for cancer-related mortality worldwide (1). The majority of lung cancer is NSCLS (non–small cell lung cancer) that includes squamous cell carcinoma, adenocarcinoma, and large cell carcinoma. One third of these patients are diagnosed with stage III disease when curative treatment is extremely limited (2). Despite the tremendous efforts and progress in lung cancer research, and the use of aggressive multimodal chemo- and radiotherapy, the overall treatment outcome for these NSCLC patients remains poor.

For patients with advanced stages of NSCLC when surgical excision is not an option, the combined treatment of radiotherapy and chemotherapy is extensively used. The first-line therapy typically includes a platinum derivative, such as cis-diaminedichloroplatinum (II; cisplatin; refs. 3, 4). Meta-analyses based on individual data from randomized clinical trials have demonstrated statistically significant benefits of combining chemotherapy with radiation for stage III NSCLC patients, and the greatest difference in efficacy was observed in the cisplatin-based trials (5). In addition, meta-analysis data also showed that concurrent chemotherapy and radiation (ChRT) significantly improved survival when compared with sequential chemotherapy and radiation, and most effective treatment observed was the immediate concurrent ChRT (5, 6).

However, most commonly used chemotherapy regimens cannot be administered at full doses safely with radical doses of thoracic radiation, and a conclusion arising from these efforts is that concurrent ChRT produces the highest cure rates but with an increased level of toxicity (7, 8). Notable, data from clinical trials with cisplatin-based concurrent ChRT showed that schedules with low-dose chemotherapy might reduce the incidence and level of side toxicities, including acute esophagitis and hematologic toxicities (8). It is therefore possible to hypothesize that further
optimization of chemotherapy regimen to be given with radiation may help for better treatment outcomes, with reduced toxicity.

In chemoradiotherapy strategies for cancer patients, the chemotherapy aim to minimize the risk of distant metastasis and the radiotherapy will preserve locoregional control (9). A chemotherapeutic drug may also act as a radiosensitizer and increase the effect of radiation treatment (10). Of note, studies have shown that cisplatin can increase radiosensitization (11). However, studies also revealed that cisplatin treatment is associated with significant toxic effects and requires fluid hydration, which can be problematic in patients with cardiovascular disease. In addition, prolonged cisplatin treatment may promote the emergence of resistant tumors (12).

In this study, we investigated the synergistic radiosensitization effects of Suberylanilide Hydroxamic Acid (SAHA) and cisplatin on human lung cancer cells. We demonstrate here that SAHA can promote the radiosensitization effects of cisplatin on human lung cancer, which may be mediated through interaction of impaired non-homologous end-joining (NHEJ or EJ) and homology-directed repair (HDR or DR) for repair of DNA double-strand break (DSB) caused by treatment of ionizing radiation (IR). Our data also showed that SAHA can induce differentiation of cancer stem cells (CSC) and reduce the invasiveness of lung cancer cells.

Materials and Methods

Reagents
SAHA and cisplatin were purchased from Sigma-Aldrich. Anti–DNA-PKcs and anti–phospho-histone H2A.X (ser-139) antibodies were from Upstate Biotechnology. Anti-RAD51, anti-KI67, anti-syndecan 3, anti-involucrin, and anti-p53 antibodies were from Santa Cruz Biotechnology. Anti-b-actin antibody was from Cell Signaling Technology. siRNA oligos for RAD51, p53 and DNA-pKcs, and control siRNA-A were also from Santa Cruz Biotechnology. Plasmids pimI-SceI and EcoR V were from New England Biolabs. pcDNA3.1 has been described previously (13). Enzymes for recombinant plasmid as previously reported (16). Briefly, cells were transiently transfected with siRNA oligos. Twenty-four hours later, cells were then treated with SAHA, cisplatin, and/or IR as described above.

Cell culture
Human NSCLC cell lines H460, A549, H1299, and MCF-10A were obtained directly from the ATCC, which has provided certification of authentication as published on their website (i.e., using Karyotype and STR Profiling analyses). A549 cells were grown in F12-K medium supplemented with 10% FBS (Omega scientific). H460 and H1299 cells were grown in RPMI-1640 medium supplemented with 10% FBS. MCF-10A cells were grown in MEGM Mammary Epithelial Cell Growth Medium (Lonza). U2OS reporter cell lines, which consist of the U2OS osteosarcoma cell line with integrated copies of the EJ-GFP, DR-GFP, and SSA-GFP reporters along with a unique I-SceI cleavage site, have been described previously (14, 15), and were maintained in DMEM medium with 2 mmol/L L-Glutamine, 4.5 g/L glucose, and 10% FBS. Hamster lung fibroblast cell lines V79 (wild-type) and VC8 (defective in the BRCA2 gene), and Chinese hamster ovary (CHO) cell lines AA8 (wild-type) and V3 (defective in DNA-PKcs expression) were maintained in DMEM medium as described previously (14). All cell lines were tested as negative Mycoplasma contamination with Cell Culture Contamination Detection Kit (Thermo Fisher Scientific), and none of the cell lines was cultured longer than 6 months.

Stable transfection
Transfections were performed with electroporation with Gene Pulse Xcell (Bio-Rad) as per the manufacturer’s instructions. Stable transfectants were selected by G418 (Sigma).

Clonogenic survival assays
Log-phase cells were treated with SAHA for 72 hours or with cisplatin for 24 hours. Cells were trypsinized and plated for colony formation. Ionizing irradiation (IR) was delivered immediately after cell plating. When combination treatment was applied, cells were pretreated with SAHA for 72 hours, and cisplatin was added into cell cultures 24 hours before trypsinization. DMSO was included as control. Survived colonies were stained with crystal violet, and colonies consisting >50 cells were directly scored. Average numbers for survival colonies were plotted versus doses of SAHA, cisplatin or IR to determine the lethal doses (LD), or survival fractions.

In experiments with siRNA, cells were transiently transfected with siRNA oligos. Twenty-four hours later, cells were then treated with SAHA, cisplatin, and/or IR as described above.

Median effect analysis
Median effect analysis was performed as described previously (16). Briefly, cells were treated with increasing total doses of cisplatin, SAHA, and IR with constant ratio of doses based on the corresponding LD50, and cell survival was determined using clonogenic survival assay. A plot of the log of the total dose versus log of the reciprocal of the fraction of cells affected minus 1 yielded linear plot. The slope and y-intercept from these plots were used to calculate the CI (17, 18).

Immunofluorescence analysis
After treatments, cells were washed twice with Ca2+/Mg2+-free PBS, and fixed in 4% paraformaldehyde. Immunofluorescence analyses for γH2AX were performed as previously reported (14). Images were acquired with LSM 510 confocal microscope (Zeiss) with ×40 objective and processed by Photoshop (Adobe). At least 100 cells from each experiment were selected at random and were counted to calculate the percentage of cells as “positive” for γH2AX foci if they displayed >5 discrete dots in nuclei.

DNA damage repair assays
A total of 1 × 105 U2OS reporter cells were plated in 12-well plate. Twenty-four hours later, cells were transfected with 0.8 μg pCBASce by using Lipofectamine 2000 transfection reagent (Life Technologies), and 1 μmol/L SAHA or 2 μmol/L cisplatin were added into culture medium 3 hours later after the initiation of the transfection. After exposure for 24 hours, cisplatin was removed from cell culture, and cells were maintained in fresh medium for cisplatin treatment or in SAHA-containing medium until analysis as described previously (14). GFP-positive cells were quantified by flow cytometric analysis. Up to 5 × 104 cells were counted for each sample. When siRNA applied, siRNA oligos were cotransfected with pCBASce.

In vivo NHEJ and HDR assays
In vivo reunion assays were based on the reactivation of linearized plasmid as previously reported (16). Briefly, cells were
pretreated with SAHA for 72 hours or with cisplatin for 24 hours. A total of $1 \times 10^5$ cells were then collected and cotransfected with 1.2 µg I-SceI–linearized EJ5-GFP or DR-GFP substrates and 0.5 µg circular pDsRed-Express2-N1 (as transfection control) by using electroporation. After replating, cells were treated with 2 Gy of IR. Flow cytometry analysis was performed 72 hours later with Fortessa Flow Cytometer (Fuojarma). The ratio of GFP-positive cells to DsRed-positive cells was used as a measure of relative activity for NHEJ or HDR.

**Flow cytometry analysis**

Cells were detached with stempro accutase (Life Technologies), and washed twice with PBS. Cells were then stained with phycocerythrin (PE)-conjugated anti-Sox2, anti-Oct3/4, anti-Nanog, or anti-CD133 antibodies, or costained with PE-conjugated anti-CD24 and FITC-conjugated anti-CD44 antibodies (BD Biosciences). In the process for staining of Sox2, Oct3/4, and Nanog, BD Perm/Wash buffer was also used as per manufacturer’s instructions. PE- or FITC-positive cells were quantified by flow cytometry.

For cell-cycle analysis, cells were collected and fixed with 80% ethanol, stained with propidium iodide, and analyzed by flow cytometry. FlowJo software (FlowJo data analysis software) was used for cell-cycle analysis.

**Immunoblot assay**

Cell lysates were prepared in RIPA buffer with mild sonication and subjected to SDS-PAGE gel for immunoblot assays. For assays of acetyl-histone H4, 1 mmol/L TSA, and 5 mmol/L nicotinamide were added into RIPA buffer.

**Invasion assay**

A total of $5 \times 10^4$ cells in growth medium containing 1% FBS were seeded in 1 × BME (Trevenen) coated 8.0-µm pore size cell culture inserts (for 24-well plate; Millipore). Complete growth medium containing 10% FBS was placed outside the chambers, and cells were allowed to invade toward the attractant of full-serum medium. Chamber filter processing and visualization/quantitation of invasion was performed as described previously (19).

**Primary tumor tissue culture**

Four primary tumor tissues were collected from surgical specimens of lung cancer patients at Affiliated Hangzhou Hospital of Nanjing Medical University. Informed consents were obtained from all patients before operation and the procedure was approved by the Medical Ethics Committee. Tumor tissues were immediately cut into 4 mm x 4 mm sections, and randomly plated into 6-well dishes precoated with 2 mL methylcellulose medium (STEMCELL Technologies). Complete medium containing SAHA and cisplatin were then added. Twenty-four hours later after cisplatin treatment, tissue sections were irradiated or left untreated. Immunofluorescence analysis for γ-H2AX staining was performed in frozen tissue sections.

**Tumor growth analysis and tumor-initiating test**

Animal protocol (Project # SCXK2008–0016) for tumor-initiating test and tumor growth assay was reviewed and approved by the Institute Animal Ethical Committee at Zhejiang Experimental Animal Center and Zhejiang Academy of Medical Sciences (Hangzhou, China).

For tumor growth assay, $1 \times 10^4$ of H460 cells in 0.2 mL of HBSS/Matrigel (Life Technologies) mixture (1:1 V/V) were inoculated subcutaneously into the right thigh of 4- to 6-week-old female athymic nude mice (Charles River Laboratories). When tumor volumes reached a size of 20 to 50 mm³, mice were randomly grouped into 6 groups (n = 6–9) and receive following treatments: (i) DMSO for 5 days as control; (ii) IR (5 Gy) x1 on day 3 after initiating the treatments; (iii) SAHA (20 mg/kg/d) for 5 days; IR was delivered on day three after SAHA injection; (iv) cisplatin (1 mg/kg/d) for 2 days at day 2 and day 3; IR was delivered on day three after cisplatin injection; (v) combination treatment of SAHA and cisplatin; (vi) combination treatment of SAHA, cisplatin, and IR. All chemicals were delivered with intraperitoneal injection, and mice were irradiated locally on the right thigh using a collimator with a 30-mm opening. Tumors were measured biweekly and tumor volumes were determined from caliper measurements of tumor length (L) and width (W) according to the formula $(L \times W^2)/2$.

Tumor-initiating test was conducted following the described in Materials and Methods (20). Briefly, H460 cells were treated with 1 µmol/L SAHA or DMSO for 72 hours. Freshly prepared cells were resuspended in serum-free RPMI1640/Matrigel mixture (1:1 v/v), and 500 cells were inoculated subcutaneously to bilateral flanks of same female NOD/SCID mouse (6 weeks old; Charles River Laboratories). The mice were euthanized 4 weeks after tumor cell injection and tumors were excised. Tumor sizes were measured using a caliper and hematoxylin and eosin (H&E) staining was performed for validation of formed tumors.

**Statistical analyses**

Statistical analyses were performed using the Student t test. A P value of <0.05 was considered as significant (⁎).

**Results**

**Cisplatin-induced and SAHA-induced radiosensitizations involve distinct compromised DNA damage repair pathways**

It has been previously reported that cisplatin treatment does not affect NHEJ activity, however, complex cisplatin-DSB lesions directly impair cellular NHEJ (11, 21). On the other hand, our studies showed that exposure to SAHA reduced expression of Rad51, a key element involved in HDR pathway, and decreased HDR activity in cells with SAHA treatment (Fig. 1A). We also observed that cisplatin and SAHA may act on distinct DNA damage repair pathways, both leading to the potential of enhancing cellular sensitivity to IR treatment.

To test this, we first evaluated the effects of cisplatin and SAHA on different DNA damage repair machineries. In a pilot study, we found that SAHA at a minimal concentration of 500 nmol/L could induce acetylation of histone H4 in U2OS-DR cells, and treatment with 2 µmol/L of cisplatin for 24 hours induced $G_2$–M accumulation in U2OS-EJ cells. However, we observed that exposure to either 2 µmol/L SAHA for 72 hours or 5 µmol/L cisplatin for 24 hours caused obvious cytotoxicity in nonmutagenic human mammary epithelial MCF-10A cells, as shown for decrease of clonogenic survival (Supplementary Fig. S1). We thus used 1 µmol/L of SAHA for 72 hours and 2 µmol/L of cisplatin for 24 hours as treatment schedules. With DNA damage repair assays using U2OS reporter cells, we detected significant reduction of HDR activity in cells with SAHA treatment (Fig. 1A). We also observed that exposure to SAHA dramatically decreased Rad51 protein level, and knocking down of Rad51 by siRNA transfection eliminated the inhibitory effect of SAHA on HDR activity.
SAHA Enhances Radiosensitization of Cisplatin in Lung Cancer

Figure 1.
Effects of SAHA and cisplatin on DNA damage repair. A, U2OS reporter cells were used to determine the effects of SAHA (1 μmol/L) or cisplatin (2 μmol/L) on distinct DNA DSB repair pathways. Repair of I-SceI-induced DSB was quantified by flow cytometry as described in Materials and Methods. B, inhibitory effect of SAHA on HDR is dependent on Rad51 protein. Top, Western blot analysis showing the effect of SAHA on Rad51 protein expression in U2OS-DR reporter cells. β-Actin was included to verify the protein equal loading; bottom, U2OS-DR reporter cells were transfected with siRNA-Rad51, and the effect of knocking-down Rad51 expression on SAHA-inhibited HDR was measured with flow cytometry. C, in vivo reunion assay for relative NHEJ activity. U2OS cells were transfected with or without siRNA-DNA-pKcs, and were then treated with 2 μmol/L cisplatin for 24 hours followed by cotransfection of linearized (with I-SceI) EJ5-GFP plasmid and control pMD18. Twenty-four hours later, cells were irradiated with 2Gy IR, and recycled EJ5-GFP was counted by flow-cytometry analysis 48 hours post-IR (left). Graphs show the changes of relative NHEJ activity. D, clonogenic survival analysis was performed to determine LD50 values for IR, cisplatin, and SAHA in paired cell lines. E and F, median effect analyses. Combination indexes (CI) were determined for combined treatment of IR with cisplatin (E) or with SAHA (F). Data represent the average of three independent experiments.

[Fig. 1B]. However, no obvious changes were detected in any reporter cell lines when cells were treated with cisplatin. In addition, we noticed that cisplatin treatment did not change the reunion frequency of transfected linear EJ5-GFP plasmid DNA, which indicates the in vivo NHEJ repair capability and is eventually affected by silencing of DNA-pKcs expression (Fig. 1C).

We next tested the radiosensitization effects of cisplatin and SAHA in NHEJ- and HDR-deficient CHO and hamster lung fibroblast cells. We performed median effect analyses in these cell lines with treating cells with constant ratios of corresponding LD50 values of IR, SAHA, and cisplatin that were determined by colony formation assay (Fig. 1D and Supplementary Fig S1). Our results showed that treatments with cisplatin and SAHA both induced synergistic radiosensitization (as indicated of CI values < 1) in control V79 and AA8 cells, and these synergistic radiosensitization effects were further enhanced correspondingly in BRCA2-defective V8 in cisplatin (Fig. 1E) and in DNA-pKcs-defective V3 cells for SAHA (Fig. 1F), with obviously reduced CI values. However, CI values stayed approaching near 1 in cisplatin-treated V3 cells and in SAHA-treated V8 cells, indicating that the synergisms for radiosensitization effects were eliminated in NHEJ-deficient cells for cisplatin and in HDR-deficient cells for SAHA.

These results validated that cisplatin can induce synergistic radiosensitization through impaired NHEJ (without affecting NHEJ activity), and SAHA compromises HDR, which leads to increased sensitivity of cells to IR treatment.

Effects of SAHA and cisplatin on DNA damage repair pathways in human lung cancer cells in response to IR treatment

In human lung cancer H460 and H1299 cells, treatment with 1 μmol/L of SAHA for 72 hours or with 2 μmol/L of cisplatin for 24 hours also showed their biologic effects on histone acetylation...
(for SAHA) or G2–M accumulation (for cisplatin, Supplementary Fig. S2). As expected, treatment with cisplatin alone had no obvious effects on expressions of NHEJ-relative DNA-pKcs, Ku70, and Ku80 proteins, or HDR-relative Rad51 protein in both H460 and H1299 cells, and did not cause any change in either HDR or NHEJ activity in these cells receiving IR treatment, as determined with in vivo reunion assay. Treatment with SAHA or SAHA combining with cisplatin, however, obviously decreased Rad51 protein expression. Exposure to SAHA also decreased HDR activity in both irradiated H460 and H1299 cells (Fig. 2A–C, Supplementary Fig. S2).

Interestingly, we noticed that SAHA treatment decreased Ku80 protein expression and reduced NHEJ activity in H460 cells. However, no such changes were observed in H1299 cells (Fig. 2A and C, Supplementary Fig. S2). Of note, H460 cells express wild-type p53 and H1299 cells express no p53 protein (Supplementary Fig. S2). Mutation of tumor-suppressor p53 gene is one of the most significant molecular events in lung cancers, occurring in about 50% of NSCLC, and clinical studies suggest that NSCLC with mutant p53 carries a worse prognosis and may be relatively more resistant to chemotherapy and radiation (22). To verify whether observed effects of SAHA on Ku80 and NHEJ activity correlated with p53 status, we used H1299 cells with engineered expression of wild-type p53, and found that expression of wild-type p53 protein resulted in decreased Ku80 expression and reduced reunion frequency for linear pimEJ5GFP in irradiated H1299 cells when cells were treated with SAHA (Fig. 2D). In addition, our results from assay using DR reporter

![Figure 2](image-url)

**Figure 2.** Effects of SAHA and cisplatin on distinct DNA damage repair pathways in human lung cancer cells. A, Western blot analysis. Cells were treated with 1 μmol/L SAHA for 72 hours or with 2 μmol/L of cisplatin for 24 hours, or with combined treatment of SAHA and cisplatin as described in Materials and Methods. B and C, in vivo reunion assays for relative HDR (B) and NHEJ (C) activity. Cells were treated with SAHA, cisplatin, or combined treatment as indicated. Cells were then cotransfected with linearized (with I-SceI) DR-GFP (B) or EJ-GFP (C) plasmid and control pDsRed. 2 Gy of IR was delivered 24 hours later after transfection. Recycled DR-GFP was counted by flow cytometry analysis 48 hours post-IR (top). Graphs showing the changes of relative HDR (B) and NHEJ (C) activity. D, effect of p53 expression on SAHA-inhibited HDR activity. H1299 cells with or without engineering expression of wild-type p53 protein were treated with 1 μmol/L SAHA for 72 hours, and Western blot analysis (top right) and in vivo reunion assay (iR) were then performed as described above. Graphs (bottom right) showing the changes of relative NHEJ activity. E, effect of knocking down p53 on SAHA-inhibited HDR activity. U2OS HDR reporter cells were cotransfected with pcDNA3.1 and siRNA-p53, or siRNA-A as control. Repair of I-SceI-induced DSB was quantified by flow cytometry as described in Materials and Methods. Western blot analysis showing the reduced p53 expression in siRNA-p53 transfected cells. Data represent the average of three independent experiments.
cell showed that siRNA knocking down of p53 did not affect SAHA-inhibited HDR activity (Fig. 2E). These results thus suggested a potential that, in addition to its effect on HDR, which is p53-independent, SAHA may also reduce NHEJ activity in irradiated cancer cells in a p53-dependent manner. It is needed to be indicated that we only observed slight but not significant decrease in NHEJ activity for SAHA treatment in EJ5-reported cells as shown in Fig. 1A, and this may be caused by the shorter exposure time of SAHA before activation of the NHEJ pathway in the assay.

SAHA enhances the radiosensitization effect of cisplatin in lung cancer cells

We and others have shown the potential cross-talks among DNA damage repair pathways and compromising both NHEJ and HDR pathways led to enhanced synergistic radiosensitivity in cancer cells (16, 23). To test whether SAHA may coordinate with cisplatin on radiosensitivity, we examined the combination effect of SAHA and cisplatin on clonogenic survivals of H460 and H1299 cells receiving a single clinical radiation dose (2 Gy). We found that pretreatment with SAHA or cisplatin significantly decreased clonogenic survival of irradiated cells: For H460, the survival fraction decreased from 74.21 ± 7.79 to 27.94 ± 5.55 for SAHA treatment (P = 0.0011), and to 35.33 ± 7.40 (P = 0.0033) for cisplatin treatment; for H1299, the survival fraction decreased from 78.40 ± 8.87 to 41.59 ± 3.14 for SAHA treatment (P = 0.0025), and to 38.85 ± 2.27 for cisplatin treatment (P = 0.0017), respectively. As expected, our results showed that combined treatment of SAHA and cisplatin further led to dramatically enhanced depression on clonogenic survival for both H460 and H1299 cells (P < 0.05 when compared with IR treatment or to the treatment of each single agent, Fig. 3A and B). In H1299 cells, however, we noticed that the enhancement effect of SAHA on cisplatin-inhibited clonogenic survival of irradiated cells was eliminated when Rad51 expression was knocked down (Fig. 3C). This result indicates a role of Rad51 expression, or HDR, in SAHA-enhanced synergistic effect of cisplatin on radiosensitivity of cells.

We further performed median effect analysis in these cell lines. In this experiment, LD₅₀ values for cisplatin and IR, and the CI values for the combination treatment of cisplatin and IR were determined in cells exposed to SAHA, or DMSO as control. Our results showed that the pretreatment with 1 μmol/L SAHA for 72 hours decreased LD₅₀ values of IR from 2.96 ± 0.31 Gy to 1.60 ± 0.26 Gy (P = 0.004) for H460 cells, and from 3.19 ± 0.19 Gy to 2.03 ± 0.18 Gy (P = 0.002) for H1299 cells, respectively. SAHA pretreatment also slightly reduced LD₅₀ values of cisplatin from 1.82 ± 0.15 μmol/L to 1.60 ± 0.10 μmol/L in H460 cells, and from 2.74 ± 0.43 μmol/L to 1.73 ± 0.10 μmol/L in H1299 cells; however, no statistical significances were detected for these changes (P = 0.0964 for H460 and P = 0.0166 for H1299, Fig. 3D). Interestingly, previous study showed that SAHA at higher concentration (2.5 μmol/L) enhanced cisplatin-inhibited tumor cell growth when cells were treated with cisplatin for 48 hours (24). The contradictory observations may be caused by the different concentrations of SAHA and treatment protocol used for cisplatin.

Median effect analysis showed that although cisplatin alone induced synergistic radiosensitization with CI values of < 1 for both H460 and H1299 cell lines, the overall CI values were further remarkably reduced for combination treatments of cisplatin and IR in cells that were with pretreatment of SAHA, indicating that SAHA pretreatment enhances synergistic radiosensitization effects of cisplatin in these cells (Fig. 3D; Table 1).

In A549 cells, we also observed that pretreatment with SAHA reduced CI values of cisplatin for its synergistic radiosensitization effect (Supplementary Fig. S3).

Effect of the combination treatment of SAHA and cisplatin on persistence of nuclear γ-H2AX foci formed in cells with IR treatment

IR produces DSBs in chromosomal DNA leading to phosphorylations of C-terminal tails of variant H2AX in chromatin and form γ-H2AX foci at DNA break sites. Persistence of γ-H2AX nuclear foci has been suggested to be an indicator of lethal DNA damage with nonrepaired DNA DSBs (25, 26). To test whether SAHA exposure affect the persistence of γ-H2AX nuclear foci in lung cancer cells treated with the combination of cisplatin and IR, we examined the kinetic changes of γ-H2AX foci in H460 cells. While unirradiated H460 cells were predominantly negative for γ-H2AX, a homogeneous pattern of discrete nuclear γ-H2AX foci was observed immediately after IR delivery, reaching almost 90% of positive foci, and then decreased slowly over time. Pretreatments with SAHA or cisplatin alone, or with combined treatment of SAHA and cisplatin, did not cause obvious basal level changes of nuclear γ-H2AX foci. However, the percentage of cells with residual γ-H2AX foci at time point of 60 hours post-IR remained higher in cells with pretreatment of SAHA (30.7 ± 5.27 vs. 11.7 ± 2.38 for control, P = 0.005) or of cisplatin (25.3 ± 5.18, P = 0.014). Notably, in cells treated with the combination of SAHA and cisplatin, more than half (60.7 ± 4.11) of the cells showed persistent nuclear γ-H2AX foci after 60 hours post-IR, with statistical significance when compared with those in cells treated with IR alone (P = 0.0001), or single agent of SAHA (P = 0.0015) or cisplatin (P = 0.0008; Fig. 4A), indicating that SAHA could further reduce the cellular capabilities to repair IR-induced DNA damage in cells with treatment of cisplatin.

In surgical lung cancer tumor tissues cultured in a three-dimensional environment, we found the number of cells with γ-H2AX nuclear foci formed after IR or combined treatment of IR with cisplatin and SAHA varied remarkably among the cases studied. However, we did observe that pretreatment with SAHA caused dramatically enhanced persistence of γ-H2AX nuclear foci in tumor tissues that were treated with IR in combination with cisplatin, as shown in Fig. 4B which illustrates the results from one case studied.

Effect of the combination treatment of SAHA and cisplatin on tumor growth of H460 xenografts receiving radiotherapy

We further determined the effect of the combination treatment of SAHA and cisplatin on the tumor growth of H460 xenografts receiving radiotherapy. In this study, the doses of SAHA and cisplatin were determined with the results of a pilot tolerances study (data not present). In consistence with the results seen in vitro, our in vivo data showed that the combination treatment dramatically increased tumor growth suppression when compared with IR alone (Fig. 4C; Supplementary Fig. S4). Of note, we did not observe any unusual symptoms such as weight loss or sickness in mice that were treated with triple therapy protocol, indicating a well tolerance for in vivo triple therapy when radiotherapy was delivered locally at tumor sites.
SAHA treatment induces differentiation of stem cell–like lung cancer cells and reduces cancer cell invasiveness

Histone deacetylase inhibitors (HDACi) have been characterized not only as anticancer drugs, but also as cytodifferentiation-inducing agents (27, 28). Of these HDACis, SAHA has also been reported to inhibit cancer cell invasion (29). We therefore tested the potential effect of SAHA on differentiation of CSCs and cancer cell invasiveness.

In H460 cells, treatment with 1 μmol/L of SAHA for 72 hours significantly decreased the percentages of cells with positive staining of embryonic stem cell marker Oct3/4 (30), Sox II (31), and Nanog (32), and putative CSCs markers CD133+ (33) and CD24low/CD44+ (34). Exposure to SAHA also increased the expressions of differentiation markers, involucrin and syndecan-3 (Fig. 5A and B and Supplementary Fig. S4; refs. 35, 36).

We further assess whether SAHA treatment could change tumorigenicity of H460 cells in vivo. In tumor-initiating test, we injected the same number of H460 cells that received SAHA treatment, or DMSO as control, subcutaneously to bilateral franks of same animal. With as few as 500 cells, both control and SAHA-treated cells formed tumors at all sites (4/4) in NOD/SCID mice. However, the average volume for tumors formed with SAHA-treated H460 cells was significantly smaller than that of control cells at day 25 (111.0 mm³ vs. 12.7 mm³; Fig. 5C). Most importantly, SAHA-treated cells had longer latency for forming tumors when compared with control cells (14 ± 4 days vs. 22 ± 2 days). Thus, these results suggested that SAHA could induce differentiation of stem cell–like cancer cells and reduce tumorigenicity of H460 cells.

With Transwell invasion assay, we observed that treatment with 1 μmol/L SAHA for 72 hours remarkably reduced invasiveness of
H460 cells, as shown with the changes of the percentage for invasive cells (Fig. 5D and Supplementary Fig. S4). Interestingly, our results showed that cisplatin treatment could also reduce the percentage of invasive cells, and this effect was further enhanced in cells with pretreatment of SAHA.

In H1299 cells, we noticed barely detectable cell fraction with positive staining of CD24<sup>low</sup>/CD44<sup>+</sup>, and no obvious changes were observed for the percentages of CD24<sup>low</sup>/CD44<sup>+</sup> and SOX II cell populations in cells treated with SAHA. However, exposure to SAHA decreased the percentages of cells with positive staining of CD133<sup>+</sup>, Oct3/4, or Nanog, and induced protein expressions of involucrin and syndecan-3. We also observed that treatment with SAHA dramatically reduced cancer cell invasiveness (Supplementary Fig. S5).

Taken together, our results present here suggest that SAHA and cisplatin compromise distinct DNA damage repair pathways, which lead to enhanced radiosensitivity in human lung cancer cells treated with combination of SAHA and cisplatin; treatment with SAHA can also induce differentiation of stem cell–like cancer cells and reduce lung cancer cell invasiveness. Of note, these potential biologic effects of SAHA are not p53 dependent.

**Discussion**

Despite the tremendous efforts and progress in lung cancer research, treatment outcomes for nonlocalized NSCLC remain poor (37). New treatment strategies are urgently needed to improve survival for advanced NSCLC patients. In this study, we uncovered a potent synergistic radiosensitization effect of cisplatin and SAHA (vorinostat), a HDACi approved as single-agent chemotherapy for refractory cutaneous T-cell lymphoma (38), and for in and SAHA (vorinostat), a HDACi approved as single-agent treatment for targeting cancer cell invasion and metastasis. In addition, the CSC differentiation induced by SAHA may also contribute to the observed enhancement of radiosensitization in cisplatin-treated cancer cells.

**Table 1.** Median effect analysis for the effect of SAHA on synergistic radiosensitization of cisplatin in human lung cancer H460 and H1299 cells.

<table>
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<tr>
<th>Cell line</th>
<th>Combination Ratio</th>
<th>10% CI value&lt;sup&gt;a&lt;/sup&gt; at inhibition of</th>
<th>50% CI value&lt;sup&gt;a&lt;/sup&gt; at inhibition of</th>
<th>75% CI value&lt;sup&gt;a&lt;/sup&gt; at inhibition of</th>
<th>90% CI value&lt;sup&gt;a&lt;/sup&gt; at inhibition of</th>
<th>10% DRI value&lt;sup&gt;b&lt;/sup&gt; at inhibition of</th>
<th>50% DRI value&lt;sup&gt;b&lt;/sup&gt; at inhibition of</th>
<th>75% DRI value&lt;sup&gt;b&lt;/sup&gt; at inhibition of</th>
<th>90% DRI value&lt;sup&gt;b&lt;/sup&gt; at inhibition of</th>
</tr>
</thead>
<tbody>
<tr>
<td>H460</td>
<td>Without SAHA</td>
<td>0.78</td>
<td>0.58</td>
<td>0.55</td>
<td>0.51</td>
<td>0.34</td>
<td>0.85</td>
<td>1.35</td>
<td>2.13 (Gy)</td>
</tr>
<tr>
<td></td>
<td>With SAHA</td>
<td>0.79</td>
<td>0.47</td>
<td>0.37</td>
<td>0.29</td>
<td>0.29</td>
<td>0.38</td>
<td>0.42</td>
<td>0.48 (Gy)</td>
</tr>
<tr>
<td></td>
<td>P values for paired CI</td>
<td>0.9081</td>
<td>0.0026</td>
<td>0.098</td>
<td>0.0002</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H1299</td>
<td>Without SAHA</td>
<td>0.93</td>
<td>0.77</td>
<td>0.70</td>
<td>0.64</td>
<td>0.80</td>
<td>1.23</td>
<td>1.52</td>
<td>1.89 (Gy)</td>
</tr>
<tr>
<td></td>
<td>With SAHA</td>
<td>0.92</td>
<td>0.66</td>
<td>0.56</td>
<td>0.48</td>
<td>0.48</td>
<td>0.67</td>
<td>0.79</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td>P values for paired CI</td>
<td>0.2984</td>
<td>0.0013</td>
<td>0.0054</td>
<td>0.0011</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>CI < 1, CI = 1 and CI > 1 indicate synergism, additive effect, and antagonism, respectively.

<sup>b</sup>DRI represents the order of magnitude (fold) of dose reduction that is allowed in combination for a given degree of effect compared with the dose of each treatment alone. Upper values are for IR (Gy) and lower values are for cisplatin (μmol/L). P values were determined with Student t test. Data from three independent experiments.

NSCLC patients (41, 42), and thus results in improved control rates. However, further improvements in the therapeutic index of radiation through dose escalation or the addition of chemotherapy agents that add to the toxicities of the conditioning regimen remain challenging, and in particular, there is a clear need for agents and strategies that will increase the radiosensitivity of cancer cells to radiation therapy without significant additional toxicity for advanced NSCLC patients.

The molecular basis of radiation response is multifactorial. However, the predominant mechanism by which therapeutic irradiation kills most tumor cells is through clonogenic death. In the process, DSBs are regarded as the specific lesions that initiate this lethal response (43, 44), and the repair of DSBs is then critical in determining radiosensitivity (45, 46). Thus, targeting DNA damage repair pathways has been a strategy for optimizing radiotherapy to improve outcomes for cancer treatment (47). To this setting, the results present here showing that SAHA and cisplatin compromise distinct DNA damage repair pathways, and the combination effect of SAHA and cisplatin on radiosensitivities of human lung cancer cells and xenograft tumors suggest a clinic impact for novel therapeutic regimens containing SAHA as radiosensitizer for treatment of NSCLC patients.

Of interest, we showed here that SAHA treatment can induce differentiation in the population of stem cell–like cancer cells or CSCs. CSCs represent a subpopulation of tumor cells endowed with self-renewal and multilineage differentiation capacity, with an innate resistance to cytotoxic agents, which thus become major clinical challenges toward the complete eradication of minimal residual disease in cancer patients (48). CSCs are also likely to play essential roles in the metastatic spread of primary tumors because of their self-renewal capability and their potential to give rise to differentiated progenies that can adapt to different target organ microenvironments (49). Thus, targeting CSCs or inducing cell differentiation in this cell population may help to challenge tumor relapse and cancer metastasis. Indeed, we found that exposure to SAHA significantly lowers the invasive potential of human lung cancer cells in vitro, indicating a potential of SAHA treatment for targeting cancer cell invasion and metastasis. In addition, the CSC differentiation induced by SAHA may also contribute to the observed enhancement of radiosensitization in cisplatin-treated cancer cells.
Figure 4.
Effect of SAHA on cisplatin-induced persistence of nuclear γ-H2A.X foci in irradiated lung cancer cell and in primary lung tumor tissues, and on tumor growth in H460 xenografts with radiotherapy. A, representative images of nuclear γ-H2A.X foci formed in irradiated H460 cancer cells. Diagram (bottom) showing the changes of cell fractions with γ-H2A.X foci in irradiated H460 cells with indicated treatments. B, representative images of nuclear γ-H2A.X foci formed in irradiated primary lung cancer tumor tissues; diagram (bottom) showing the changes of cell fractions with γ-H2A.X foci in irradiated primary lung cancer tumor tissues with indicated treatments. C, effect of combined treatment of SAHA and cisplatin on tumor growth in H460 xenografts in response to radiotherapy of 5 Gy IR. The growth curves represent the average value in each group of 6 to 9 mice. Error bars, one SE.
SAHA induces differentiation of stem cell-like cancer cells and inhibits cancer cell invasion of H460 cells treated with cisplatin. H460 cells were treated with 1 μmol/L of SAHA or DMSO as control for 72 hours, and collected for flow cytometry, Western blot analysis, and tumor-initiating test, or were replated for invasion assay. A, flow cytometry analysis for putative cancer stem cell markers. B, Western blot analyses for expressions of involucrin and syndecan-3 in H460 cells with or without SAHA treatment. C, tumorigenesis of H460 cells with or without SAHA treatment. Top, images for collected tumors from tumor-initiating test; bottom, H&E staining results for formed tumors. D, representative images of Transwell invasion assay; 2 μmol/L of cisplatin was added to cells 24 hours before plating for invasion assay.

Figure 5.
However, it needs to be indicated that SAHA may cause epigenetic regulation for gene expression, as we observed that SAHA treatment resulted in multiple changes of protein expression in this study. Indeed, HDACi, including SAHA, has shown promise in clinical trials as epigenetic therapy for human malignancies (50). These facts thus necessitate future studies to rule out/rule in alternative mechanisms of cisplatin + SAHA synergy in addition to effects on DNA damage repair and on CSC differentiation. On the other hand, the detail for the role of p53 in SAHA-regulated DNA damage NHEJ repair also needs to be determined.

Nevertheless, our data demonstrate the synergistic radiosensitization effects of SAHA and cisplatin in NSCLC cells and lung cancer xenografts; SAHA can also induce CSC differentiation and reduce cancer cell invasion, indicating potential therapeutic strategies with SAHA-containing chemoradiotherapy may further reduce cancer cell invasion, indicating potential therapeutic strategies with SAHA-containing chemoradiotherapy may further benefit those patients with nonresectable NSCLC in advanced stages (IIIB and IV). However, future studies are needed to evaluate the clinical applicability for SAHA as a part of the chemoradiotherapy regimen and the overall role of Rad51 for this potential regimen should be further determined.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Disclaimer
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the article.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Feng, S. Zhang, K. Wu, H. Jiang, R. Xu, X. Chen
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Feng, S. Zhang, K. Wu, L. Ying, X. Zheng, X. Chen, S. Ma
Study supervision: X. Chen, S. Ma

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Molecular Cancer Therapeutics

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