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ORIGINAL ARTICLE

RAD51 potentiates synergistic effects of chemotherapy with PCI-24781 and *cis*-diamminedichloroplatinum on gastric cancer

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Abstract

AIM: To explore the efficacy of PCI-24781, a broad-spectrum, hydroxamic acid-derived histone deacetylase inhibitor, in the treatment of gastric cancer (GC).

METHODS: With or without treatment of PCI-24781 and/or *cis*-diamminedichloroplatinum (CDDP), GC cell lines were subjected to functional analysis, including cell growth, apoptosis and clonogenic assays. Chromatin immunoprecipitation and luciferase reporter assays were used to determine the interacting molecules and the activity of the enzyme. An *in vivo* study was carried out in GC xenograft mice. Cell culture-based assays were represented as mean ± SD. ANOVA tests were used to assess differences across groups. 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. Significance was set at $P < 0.05$.

RESULTS: PCI-24781 significantly reduced the growth of the GC cells, enhanced cell apoptosis and suppressed clonogenicity, and these effects synergized with the effects of CDDP. PCI-24781 modulated the cell cycle and significantly reduced the expression of RAD51, which is related to homologous recombination. Depletion of RAD51 augmented the biological functions of PCI-24781, CDDP and the combination treatment, whereas overexpressing RAD51 had the opposite effects. Increased binding of the transcription suppressor E2F4 on the RAD51 promoter appeared to play a major role in these processes. Furthermore, significant suppression of tumor growth and weight *in vivo* was obtained following PCI-24781 treatment, which synergized with the anticancer effect of CDDP.

CONCLUSION: These data suggest that RAD51 potentiates the synergistic effects of chemotherapy with PCI-24781 and CDDP on GC.

Key words: Chemotherapy; Combination; Gastric cancer; Histone deacetylase inhibitor; Homologous recombination

Core tip: This is the first study to show that PCI-24781 synergizes with the chemotherapeutic effect of *cis*-diamminedichloroplatinum in gastric cancer *in vivo* and *in vitro*, and PCI-24781-induced RAD51 repression may be one of the mechanisms. PCI-24781 could be a potential drug and novel therapeutic strategy for the treatment of gastric cancer.

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INTRODUCTION

Gastric cancer (GC) is one of the most aggressive malignancies, especially in South Asia. It ranks as the second leading cause of cancer mortality in China[1]. Despite curative surgery and postoperative adjuvant therapy, nearly 60% of patients succumb to the disease[2,3]. While the utility of classical chemotherapy agents has been thoroughly explored, advances have been slow, and the efficacy of these agents has reached a plateau[4]. Recent studies have suggested that histone deacetylase inhibitors may be attractive anticancer drugs because histone deacetylases (HDACs) are frequently upregulated in cancers, and these drugs can be less toxic to patients[5-7]. Among these HDAC inhibitors (HDA*Cis*), suberoylanilide hydroxamic acid (SAHA, Vorinostat) was first approved by the US Food and Drug Administration for the treatment of T-cell lymphoma. *In vitro* chemosensitivity of gastric adenocarcinomas to SAHA has been reported[8]. Similarly, PCI-24781 (Pharmacyclics, Inc.), a broad-spectrum, hydroxamic acid-derived HDACi currently being evaluated in phase I clinical trials[9], has shown significant anticancer activity in soft tissue sarcomas[10] and gallbladder carcinomas[11], as well as other tumor cell lines including colon carcinomas[12], glioblastomas[13], breast cancers[14], and bone sarcomas[15]. However, information about the efficacy of PCI-24781 in the treatment of gastric cancer is limited.

Despite HDA*Cis* showing promise as single agents, several recent studies have suggested that the optimal use of HDA*Cis* is likely in combination with other chemotherapeutic agents[10,15,16]. *Cis*-diamminedichloroplatinum (CDDP) is a classic chemotherapeutic drug frequently used in GC treatment. CDDP exerts its effect mainly by causing DNA damage. However, DNA damage can be repaired through homologous recombination (HR) or through non-homologous end joining[17], which can lead to chemotherapy resistance. HR usually occurs during and shortly after DNA replication during the S and G2 phases of the cell cycle, when sister chromatids are more easily available[18]. RAD51 is a key protein involved in and is regarded as a biomarker for homologous recombination[19]. Studies have suggested that RAD51, which highly correlates with GC[20], is involved in the repair of DNA double-strand breaks (DSBs) produced by CDDP and other platinum agents[21]. Adimoolam *et al*[22] also confirmed that PCI-24781 could decrease RAD51 expression and suppress HR in colon tumor cells. Thus, in this study we aimed to evaluate the efficacy of PCI-24781 on GC and its combinational effect of chemotherapy with CDDP and elucidate underlying mechanisms to find out whether RAD51 is involved in the process.

MATERIALS AND METHODS

Cell culture

The human GC cell line AGS (most common gastric adenocarcinoma), HGC27 (undifferentiated carcinoma with high malignancy) was obtained from the Committee of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). GC cell lines were cultured in RPMI 1640 medium (Gibco), supplemented with 10% fetal calf serum (HyClone, Logan, UT, United States) at 37 ℃ under a humidified atmosphere of 5% CO_2. The HDAC inhibitor PCI-24781 was purchased from Pharmacyclics and dissolved in DMSO to create stock solutions.

Measurement of cell growth

Cell growth assays were done using CellTiter96 cell proliferation assay kit (Promega)[23]. Cells were plated at concentrations of 2×10^3 cells per well in 96-well plates. The next day, cells were treated with either 0.1% DMSO as control or different concentrations of PCI-24781 or/and CDDP for 48 h. Absorbance was measured at a wave length of 490 nm. Drug concentrations required to inhibit cell growth by 50% (IC_{50}) were determined by interpolation of dose-response curves. Isobologram analysis was introduced to evaluate the synergistic effect[24]. Briefly, when the combination is synergistic, the data points from the combination will be depicted at the left side of the curve that generated by IC_{50} of two drugs, while the combination is antagonistic when these points are at the right side of the curve.

Apoptosis assay

Apoptosis was measured using flow cytometry (FCM) stained with Annexin V as well as Quantum Dot Probing cleaved Caspase-3[25]. As a standard, DMSO or drug treated cells were stained with Annexin V and Qdot con-

jugation before subjected to flow cytometry or fluorescent microscopy. Fluorescent positive rates were counted five randomly selective areas and analyzed independently by three reviewers.

Clonogenic assay

GC cells were treated in culture dishes with 0.1% DMSO (control), PCI-24781 (0.25 μmol/L), CDDP (2.5 μmol/L) or a combination of PCI-24781 plus CDDP for 24 h. One hundred cells per well were replated, then allowed to grow in corresponding drug-added 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 were washed with deionized H_2O. Individual colonies retaining staining solution were counted.

Protein extraction and immunoblotting

The procedures were as previously described[26,27]. Briefly, frozen tissue samples were solubilized in lysis buffer, containing 7 mol/L urea, 2 mol/L thiourea, 2% CHAPS, 0.1 mol/L DTT, 0.1% NP40, 40 mmol/L Tris-HCl, using a polytron homogenizer following centrifugation (100000 *g*) for 30 min at 4 ℃. For cell lines, lysates were harvested by centrifugation (12000 rpm) at 4 ℃ for 15 min. The supernatants were separated, and protein concentration was assessed by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). After heating for 10 min at 100 ℃, equal amounts of denatured protein were resolved by 12% SDS-PAGE and electrophoretically transferred on to PVDF membranes. The blots were probed with primary antibodies. After completion, membranes were stripped and reprobed with β-actin antibody as a control. The bands were visualized using the enhanced chemiluminescence detection system (GE Healthcare, United States) and the intensity was quantified by densitometry.

Reverse transcription polymerase chain reaction and real-time quantitative polymerase chain reaction

The procedure was slightly modified according to our previous report[28]. The expression of mRNA in GC cells with different treatment was assessed with reverse transcription polymerase chain reaction (RT-PCR) using 0.5 μg of total RNA extracted by an RNeasy kit (Qiagen, Hilden, Germany). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was chosen as an endogenous standard. Primer sets were constructed by Invitrogen as follows: GAPDH Forward AATCCCATCACCATCTTCCAG and Reverse AAATGAGCCCCAGCCTTC. RAD51 Forward GGGAGAATCACTTAAGCCTGG and Reverse CTGTTTACTTGCCCTCTGAAATG. Reaction conditions were: 1 cycle of 95 ℃ for 10 min, followed by 35 cycles at 95 ℃ for 1 min for denaturation, 55 ℃ for 1 min for annealing, 72 ℃ for 1 min for extension, and finally 1 cycle of 72 ℃ for 5 min. Gene-specific amplification was analyzed on 1% agarose gel and visualized by ethidium bromide staining method, and then performed in real-time quantitative PCR using a Light Cycler Real-time Detection System (Roche, Switzerland). All reactions were carried out with SYBR Green Master (Roche) according to the manufacturer's protocol. Reaction conditions were similar to those described above, except that the first cycle of 95 ℃ was 3 min, followed by 45 cycles of each temperature for 30 s for each reaction. The C_t (threshold cycle) value of each sample was calculated, and the relative mRNA expression was normalized to the GAPDH value.

Manipulation of RAD51

The procedures were similar to those described previously[28]. Commercial siRNA targeting RAD51 reagent was used for knocking down its expression. For the overexpression system, human RAD51 cDNA was cloned into pcDNA3.1 (Invitrogen) to yield plasmid pcDNA-RAD51. Cells with or without transfection were subjected to treatment of DMSO, PCI-24781, DDP or combination. Then the expressions of RAD51 and cell apoptosis were evaluated by Western blotting or flow cytometry respectively.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays[29] were performed using ChIP assay kits (Upstate Technology) according to the instructions. Appropriate treated GC cells were fixed in 1% formaldehyde and incubated in 0.125 mol/L glycine to stop cross-linking, then washed and recovered, followed by lysis in SDS buffer. Lysates were sonicated, yielding genomic DNA fragments with a bulk size of 200-1000 bp before centrifugation. Supernatants were diluted and precleared with salmon sperm DNA/protein G-agarose. Lysates were immunoprecipitated with relevant antibodies. Antibody-nucleoprotein complex mixtures were incubated overnight and recovered by incubation with 60 μL salmon sperm DNA/protein G-agarose for 1 h at 4 ℃. 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 ℃, followed by 1.5-h digestion with RNAse A and proteinase K at 50 ℃. DNA fragments were recovered by phenol/chloroform extraction and ethanol precipitation and analyzed by qPCR.

Luciferase reporter assay

RAD51 promoter sequence (Entrez GeneID 5888, from -403 to +63 bp) was cloned into pGL3 and then transfected into HGC27 or AGS cells using Fugene 6 transfection reagent (Roche) according to the manufacturer's instructions. The RAD51 reporter construct was further mutated at the E2F binding site using Stratagene Quick Change mutagenesis kit (Stratagene) per the manufacturer's instruction and used for transfection as above. Cells were harvested after incubation with 0.1% DMSO or

PCI-27481 (0.5 μmol/L) for 24 h. Luciferase assays were done using dual-luciferase assay reagents (Promega) and assessed with a luminometer.

Immunostaining analysis

For *in vivo* tumor samples, immunohistochemistry PCNA (Sigma) for cell proliferation and TUNEL assay (Sigma) for cell apoptosis were used. Staining distribution (% positive stained tumor cells) and intensity (0 = no staining, 1 = low, 2 = high) counts were evaluated and scored by three independent reviewers. Cells stained with FK2 and RAD51 antibodies after IR or drugs were examined by confocal laser scanning microscopy (CLSM). Cells stained with FK2 (Life technologies) and RAD51 (Santa Cruz Biotechnology) antibodies after ionizing radiation (IR) or drugs were examined by CLSM.

In vivo therapeutic studies

All animal procedures and care were approved by the Hospital Animal Care and Usage Committee according to NIH "Guide for the Care and Use of Laboratory Animals." Animal models were utilized as previously described[30]. Viable HGC27 cells (1×10^6/0.1 mL HBSS/mouse) were injected into the flank (*sc*) of 6-wk-old female SCID mice ($n = 40$/experiment), growth was measured twice weekly. When average tumor volumes reached about 100 mm^3, the mice were assigned to treatment of either vehicles (negative control, $n = 8$), PCI-24781 (50 mg/kg per day × 5 d/wk, *ip*; according to company recommendations and previous study[10]; $n = 8$), *cis*-diamminedic-hloroplatinum (10 mg/kg per day × 5 d/wk, *ip*; as a positive control[31,32]; $n = 8$). The mice were followed for tumor size and body weight and sacrificed 6 wk later. Tumors were resected, weighed, and frozen for detection of RAD51 expression or fixed in paraformaldehyde and paraffin-embedded for immunohistochemical studies.

Statistical analysis

Cell culture-based assays were represented as mean ± SD. ANOVA tests were used to assess differences across groups. Tumor volume was logarithmically transformed for further statistical analyses. A linear mixed model was used to assess the effect of treatment on tumor growth over time and a linear regression model for tumor weights. 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. Significance was set at $P < 0.05$.

RESULTS

PCI-24781 suppressed human gastric cancer cell function, synergizing the effects of CDDP

The efficacy of PCI-24781 in the treatment of gastric cancer was evaluated by cell growth, apoptosis and clonogenic assays. GC cell growth was abrogated by pretreatment with PCI-24781 (IC_{50} = 0.35 μmol/L in HGC27, 0.31 μmol/L in AGS) or CDDP (IC_{50} = 8.00 μmol/L in HGC27, 7.28 μmol/L in AGS) in a dose-dependent manner (Figure 1A-D). Isobologram analysis revealed a synergistic effect when the two treatments were combined (Figure 1E, F). Furthermore, low dose treatment (0.2 μmol/L PCI-24781 and 2.5 μmol/L CDDP) increased the apoptotic cell ratio (Annexin V positive by FCM) from 14% ± 3.9% for PCI-24781 and 16% ± 2.5% for the CDDP to 50% ± 3.6% (Figure 2A). Quantum dot probing for cleaved caspase-3 also showed similar results (Figure 2B). In addition, the clonogenicity was dramatically impaired when cells were treated with the combination therapy (8 ± 2) compared to cells treated with PCI-24781 (20 ± 4.2), CDDP (22 ± 3.5) or untreated cells (57 ± 5.6) (Figure 2C, D).

PCI-24781 modulated the cell cycle and genes related to DNA damage and repair

To explore how PCI-24781 synergized with CDDP, we further checked whether PCI-24781 could modulate the cell cycle and affect the DNA damage repair mechanism. Indeed, treatment with 0.5 μmol/L PCI-24781 for 24 h resulted in a decreased number of cells in S phase and a G2 cell cycle arrest (Figure 3A), which is consistent with a previous report[10]. The increased sub-G1 population also indicated more apoptotic cells. Meanwhile, we found the expression of RAD51, one of the most important mediators of HR, was decreased in a time-dependent manner at both the mRNA and protein levels (Figure 3B, C). We then tried to determine what role the PCI-24781-induced reduction of RAD51 plays in the DNA damage conditions. It has been well documented that DSBs elicit a signaling cascade that modifies the chromatin surrounding the break, first by ATM-dependent phosphorylation and then by chromatin ubiquitination[33]. Here, we utilized classic IR-induced DNA damage to test the potential effect of PCI-24781 on HR. Both ubiquitin conjugation on the chromatin, as tagged by an anti-FK2 antibody, and RAD51-containing subnuclear repair foci were visible in HGC27 cells 16 h after radiation exposure. With pretreatment of a low dose of PCI-24781 (0.1 μmol/L), which did not result in significant apoptosis, for 24 h, the number of DNA damage foci (FK2 positive) significantly increased while the number of repair foci (RAD51 positive) decreased markedly (Figure 3D, E). These data suggested that HR-related RAD51 may play a central role in the potency of PCI-24781.

RAD51 participates in the effects of PCI-24781, CDDP and the combination treatment

RAD51 dysfunction may be a key event leading to genomic instability and tumorigenesis[34]. To validate the role of RAD51 in the potency of PCI-24781, we manipulated the expression of RAD51 in gastric cancer cells. When RAD51 was depleted (Figure 4A, B), we observed a significant increase in the number of apoptotic cells in the PCI-24781- or CDDP-treated cells, with the highest ratio in the combination treatment (Figure 4C).

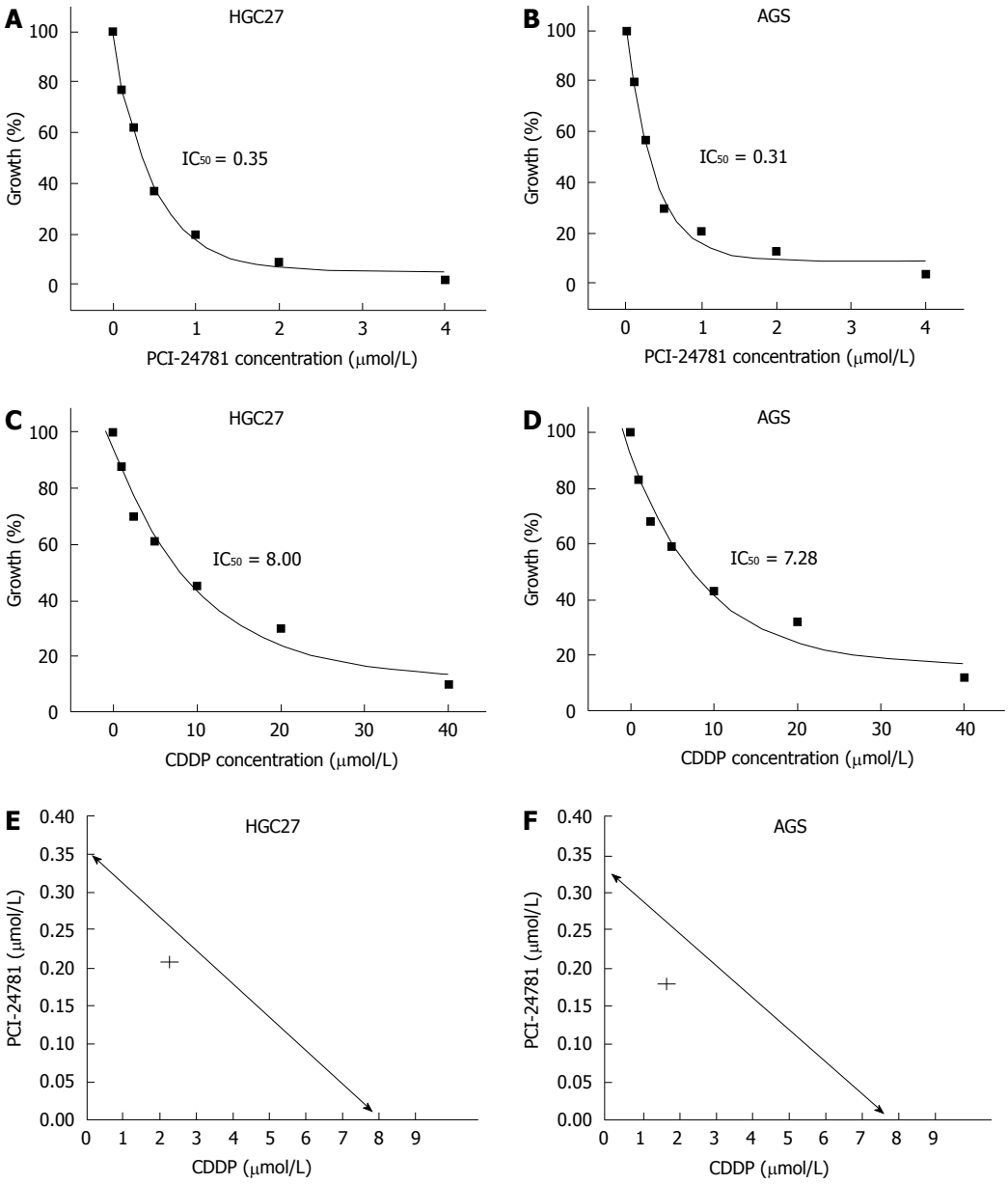


Figure 1 PCI-24781 suppressed gastric cancer cell growth and synergized with the effect of *cis*-diamminedichloroplatinum. The gastric cell lines HGC27 and AGS were plated at densities of 2×10^3 cells per well in 96-well plates and were subjected to cell growth assays. The cells were incubated with various concentrations of PCI-24781 and/or *cis*-diamminedichloroplatinum (CDDP) for 48 h, and the inhibition ratio was determined (IC_{50}). Cell growth assays showed the significant cell growth inhibition of the GC cells in response to PCI-24781 (A, B) and CDDP (C, D) treatment. Isobologram analysis was used to evaluate the synergistic effect. The data point (IC_{50}) from the combination treatment is shown on the left side of the curve and was generated by IC_{50} of the two drugs; this suggests that the combination treatment (concentration constant ratio PCI-24781:CDDP = 1:10) as synergistic (E, F).

In contrast, overexpression of RAD51 suppressed the pro-apoptotic effects of PCI-24781 and CDDP (Figure 4D-F). These data suggested an essential role of RAD51 in the anti-GC effect of PCI-24781, CDDP and combination treatment.

PCI-24781 induces RAD51 transcriptional repression

To determine the mechanism by which PCI-24781 decreases the expression of RAD51, we pretreated the cells with actinomycin D for 30 min to block mRNA transcription and then added PCI-24781 to the medium. RAD51 mRNA was monitored via quantitative RT-PCR, and we found no significant difference in the half-lives (6-8 h) of the mRNA from the PCI-24781-treated and untreated samples, suggesting that PCI-24781 does not directly induce RAD51 mRNA degradation. Thus, we proposed that PCI-24781 treatment might result in RAD51 transcription repression. The results showed that treatment with PCI-24781 for 24 h significantly decreased the association of RNA polymerase Ⅱ (Poly Ⅱ) and slightly increased the association of acetylated histones H3 and H4 with the RAD51 gene (Figure 5A, B). To investigate the translational activity of the promoter, a RAD51 luciferase reporter, which contained the -403 to +63 RAD51 promoter region, was transiently transfected into GC cells. As shown in Figure 5C, PCI-24781

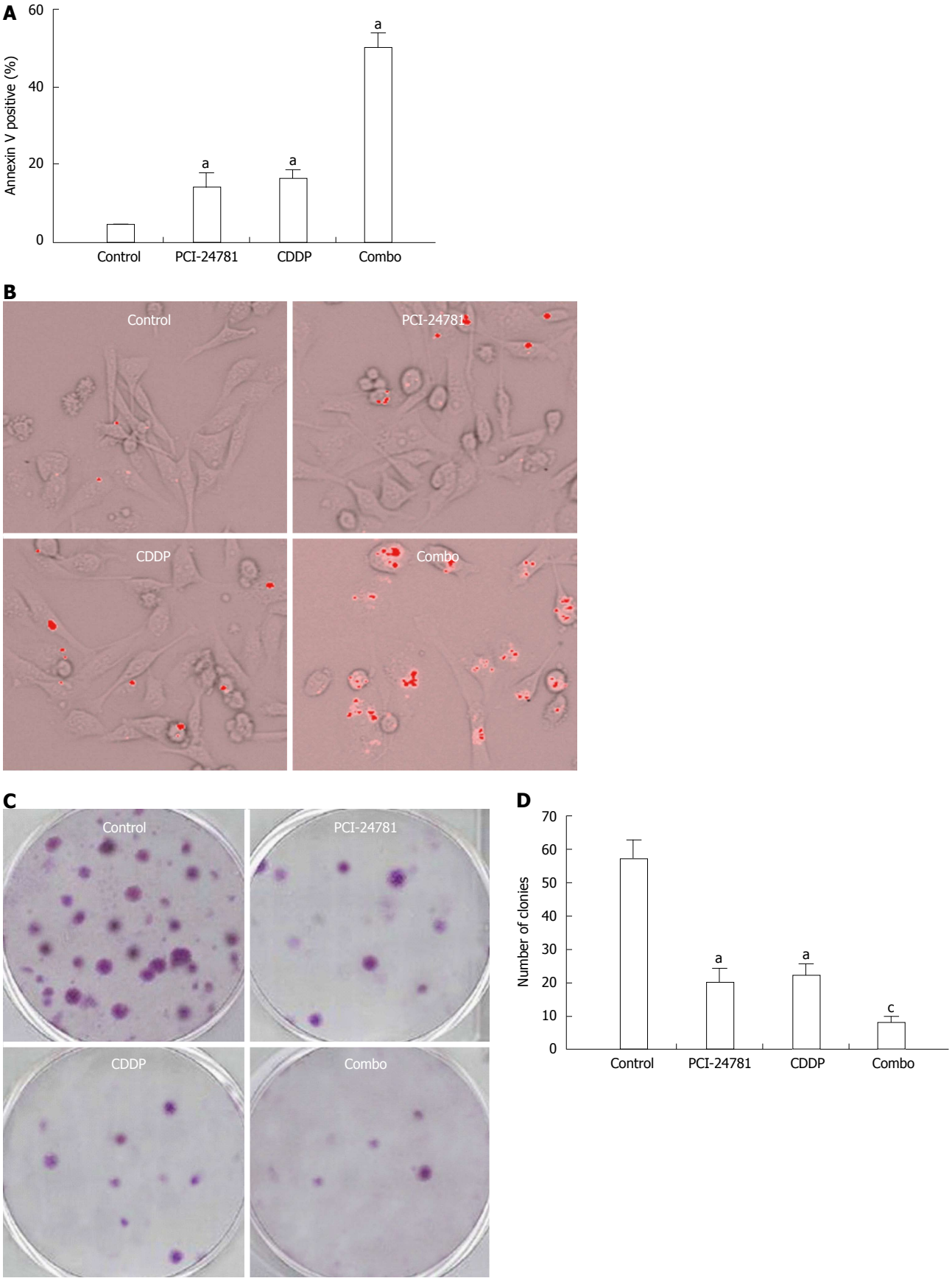
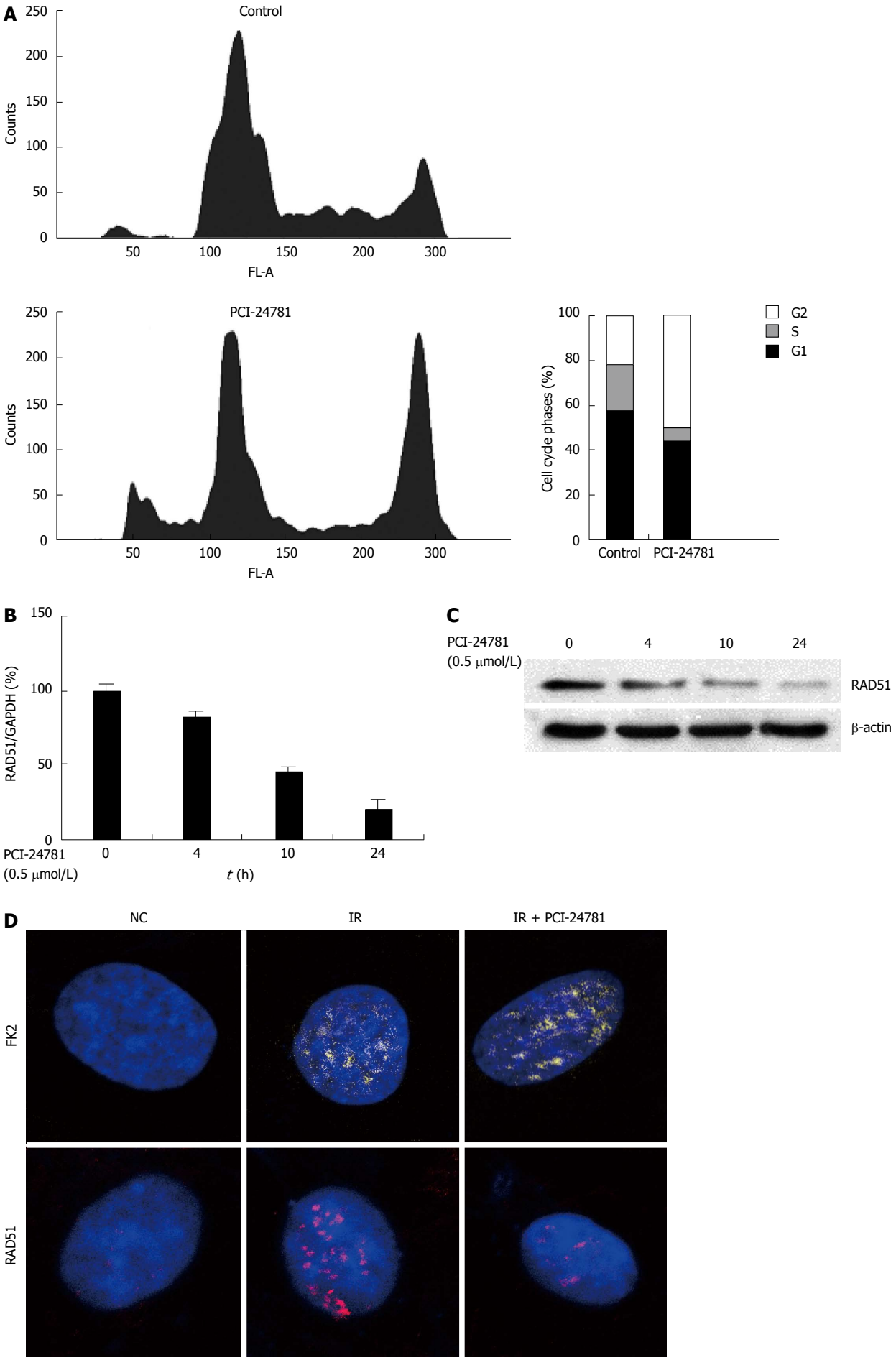


Figure 2 PCI-24781 synergizes with the *cis*-diamminedichloroplatinum-induced gastric cancer cell apoptosis and clonogenic inhibition. A, B: After appropriate treatment [PCI-24781, *cis*-diamminedichloroplatinum (CDDP) and combination] for 48 h, the apoptosis of the HGC27 cells was measured using flow cytometry of annexin V labeled cells (A) and the quantum dot analysis of cleaved caspase-3 (B). The number of apoptotic cells was remarkably increased in the PCI-24781- or CDDP-treated groups, with a synergistic effect in the combination treated group; C, D: PCI-24781 or CDDP partially abrogates the colony formation capacity of the GC cells (10 d). The combination treatment exhibited a synergistic effect. Combo: Combination treatment. $^{a}P < 0.05$ *vs* the control; $^{c}P < 0.05$ *vs* PCI-24781 or CDDP.


A
Control
Counts
FL-A
PCI-24781
Cell cycle phases (%)
G2
S
G1
Control
PCI-24781
B
RAD51/GAPDH (%)
PCI-24781
(0.5 μmol/L)
t (h)
C
PCI-24781
(0.5 μmol/L)
RAD51
β-actin
D
NC
IR
IR + PCI-24781
FK2
RAD51

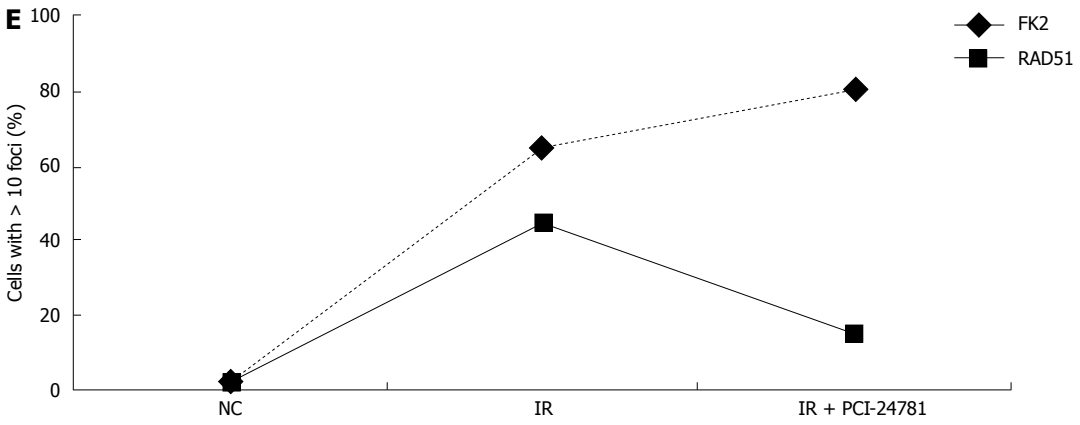


Figure 3 PCI-24781 modulates the genes related to DNA damage and repair. A: Propidium iodide/fluorescence activated cell sorting analysis showed the effect of the histone deacetylase inhibitor (0.5 μmol/L PCI-24781, 24 h) on HGC27 cell cycle progression. A significant ($P < 0.05$) reduction in the number of S phase cells and a G2 arrest was observed; The increased sub-G1 population also indicated more apoptotic cells; B, C: Treatment with 0.5 μmol/L PCI-24781 induced a time-dependent decrease in RAD51, both at the mRNA and protein levels; D, E: The confocal laser scanning microscopy immunofluorescence images show HGC27 cells stained with an anti-FK2 antibody (DNA damage marker) and an anti-RAD51 antibody (homologous recombination marker) after pretreatment with 0.1 μmol PCI-24781 for 24 h, followed by 5 Gy irradiation (IR); images were captured 16 h after irradiation. PCI-24781 notably increased the amount of DNA damage-induced ubiquitin conjugation on chromatin (FK2 positive) and suppressed RAD51 foci formation. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

treatment for 24 h resulted in a significant suppression of RAD51 transcription ($P < 0.05$). Because -50 Luc was previously reported to regulate the transcription of RAD51, which contains an E2F binding-site[35], a mutation in this region was introduced to identify whether this *cis* element mediated the aforementioned repressive effect. Indeed, the PCI-24781-induced luciferase reduction in the wild-type promoter construct was abolished when this element was mutated (Figure 5D). We also observed a significant increase in E2F4 binding, whereas slightly decreased E2F1 binding to the RAD51 promoter was observed in response to PCI-24781 treatment (Figure 5E, F); however, there was no significant difference in the levels of these two proteins (data not shown). To elucidate the role of E2F4 in this process, siRNA knockdown was introduced. When E2F4 was depleted (Figure 5G), RAD51 transcription increased approximately 1.5-fold, and the PCI-24781-induced repression was abolished (Figure 5H, I), indicating that E2F4 mediates the efficacy of PCI-24781 as a transcription repressor of RAD51. These data suggested that PCI-24781 transcriptionally represses the expression of RAD51 mainly by increasing E2F4 binding to the promoter of the *RAD51* gene, which accounts for its anti-GC effect.

PCI-24781 synergized with the anti-GC effects of CDDP in vivo

To explore the anti-tumor effect of PCI-24781 *in vivo*, we used SCID mice with hypodermal gastric cancer. A linear mixed model was used to assess tumor growth (tabulated as the log-transformed tumor volume) across the treatment groups over time, and a linear regression model was used to assess the tumor weights. Therapy was initiated after the tumor was established (initial tumor sizes of control, PCI-24781, CDDP and combinational treatment were 112 ± 1.1, 104 ± 1.1, 100 ± 1.0 and 107 ± 1.2 mm^3, respectively). Both PCI-24781 and CDDP treatment significantly repressed tumor growth indicated as reduction in the volume of tumor (Figure 6A) and decreased tumor weight versus the control mice (Figure 6B; $P < 0.05$). The average group tumor weights at the termination of the study were 2.01 ± 0.57 g for the control, 1.26 ± 0.35 g for the PCI-24781-treated, 1.36 ± 0.34 g for the CDDP-treated, and 0.68 ± 0.27 g for the combination mice (Figure 6B). The largest reduction of tumor growth and weight were obtained with the combination treatment. Immunohistochemical analysis revealed that the synergy decreased tumor cell proliferation and enhanced cell apoptosis, which might account for this outcome (Figure 6C-F). In the combination therapy group, cell proliferation (PCNA positive ratio) dropped to 22% ± 4.5% compared to the 85% ± 6.2% found in the control group, 57% ± 3.6% in the PCI-24781 alone group, and 60% ± 8.2% in the CDDP alone group; additionally, apoptosis (TUNEL positive ratio) increased to 52% ± 4.1% compared to the 5% ± 1.2% for the control group, 26% ± 3.3% for the PCI-24781 alone group, and 24% ± 3.1% for the CDDP alone group. These data suggested that PCI-24781 could synergize with the anti-GC effects of CDDP *in vivo*. Additionally, consistent with the *in vitro* data, PCI-24781 also inhibited the expression of RAD51 *in vivo*.

DISCUSSION

In this study, we provided the first evidence that the HDACi PCI-24781 synergizes with the therapeutic effects of CDDP in GC. PCI-24781-induced downregulation of RAD51, which closely relates with HR and DNA repair mechanism, may potentiate these effects.

HDA*Cis* are a new class of anticancer therapeutics whose mechanisms have yet to be elucidated in GC. Previous studies have demonstrated the antitumor effect of PCI-24781 on leukemia[36], lymphoma[37], glioblastoma[13],

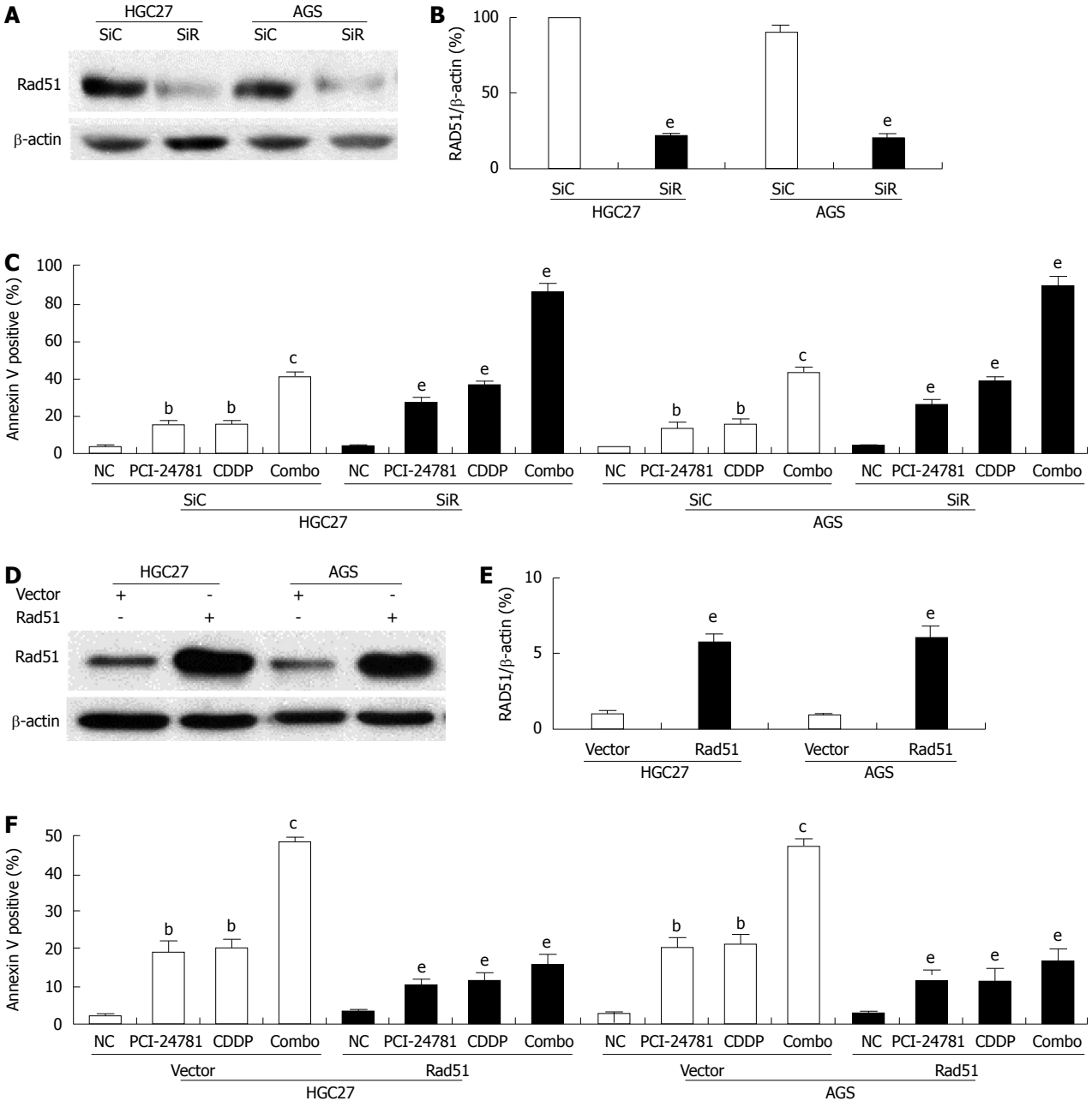


Figure 4 RAD51 mediates the effect of PCI-24781. A, B: siRNA targeting RAD51 dramatically decreased the expression of RAD51; C: Depletion of RAD51 significantly increased the apoptotic cells ratio both in the PCI-24781- and *cis*-diamminedichloroplatinum (CDDP)-treated cells. A synergistic effect was observed in the combination treatment; D, E: For the overexpression system, human RAD51 cDNA was cloned into pcDNA3.1 to yield the pcDNA-RAD51 plasmid. The empty vector served as a control. The cells transfected with pcDNA-RAD51 showed a notable increase in RAD51 expression; F: Overexpression of RAD51 significantly decreased the apoptotic cells ratio both in the PCI-24781- and CDDP-treated cells. A synergistic effect was observed in the combination treatment. SiC: siRNA-control; SiR: siRNA-RAD51; Combo: Combination treatment. $^{b}P < 0.01$ *vs* the control; $^{c}P < 0.05$ *vs* PCI-24781 or CDDP; $^{e}P < 0.05$ *vs* SiC or vector.

and malignant peripheral nerve sheath tumors[38], as well as showing its adjuvant chemo-function in multidrug-resistant sarcoma cell lines[16], bone sarcoma cells[15], and soft tissue sarcomas[10]. However, evidence is limited concerning its effect in GC. *In vitro*, we found that PCI-24781 could abrogate GC cell growth, suppress clonogenicity and enhance cell apoptosis (Figures 1 and 2). These effects were greatly enhanced when combined with CDDP treatment. We also confirmed that PCI-24781 treatment resulted in a significant reduction of RAD51, as well as a drastic suppression of DNA damage-induced ubiquitin conjugation on chromatin and RAD51 foci formation (Figure 3). This suggested that PCI-24781 may affect HR by modulating RAD51. The results from Adimoolam *et al*[22] robustly support this concept. They found that level of RAD51 was reduced to 20% after 24 h treatment with PCI-24781. They transfected DRAA8/CHO cells with the I-SceI-expressing plasmid to monitor recombination frequency. Results showed that the rate of HR dropped from 0.72% to 0.27% upon the addition of 2.0 μmol/L PCI-24781 6 h after transfection. Furthermore, PCI-24781 could in-

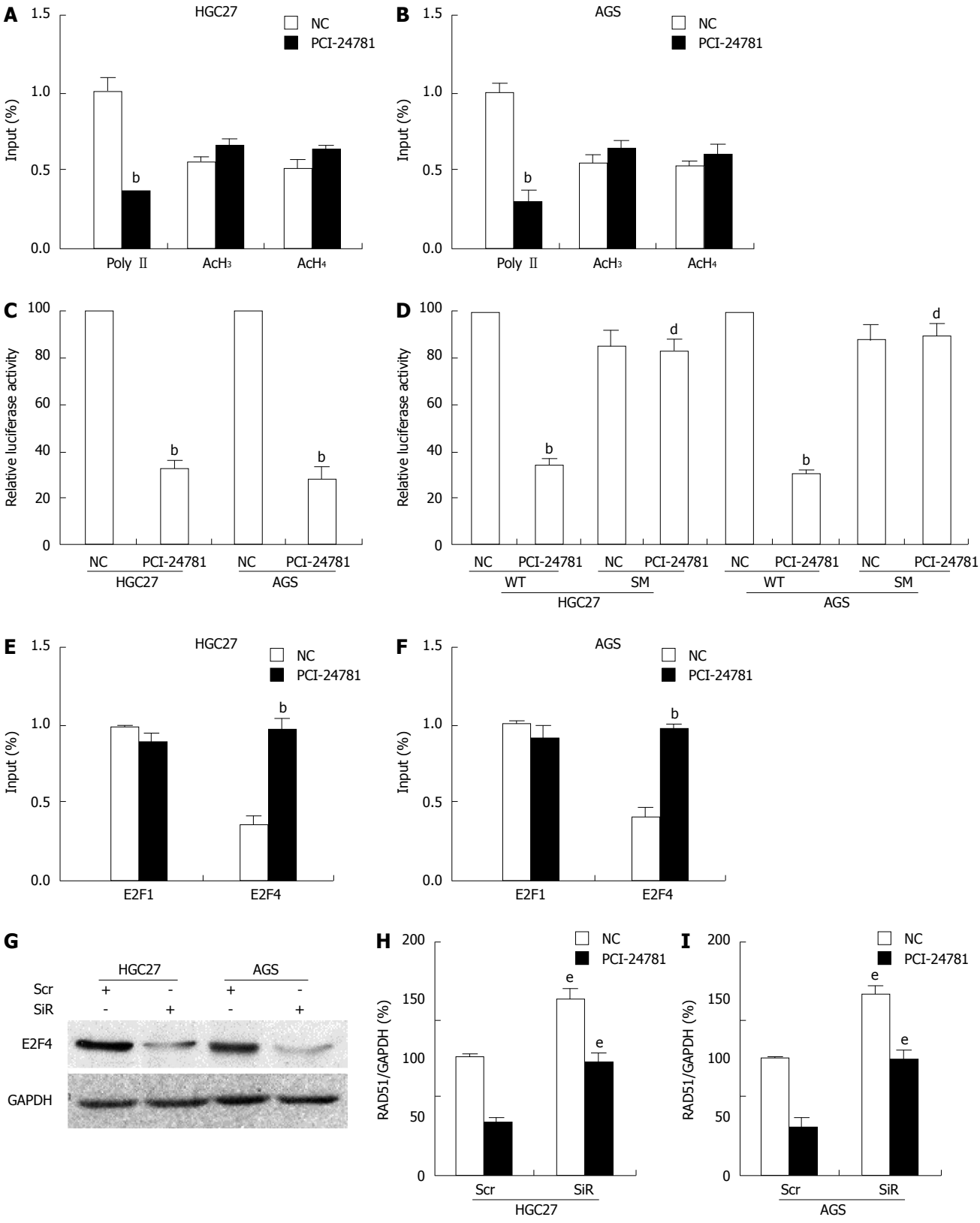


Figure 5 PCI-24781 induces RAD51 transcriptional repression. A, B: Chromatin immunoprecipitation assays showed a decrease in Polymerase Ⅱ binding to the transcribed region of the proximal RAD51 gene in gastric cancer (GC) cells in response to treatment with 0.5 μmol/L PCI-24781 for 24 h; binding of acetylated histones 3 and 4 to the RAD51 region was slightly enhanced. No significant nonspecific IgG binding was observed, and input DNA was used as a loading control; C: Luciferase assays demonstrated that treatment with 0.5 μmol/L PCI-24781 for 24 h resulted in the suppression of RAD51 promoter activity; D: Mutation of the E2F binding site located within the first 50 bp of the RAD51 promoter construct abrogated the aforementioned repression; E, F: ChIP analysis showed significantly enhanced E2F4 binding and slightly decreased E2F1 binding to the *cis* element in the RAD51 promoter in response to PCI-24781 treatment (0.5 μmol/L for 24 h); G: siRNA targeting E2F4 dramatically decreased the expression of E2F4; H, I: Depletion of E2F4 significantly increased the expression of RAD51 and reversed the PCI-24781-induced decrease in RAD51 expression. SM: E2F binding-site mutation; Scr: Scramble siRNA; SiR: siRNA-E2F4. $^{b}P < 0.01$ *vs* the control; $^{d}P < 0.01$ *vs* wild-type (WT); $^{e}P < 0.05$ *vs* Scr.

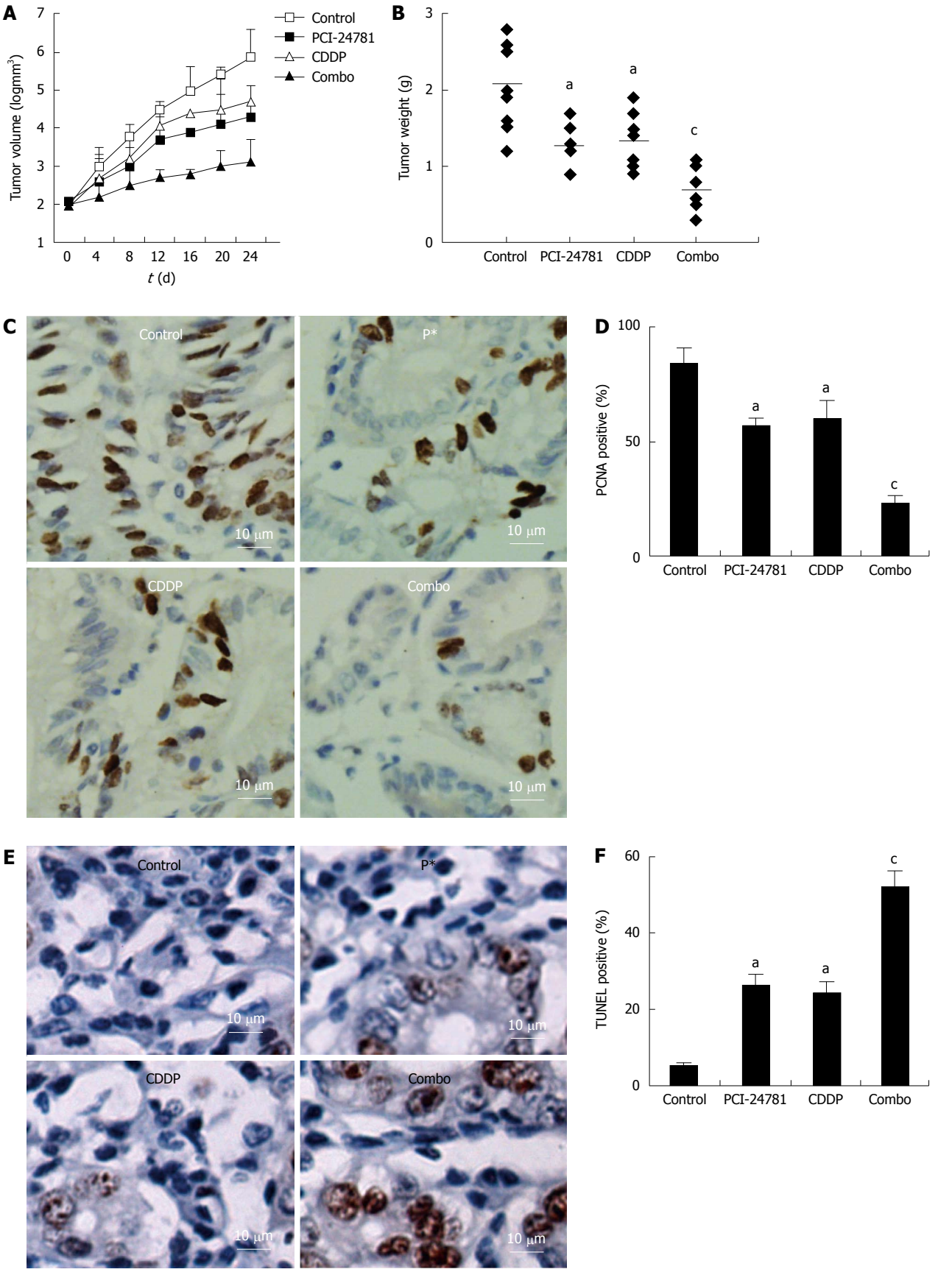


Figure 6 PCI-24781 synergized with the chemotherapeutic effect of *cis*-diamminedichloroplatinum in a xenograft model. A, B: Tumor growth curves showed a significant difference between the PCI-24781- or *cis*-diamminedichloroplatinum (CDDP)-treated mice and the negative control. PCI-24781 or CDDP also dramatically decreased the tumor weight. The combination treatment obtained the smallest and lightest tumors compared to the single treatment or negative control groups. Immunohistochemical analysis of the GC xenograft specimens demonstrated the significantly decreased tumor proliferation (C, D; PCNA positive) and increased apoptosis (E, F; TUNEL positive) in the PCI-24781- or CDDP-treated tumors, with a synergistic effect in the combination treatment. CDDP: *Cis*-diamminedichloroplatinum; Combo: Combination treatment. $^{a}P < 0.05$ *vs* the control; $^{c}P < 0.05$ *vs* PCI-24781 or CDDP. Bar = 10 μm.

hibit expression of RAD51 both *in vitro* and *in vivo* (Figure 3B, C). Depletion of RAD51 augmented the apoptotic effect of PCI-24781, CDDP and the combination treatment, whereas overexpressing RAD51 had the opposite effects (Figure 4). In agreement with these findings, studies have reported that defects in either recombination mediators or co-mediators, including BRCA1 and BRCA2, lead to impaired HR[39-41], which can be genetically complemented by overexpression of RAD51[42]. Conversely, administration of a RAD51 inhibitor disrupts homologous recombination[43]. Consistent with *in vitro* data, PCI-24781 also exerted comparable anti-GC effects with the conventional chemotherapeutic CDDP *in vivo*. Combination treatment of PCI-24781 and CDDP gained most favorable therapeutic outcomes (Figure 6).

The exact mechanism of the HDACi-induced decreased gene expression remains uncertain. The decreased expression may be due to HDACi-induced transcriptional repression and/or decreased mRNA stability[44,45]. Alternatively, HDA*Cis* may induce the acetylation of non-histone proteins, including several transcription factors, thereby altering their function and potentially negatively affecting oncogenic target gene transcription[5]. In this study, PCI-24781 appeared to transcriptionally repress RAD51 rather than affect its mRNA stability. Although a slight increase in the association of acetylated histones H3 and H4 and RAD51 was observed, which may promote transcription, the significant decrease in Pol II association seemed to play the dominant negative role here (Figure 5A, B). Luciferase and site mutation assays also revealed that the E2F binding-site in the RAD51 promoter was essential for potency of PCI-24781 (Figure 5E, F). Researchers have indicated that E2F4, as a transcription repressor, may specifically inhibit RAD51 transcription in response to hypoxia[46], whereas E2F1 is a positive transcriptional regulator[47]. We also observed that the binding of E2F4 to the RAD51 promoter significantly increased, while the binding of E2F1 slightly decreased. Depletion of E2F4 using siRNA led to an increase in RAD51 expression and reversed the PCI-24781-induced decrease in RAD51 (Figure 5G-I). Overall, our findings suggest an anti-GC role of PCI-24781 and the potential mechanism.

To the best of our knowledge, this is the first study to show that PCI-24781 synergizes with the chemotherapeutic effect of CDDP in gastric cancer *in vivo* and *in vitro*, and PCI-24781-induced RAD51 repression may be one of the mechanisms. PCI-24781 could be a potential drug and novel therapeutic strategy for the treatment of gastric cancer.

COMMENTS

Background

Gastric cancer (GC) is one of the most aggressive malignancies, especially in South Asia. It ranks as the second leading cause of cancer mortality in China. Despite curative surgery and postoperative adjuvant therapy, nearly 60% of patients succumb to the disease. Recent studies have suggested that histone deacetylase (HDAC) inhibitors may be attractive anticancer drugs. PCI-24781, a broad-spectrum, hydroxamic acid-derived HDAC inhibitor (HDACi), shown significant anticancer activity in various tumors including soft tissue sarcomas, gallbladder carcinomas, colon carcinomas, glioblastomas, breast cancers, and bone sarcomas. However, information about the efficacy of PCI-24781 in the treatment of gastric cancer is limited.

Research frontiers

Despite HDA*Cis* showing promise as single agents, several recent studies have suggested that the optimal use of HDA*Cis* is likely in combination with other chemotherapeutic agents, like *cis*-diamminedichloroplatinum (CDDP). In the area of cancer chemotherapy, the research hotspot is discovering and revealing mechanisms of new drugs with or without combining conventional chemotherapeutic agent, by which authors are hoping to improve effectiveness of chemotherapy and simultaneously reduce its adverse reactions.

Innovations and breakthroughs

In this study, authors provide the first evidence that the HDACi PCI-24781 synergizes with the therapeutic effects of CDDP in GC. Not only in GC cell lines but also in xenograft mouse model, PCI-24781 shows decent suppressive effect. When combining with conventional agent CDDP, chemotherapy gains synergistic benefit. To one step further, they attempt to reveal the potential underlying mechanisms. Previous studies demonstrated that DNA damage can be repaired through homologous recombination or through non-homologous end joining, which can lead to chemotherapy resistance. RAD51 is a key protein involved in and is regarded as a biomarker for HR. Interestingly, they found that PCI-24781 can down-regulate RAD51 in a time and dose dependent fashion; RAD51 potentiates synergistic effects of chemotherapy with PCI-24781 and CDDP on gastric cancer.

Applications

The study results suggest that PCI-24781 could be a potential drug and novel therapeutic strategy for the treatment of gastric cancer.

Terminology

Homologous recombination: Homologous recombination is a type of genetic recombination in which nucleotide sequences are exchanged between two similar or identical molecules of DNA. It is most widely used by cells to accurately repair harmful breaks that occur on both strands of DNA, known as double-strand breaks. Non-homologous end joining is a pathway that repairs double-strand breaks in DNA. It typically utilizes short homologous DNA sequences called microhomologies to guide repair. These microhomologies are often present in single-stranded overhangs on the ends of double-strand breaks.

Peer review

The manuscript reported a series of evaluations for the efficacy of PCI-24781 on gastric cancer. Each experiment is well designed.

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