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Radiosensitization of p53 Mutant Cells by PD0166285, a Novel G_2 Checkpoint Abrogator

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ABSTRACT

The lack of functional p53 in many cancer cells offers a therapeutic target for treatment. Cells lacking p53 would not be anticipated to demonstrate a G_1 checkpoint and would depend on the G_2 checkpoint to permit DNA repair prior to undergoing mitosis. We hypothesized that the G_2 checkpoint abrogator could preferentially kill p53-inactive cancer cells by removing the only checkpoint that protects these cells from premature mitosis in response to DNA damage. Because Wee1 kinase is crucial in maintaining G_2 arrest through its inhibitory phosphorylation of Cdc2, we developed a high-throughput mass screening assay and used it to screen chemical library for Wee1 inhibitors. A pyridopyrimidine class of molecule, PD0166285 was identified that inhibited Wee1 at a nanomolar concentration. At the cellular level, 0.5 μM PD0166285 dramatically inhibits irradiation-induced Cdc2 phosphorylation at the Tyr-15 and Thr-14 in seven of seven cancer cell lines tested. PD0166285 abrogates irradiation-induced G_2 arrest as shown by both biochemical markers and fluorescence-activated cell sorter analysis and significantly increases mitotic cell populations. Biologically, PD0166285 acts as a radiosensitizer to sensitize cells to radiation-induced cell death with a sensitivity enhancement ratio of 1.23 as shown by standard clonogenic assay. This radiosensitizing activity is p53 dependent with a higher efficacy in p53-inactive cells. Thus, G_2 checkpoint abrogators represent a novel class of anticancer drugs that enhance cell killing of conventional cancer therapy through the induction of premature mitosis.

INTRODUCTION

The driving force for G2→M progression is the Cdc2/cyclin B1 protein complex (1–3). In addition to association with cyclin B1, Cdc2 is also subjected to both positive and negative phosphorylation controls. Thr-161 phosphorylation, catalyzed by cyclin-dependent kinase activating kinase, is required for Cdc2 kinase activity (1). On the other hand, Thr-14 and Tyr-15 phosphorylations on Cdc2 inhibit its kinase activity. Wee1 is the major kinase phosphorylating Cdc2 on Tyr-15 (2–4), therefore inhibiting Cdc2 activity. At the onset of mitosis, the inhibitory phosphates are removed by a dual-specific phosphatase Cdc25C, leading to activation of Cdc2 kinase. Thus, Cdc25 phosphatase plays a key role in normal cell cycle progression between G_2 and M phases. DNA damage can cause Cdc25 inactivation, resulting in a G_2 arrest and allowing the damaged DNA to be repaired. The mechanism of Cdc25 inactivation is through its phosphorylation on Ser-215, catalyzed by Chk1/Chk2 or C-TAK1 kinases (5–7). This phosphorylation creates a binding site for 14-3-3. The interaction between Cdc25 and 14-3-3 results in nuclear exporting of Cdc25 and its cytoplasmic accumulation (8). The upstream kinase that activates Chk2 is ATM,[2] which can be activated by DNA damage (9–12). Therefore, DNA damage activates a G_2 checkpoint by activation of ATM/ATR, followed by Chk1/2 activation and Cdc25 and Cdc2 inactivation.

We hypothesized that a strategy could be developed that would permit us to exploit the G_2 checkpoint to obtain a therapeutic index in the treatment of cancers lacking a G_1 checkpoint. The lack of a G_1 checkpoint is common in >50% of cancers containing p53 mutations. In this strategy, normal cells arrest in G_1 after DNA damage from irradiation (or chemotherapy), whereas cancer cells with a defective G_1 checkpoint would progress through S-phase and into G_2. Therefore, abrogation of G_2 checkpoint will be more detrimental to cancer than normal cells. In an effort to search for specific G_2 checkpoint abrogators, Wee1 kinase was selected as a anticancer target for following reasons: (*a*) Wee1 is a negative regulator of Cdc2 kinase activity, and expression of Cdc2AF mutant, a mutant that cannot be phosphorylated by Wee1 and Myt1 kinases, caused premature mitosis (13–15); (*b*) Wee1 was down-regulated in p53-positive cells after DNA damage (16); (*c*) Wee1 was degraded in Fas-ligand induced apoptosis, involving caspase-dependent activation of Cdc2 (17); and (*d*) Wee1 overexpression rescues apoptosis (14, 18). In this report, we show the identification of a Wee1 kinase inhibitor PD0166285 with an IC_{50} at nanomolar concentration. Interestingly, the compound also has nanomolar IC_{50} for Myt1 kinase. PD0166285 inhibits Cdc2 phosphorylation on both Tyr-15 and Thr-14 *in vivo* in seven tumor cell lines tested. It also abrogates radiation-induced G_2 arrest as measured by biochemical markers and mitotic index. Furthermore, PD0166285 sensitizes radiation-induced cell killing in p53 mutant HT29 cells and in the E6-transfected, p53-null ovarian cancer cell line PA-1 but to a lesser extent in p53 wild-type PA-1 cells. Our observations support the concept that abrogation of G_2 checkpoint potentiates cancer cells, particularly those with a functional inactive p53 to DNA damage-induced cell killing. Therefore, the G_2 checkpoint abrogator presents a new class of anticancer drug functioning as a radiosensitizer.

MATERIALS AND METHODS

Compounds. PD0166285 was synthesized at Pfizer Global Research and Development. UCN-01 was obtained from National Cancer Institute. Caffeine was purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. Ovarian carcinoma PA-1 cells, transfected with the vector control (PA-1/neo, p53wt) or E6 (PA-1/E6, p53-null) were kindly provided by Dr. El Deiry (University of Pennsylvania, Philadelphia, PA) and cultured with Basic Medium Eagle with 10% FBS. All other cell lines are from American Tissue Culture Collection. HT29 (human colon carcinoma cell line with p53 mutation) and HeLa (human cervical cancer cell line with p53 wt/hpv) cells were cultured in high glucose DMEM with 10% FBS. HCT8 (human colon carcinoma cell line with wild-type p53 status) cells were cultured in RPMI 1640 with 10% FBS. HCT116 (human colon carcinoma cell line with wild-type p53) cells were cultured in McCoy's 5a medium with 10% FBS. DLD-1 (human colon carcinoma cell line with mutant p53) cells were cultured in Eagle's MEM with 10% FBS. H460 (human lung carcinoma cell line with wild-type p53) cells were cultured in RPMI 1640 with sodium pyruvate and 10% FBS. C26 (mouse colon carcinoma cell line with wild-type p53)[3] cells were cultured in DMEM/F12 and 10% FBS. All cell culture media were from Life Technologies, Inc.

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[2] The abbreviations used are: ATM, ataxia telangiecstasia mutated; PD0166285, 6-aryl-pyrido[2,3-*d*] pyrimidine; UCN-01, 7-hydroxy staurosporine; FBS, fetal bovine serum; FACS, fluorescence-activated cell sorter; SER, sensitizing enhancement ratio.

[3] Y. Sun *et al.*, unpublished observation.

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Wee1 Mass Screening. Wee1 mass screening was performed using Amersham's p34^{cdc2} kinase SPA (scintillation proximity assay) kit with some modifications. Briefly, 45–60 nM full-length Wee1 kinase was incubated with 25 μM compounds, 20 μM ATP, and 122–441 nM Cdc2/cyclin B in a final volume of 50 μl of enzyme dilution buffer [50 mM Tris (pH 8.0), 10 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, and 0.1 mM Na_3VO_4]. After 30 min incubation at 30°C, 30 μl of [^{33}P]ATP containing kinase buffer [67 mM Tris (pH 8.0), 40 mM NaCl, 13 mM $MgCl_2$, 1 mM DTT, and 0.13 mM Na_3VO_4] containing 1 μM biotinylated peptide, and 0.25 μCi of [γ-^{33}P]ATP was added to the reaction and incubated for another 30 min at 30°C. The reaction was stopped by adding 200 μl of stop buffer [50 μM ATP, 5 mM EDTA, 0.1% Triton X-100, and 1.25 mg/ml SPA beads in PBS]. After centrifugation at 2400 rpm for 15 min, the plate was counted with Wallac's Microbeta counter.

Immunoblots. Cells were irradiated with the indicated dose of X-ray irradiation. Sixteen h after radiation, cells were treated with compounds in the presence of 50 ng/ml nocodazole for 4 h. Cells were then lysed in buffer containing 50 mM HEPES (pH 7.5), 250 mM NaCl, 0.1% NP40, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM Na_3VO_4, and 40 μl/ml proteinase inhibitor mixture (Roche Biochemicals). The lysates were then clarified by centrifugation at 14,000 rpm for 10 min at 4°C. Fifteen μg of each cell lysate were then resolved on 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Novex). The membranes were blocked with 3% BSA (CalBiochem) in TBST [50 mM Tris (pH 8.0), 150 mM NaCl, and 0.1% Tween 20] containing 1 mM NaF, 0.1 mM Na_3VO_4, and 10 mM β-glycerophosphate for 1 h and then incubated with anti-Cdc2 (1:5,000 dilution), anti-Cdc2Y15 (1:10,000 dilution), anti-Cdc2T14 (1:2000 dilution), anti-phosphoPP1 (1:3,000 dilution), or anti-Myt1 (1:3,000 dilution) antibodies, respectively. All antibodies were from Onyx Pharmaceuticals (Richmond, CA). After an extensive wash with TBST, the membrane was incubated with horseradish peroxidase-conjugated antirabbit antibody (Bio-Rad; 1:5,000 dilution) for 1 h. The blot was then developed with ECL Western blotting detection reagent (Amersham) according to the manufacturer's instruction.

Mitotic Index. Cells were irradiated and treated with compounds as described above. The cells were then harvested by washing the plate once with PBS and incubated with 5 mM EDTA in PBS for 5 min at 37°C. Both attached and detached cells were combined and collected by centrifugation at 3000 rpm for 5 min. Each cell pellet was first resuspended in 100 μl of PBS, and then 1 ml of 0.8% sodium citrate was added. After incubation on ice for 1 min, cells were subjected to 3000 rpm centrifugation for 5 min. The cell pellets were resuspended in 100 μl of 0.8% sodium citrate and fixed in 1 ml of Carnoy's fixative (glacial acetic acid:methanol, 1:3) for 5 min at room temperature. The fixed cells were centrifuged and resuspended in 100 μl of Carnoy's fixative and then dropped to a clean glass slide from an arm's-length distance. The glass slides were air-dried and stained with 4′6-diamidino-2-phenylindole (Molecular Probes; M-7006) with 1:20 dilution for 30 min.

Flow Cytometry. Cells were irradiated with the indicated doses of X-ray. Compounds were added 16 h after radiation. Four h after the drug treatment, both detached and attached cells were harvested by centrifugation at 3000 rpm for 5 min. The cells were washed twice with PBS and fixed with 10 ml of 85% ice-cold ethanol. Prior to analysis by flow cytometry, the cells were washed with PBS, treated with 0.25 mg/ml RNase A and 50 μg/ml propidium iodide for 30 min at 37°C. The treated cells were then passed through 12 × 75-mm falcon tube with 35-μm strainer cap. Cell cycle distribution assessments were performed using a Becton Dickinson's FACScan flow cytometer. Events (25,000) were collected for each sample (19).

Clonogenic Assays. For standard clonogenic assay, cells were plated on six-well plates with 300–20,000 cells/well based upon the dose of radiation and the concentration of the compound to achieve 20–200 colonies/well. One day after cell plating, the plates were irradiated, and the compound was given from 0 to 4 h after radiation. The cells were then grown from 7 to 10 days to allow the colony formation. Colonies consisting of 50 or more cells were counted with an imaging microscope. The clonogenic assay data were analyzed with computer software as described previously (20). To increase the throughput of the assay, a 24-well clonogenic assay was developed. This assay was performed in a similar way as the 6-well plate assay, except high-density cells were seeded and the growth area, instead of number of colonies, was measured with an imaging microscope. Specifically, cells were plated from 2,500 to 40,000/well on a 24-well plate, depending on the dose of radiation and the condition of compound treatment to achieve a growth area that has linear correlation with the cells seeded. The radiation and compound treatment were given in the same time schedule as described in the standard clonogenic assay. The cells were fixed after 7 days growth, and the growth areas (excluding areas that had <50 cells) were counted. For the combination study of 0–24 h treatment, compounds were given at the same time with X-irradiation. Twenty-four h after compound treatment, cells were fed with fresh medium and grown for 7 days from the time of plating.

For PA-1 cell clonogenic assay, growing cells were irradiated with the indicated dose of γ-radiation (2–8 Gy). Cells were subsequently cultured in the original medium for 12 h, followed by treatment of 0.4 μM PD0166285 or DMSO control for another 4 h. Both drug-treated or DMSO-control cells were washed with PBS and trypsinized. Five hundred to 10,000 cells were seeded in triplicate in 60-mm tissue culture dishes with 5 ml of medium to form colony numbers at range of 20–200 in each dish. Surviving cells resulted in colonies (>50 cells) in 9–12 days, and these colonies were washed and fixed with 10% acidic acid in methanol and stained with methylene blue (0.05%). The surviving fraction was determined by the proportion of seeded cells after irradiation and the drug treatment to form colonies relative to irradiated and DMSO-control cells. The plating efficiency for PA-1/E6 was ~25%, whereas it was ~40% for PA-1/Neo.

RESULTS

Identification of a Pyridopyrimidine Class of Wee1 Inhibitor, PD0166285. In an attempt to discover novel drugs that disrupt the G_2 checkpoint in cancer cells, we set up a mass-screening assay for rapid screening of chemical library for Wee1 inhibitors (Fig. 1*A*). In this assay, Wee1 kinase phosphorylates and inhibits Cdc2 kinase and therefore decreases the phosphorylation of the biotinylated histone H1 peptide that is captured by streptavidin-conjugated SPA beads. The level of histone H1 peptide phosphorylation is detected with the SPA[^{33}P]p34^{cdc2} kinase assay kit (Amersham). PD0166285, a pyridopyrimidine class compound, was identified with an IC_{50} of 24 nM. The inhibition was found to be ATP competitive (data not shown). The compound also inhibits Myt1 kinase with an IC_{50} of 72 nM and Chk1 kinase with an IC_{50} of 3.4 μM. As a control, the IC_{50} of UCN-01, a known G_2 checkpoint abrogator, against Wee1 and Chk1 is 18.8 μM and 7 nM, respectively (Fig. 1*B*).

PD0166285 Inhibits Cdc2 Phosphorylation on Both Y15 and T14 *in Vivo*. It has been shown that the Cdc2 T14Y15 phosphorylation level is cell cycle dependent. The inhibitory phosphorylation peaks prior to the onset of mitosis and is removed by the dual-specific phosphatase Cdc25C at the onset of mitosis (21–23). If Wee1 is the major kinase phosphorylating Cdc2Y15 *in vivo*, inhibition of Wee1 could decrease Cdc2Y15 phosphorylation in a cell-based assay. To test this hypothesis, Cdc2Y15 phosphorylation was examined after DNA damage in human cancer cell lines derived from carcinomas of colon (HCT116, HT29, DLD-1, and HCT8), lung (H460), and cervix (HeLa) as well as the mouse colon carcinoma line C26. Indeed, as shown in Fig. 2*A,* PD0166285 at 0.5 μM concentration can inhibit Cdc2Y15 phosphorylation in all cell lines tested, regardless of their p53 status. Although less potent, the compound also inhibits Myt1 kinase at nanomolar concentration in our *in vitro* assay. The effect of PD0166285 on Cdc2T14 phosphorylation was therefore determined *in vivo*. As shown at Fig. 2*B,* PD0166285 at 0.5 μM concentration also can inhibit T14 phosphorylation in all cell lines tested.

PD0166285 Abolishes Cdc2 Phosphorylation at Tyr-15, Induces Supershift of Myt1, and Increases PP1 Phosphorylation in HT29 Cells. To test G_2 checkpoint abrogation activity of PD0166285, HT29 cells were first irradiated to activate the G_2 checkpoint. Sixteen h after DNA damage, cells were treated for 4 h with PD0166285, along with UCN-01 or caffeine, two positive controls known to abrogate G_2 checkpoint. As shown in Fig. 3, similar to UCN-01 and caffeine, PD0166285 is able to inhibit Cdc2Y15 phosphorylation (a marker for G_2 phase; Ref. 24), to increase phosphatase 1 phosphory-

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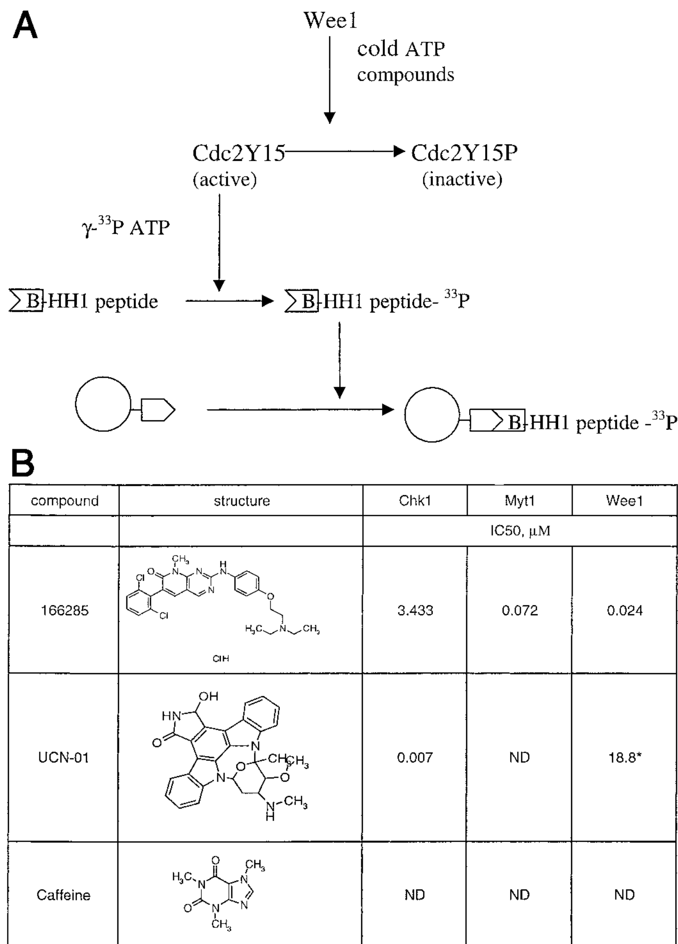

compound	structure	Chk1	Myt1	Wee1
		IC50, μM		
166285		3.433	0.072	0.024
UCN-01		0.007	ND	18.8*
Caffeine		ND	ND	ND

Fig. 1. *A,* Wee1 kinase scintillation proximity assay (SPA). The assay has two incubation steps. In the first step, cold ATP, compounds, Wee1, and Cdc2/B were mixed and incubated at 30°C for 30 min. At the second step, biotinylated (∑]) histone H1 peptide (PKTPKKAKKL), containing a Cdc2 phosphorylation site, and [γ-^{33}P]ATP were added and incubated at 30°C for another 30 min. After the reaction was stopped, the streptavidin-conjugated SPA beads were added to the reaction to capture the biotinylated histone H1 peptide. The ^{33}P signal captured by the SPA beads were read on Wallac's Microbeta counter (Wallac). *B,* structure of compounds and IC_{50}s for Wee1, Myt1, and Chk1 kinases. The chemical structures of three compounds used in this study are shown, along with the IC_{50} for their inhibitory activity against Wee1, Myt1, and Chk1.

lation (a marker for mitosis; Ref. 25), as well as to increase Myt1 phosphorylation and mobility shift (a marker for mitosis; Ref. 26).

PD0166285 Abrogates Irradiation-induced G_2-M Arrest. Having established that PD0166285 abolishes Cdc2 phosphorylation at Y15 as well as at T14, which would activate Cdc2 activity, we next examined whether the compound would abrogate G_2 arrest and promote mitosis entry by FACS analysis. As shown in Fig. 4, DNA damage induced by 7.5 Gy of X-ray radiation caused G_2 arrest in HT29 cells (*left panels*) as evident by an increase of G_2-M population from 19% to 66–69%. Four-h treatment of PD0166285 as well as UCN-01 or caffeine (*right panels*) decreased the G_2-M cell population to 37, 28, or 33%, respectively, and increased the G_1-G_0 cell population to 52, 64, and 60%, respectively, clearly indicating the compound abrogates the DNA damage-induced G_2 checkpoint.

PD0166285 Increases Mitotic Index. Because FACS analysis could not distinguish G_2 cells from M-phase cells, mitotic index measurement was used to further determine whether the compounds treatment produced a high percentage of M-phase population. Cells were irradiated with 5 or 10 Gy and were treated with the indicated amount of compounds for an additional 4 h in the presence of nocodazole to block cells at M-phase. The cells were then harvested, and mitotic index was measured. As shown in Fig. 5, in the absence of DNA damage, there are about 10–15% cells at M-phase. PD0166285 abrogated DNA damage-induced G_2 arrest and increased mitotic cell population from 16 to 28% at 5 Gy of radiation. This G_2 checkpoint abrogation effect was more dramatic at 10 Gy, as evidenced by an increase of M phase cells from 8 to 45%. The degree of

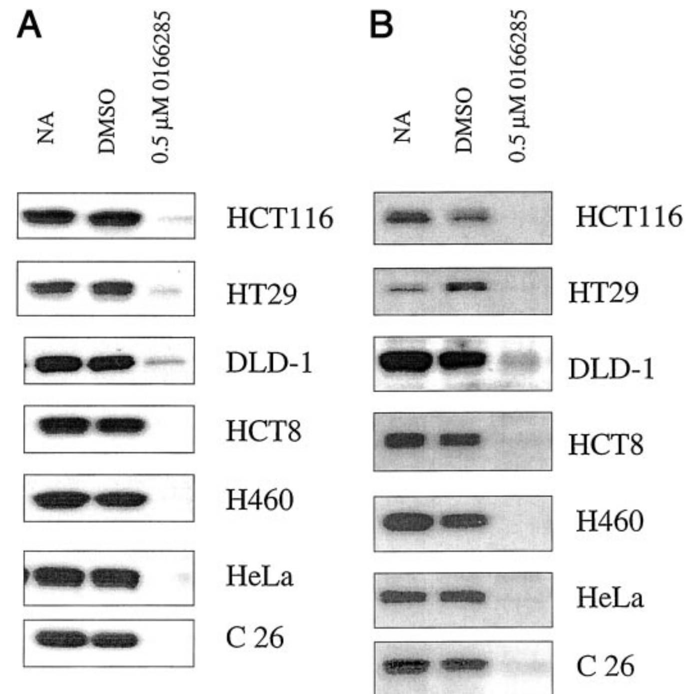

Fig. 2. PD0166285 inhibits Cdc2Y15 and CdcT14 phosphorylation in both human and mouse cancer cell lines. The indicated cell lines were plated on day 1 and irradiated on day 2 with 5 Gy of X-ray. Sixteen h after radiation, cells were treated with 50 ng/ml nocodazole alone (*NA*), nocodazole plus DMSO (*DMSO*) or nocodazole with 0.5 μM PD0166285 (0.5 μM 0166285). Four h after compound treatment, cells were harvested, and 15 μg of cell lysate/lane were loaded on 12% SDS-PAGE for immunoblots, as detailed in "Materials and Methods." *A,* immunoblot with anti-CdcY15. *B,* immunoblot with anti-Cdc2T14.

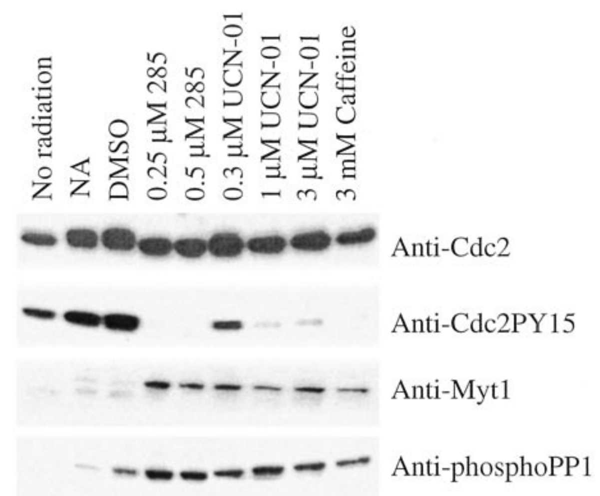

Fig. 3. PD0166285 abolishes phosphorylation of Cdc2 at Tyr-15, induces Myt1 supershift, and increases PP1 phosphorylation. Human colon carcinoma cell line HT29 was plated on day 1 and irradiated on day 2 with 7.5 Gy of X-ray. Sixteen h after radiation, cells were left untreated (*NA*) or treated with DMSO control or the indicated compounds in the presence of 50 ng/ml nocodazole for 4 h. Fifteen μg of cell lysate were loaded on 12% SDS-PAGE and immunoblotted with anti-Cdc2, anti-Cdc2PY15, anti-Myt1, and anti-phosphoPP1 as described in "Materials and Methods."

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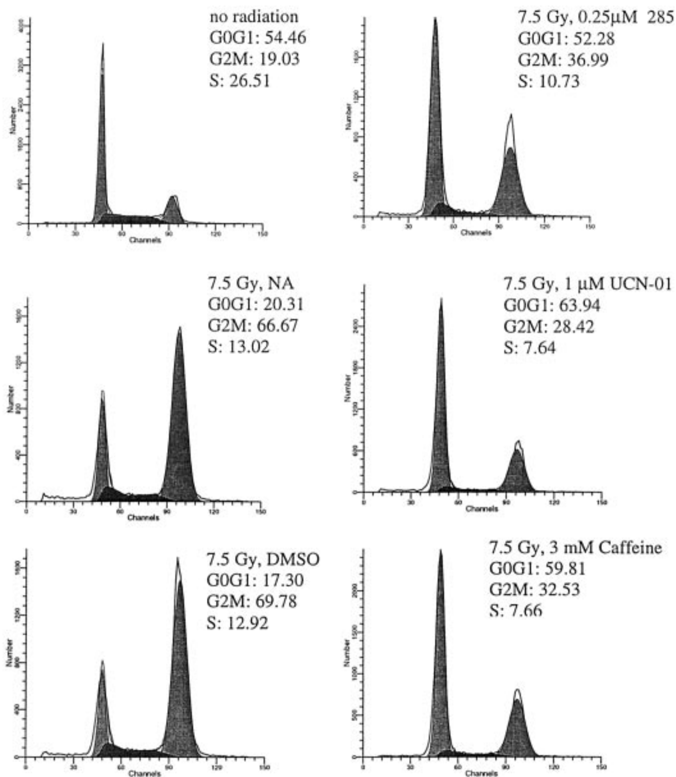

Fig. 4. PD0166285 abolishes irradiation-induced G_2 arrest. Cells were treated in the same way as described in the Fig. 3 legend, except that no nocodazole was added at the time of the compound treatment. Both attached and floating cells were harvested, and the cell cycle distribution was analyzed with FACScan as described in "Materials and Methods."

increase of the mitotic cell population appears to be directly correlated with the dose of radiation. The effect of PD0166285 is comparable with that of UCN-01. Thus, both biochemical and biological end points support the concept that PD0166285, similar to UCN-01 and caffeine, can abrogate radiation-induced G_2 arrest and induce premature mitosis.

PD0166285 Enhances Radiation-induced Cell Killing in HT29 Cells. DNA damage-induced G_2 arrest is a critical step for cells to repair damage before entering mitosis. Abrogation of the G_2 checkpoint would promote mitosis entry prematurely and eventually lead to cell death. To test this hypothesis, HT29 cells were irradiated with 0, 1.5, 3, 5, or 8 Gy with or without 0.5 μM PD0166285 for 4 h immediately after DNA damage. PD0166285 at 0.5 μM final concentration can sensitize radiation-induced cell killing with a SER of 1.23 for standard clonogenic assay (Fig. 6*A*) and 1.38 for 24-well assay (Fig. 6*B*). As the controls, UCN-01 and caffeine, two known G_2 checkpoint abrogators, were also evaluated for their sensitizing enhancement activities. HT29 cells were treated for 24 h with the drugs at the time of X-ray radiation. As shown in Fig. 6*C,* UCN-01 and caffeine enhanced radiation-induced cell killing with SERs of 1.58 and 2.05, respectively.

Radiosensitizing Activity of PD0166285 Is p53 Dependent. We had hypothesized that the radiosensitizing activity of Wee1 inhibitor would be greater in cells missing p53. To assess more directly the role of p53, wt p53-containing ovarian tumor cell line PA-1, transfected with E6 to degrade p53 (PA-1/E6, p53-null) and the vector control (PA-1/neo, p53-positive; Ref. 27) were used in the study. We first examined p53 status of the cells and its response to DNA damage. As shown in Fig. 7, a low level of endogenous p53 was detectable in the neo control but not in the E6-transfected cells. Both p53 and the p53 downstream target gene, *MDM2,* were induced after DNA damage in PA-1/Neo control but not in the E6-transfected PA-1. The results confirmed that PA-1 neo cells contain functional active p53, whereas E6-transfected PA-1 cells are p53-null. We next performed clonogenic assay of these cells after γ-irradiation, and results are shown in Fig. 8. PD0166285 increased radiation-induced cell killing with a SER of 1.2 in E6-transfected cells but not in neo control cells. Thus, radiosensitizing activity of PD0166285 is p53 dependent with a higher activity in p53-null cells.

DISCUSSION

Effective anticancer therapies (chemotherapeutic agents and ionizing radiation) kill proliferating cancer cells by damaging their DNA and inducing apoptosis. A significant limitation for these treatments is

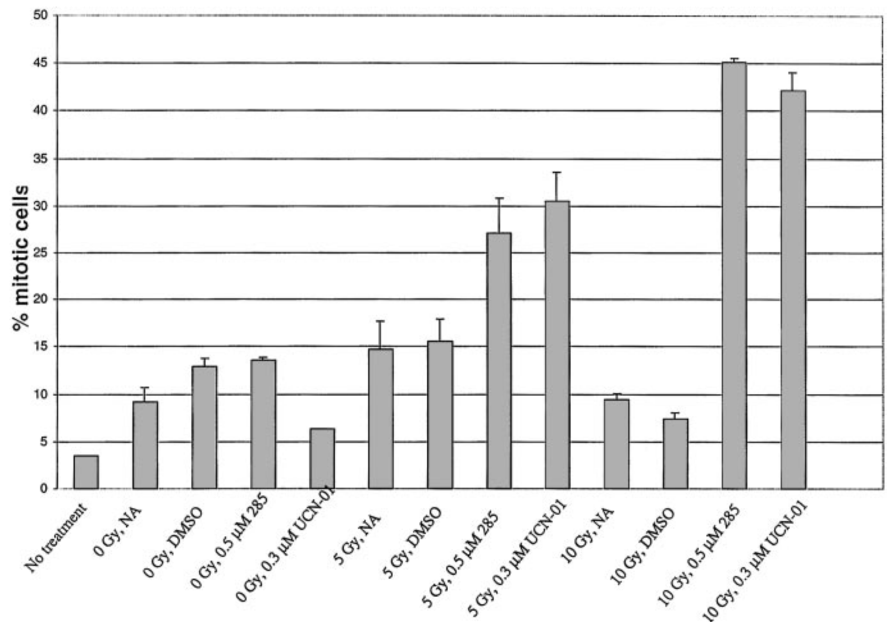

Fig. 5. PD0166285 increases mitotic index. HT29 cells were treated as described in the Fig. 3 legend with the indicated doses of irradiation and concentrations of the compounds. Both attached and detached cells were combined and subjected to mitotic index analysis as described in "Materials and Methods."

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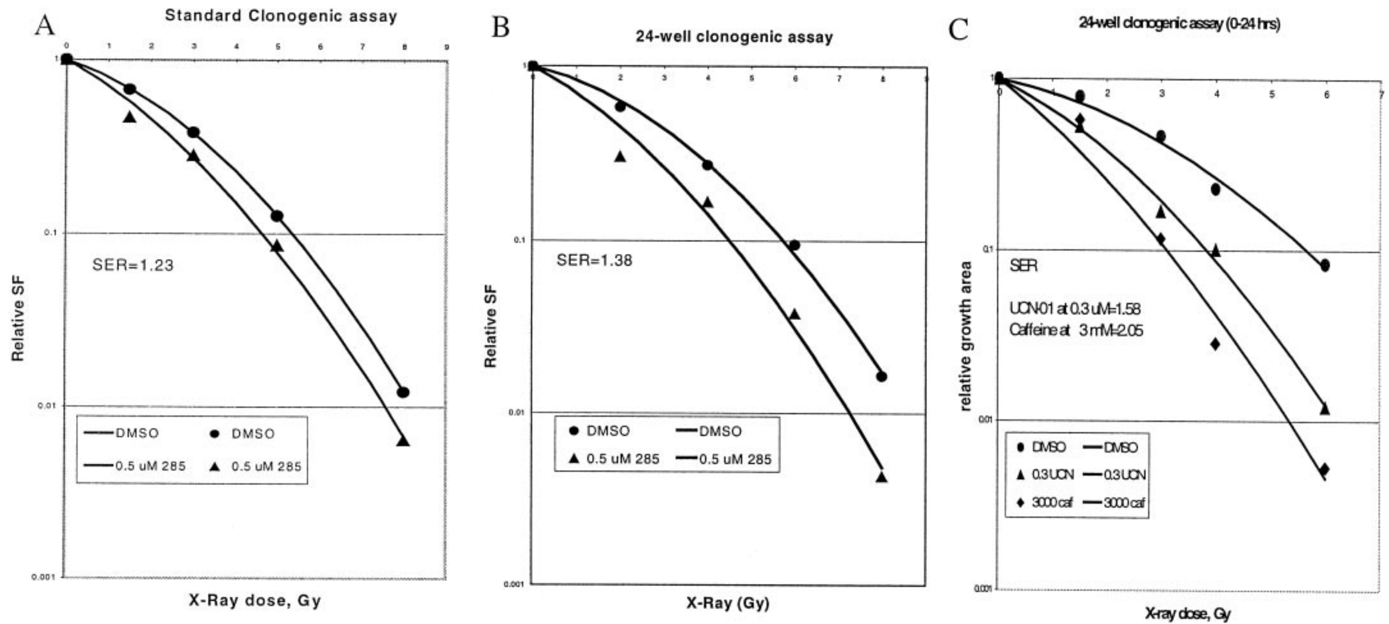

Fig. 6. PD0166285 sensitizes HT29 cells to irradiation-induced cell killing. Standard clonogenic assay in combination with X-irradiation and 4 h of PD0166285 treatment was performed in the 6-well plates (*A*) as well as in the 24-well plates for high throughput (*B*) as described in "Materials and Methods." For the 24-well clonogenic assay, 2,500 to 40,000 cells were seeded/well, depending upon the treatment. The growth areas, instead of the individual colonies, were counted. Standard clonogenic assay was performed in combination with X-irradiation and UCN-01 and caffeine (*C*). Cells were treated with compounds starting at the time of irradiation for 24 h. PD0166285 was not tested under this condition because of its toxicity.

that many tumor cells developed resistance to DNA damage-induced cell killing. One of the major mechanisms involved is that >50% of human cancers have mutations in the tumor suppressor gene *p53* (28), which is a critical component for induction of apoptosis in response to DNA damage (29, 30). The cellular response to DNA damage involved cell cycle arrest, mainly at G_1 and G_2 phases. The cell cycle arrests allow cells to repair the damage before entering S-phase for DNA replication and M-phase for mitosis and, therefore, are critical for cells to maintain their genetic integrity. Failure to repair the DNA damage will cause mutations and eventually cell death. The G_1 arrest requires functional p53, which in turn induces the cyclin-dependent kinase inhibitor p21 expression. p21 is required for p53-dependent G_1 arrest (31, 32). The function of p53 at G_2 arrest is less well understood. It has been reported that 14-3-3σ, a downstream target of p53, was induced after DNA damage, and this induction is required for prolonged G_2 arrest (33). On the other hand, functional p53 may also accelerate mitosis entry after DNA damage. This accelerated G_2-M transition induced by p53 was associated with enhanced cytotoxicity and apoptosis (16).

The G_2 checkpoint involves Cdc2 inactivation by negative phosphorylation. Premature activation of Cdc2 kinase by dephosphorylation has been shown to cause apoptosis (14, 34, 35). Therefore, pharmacological activation of Cdc2 kinase to induce premature mitosis and apoptosis could be an effective approach to circumvent the resistance of p53-deficient cancer cells to genotoxic agents. Normal cells, on the other hand, might be more tolerate to the G_2 checkpoint abrogator because of their intact G_1 checkpoint. Indeed, caffeine and UCN-01, which have been shown previously to abrogate the G_2 checkpoint, can sensitize p53 inactive cells to apoptosis (36, 37). However, the molecular target(s) of these agents were not very clear at that time. UCN-01 was initially identified as a protein kinase C inhibitor and has anticancer activity. Subsequent investigation showed that UCN-01 inhibits Chk1 (36, 38). Caffeine has been studied for over 20 years and was able to abrogate the G_2 checkpoint at mM range (37). Recently, data has shown that caffeine inhibits ATM kinase and ATM-related kinase, ATR, at a concentration similar to that inducing G_2 checkpoint abrogation (39).

In search for a more specific G_2 checkpoint abrogator, PD0166285, a pyridopyrimidine class of compound, was identified from Wee1 mass screen. It inhibits Wee1 and Myt1 with an IC_{50} of 24 and 72 nM, respectively. It does not inhibit Cdc2/cyclin B (data not shown) but inhibits Chk1 kinase at a much higher concentration (3433 nM). Although it is not Wee1/Myt1 specific, PD0166285 does target different molecules from what UCN-01 and caffeine target for their G_2 checkpoint abrogation activity. At the cellular level, the compound inhibits Cdc2Y15 and Cdc2T14 phosphorylation in seven tumor cell lines tested. In a further detailed study with HT29 cells, it was demonstrated that PD0166285 is able to abrogate the G_2 checkpoint and sensitize HT29 cells to radiation-induced cell killing. Our data are consistent with a model that through the inhibition of Wee1 and Myt1 kinases, PD0166285 induces premature activation of Cdc2 activation,

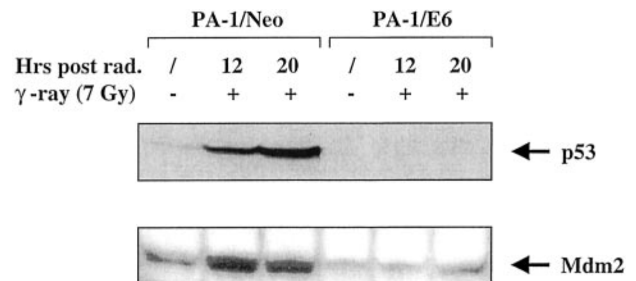

Fig. 7. Lack of functional p53 in PA-1/E6 cells. Growing cells were either not irradiated or γ-irradiated with 7 Gy. Cells were incubated for hours as indicated after irradiation (*Hrs post rad.*). Whole cell extracts were prepared by washing cells with PBS once and were directly lysed with a sample buffer containing 0.125 M Tris (pH 6.8), 20% glycerol, 4% SDS (w/v), 0.05 M DTT, and 0.1% bromphenol blue. Equal amounts of protein (30 μg) were resolved in 4–20% SDS-polyamide gels and electrophoresed at 150 V. After being transferred to polyvinylidene difluoride membranes, proteins were immunoblotted with p53 antibody. The same membrane was washed with 1× Western reprobe buffer and then reprobed with MDM2 antibody.

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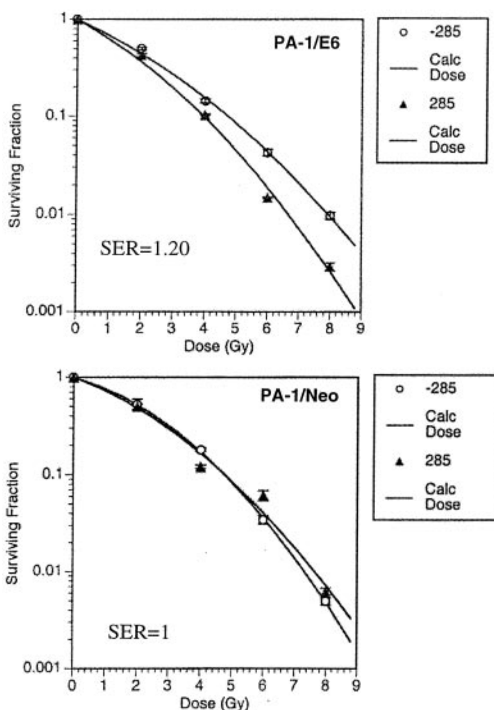

Fig. 8. p53-null cells are more sensitive to PD0166285. Growing cells were irradiated with indicated doses of γ-ray (2–8 Gy). Cells were incubated in the original medium for 12 h after irradiation and then either treated or not treated with 0.4 μM PD166285 for another 4 h. Both drug treated and untreated cells were washed with PBS and trypsinized. Cells (500–10,000) were seeded in triplicate in 60-mm tissue culture dishes with 5 ml of medium. Surviving cells resulted in colonies (>50 cells) in 9–12 days, and these colonies were washed and fixed with 10% acidic acid in methanol and stained with methylene blue (0.05%). The surviving fraction was determined by the proportion of colony numbers in irradiated- and the drug-treated cells relative to those in unirradiated- and drug-untreated cells. *Top panel,* PA-1/E6; *bottom panel,* PA-1/Neo. *Bars,* SE.

abrogates the radiation-induced G_2 checkpoint, and thus enhances radiation-induced cell killing.

Because the compound inhibits both Wee1 and Myt1 in the nanomolar range, it is not clear from this study whether the G_2 checkpoint abrogation activity is solely attributable to the Wee1 inhibition. It is noteworthy that the radiosensitizing activity of PD0166285 is modest compared with reported G_2 checkpoint abrogators, UCN-01 and caffeine (Fig. 5). This appears to be attributable to cellular toxicity induced by the compound in the absence of DNA damage, particularly in the long-term survival assay such as clonogenic assay. To limit the compound's toxicity at a level <40% of the cell population, the highest dose of PD0166285 can be used is 0.5 μM for a period of no more than 4 h. Because the compound's IC_{50} to inhibit Wee1 kinase activity *in vivo* is 0.269 $\pm$ 0.222 μM,[4] the therapeutic window of the cellular effectiveness *versus* cellular toxicity of the compound is quite low. In contrast, UCN-01 and caffeine are much less toxic to cells at their active concentration and can be used for up to 24 h. It is not clear at the present time whether the toxicity of PD0166285 is attributable to its Wee1/Myt1 inhibition or attributable to its inhibition of other tyrosine kinases such as c-Src, fibroblast growth factor receptor, epidermal growth factor receptor, or platelet-derived growth factor receptor (40).

The data presented in this report, using paired p53-positive and p53-negative cancer cells, support the hypothesis that abrogation of G_2 checkpoint by targeting Wee1 and Myt1 kinases is more effective against p53-null cancer cells and raise the hope for a potential therapeutic window. With the development of more potent and selective Wee1 inhibitors, these G_2 checkpoint abrogators, when used in combination with cytotoxic agents or radiation, may greatly benefit cancer patients, particularly those whose cancer contain functional-inactive p53.

In summary, we have tested and confirmed our hypothesis that G_2 checkpoint abrogation would preferentially kill the cancer cells that lack a functional p53 and/or G_1 checkpoint control. Because normal cells have intact checkpoints at both the G_1 and G_2 phases of the cell cycle and would be more resistant to G_2 checkpoint abrogation, the abrogators targeting the G_2 checkpoint would therefore kill cancer cells more efficiently than do the normal cells, providing a therapeutic window for cancer treatment. Thus, G_2 checkpoint abrogators represent a novel class of anticancer drug that selectively enhances cancer cell killing by conventional cancer therapies.

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