HDAC inhibitor PCI-24781 decreases RAD51 expression and inhibits homologous recombination

Shanthi Adimoolam*, Mint Sirisawad*, Jun Chen*, Patti Thiemann*, James M. Ford†, and Joseph J. Buggy*‡

*Pharmacyclics, Inc., Sunnyvale, CA 94085-4521; and †Division of Oncology, Stanford University School of Medicine, Stanford, CA 94305

Edited by Richard B. Setlow, Brookhaven National Laboratory, Upton, NY, and approved October 3, 2007 (received for review August 20, 2007)

Histone deacetylase (HDAC) inhibitors such as the phenyl hydroxamic acid PCI-24781 have emerged recently as a class of therapeutic agents for the treatment of cancer. Recent data showing synergy of HDAC inhibitors with ionizing radiation and other DNA-damaging agents have suggested that HDAC inhibitors may act, in part, by inhibiting DNA repair. Here we present evidence that HDAC enzymes are important for homologous recombinational repair of DNA double-strand breaks. Combination studies of PCI-24781 with the poly(ADP-ribose) polymerase inhibitor PJ34, an agent thought to produce lesions repaired by homologous recombination (HR), resulted in a synergistic effect on apoptosis. Immunofluorescence analysis demonstrated that HDAC inhibition caused a complete inhibition of subnuclear repair foci in response to ionizing radiation. Mechanistic investigations revealed that inhibition of HDAC enzymes by PCI-24781 led to a significant reduction in the transcription of genes specifically associated with HR, including RAD51. RAD51 protein levels were significantly decreased after 24 h of drug exposure both *in vitro* and *in vivo*. Consistent with inhibition of HR, treatment with PCI-24781 resulted in a decreased ability to perform homology directed repair of I-SceI-induced chromosome breaks in transfected CHO cells. In addition, an enhancement of cell killing was observed in Ku mutant cells lacking functional nonhomologous end joining compared with WT cells. Together these results demonstrate that HDAC enzymes are critically important to enable functional HR by controlling the expression of HR-related genes and promoting the proper assembly of HR-directed subnuclear foci.

Chromatin structure is regulated, in part, by affecting the acetylation of lysine residues on the amino-terminal tails of nucleosomal histones. The acetylation state of histones is maintained by the opposing actions of histone acetyl transferase and histone deacetylase (HDAC) enzymes. There are 11 known isoforms in the classic HDAC family, denoted HDAC 1–11 (1). In addition to histones, HDAC enzymes are known to deacetylate other proteins, including α-tubulin (2), suggesting complex, multifunctional roles for HDACs *in vivo*.

PCI-24781 (formerly CRA-024781) is a broad-spectrum phenyl hydroxamic acid HDAC inhibitor currently being evaluated in phase I clinical trials in patients with neoplastic disease (3). The compound is a specific inhibitor of multiple HDAC isoforms that potently inhibits tumor growth *in vivo* with acceptable toxicity. PCI-24781, along with other HDAC inhibitors also in clinical development, represent a promising class of anticancer therapy agents (4, 5). In addition to evidence of efficacy as a monotherapy, some HDAC inhibitors have been shown to inhibit tumor growth synergistically when administered together with ionizing radiation (IR) or with DNA-interacting cancer drugs in preclinical models (6–12). It has been suggested that the mechanism of the synergy may involve the inhibition of DNA double-strand break (DSB) repair, because after cellular irradiation, HDAC inhibition enhances and prolongs the phosphorylation of histone H2AX, a well characterized marker of DNA DSBs (13–17).

In mammalian cells, DSBs are repaired by one of two genetically distinct processes, known as nonhomologous end joining (NHEJ) or homologous recombination (HR) (18). NHEJ is the simpler, but more error-prone mechanism, in which the DNA ends are recognized and bound by the Ku heterodimer, which recruits DNA–PK and other proteins to directly ligate the two DNA termini. By comparison, HR is a process of greater accuracy and complexity, requiring the presence of a sister chromatid to serve as a template for repair. HR begins with strand recognition and nucleolytic processing by the MRE11–RAD50–NBS1 (MRN) complex, followed by strand invasion, branch migration, and Holliday junction formation (18, 19). Both strand invasion and branch migration are initiated by RAD51, a recA homolog that binds MRN-generated ssDNA, forming nucleoprotein filaments essential for recombinational repair. After exposure to IR, RAD51 rapidly forms a complex with BRCA2 and other proteins that stimulate RAD51-mediated strand exchange and the assembly of subnuclear foci characteristic of HR (20, 21). Cells lacking functional RAD51 are unable to form foci and are significantly more sensitive to IR (22). In addition to repair of IR-induced DNA damage, recent evidence suggests that RAD51 (and HR) is involved in the repair of DNA DSBs produced by cisplatin and other platinum agents (23, 24) and also in the repair of DSBs produced by stalled replication forks (25), such as those produced by inhibitors of poly(ADP-ribose) polymerase (PARP) (26–28).

In the present report, we provide evidence that HDAC enzymes are important for homologous recombinational repair of DNA DSBs and the proper assembly of RAD51 subnuclear foci. We show that HDAC inhibition results in a synergistic increase in apoptosis after treatment with inhibitors of PARP and a decrease in RAD51 expression *in vitro* and *in vivo*. As a result, PCI-24781 treatment results in a decrease in homology-directed repair of DSBs and an inhibition of colony-forming ability in combination with IR or in Ku mutant cells lacking functional NHEJ. Together, these results demonstrate that HDAC enzymes are critically important in enabling functional HR by controlling the expression of HR related genes and promoting the proper assembly of RAD51 subnuclear foci. These findings suggest a potential therapeutic utility of HDAC inhibitors in cancer patients with tumors that have overactive HR or in combination with chemotherapeutic agents that induce damage repaired by HR.

Results

Synergistic Effect on Apoptosis in the HCT116 Colon Tumor Cell Line After HDAC and PARP Inhibition. A systematic screening effort of anti-cancer agents in combination with PCI-24781 in the colon tumor cell line HCT116 revealed that one of the strongest synergies occurred with PARP-selective inhibitors, including PJ34 [*N*-(6-oxo-5,6-dihydrophenanthridin-2-yl)-*N*,*N*-dimethylacetamide·HCl]. As shown in Fig. 1, HCT116 cells treated with both PCI-24781 and PJ34 in combination resulted in a significantly more than additive

Author contributions: S.A. and J.J.B. designed research; S.A., M.S., J.C., and P.T. performed research; S.A. and J.M.F. contributed new reagents/analytic tools; S.A., M.S., and J.C. analyzed data; and S.A. and J.J.B. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

‡To whom correspondence should be addressed at: Department of Cancer Biology, Pharmacyclics, Inc., 995 East Arques Avenue, Sunnyvale, CA 94085-4521. E-mail: jbuggy@pcyc.com.

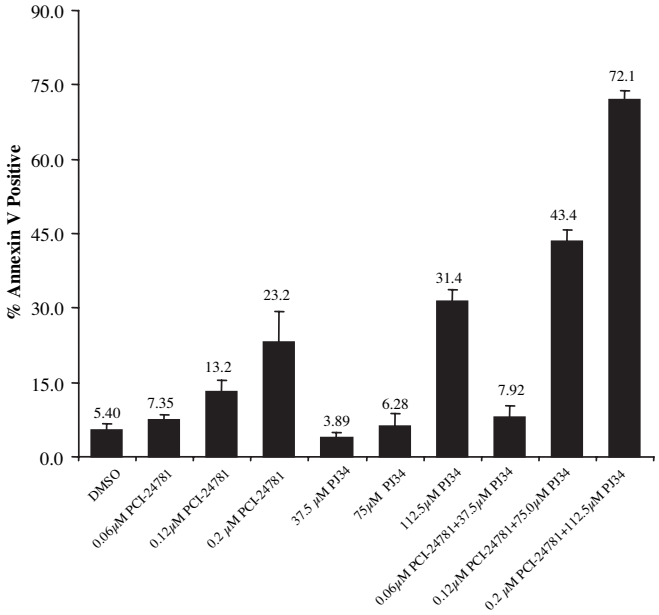

Fig. 1. Synergy of HDAC and PARP inhibition. HCT116 cells were treated with either PCI-24781 or PJ34 alone or in combination at the indicated doses. After treatment for 96 h, cell toxicity was assayed by Annexin V–FITC staining and quantitated by flow cytometry. The percentage of apoptosis is represented on the ordinate. The combined effect of both drugs was quantitatively evaluated by the median effects method as described in *Materials and Methods*. A CI of <1.0 was obtained, indicating strong synergism.

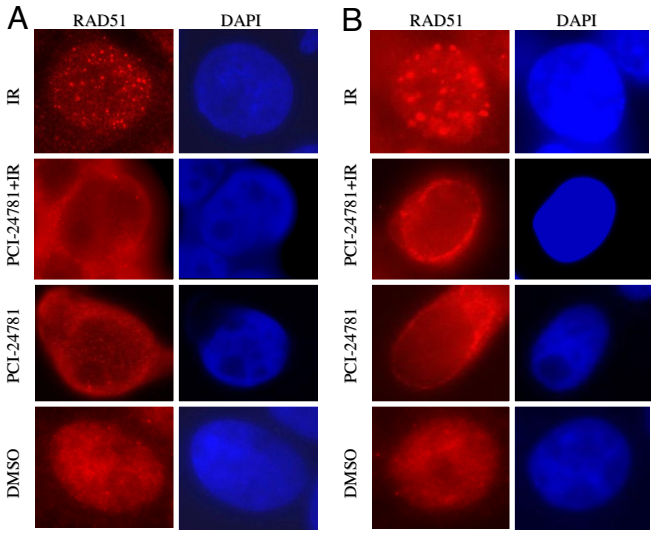

Fig. 2. Inhibition of subnuclear repair foci by PCI-24781. Shown are immunofluorescence images of HCT116 cells stained with anti-RAD51 antibody after 24 h of pretreatment with PCI-24781 and at either 1 h (*A*) or 16 h (*B*) after irradiation (IR). DAPI shows nuclear staining.

effect on apoptosis as assayed by Annexin V staining compared with either agent alone. The combination of 0.12 μM PCI-24781 with 75 μM PJ34 resulted in 43.4% apoptosis compared with 13.2 and 6.3% for each individual agent, respectively. Similarly, the combination of 0.2 μM PCI-24781 with 112.5 μM PJ34 resulted in 72.1% Annexin V-positive cells compared with 23.2 and 31.4% for each agent. The effects of the treatments were further determined by calculating the combination index (CI) by using the median effects method, as described by Chou and Talalay (29). A CI of <1.0 was obtained indicating a synergistic interaction between these two agents.

Subnuclear Repair Foci Are Inhibited by PCI-24781. PARP inhibition leads to the accumulation of unrepaired single-strand breaks (26). Because of stalled replication forks, DSBs arise at these sites that are repaired by HR (26). As a consequence, cells from tumors lacking HR are known to be exquisitely sensitive to PARP inhibition (30, 31), suggesting an indirect role for PARP enzymes in HR (32). We hypothesized that the mechanism of the observed synergistic drug activity may involve an inhibition of HR by PCI-24781. To test this, our first approach was to determine the effect of PCI-24781 on the ability of HCT116 cells to form subnuclear repair foci in response to IR by using immunofluorescence with antibodies against RAD51, a key enzyme in HR (33–35). RAD51-containing subnuclear foci were visible in HCT116 cells both 1 h (Fig. 2*A*) and 16 h (Fig. 2*B*) after 10 Gy of radiation. Foci at 16 h were present in 98.0% of the cells examined and were larger and fewer (20–30 foci per cell) when compared with 1 h after irradiation (>100 foci per cell), as has been reported in ref. 33, and foci were visualized in 96.1% of the cells. To determine the effect of HDAC inhibition on foci formation, we pretreated cells with 0.2 μM PCI-24781, a concentration at which synergy has been observed previously (Fig. 1) and by itself does not cause significant apoptosis over 24 h of treatment (Table 1), as corroborated by normal cell morphology and DAPI staining for intact nuclei under this condition (Fig. 2). After a 24-h pretreatment of cells with 0.2 μM PCI-24781, 100% inhibition of RAD51 foci formation was noted in 93.4 and 96.8% of the cells at 1 and 16 h after radiation, respectively. To quantitate the RAD51 foci for each treatment, five fields were counted, totaling a minimum of 100 cells. Thus, PCI-24781 treatment resulted in loss of the ability of the cell to form proper subnuclear repair foci after irradiation.

HDAC Enzymes Regulate the Expression of Homologous Recombinational Repair Genes. We next examined whether exposure to PCI-24781 affects the expression level of genes involved in HR. Total RNA from HCT116 cells treated with 0.2 μM PCI-24781 was isolated at 4, 6, 16, and 24 h, and gene expression changes were quantified by TaqMan analysis for the HR-related genes BRCA1, BRCA2, and RAD51, and the non-HR-associated gene GADD45γ was used as a transcriptional control. As seen in Fig. 3, transcript levels of all three HR-associated genes were down-regulated, with RAD51 and BRCA2 levels reduced to 20% of normal by 24 h. However, GADD45γ transcripts showed an increase over control at 24 h, similar to what has been reported in refs. 36 and 37.

RAD51 is a centrally important factor in HR, because mutant cells lacking RAD51 lack all functional HR (34, 35). To determine whether transcriptional down-regulation of RAD51 affected protein levels similarly, RAD51 protein levels were measured in HCT116 cells at 6 and 24 h after treatment with 0.2, 0.5, or 1.0 μM PCI-24781. As shown in Fig. 4*A*, RAD51 protein levels decreased significantly by 24 h, whereas only a slight decrease was observed at 6 h. Quantitation of band intensity normalized to actin indicated that RAD51 levels were reduced to 47 and 35% of control with 0.2 and 1.0 μM PCI-24781, respectively, at 24 h. The concentrations of 0.2, 0.5, and 1.0 μM also led to HDAC inhibition in these cells, as shown by the accumulation of acetylated tubulin and acetylated histones.

Table 1. Apoptosis in response to 24-h treatment

HCT116 treatment	Apoptotic, %
DMSO	2.9
0.2 μM PCI-24781	7.0
0.5 μM PCI-24781	17.0
1.0 μM PCI-24781	20.0

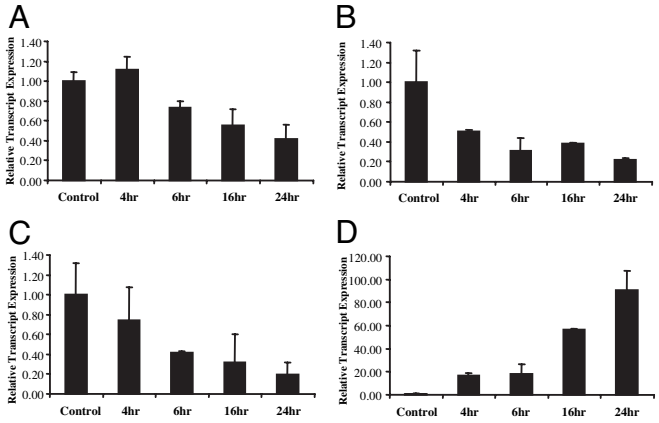

Fig. 3. PCI-24781 treatment decreases transcript levels of HR genes. The effect of PCI-24781 (0.2 μM) treatment for various times on the transcript levels of selected DNA-repair-associated genes [BRCA1 (*A*), BRCA2 (*B*), RAD51 (*C*), and GADD45γ (*D*)] in HCT116 cells was quantified by TaqMan analysis and is shown relative to the DMSO control.

Because PCI-24781 is known to activate caspases and induce apoptosis in HCT116 cells, and RAD51 is a known substrate of caspase 3 (38), we wished to assess the contribution of cleavage by caspases on levels of RAD51. PARP cleavage was observed after 24 h of treatment with 0.5 and 1.0 μM PCI-24781, indicating the presence of some degree of apoptosis under these conditions (Fig. 4*B*). Preincubation with the pan-caspase inhibitor quinoline–valine–aspartic–CH_2-*O*-phenyl (Q-VD-OPh) before exposure to PCI-24781 inhibited the PARP cleavage but showed the same decrease in RAD51 levels, indicating that caspase cleavage does not contribute to the observed decrease in RAD51 levels.

To determine whether the reduction in RAD51 occurs *in vivo*, mice bearing HCT116 tumor xenografts were treated orally with 200 mg/kg doses of PCI-24781, and RAD51 levels were examined in the tumors at necropsy. Data from previous pharmacokinetic studies were used to choose the 200 mg/kg dose, which was projected assuming dose-exposure linearity to achieve a peak plasma concentration of 1.4 μM and to sustain plasma levels >0.2 μM for 240 min after each dose. Mice were treated once over a 4-h period (1×), twice over a 22-h period (2×), or three times over a 28-h period (3×). Similar to the *in vitro* results, RAD51 protein levels were reduced in tumors from treated mice, and a 69% decrease was observed when the total time of exposure to drug was >24 h (Fig. 4*C*).

Homologous Recombinational Repair Is Inhibited by PCI-24781. To determine whether PCI-24781 directly inhibits the process of homologous recombinational repair in cells, we used an intrachromosomally based GFP reporter assay system in DRAA8/CHO cells that has been described in ref. 39. In brief, a single DSB is introduced into an integrated GFP gene by using SceI endonuclease, and repair of the break by endogenous HR results in functional GFP expression, as measured by flow cytometry. Compared with HCT116, DRAA8/CHO cells are less sensitive to PCI-24781, requiring a 2.0 μM dose of the drug to achieve a measurable decrease in RAD51 (Western blot in Fig. 5); therefore, 2.0 μM PCI-24781 is used in this experiment. Transfection of DRAA8/CHO cells with the I-SceI-expressing plasmid resulted in a 0.72% recombination frequency (Fig. 5). Addition of 2.0 μM PCI-24781 6 h after transfection reduced the recombination frequency to 0.27%, and 3.0 μM PCI-24781 reduced the recombination frequency even further to 0.16%. These results indicate that HR activity is inhibited as a consequence of HDAC inhibition and is sufficient to explain the observed synergy with PJ34.

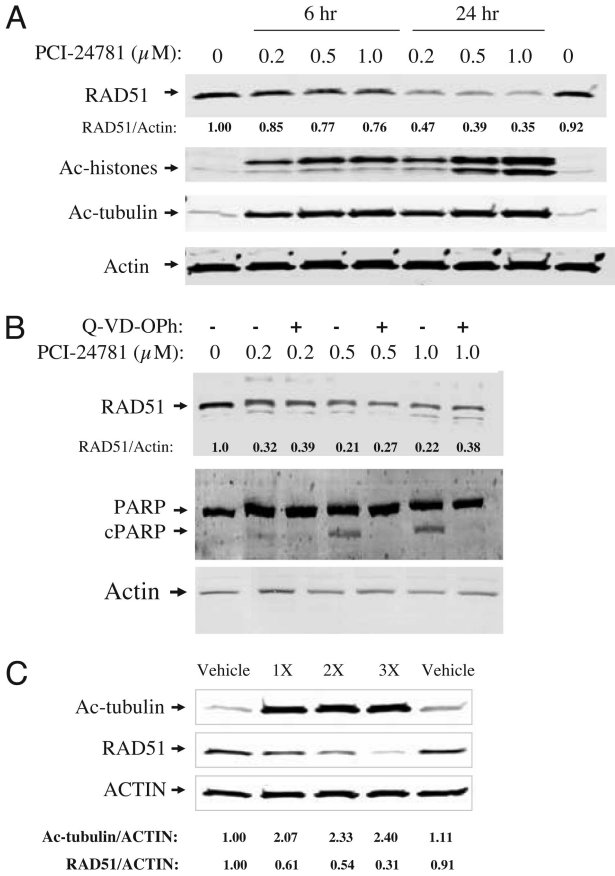

Fig. 4. PCI-24781 decreases RAD51 protein levels. (*A*) Effect of PCI-24781 on RAD51 protein expression, acetylated tubulin, and acetylated histones for 6 or 24 h was evaluated in HCT116 cells by Western blotting. (*B*) RAD51 down-regulation is not dependent on caspase activity. RAD51 and full-length and cleaved PARP protein levels were assessed by Western blotting after a 1-h pretreatment of HCT116 cells with 10 μM Q-VD-OPh, a pan-caspase inhibitor, and 24-h treatment with the indicated doses of PCI-24781. (*C*) An oral 200 mg/kg dose of PCI-24781 reduces RAD51 in HCT116 tumor xenografts *in vivo*. RAD51, acetylated tubulin, and actin levels after extraction of tumor from HCT116 mouse xenografts with different dosing regimens are shown. 1X animals received a single oral dose 4 h before the end of the study; 2X animals received one oral dose 28 h before the end of the study and received a second dose 6 h later; 3X animals were dosed as in the 2X but also received a third dose the following morning, 24 h after the first dose was administered and 4 h before the end of the study. Fold changes in protein levels were quantitated by using Odyssey software and were normalized to the levels of the actin loading control.

HDAC Inhibition by PCI-24781 Enhances Radiosensitivity of Tumor Cell Lines. Inactivation of RAD51 renders cells highly sensitive to IR (40). To investigate the effects of PCI-24781 on radiosensitivity, the HCT116 colon tumor cell line was treated with PCI-24781, for 2, 6, 16, or 24 h before cellular irradiation, and surviving cells were quantified by their ability to form colonies. HDAC inhibition by PCI-24781 led to a decrease in the number of cells able to form colonies after irradiation compared with irradiation alone (Fig. 6), with the most prominent effects observed after 16 or 24 h of treatment compared with 2 or 6 h of treatment, consistent with the kinetics of RAD51 down-regulation and inhibition of subnuclear repair foci. Similar effects were observed with the NCI-H460 lung and A549 lung tumor cell lines, suggesting that radiation sensitivity is not limited to HCT116 cells.

NHEJ Mutant Cells Are Hypersensitive to PCI-24781. Because HDAC inhibition appears to disrupt the HR pathway, we hypothesized that cells lacking a functional NHEJ pathway should be particularly

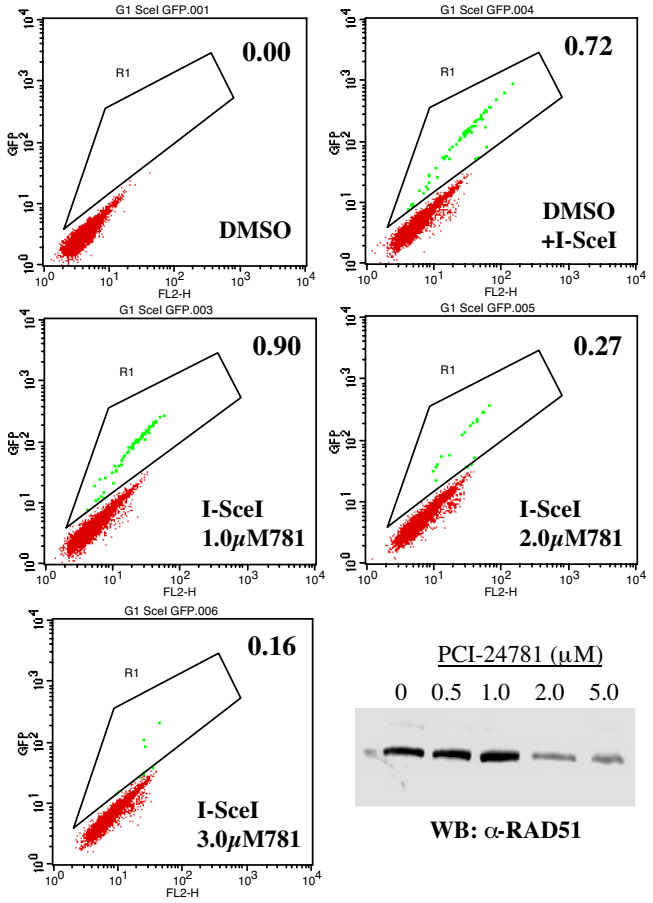

Fig. 5. PCI-24781 inhibits homologous recombinational repair. AA8 cells containing the DR-GFP recombination substrate (DRAA8/CHO) were used to determine the effect of PCI-24781 on homologous recombinational repair activity. Repair of I-SceI-induced DSB is quantified by green fluorescence. PCI-24781 was added at the indicated concentrations. The numbers shown in the upper right-hand corners of the graphs are the percentages of HR-positive cells (gated green cells) relative to the total live cell population (red and green). The Western blot (WB) shows levels of the HR protein RAD51 after treatment with the indicated doses of PCI-24781.

sensitive to PCI-24781. To test this, we used a previously described Ku86 mutant derivative of CHO cells that lack functional NHEJ (41). Disruption of HR by the HDAC inhibitor resulted in a 5.3-fold reduction in colony formation in the absence of functional Ku compared with WT CHO-K1 (Fig. 7) at 2.0 μM, a dose previously shown to decrease RAD51 expression and affect HR in CHO cells (Fig. 5). The increased sensitivity of the NHEJ mutant line is consistent with the hypothesis that inhibition of HR by PCI-24781 results in a severely repressed ability to repair DNA DSBs, leading to cell death.

Discussion

The present work provides evidence that HDACs are important for the process of HR by regulating the expression of RAD51 and assisting the proper formation of repair complexes. The specific HDAC inhibitor PCI-24781, by virtue of its ability to block HR, enhanced apoptosis in combination with a PARP inhibitor and inhibited colony formation in combination with γ irradiation, two agents that induce lesions repaired by HR.

HDAC inhibitors as a class have shown efficacy as single agents, but much of their value as therapeutic agents may be in combination with other drugs, including conventional chemotherapeutic agents and kinase and proteasome inhibitors (4). In an attempt to understand the role of HDAC inhibition in combination chemotherapy, we first evaluated the efficacy of PCI-24781 in combination studies with other DNA-damaging agents, and this led to the identification of a synergy between PCI-24781 and PARP inhibitors. PARP inhibitors have received much attention after the recent findings of Farmer *et al.* (31) that BRCA1 or BRCA2 dysfunction results in hypersensitivity to the inhibition of PARP enzyme function. Inhibition of PARP generates single-strand breaks that are subsequently encountered by the replication fork, which gives rise to DSB substrates for homologous recombinational repair (26). Sensitivity to PARP inhibitors is not limited to BRCA1 or BRCA2 defects but to defective homologous recombinational repair in general (42). The observation of synergistic apoptosis in cell lines treated with PCI-24781 in combination with PARP inhibitors, therefore, stimulated our interest because it suggested (*i*) utility for clinical trials by using HDAC inhibitors in combination with PARP inhibitors and (*ii*) that HDACs may be necessary for repair of DSBs generated

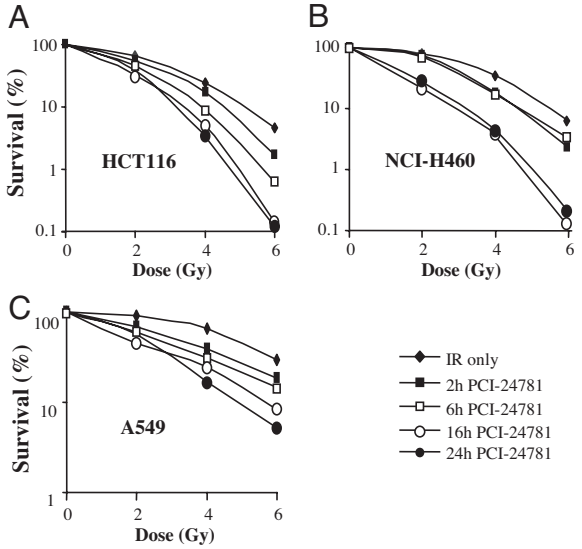

Fig. 6. Pretreatment with PCI-24781 enhances sensitivity to radiation in three tumor cell lines. Colony-formation assays were performed by pretreating cells with 1.0 μM PCI-24781 for various lengths of time, followed by exposure to the indicated doses of γ-irradiation (IR) in HCT116 (*A*), NCI-H460 (*B*), and A549 (*C*) cells. The percentage of survival is represented logarithmically on the ordinate.

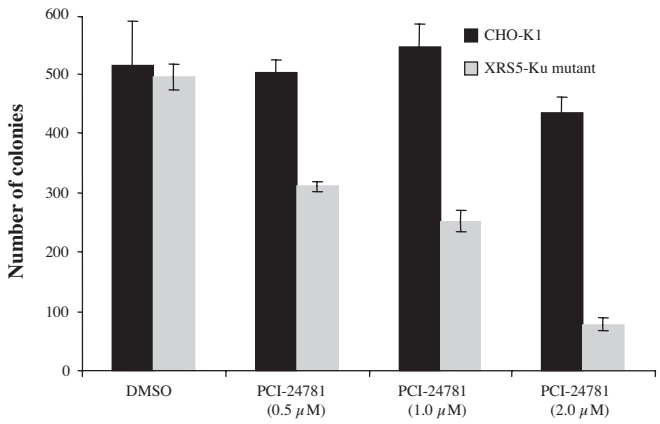

Fig. 7. NHEJ-defective cells are hypersensitive to the effects of PCI-24781. Clonogenic survival for CHO-K1 and its Ku86 mutant derivative after 0.5, 1.0, and 2.0 μM PCI-24781 treatment was assayed. Cells (10^3) were plated in 10-cm dishes, allowed to adhere, treated with the indicated doses of PCI-24781 for 24 h, and replaced with drug-free medium.

by PARP inhibition. This prompted us to evaluate whether regulation of homologous recombinational repair activity is altered after exposure to PCI-24781. Increasing doses of PCI-24781 led to a progressive decrease in HR activity, as assayed by using an intrachromosomal GFP reporter HR assay system (39) (Fig. 5). Direct inhibition of HR has never been described previously for an HDAC inhibitor, and it provides a reasonable explanation for the observation of synergy with a PARP inhibitor or DNA-damaging agents.

We have shown that PCI-24781 is able to inhibit the formation of subnuclear repair foci visualized by using RAD51 antibodies in HCT116 cells (Fig. 2). Such foci are thought to represent "repairosomes", where active repair has been initiated (33). Pretreatment with PCI-24781 inhibited the formation of RAD51-containing nuclear foci in >90% of treated cells. Overall nuclear staining was diminished when compared with control, although in many cases, some RAD51 appeared to be associated with the nuclear membrane in treated cells, but the significance of this was not clear. The marked decrease in subnuclear repair foci can be partly explained by decreased RAD51 protein levels (Fig. 4*A*), although this alone fails to explain a complete loss of RAD51 foci in >90% of cells because RAD51 levels, as measured by Western blotting decrease only to 30–40% of control levels. Indeed, some RAD51 staining was still present in treated cells even though foci were disrupted. It should be noted that the formation of subnuclear repair complexes is also dependent on functional BRCA2 (43) and probably other components of the HR repairosome as well. TaqMan analysis demonstrated a significant transcriptional down-regulation of at least three HR-specific genes, RAD51, BRCA1, and BRCA2 (Fig. 3). PCI-24781-mediated down-regulation of BRCA1 and BRCA2 transcript levels may further affect the proper assembly of the repair complex and, along with effects on RAD51, could account for the dramatic loss of repair foci. Decreases in tumor RAD51 were also observed *in vivo* after one, two, or three consecutive oral doses delivered to HCT116 colon-tumor-bearing mice, with a maximal effect observed after three consecutive doses over a 28-h period. This is consistent with the kinetics of RAD51 protein down-regulation observed *in vitro*, where a significant decrease in RAD51 protein required a 24-h pretreatment rather than a 6-h pretreatment (Fig. 4*A*). The 200 mg/kg dose was chosen based on previous efficacy studies and pharmacokinetic data in mice (data not shown). In a previous experiment, an oral dose of 200 mg/kg did not cause toxicity as measured by body weight loss when dosed for 3 consecutive days. The dose of 200 mg/kg in mice was also chosen because it is similar to the plasma area under the concentration–time curve expected in an ongoing clinical trial after an oral dose of 2.0 mg/kg.

Specific inhibition of HR leads to the prediction that cells lacking functional NHEJ will be more sensitive to the effect of inhibiting HR by PCI-24781 compared with WT cells. For example, cells from mice lacking both RAD54 and Ku70 have compromised survival and indications of a large increase in spontaneous DNA damage (44, 45). As predicted from this, CHO cells lacking Ku86 and functional NHEJ were observed to be significantly more sensitive to HR inhibition by PCI-24781 compared with the matched parental control cell line (Fig. 7).

Levels of RAD51 are elevated in various tumor cell lines and primary tumors (46) and are associated with increased HR-mediated DSB repair, resistance to drug and radiation therapy, tumorigenesis, tumor recurrence (22), and poor prognosis (22, 47–49). In addition, the function of several established tumor suppressor genes has been linked to elevated RAD51 levels, indicating that increased RAD51 activity offers an all-around advantage to cells from tumors (22, 46). The observation that PCI-24781 significantly lowers RAD51 levels and inhibits RAD51 function and homologous recombinational repair activity suggests that RAD51 protein levels may represent a convenient clinical biomarker of PCI-24781 in predicting clinical efficacy both as single agent and in combination with radiation. Knowledge of the timing of RAD51 decrease *in vitro* and *in vivo* will enable the design of efficient clinical trials of HDAC inhibitors used in combination with other DNA-damaging chemotherapeutic agents.

Materials and Methods

Cell Lines and Reagents. A549, CHO-K1, CHO-XRS5, NCI-H460, and HCT116 cell lines used in this study were obtained from American Type Culture Collection. HCT116 cells were cultured in McCoy's 5A medium supplemented with 10% FBS and penicillin/streptomycin antibiotics. CHO-K1 cells were cultured in Ham's F12-K medium containing 10% FBS and antibiotics. The Ku-deficient XRS5 (CHO-derived) cell line was grown in Alpha MEM lacking ribonucleosides and deoxyribonucleosides and containing 10% FBS and antibiotics. NCI-H460 and A549 lung carcinoma cell lines were grown in complete RPMI medium 1640. The DRAA8 cell line was a generous gift from Maria Jasin (Memorial Sloan-Kettering Cancer Center, New York) and was cultured in DMEM supplemented with 10% FBS and antibiotics. PCI-24781 (M_r 434) was synthesized at Pharmacyclics, Inc. as an HCl salt. The structure of PCI-24781 has been reported in ref. 3.

Apoptosis Assay by Annexin V Staining. To determine potential synergy between PCI-24781 and the PARP inhibitor PJ34 (EMD Biosciences) in HCT116 cells, cytotoxicity was evaluated by assaying Annexin V–FITC (Biosource) binding after 96 h treatment with specified doses of the agents alone or in combination. The doses were adjusted to keep the ratio between the two drugs constant for the two combination treatments. Apoptosis was quantitated by using a FACSCalibur instrument (Becton Dickinson). The CalcuSyn program (Biosoft) was used to generate a median effects plot based on the CI calculated as described in ref. 29. A CI of >1 indicates antagonism, a CI of 1 indicates additivity, and a CI of <1 indicates synergy. To determine the percentage of apoptosis at 24 h in HCT116 cells, cells were treated with 0.2, 0.5, and 1.0 μM PCI-24781, and Annexin V binding was evaluated 24 h later.

Immunofluorescence. HCT116 cells were grown on chamber slides and treated with 0.2 μM PCI-24781 for 24 h. Cells were then exposed to 10 Gy irradiation and incubated for 1 or 16 h after irradiation. Cells were then fixed and permeabilized in buffer containing 2% paraformaldehyde, 0.2% Triton X-100 in PBS. Cells were blocked in 2% BSA in PBS and probed with anti-RAD51 polyclonal antibody, followed by AlexaFluor594-conjugated secondary antibody. After staining, coverslips were mounted by using the Vecta shield mounting medium with DAPI (Vectalabs) and visualized by using fluorescence microscopy.

TaqMan Gene Expression Assays. HCT116 cells were treated with PCI-24781 for various times, and total RNA was extracted by using the RNeasy kit (Qiagen). Total RNA was quantitated by using the Ribogreen RNA quantitation kit (Molecular Probes). One-step RT-PCR assays were set up in triplicate according to the instructions of the manufacturer by using the TaqMan master mix (Applied Biosystems, Inc.) and 50 ng of total RNA as template. The amount of RNA in each reaction well was requantitated and used for normalizations. Gene expression assay probe sets for BRCA1, BRCA2, RAD51, and GADD45γ were purchased from Applied Biosystems, and assays were performed on an ABI PRISM 7300 instrument according to standard protocols.

Western Blot Analysis. Cells were treated with PCI-24781 for the designated times and lysed, and total protein was quantitated by using the BCA protein assay kit (Pierce). An equivalent amount of protein was loaded in each lane. The pan-caspase inhibitor Q-VD-OPh, which is used for blocking caspase cleavage, was purchased from MP Biomedicals. Total protein (30 μg) for each treatment was resolved on 4–15% gradient SDS/PAGE gels (Bio-Rad). Proteins were transferred to PVDF membrane and probed with the appro-

priate primary antibodies and secondary antibodies conjugated to AlexaFluor680 and IFdye800 (Rockland) were used. Imaging was performed by using an Odyssey scanner (LI-COR). RAD51 and Actin antibodies were from Santa Cruz Biotechnology. PARP antibody was from Cell Signaling.

***In Vivo* Studies.** BALB/c female nude mice were implanted s.c. with 3×10^6 HCT116 cells in a 1:1 ratio with Matrigel into the right-hand flank 24 h after administering 4.5 Gy (whole-body irradiation). Mice were entered into study ≈10 days after implantation or when tumor volumes reached a minimum of 75–100 mm^3. Four treatment regimens were followed with three animals in each group, each receiving a dose of 200 mg/kg: (*i*) vehicle; (*ii*) animals received a single oral dose 4 h before the end of the study (1×); (*iii*) animals received one oral dose 28 h before the end of the study and a second dose 6 h later (2×); and (*iv*) animals were dosed as in the 2× group but also received a third dose the following morning, 24 h after the first dose was administered and 4 h before the end of the study (3×). Animals were humanely killed, tumors were extracted, and total protein was isolated and quantitated by using the BCA protein assay kit. Total protein (30 μg) was loaded for each tumor, and Western blotting was performed to determine RAD51, acetylated tubulin, and actin levels.

Homologous Recombinational Repair Assay. Homologous recombinational repair activity was measured in DRAA8 Chinese hamster ovary cells as described in ref. 39. Briefly, DRAA8/CHO cells contained a nonfunctional GFP sequence and an internal GFP (iGFP) sequence integrated into the hprt locus. The mutant GFP sequence contained an 18-bp I-SceI recognition site, and DSBs were introduced by expression of the I-SceI endonuclease. The I-SceI expression vector (3.5 μg) was transfected by using nucleofection (Amaxa), and DSB-induced gene conversion by using the downstream iGFP repeat was quantitated by using FACS by measuring GFP signal. PCI-24781 was added at the appropriate concentrations 6 h after transfection, the live cells were gated based on 7-amino-actinomycin D (7-AAD) signal, and GFP expression in the gated live population was analyzed 36 h later.

Clonogenic Survival Assays. To determine the effects of PCI-24781 treatment on colony formation in CHO-K1 and the NHEJ-deficient Ku80 mutant XRS5 cell lines, cells at the appropriate plating density were plated in a 10-cm dish and allowed to attach. Cells were exposed to 0.5, 1.0, or 2.0 μM concentrations of drug for 24 h, after which the cells were placed in fresh growth media without drug and allowed to incubate for 7–10 days. Colonies were fixed with 100% isopropyl alcohol and stained with 1% crystal violet. For each condition, the assay was performed in triplicate. In HCT116, NCI-H460, and A549 lung tumor cell lines, to determine the appropriate time of PCI-24781 treatment for synergy with radiation, cultures were pretreated with 1 μM PCI-24781 for various lengths of time, ranging from 2 to 24 h. Cells were exposed to 0, 2, 4, or 6 Gy irradiation (Gammacell 40, Atomic Energy of Canada, Ltd.), and clonogenic survival was assessed after 7–12 days. From the plating efficiencies at each dose, the percentage of colony-forming ability was calculated.

We thank Quan Zheng for assistance with the *in vivo* animal xenograft studies and Drs. Darren Magda, Sriram Balasubramanian, Lee Honigberg, and Richard Miller for critical evaluation of the manuscript.

1. De Ruijter AJM, van Gennip AH, Caron HN, Kemp S, van Kuilenburg ABP (2003) *Biochem J* 370:737–749.
2. Matsuyama A, Shimazu T, Sumida Y, Saito A, Yoshimatsu Y, Seigneurin-Berny D, Osada H, Komatsu Y, Nishino N, *et al.* (2002) *EMBO J* 21:6820–6831.
3. Buggy JJ, Cao ZA, Bass KE, Verner E, Balasubramanian S, Liu L, Schultz BE, Young PR, Dalrymple SA (2006) *Mol Cancer Ther* 5:1309–1317.
4. Bolden JE, Peart MJ, Johnstone RW (2006) *Nat Rev Drug Discov* 5:769–784.
5. Drummond DC, Noble CO, Kirpotin DB, Guo Z, Scott GK, Benz CC (2004) *Annu Rev Pharmacol Toxicol* 45:495–528.
6. Camphausen K, Cerna D, Scott T, Sproull M, Burgan WE, Cerra MA, Fine H, Tofilon PJ (2005) *Int J Cancer* 114:380–386.
7. Cerna D, Camphausen K, Tofilon PJ (2006) *Curr Top Dev Biol* 73:173–204.
8. Chinnaiyan P, Vallabhaneni G, Armstrong E, Huang SM, Harari PM (2005) *Int J Radiat Oncol Biol Phys* 62:223–229.
9. Flatmark K, Nome RV, Folkvord S, Bratland A, Rasmussen H, Ellefsen MS, Fodstad O, Ree AH (2006) *Radiat Oncol* 1:25–35.
10. Jung M, Velena A, Chen B, Petukhov PA, Kozikowski AP, Dritschilo A (2005) *Radiat Res* 163:488–493.
11. Karagiannis TC, El-Osta A (2006) *Oncogene* 25:3885–3893.
12. Zhang Y, Adachi M, Zhao X, Kawamura R, Imai K (2004) *Int J Cancer* 110:301–308.
13. Geng L, Cuneo KC, Fu A, Tu T, Atadja PW, Hallahan DE (2006) *Cancer Res* 66:11298–11304.
14. Camphausen K, Burgan W, Cerra M, Oswald KA, Trepel JB, Lee MJ, Tofilon PJ (2004) *Cancer Res* 64:316–321.
15. Zhang Y, Adachi M, Zou H, Hareyama M, Imai K, Shinomura Y (2006) *Int J Radiat Oncol Biol Phys* 65:859–866.
16. Munshi A, Kurland JF, Nishikawa T, Tanaka T, Hobbs ML, Tucker SL, Ismail S, Stevens C, Meyn RE (2005) *Clin Cancer Res* 11:4912–4922.
17. Munshi A, Tanaka T, Hobbs ML, Tucker SL, Richon VM, Meyn RE (2006) *Mol Cancer Ther* 5:1967–1974.
18. Sancar A, Lindsey-Boltz LA, Unsal-Kacmaz K, Linn S (2004) *Annu Rev Biochem* 73:39–85.
19. Lombard DB, Chua KF, Mostoslavsky R, Franco S, Gostissa M, Alt FW (2005) *Cell* 120:497–512.
20. Pellegrini L, Yu DS, Lo T, Anand S, Lee M, Blundell TL, Venkitaraman AR (2002) *Nature* 420:287–293.
21. Shivji MK, Davies OR, Savill JM, Bates DL, Pellegrini L, Venkitaraman AR (2006) *Nucleic Acids Res* 34:4000–4011.
22. Henning W, Sturzbecher HW (2003) *Toxicology* 193:91–109.
23. Nojima K, Hochegger H, Saberi A, Fukushima T, Kikuchi K, Yoshimura M, Orelli BJ, Bishop DK, Hirano S, Ohzeki M, *et al.* (2005) *Cancer Res* 65:11704–11711.
24. van Waardenburg RC, de Jong LA, van Delft F, van Eijndhoven MA, Bohlander M, Bjornsti MA, Brouwer J, Schellens JH (2004) *Mol Cancer Ther* 3:393–402.
25. Saleh-Gohari N, Bryant HE, Schultz N, Parker KM, Cassel TN, Helleday T (2005) *Mol Cell Biol* 25:7158–7169.
26. Haince JF, Rouleau M, Hendzel MJ, Masson JY, Poirier GG (2005) *Trends Mol Med* 11:456–463.
27. Schreiber V, Dantzer F, Ame JC, de Murcia G (2006) *Nat Rev Mol Cell Biol* 7:517–528.
28. Hochegger H, Dejsuphong D, Fukushima T, Morrison C, Sonoda E, Schreiber V, Zhao GY, Saberi A, Masutani M, Adachi N, *et al.* (2006) *EMBO J* 25:1305–1314.
29. Chou TC, Talalay P (1984) *Adv Enzyme Regul* 22:27–55.
30. Bryant HE, Schultz N, Thomas HD, Parker KM, Flower D, Lopez E, Kyle S, Meuth M, Curtin NJ, Helleday T (2005) *Nature* 434:913–917.
31. Farmer H, McCabe N, Lord CJ, Tutt AN, Johnson DA, Richardson TB, Santarosa M, Dillon KJ, Hickson I, Knights C, *et al.* (2005) *Nature* 434:917–921.
32. Schultz N, Lopez E, Saleh-Gohari N, Helleday T (2003) *Nucleic Acids Res* 31:4959–4964.
33. van Veelen LR, Cervelli T, van de Rakt MW, Theil AF, Essers J, Kanaar R (2005) *Mutat Res* 574:22–33.
34. Sung, P (1994) *Science* 265:1241–1243.
35. Baumann P, Benson FE, West SC (1996) *Cell* 87:757–766.
36. Chen Z, Clark S, Birkeland M, Sung CM, Lago A, Liu R, Kirkpatirck R, Johanson K, Winkler JD, Hu E (2002) *Cancer Lett* 188:127–140.
37. Hirose T, Sowa Y, Takahashi S, Saito S, Yasuda C, Shindo N, Furuichi K, Sakai T (2003) *Oncogene* 22:7762–7773.
38. Huang Y, Nakada S, Ishiko T, Utsugisawa T, Datta R, Kharbanda S, Yoshida K, Talanian RV, Weichselbaum R, Kufe D, *et al.* (1999) *Mol Cell Biol* 19:2986–2997.
39. Pierce AJ, Jasin M (2005) in *Methods in Molecular Biology*, eds Keohavong P, Grant SG (Humana, Totowa, NJ), pp 373–384.
40. Saeki T, Machida I, Nakai S (1980) *Mutat Res* 73:251–265.
41. Rathmell WK, Chu G (1994) *Proc Natl Acad Sci USA* 91:7623–7627.
42. McCabe N, Turner NC, Lord CJ, Kluzek K, Bialkowska A, Swift S, Giavara S, O'Connor MJ, Tutt AN, Zdzienicka MZ, *et al.* (2006) *Cancer Res* 66:8109–8115.
43. Davies AA, Masson JY, McIlwraith MJ, Stasiak AZ, Stasiak A, Venkitaraman AR, West SC (2001) *Mol Cell* 7:273–282.
44. Takata M, Sasaki MS, Sonoda E, Morrison C, Hashimoto M, Utsumi H, Yamaguchi-Iwai Y, Shinohara A, Takeda S (1998) *EMBO J* 17:5497–5508.
45. Couedel C, Mills KD, Barchi M, Shen L Olshen A, Johnson RD, Nussenzweig A, Essers J, Kanaar R, Li GC, *et al.* (2004) *Genes Dev* 18:1293–1304.
46. Raderschall E, Stout K, Freier S, Suckow V, Schweiger S, Haaf T (2002) *Cancer Res* 62:219–225.
47. Connell PP, Jayathilaka K, Haraf DJ, Weichselbaum RR, Vokes EE, Lingen MW (2006) *Int J Oncol* 28:1113–1119.
48. Han H, Bearss DJ, Browne LW, Calaluce R, Nagle RB, Von Hoff DD (2002) *Cancer Res* 62:2890–2896.
49. Maacke H, Jost K, Opitz S, Miska S, Yuan Y, Hasselbach L, Luttges J, Kalthoff H, Sturzbecher HW (2000) *Oncogene* 19:2791–2795.