Radiotherapy is a common method of cancer treatment, and it is known that the G2-M checkpoint plays a crucial role in the cell cycle. This checkpoint is activated by DNA damage and prevents cells from entering mitosis until the DNA is repaired. However, in cancer cells, this checkpoint is often dysfunctional, allowing the cells to continue dividing even with damaged DNA.

**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 G1 checkpoint and would depend on the G2 checkpoint to permit DNA repair prior to undergoing mitosis. We hypothesized that the G2 checkpoint abrogator could preferentially kill p53-negative 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 G2 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 G2 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-negative cells. Thus, G2 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 (4). 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 (2–4), therefore inhibiting Cdc2 activity. Wee1 overexpression rescues apoptosis (14, 18). In this report, we show the identification of a Wee1 kinase inhibitor PD0166285 with an IC50 at nanomolar concentration. Interestingly, the compound also has nanomolar IC50 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 G2 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 G2 checkpoint potentiates cancer cells, particularly those with a functional inactive p53 to DNA damage-induced cell killing. Therefore, the G2 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 Type Culture Collection. HT29 (human colon carcinoma cell line with p53 mutation) and HeLa (human cervical cancer cell line with p53 mutation) were cultured in 10% FBS. HCT116 (human colon carcinoma cell line with wild-type p53) cells were cultured in RPMI 1640 with 10% FBS. HCT29 (human colon carcinoma cell line with wild-type p53 status) cells were cultured in RPMI 1640 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.

Received 6/19/01; accepted 9/13/01.

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

3 Y. Sun et al., unpublished observation.
Wee1 Mass Screening. Wee1 mass screening was performed using Amersham’s p34<sup>cdc2</sup> 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 μM of enzyme dilution buffer [50 mM Tris (pH 8.0), 10 mM NaCl, 10 mM MgCl<sub>2</sub>, 1 mM DTT, and 0.1 mM Na<sub>2</sub>V<sub>3</sub>O<sub>5</sub>]. After 30 min incubation at 30°C, 30 μl of [γ-<sup>32</sup>P]ATP containing kinase buffer [67 mM Tris (pH 8.0), 40 mM NaCl, 13 mM MgCl<sub>2</sub>, 1 mM DTT, and 0.13 mM Na<sub>2</sub>V<sub>3</sub>O<sub>5</sub>] containing 1 μM biotinylated peptide, and 0.25 μCi of [γ-<sup>32</sup>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 plaques were washed twice with albumin/ultrafiltration’s Microbelex biotinylated antibodies. 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 G2 checkpoint in cancer cells, we set up a mass-screening assay for rapid screening of chemical library for Wee1 inhibitors (Fig. 1A). 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<sup>33</sup>P<sup>p34</sup>cdc2 kinase assay kit (Amersham). PD0166285, a pyridopyrimidine class compound, was identified with an IC<sub>50</sub> of 24 nM. The inhibition was found to be ATP competitive (data not shown). The compound also inhibits Myt1 kinase with an IC<sub>50</sub> of 72 nM and Chkl kinase with an IC<sub>50</sub> of 3.4 μM. As a control, the IC<sub>50</sub> of UCN-01, a known G<sub>2</sub> checkpoint abrogator, against Wee1 and Chkl is 18.8 μM and 7 nM, respectively (Fig. 1B).

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. 2A, 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. 2B, 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 PPI Phosphorylation in HT29 Cells. To test G2 checkpoint abrogation activity of PD0166285, HT29 cells were first irradiated to activate the G2 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 G2 checkpoint. As shown in Fig. 3, similar to UCN-01 and caffeine, PD0166285 is able to inhibit Cdc2Y15 phosphorylation (a marker for G2 phase; Ref. 24), to increase phosphate 1 phosphorylation.
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 G2-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 G2 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 G2 arrest in HT29 cells (left panels) as evident by an increase of G2-M population from 19% to 66%–69%. Four-h treatment of PD0166285 as well as UCN-01 or caffeine (right panels) decreased the G2-M cell population to 37, 28, or 33%, respectively, and increased the G1-G0 cell population to 52, 64, and 60%, respectively, clearly indicating the compound abrogates the DNA damage-induced G2 checkpoint.

**PD0166285 Increases Mitotic Index.** Because FACS analysis could not distinguish G2 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 G2 arrest and increased mitotic cell population from 16 to 28% at 5 Gy of radiation. This G2 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

**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 PD0166285). Four h after compound treatment, cells were harvested, and 15 μg of cell lysate were loaded on 12% SDS-PAGE for immunoblots, as detailed in "Materials and Methods." A, immunoblot with anti-Cdc2. B, immunoblot with anti-Cdc2T14.

**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, anti-phosphoPP1 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 endpoints 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 $\mu$M PD0166285 for 4 h immediately after DNA damage. PD0166285 at 0.5 $\mu$M final concentration can sensitize radiation-induced cell killing with a SER of 1.23 for standard clonogenic assay (Fig. 6A) and 1.38 for 24-well assay (Fig. 6B). 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. 6C, 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 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...
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 G1 and G2 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 G1 arrest requires functional p53, which in turn induces the cyclin-dependent kinase inhibitor p21 expression. p21 is required for p53-dependent G1 arrest (31, 32). The function of p53 at G2 arrest is less well understood. It has been reported that 14-3-3, a downstream target of p53, was induced after DNA damage, and this induction is required for prolonged G2 arrest (33). On the other hand, functional p53 may also accelerate mitosis entry after DNA damage. This accelerated G2-M transition induced by p53 was associated with enhanced cytotoxicity and apoptosis (16).

The G2 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 tolerant to the G2 checkpoint abrogator because of their intact G1 checkpoint. Indeed, caffeine and UCN-01, which have been shown previously to abrogate the G2 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 G2 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 G2 checkpoint abrogation (39).

In search for a more specific G2 checkpoint abrogator, PD0166285, a pyridopyrimidine class of compound, was identified from Wee1 mass screen. It inhibits Wee1 and Myt1 with an IC50 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 G2 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 G2 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,
abrogates the radiation-induced G₂ 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 G₂ checkpoint abrogation activity is solely attributable to the Wee1 inhibition. It is noteworthy that the radiosensitizing activity of PD0166285 is modest compared with reported G₂ 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 IC₅₀ to inhibit Weel kinase activity in vivo is 0.269 ± 0.222 μM, 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 Weel/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 G₂ checkpoint by targeting Weel 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 G₂ 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 G₂ checkpoint abrogation would preferentially kill the cancer cells that lack a functional p53 and/or G₂ checkpoint control. Because normal cells have intact checkpoints at both the G₁ and G₂ phases of the cell cycle and would be more resistant to G₂ checkpoint abrogation, the abrogators targeting the G₂ checkpoint would therefore kill cancer cells more efficiently than do the normal cells, providing a therapeutic window for cancer treatment. Thus, G₂ checkpoint abrogators represent a novel class of anticancer drug that selectively enhances cancer cell killing by conventional cancer therapies.

ACKNOWLEDGMENTS

We thank Dr. Wafik El-Diery at University of Pennsylvania for providing us with PA-1/neo and PA-1/E6 transfectants.

REFERENCES

34. Krek, W., and Nigg, E. A. Mutations of p34cdc2 phosphorylation sites induce premature mitotic events in HeLa cells: evidence for a double block to p34cdc2 kinase activation in vertebrates. EMBO J., 10: 3331–3341, 1991.
Radiosensitization of p53 Mutant Cells by PD0166285, a Novel G₂ Checkpoint Abrogator

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