Breaching the DNA damage checkpoint via PF-00477736, a novel small-molecule inhibitor of checkpoint kinase 1

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Abstract

Checkpoints are present in all phases of the cell cycle and are regarded as the gatekeepers maintaining the integrity of the genome. Many conventional agents used to treat cancer impart damage to the genome and activate cell cycle checkpoints. Many tumors are defective in the tumor suppressor p53 and therefore lack a functional G1 checkpoint. In these tumors, however, the S-G2 checkpoints remain intact and, in response to DNA damage, arrest cell cycle progression allowing time for DNA repair. Checkpoint kinase 1 (Chk1) is a key element in the DNA damage response pathway and plays a crucial role in the S-G2-phase checkpoints. Inhibiting Chk1 represents a therapeutic strategy for creating a “synthetic lethal” response by overriding the last checkpoint defense of tumor cells against the lethal damage induced by DNA-directed chemotherapeutic agents. Chk1 inhibition is consistent with emerging targeted therapies aiming to exploit molecular differences between normal and cancer cells. Adding a Chk1 inhibitor to DNA-damaging cytotoxic therapy selectively targets tumors with intrinsic checkpoint defects while minimizing toxicity in checkpoint-competent normal cells. PF-00477736 was identified as a potent, selective ATP-competitive small-molecule inhibitor that inhibits Chk1 with a K_i of 0.49 nM. PF-00477736 abrogates cell cycle arrest induced by DNA damage and enhances cytotoxicity of clinically important chemotherapeutic agents, including gemcitabine and carboplatin. In xenografts, PF-00477736 enhanced the antitumor activity of gemcitabine in a dose-dependent manner. PF-00477736 combinations were well tolerated with no exacerbation of side effects commonly associated with cytotoxic agents. [Mol Cancer Ther 2008;7(8):2394 - 404]

Introduction

Cancer is a multiplicity of diseases characterized by a range of molecular defects leading to unregulated, aberrant cell growth. Recent improvements in understanding the molecular basis of cancer progression, improved diagnostics, and the emergence of new classes of therapeutics have offered promise to better manage the disease. Chemotherapeutic agents that induce cytotoxicity by damaging DNA have been the mainstay of cancer treatment for many decades. Despite their effectiveness, there are several limitations, most notably a narrow therapeutic window due to lack of selectivity toward cancer cells. A new generation of chemotherapeutic agents commonly called “targeted therapies” is emerging and aims to impart selectivity to cancer cells by exploiting molecular differences between normal and cancer cells. In this report, we describe a novel kinase targeted therapy with a distinct mechanism of action, checkpoint kinase 1 (Chk1). Inhibition of Chk1 represents a molecularly targeted approach to selectively enhance the cytotoxicity of DNA-damaging agents in tumor cells with intrinsic checkpoint defects (mutated p53) while minimizing toxicity in normal cells that have a checkpoint-competent molecular phenotype (wild-type p53).

Checkpoints are present in all phases of the cell cycle and are regarded as the gatekeepers maintaining the integrity of the genome (1–4). Many conventional agents used to treat cancer impart damage to the genome and activate cell cycle checkpoints. p53 is the major player in the checkpoint that arrests cells at the G1-S boundary, whereas Chk1 is critical for the S and G2-M checkpoints (5–7). Dysfunction in cell cycle checkpoints appears to be a universal phenomenon in human cancers. The p53 tumor suppressor gene is mutated more often in human cancers than any other gene yet reported (8). These cancers lack a functional G1 checkpoint; however, the S-G2-M checkpoints remain intact and, in response to DNA damage, arrest cell cycle progression allowing time for DNA repair. The essential and indispensable role of Chk1 in initiating the S-G2-phase checkpoints has been shown through biochemical and genetic studies (9, 10). A recent review summarizes key findings validating Chk1 as a therapeutic target (11). Inhibiting Chk1 represents a therapeutic strategy for creating a “synthetic lethal” response by overriding the last checkpoint defense of the tumor cell against the lethal damage induced by DNA-directed chemotherapeutic
agents (8–10). The present studies describe the identification and characterization of PF-00477736, a potent, selective ATP-competitive small-molecule inhibitor of Chk1. PF-00477736 potently inhibited Chk1 enzymatic activity and exhibited a high degree of kinase selectivity. PF-00477736 specifically abrogated DNA damage–induced cell cycle arrest and enhanced the cytotoxicity of several DNA-damaging agents across a broad spectrum of cell lines. Further, the checkpoint abrogating activity and cytotoxic activity attributed to PF-00477736 in combination with chemotherapeutic agents showed selectivity for p53-defective cancer cell lines over p53-competent normal cells in vitro. In vivo, PF-00477736 showed dose-dependent potentiation of gemcitabine antitumor efficacy in xenographs models with no enhancement of systemic toxicity. Collectively, these results show the therapeutic potential of targeting Chk1 with selective small-molecule inhibitors. Combining DNA-damaging agents with a Chk1 inhibitor represents a therapeutic strategy to selectively target tumors with intrinsic checkpoint defects while minimizing toxicity in normal cells. This new generation of combination therapies could significantly improve the efficacy and selectivity of chemotherapeutic agents.

Materials and Methods

Dot Blot Assay—Histone H3 Phosphorylation Assay. CA46 cells were seeded at exponentially growing density in a 24-well plate (Corning). Cells were grown for 16 h followed by addition of 50 nM camptothecin to appropriate wells for 24 h to initiate DNA damage. PF-00477736 was then added at increasing concentrations in the presence of nocodazole at 0.1 μg/mL to block cells from exiting mitosis. Cells were harvested 16 h post-treatment with PF-00477736 by lysing in the plate with cold lysis buffer [50 nM Tris (pH 8.3), 100 nM β-glycerophosphate, 5 nM EDTA, 10 nM BME, 0.1% SDS] and vigorous shaking for 5 min. Cell lysates were transferred to 96-well Filtration Manifold System (Life Technologies) attached to vacuum source with Amersham Hybond-C Extra Nitrocellulose Membrane (GE Healthcare) and suction was applied. Membrane was blocked with 1% Western Blocking Reagent (Roche Diagnostics) in TBS-T (1× TBS + 1% Tween 20) for 30 min and then probed with anti-phospho-histone H3 (Upstate). Horseradish peroxidase–conjugated secondary antibody was used (GE Healthcare). Protein was visualized using SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology) and dot density was determined using SpectraMax (Molecular Devices) plate reader at 690 nm.

IC50 MTT. The proliferation assays were done using an MTT assay. The IC50 assay measures the antiproliferative effects of PF-00477736 on p53-defective human cancer cell lines. Cells in each line were seeded in complete medium at an exponentially growing density in 96-well assay plate and allowed to attach for 16 h. Serial dilutions of PF-00477736 were then done, and appropriate controls were added to each plate. Cells were incubated with drug for 96 h. After incubation, MTT working stock diluted in complete medium was added to each well, and cells were incubated for 4 h. After centrifugation and supernatant removal, DMSO was added to each well and plates were read on SpectraMax plate reader at 540 nm. IC50 and PF50 results were calculated by concentration-response curve fitting using a Microsoft Excel–based four-variable analysis method.

PF50 MTT. The agents (gemcitabine, SN-38, carboplatin, doxorubicin, and mitomycin C) were used at a concentration that caused minimal or no toxicity (<10%) in the absence of PF-00477736 (data not shown). PF-00477736 was used at 8× EC50 (360 nM) in all cell lines, except K562, where it was used at 4× EC50 (180 nM). Serial dilutions of each agent were done followed by addition of PF-00477736. Appropriate controls were added to each plate, and cells were incubated at 37°C for 96 h. The rest of the assay was done as described for IC50 MTT. Antiproliferative effects were compared relative to maximal effect of DNA-damaging agents.

Cell Survival. HT29 or human umbilical vein endothelial cells were treated with gemcitabine (15 nM) or camptothecin (25 nM) for 16 h. PF-00477736 was subsequently added at varying concentrations. At 4 to 48 h after the addition of PF-00477736, the drug-containing medium was removed and cells were incubated in drug-free medium. When the vehicle-treated control cells were 90% confluent (8 days from plating), cells were harvested and counted by Coulter counter.

In vivo Studies

Animals. Female nu/nu mice (5-8 weeks old) were purchased from Charles River Laboratories kept under clean room conditions in sterile filter-top cages with Alpha-Dri bedding and housed in HEPA-filtered ventilated racks. Animals received sterile rodent chow and water ad libitum. All of the procedures were conducted in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and with Pfizer Animal Care and Use Committee guidelines.

Pharmacokinetics of PF-00477736. Female nu/nu mice received a single dose of PF-00477736 via i.p. injection at doses of 4, 20, and 40 mg/kg. Male Sprague-Dawley rats received a single dose of PF-00477736 as a bolus i.v. injection at 4 mg/kg. The compound was administered as a solution in 50 nM sodium acetate buffer and 4% dextrose (pH 4). All rats were instrumented with jugular vein cannula for blood collection. The blood samples were collected at specific time points. Each blood sample was placed in a tube containing heparin and centrifuged to obtain plasma. The plasma samples were frozen immediately and stored at approximately −80°C until samples were analyzed using the liquid chromatography-tandem mass spectrometry method summarized below.

Liquid Chromatography-Tandem Mass Spectrometry Analysis of PF-00477736. Study plasma samples (50 μL) or blank plasma (50 μL) were transferred to 96-well plate tubes. A 200 μL aliquot of acetonitrile/methanol (1:1, v/v) containing 0.1 μM buspirone as an internal standard was
added to each plasma sample. Samples were then vortexed for 1 min and centrifuged at 3,000 to 4,000 rpm for 5 min to precipitate proteins. Supernatant (50 µL) from each tube was transferred into 96-well autosampler plate wells containing 100 µL deionized water. The liquid chromatography-tandem mass spectrometry system included a Shimadzu LC-10A high-performance liquid chromatography system and a Sciex API 4000 triple quadrupole mass spectrometer. The chromatography was done on a reverse-phase column, Zorbax SB-Phenyl (2.1 x 50 mm, 5 µm), using a gradient elution method at a flow rate of 400 µL/min. The mobile phase consisted of A [0.1% formic acid in 80:20 (v/v) water/acetoniitrile] and B [0.1% formic acid in 80:20 (v/v) acetonitrile/water]. A linear gradient of B from 5% to 60% over 3.5 min was used. The mass spectrometer was operated using electrospray in positive ion mode with multiple reactions monitoring of the transitions m/z 420.1→280.9 for PF-00477736 and m/z 386.2→122.2 for buspirone (internal standard). A calibration curve was constructed by Analyst 1.4 software using linear regression of the peak area ratios of the analyte relative to the internal standard.

S.c. Xenograft Models in Athymic Nude Mice. Colo205 cells for implantation into athymic mice were harvested and pelleted by centrifugation at 1,400 rpm for 5 min. The cell pellets were resuspended in sterile serum-free medium supplemented with 50% Matrigel (BD Biosciences). Cells (2 x 10^6 in 200 µL) were implanted s.c. into the right hind flank region of each mouse.

Antitumor Efficacy Studies. Chemotherapy agents or PF-00477736 were administered by i.p. injection when tumors were 100 to 150 mm³ in volume over the designated treatment schedules. Gemcitabine was administered over a range of doses including the maximum tolerated dose (MTD) in mice according to a once every 3 days for four treatments (q3d x 4) schedule (12). PF-00477736 was administered over a range of doses (4-60 mg/kg) according to the q3d x 4 schedule beginning 24 h after gemcitabine. MTD of PF-00477736 was determined to be 40 mg/kg considering the severity of the behavioral response on i.p. administration and body weight loss of 5% to 10%. For cytotoxic agents, MTD is the occurrence of mean body weight loss of 5% to 10%.

Tumor growth inhibition (TGI) was assessed 2 days after administration of the last dose of PF-00477736. Potentiation of TGI was determined by comparing the TGI of the cytotoxic plus PF-00477736 combination to the cytotoxic alone using the formula 100 x [1 - (TVf - Tv0)/Tv0] x 100%. Time-to-progression endpoint and associated tumor growth delay determinations were calculated using median days to reach two doublings of initial tumor size (800 mm³). Comparisons were made using one-way ANOVA with Dunnett’s post-tests. Kaplan-Meier survival curves were compared using Mantel-Haenszel log-rank test and where appropriate hazard ratio analysis was done.

Immunohistochemistry Studies. Immunohistochemical staining assays were done as directed by manufacturer (Cell Signaling Technologies). Samples were analyzed with microscope, and images were taken and quantified using Image-Pro Plus software (Media Cybernetics).

Results

PF-00477736 Potency and Kinase Selectivity

PF-00477736 is a selective, potent, ATP-competitive inhibitor of Chk1 (Supplementary Fig. S1). The in vitro kinase activity of Chk1 (1-289) catalytic domain was inhibited by PF-00477736 with a Ki of 0.49 ± 0.29 nM. PF-00477736 was a poor inhibitor of CDK1 activity (Ki = 9.9 µM, 20,000-fold versus Chk1). PF-00477736 significantly inhibited the activity of Chk2 (Ki = 47 ± 9 nM), although the selectivity ratio for Chk1 versus Chk2 is essentially equal to 100-fold. PF-00477736 selectivity was further characterized in a kinase screening panel representing >100 protein kinases, only seven kinases were inhibited by PF-00477736 with <100-fold selectivity (based on IC50 to Ki ratio): VEGFR2, Aurora-A, FGFR3, FH3, Fms (CSF1R), Ret, and Yes (Supplementary Table S1). To determine if PF-00477736 was inhibiting Aurora kinase, we did several flow cytometry experiments, comparing the Chk1 inhibitor with an Aurora inhibitor. We showed that PF-00477736 neither induced block of cytokinesis (cell division) resulting in the formation of polyploid multinucleated cells nor a block in cell proliferation, both typical hallmarks of Aurora inhibition.

PF-00477736 Abrogates the Camptothecin-Induced DNA Damage Checkpoint in a Dose-Dependent Manner

The histone H3 phosphorylation assay detects cells entering mitosis and represents the primary in vitro cell-based assay used to measure the cellular potency of PF-00477736 in abrogating the G2 checkpoint induced by DNA damage. In a dot-blot assay done in p53-mutated human lymphoma CA46 cells, PF-00477736 was found to inhibit camptothecin-induced G2 arrest. The EC50 value was 45 nM, as measured by an increase in histone H3 phosphorylation on Ser10, a marker of entry into mitosis (Fig. 1A). HeLa cells were also evaluated under similar conditions using high-content imaging with an algorithm created to quantify the mitotic index based on phospho-histone H3 expression. Here, the EC50 for PF-00477736 was calculated to be 38 nM (Fig. 1B). In addition, HT29 cells were also evaluated in the same system and showed an EC50 value of 42 nM (data not shown).

PF-00477736 Abrogates the Gemcitabine-Induced S-Phase Arrest and Induces Increase in Apoptotic Cell Death

The in vitro analysis of the cell cycle effects induced by PF-00477736 in combination with gemcitabine was done in HT29 cells (Fig. 2). In the absence of DNA damage, PF-00477736 did not induce any change in the cell cycle profile compared with the control cells. Gemcitabine is a nucleoside analogue that is incorporated into replicating DNA.

6 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
This results in partial chain termination and stalling of replication forks, which induces a S-phase arrest. In our study, gemcitabine induces a prominent S-phase arrest at 16 h post-treatment. On combination with gemcitabine, flow cytometry analysis shows abrogation of gemcitabine induced S-phase arrest with PF-00477736 (Fig. 2A). The time-dependent decrease in the S-phase cells induced by PF-00477736 corresponded to an increase in the G2-M and G0-G1 cell populations, showing that cells are entering mitosis and attempting to reenter the cell cycle. In addition, an increase in the percentage of a population of “nongated” cells was detected in the combination treated cells. A terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling–based flow cytometry study was done to evaluate the effect of the combination treatment on apoptosis. The result from this study confirmed that the checkpoint abrogation induced by the combination treatment of gemcitabine and PF-00477736 translates in apoptotic cell death (Fig. 2B).

PF-00477736 Enhances Gemcitabine Cytotoxicity in Dose- and Time-Dependent Manner

The activity of PF-00477736 in enhancing the cytotoxic effect of gemcitabine was tested in HT29 cells in a cell survival assay (Fig. 3A). Cells were treated with gemcitabine with or without PF-00477736 for different lengths of time. The drug-containing medium was then removed and cells were incubated in drug-free medium until analysis was done. With 24 h exposure at up to 12× EC50, PF-00477736 alone caused no significant effect on cell viability compared with control cells. A concentration of

![Figure 1](image1.png)

**Figure 1.** PF-00477736 abrogates the camptothecin-induced DNA damage checkpoint in a dose-dependent manner. **A,** CA46 cells were treated first with camptothecin for 8 h followed by PF-00477736 at increasing concentrations in the presence of nocodazole. Cells were harvested 16 h post-treatment with PF-00477736, cell lysates were prepared, and samples were analyzed by dot-blot assay for the levels of phospho-histone H3 (on Ser10), a marker of mitosis. Positive controls used: nocodazole (Noc); camptothecin (Cpt) in combination with caffeine. Negative controls: camptothecin with nocodazole; PF-00477736 alone. EC50 is defined as the drug concentration that induces histone H3 phosphorylation in 50% of the cell population compared with the nocodazole positive control. **B,** HeLa cells were treated first with camptothecin for 8 h followed by 128 nM PF-00477736 or 1.0% DMSO. Cells were fixed, stained with CMFDA-AM and Hoechst 33342, and analyzed by high-content imaging using algorithms designed to quantify the mitotic index based on phospho-histone H3 expression. **Left,** camptothecin + PF-00477736; **middle,** camptothecin only; **right,** curve representing the mitotic index induced by PF-00477736 and camptothecin in HeLa cells.
gemcitabine that induced minimal (21%) cell growth inhibition was used. The gemcitabine and PF-00477736 combination induced significant potentiation of the cytotoxic activity of gemcitabine (up to 89% increase in cell growth inhibition compared with gemcitabine alone), indicating that the combination treatment caused a permanent cellular damage that cannot be overcome even after drug removal. The cell kill induced by the combination treatment is time and dose (PF-00477736) dependent.

PF-00477736 Selectively Targets Tumor Cells

Based on the mechanism of action of Chk1 inhibitor, PF-00477736 in combination with chemotherapy is expected to selectively target p53-defective cancer cells (due to their deficiency in the G1 checkpoint) while having minimal cytotoxic effects on normal (p53-competent) cells. To assess the cytotoxic effect of PF-00477736 in combination with chemotherapy agents in normal cells, a cell survival assay was done in human umbilical vein endothelial cells (Fig. 3B). PF-00477736 was used in combination with either gemcitabine or camptothecin, both used at a fixed concentration that induces minimal cell toxicity (<10%). The highest concentration (12 × EC50, 540 nM) of PF-00477736 in combination causes 31.2% or 21.7% increase in cell kill compared with gemcitabine or camptothecin alone, respectively. The cytotoxic effect induced by the combination treatment in human umbilical vein endothelial cells is negligible compared with the cytotoxicity induced by the same treatment in tumor cells. The minimal toxicity induced by PF-00477736 in p53-competent nontumor cells in combination with chemotherapy provides evidence to support its selectivity for p53-defective cancer cells and potential to have minimal adverse effects in normal cells.

Chemopotentiation of PF-00477736 in Combination with Different Cytotoxic Agents in Selected Cancer Cell Lines

PF-00477736 was shown to potentiate the growth-inhibitory activity of a panel of chemotherapeutic agents across a broad spectrum of p53-deficient human cancer cell lines in the MTT assay (Fig. 4A and B; ref. 13). The agents selected (gemcitabine, SN-38, carboplatin, doxorubicin, and mitomycin C) were representatives from multiple classes of chemotherapeutic agents distinguished by their different mechanisms of DNA damage. To determine the PF50, increasing concentrations of each DNA-damaging agent were used in combination with PF-00477736 used at a fixed concentration that induces no or minimal cell toxicity (<10%) when used alone. PF-00477736 induced robust and consistent potentiation with most agents, across various types of cancer cell lines (colon, breast, prostate, and leukemia).

The results obtained in both MTT and cell survival assays provide strong evidence for the in vitro efficacy of PF-00477736 in enhancing the cell-killing activity of a variety of DNA-damaging agents in p53-defective cancer cells with minimal single agent activity attributed to PF-00477736 alone.

PF-00477736 Modulation of DNA Damage Pathway Proteins

In response to DNA damage, Chk1 phosphorlates and inhibits the protein phosphatase Cdc25C, thus preventing activation of the CDK1/cyclin B complex and mitotic entry (14). In human cells, the DNA damage–induced G2 delay has been shown to be largely dependent on inhibitory phosphorylation of CDK1 (15–17). A series of experiments...
were done to characterize the modulation of key elements of the G2 checkpoint pathway as indicators of checkpoint abrogation. A time-course assay was done using gemcitabine to induce DNA damage (Fig. 5A).

The cell cycle arrest induced by gemcitabine caused elevated levels of CDK1 phosphorylation at Tyr15 compared with untreated or PF-00477736-only-treated cells. The combination of gemcitabine and PF-00477736 caused a significant, time-dependent decrease in the phosphorylation of CDK1, consistent with checkpoint abrogation and cell entry into mitosis. Mitotic cells collected at 10 h after treatment with PF-00477736 were enriched in the mitotic, slower migrating, form of Cdc25C. This was confirmed by the presence of high levels of phospho-histone H3. The significant increase in cyclin B levels up to 10 h after the addition of PF-00477736 followed by its complete disappearance at 24 h provided further evidence of checkpoint abrogation, mitotic entry, and cell cycle progression through metaphase of mitosis.

PF-00477736 Increases γH2AX

The nucleosome, made up of four core histone proteins (H2A, H2B, H3, and H4) is the primary building block of chromatin (18–20). The histone variant, H2AX, is phosphorylated on Ser139, (γ-H2AX) and forms nuclear foci in response to different types of DNA damage. H2AX becomes phosphorylated and forms nuclear foci in response to stalling of replication forks and is further phosphorylated due to checkpoint abrogation-induced apoptosis. Treatment with gemcitabine at a concentration (30 nM) that induces S-phase arrest did not cause phosphorylation of H2AX (Fig. 5B). Addition of PF-00477736 to gemcitabine-arrested cells induced a dramatic increase in the intensity of H2AX phosphorylation, reflecting a greater number of γ-H2AX molecules near sites of DNA damage. The increase in DNA damage is presumably due to checkpoint abrogation and lack of DNA repair. PF-00477736 alone had minimal effects on H2AX phosphorylation.

![Graph](image-url)

**Figure 3.** A, PF-00477736 enhances gemcitabine-induced cytotoxicity in a time- and dose-dependent manner in HT29 cells as determined by cell survival assay. HT29 cells were treated with gemcitabine followed by PF-00477736 in selected plates. Cells were harvested and counted at indicated times. Mean ± SD (n = 3). B, PF-00477736 induces minimal cytotoxicity in combination with gemcitabine or camptothecin in human umbilical vein endothelial cells. Human umbilical vein endothelial cells were treated with gemcitabine or camptothecin in human umbilical vein endothelial cells. Human umbilical vein endothelial cells were treated with gemcitabine or camptothecin followed by PF-00477736. Twenty-four hours after the addition of PF-00477736, the drug-containing medium was removed and cells incubated in drug-free medium until control samples reached confluence. Cells were harvested and counted by Coulter counter. Mean ± SD (n = 3).
Pharmacokinetics of PF-00477736 in Preclinical Species

The pharmacokinetic variables of PF-00477736 in mouse and rat are presented in Supplementary Table S2. Following i.v. administration of PF-00477736 to intact male rats, systemic plasma clearance was low and accounted for ~20% of hepatic blood flow. Volume of distribution at steady state was distributed into body tissues in rats. The terminal half-life was 2.9 h. The pharmacokinetics in mice appears to be dose dependent following i.p. administration of PF-00477736. Greater than dose-proportional increase in AUC was observed in mice between 20 and 40 mg/kg probably due to saturation of the elimination pathways. PF-00477736 is stable in plasma for the duration of storage before liquid chromatography-mass spectrometry analysis.

Potentiation of Gemcitabine Antitumor Activity by PF-00477736 in Colo205 Xenografts

Gemcitabine combination studies were conducted in Colo205 xenografts. Dose and schedule are critical determinants of the efficacy and side effects of chemotherapy. Gemcitabine was administered over a range of doses including the MTD in mice according to a once every 3 days for four treatments (q3d × 4) schedule (12). PF-00477736 was administered over a range of doses (4-60 mg/kg) as once a day (s.i.d.) or twice a day (b.i.d.) injections according to saturation of the elimination pathways. PF-00477736 is stable in plasma for the duration of storage before liquid chromatography-mass spectrometry analysis.
to the q3d × 4 schedule beginning 24 h after gemcitabine. The optimal combination regimen for gemcitabine and PF-00477736 was sequential administration with a 24-h interval between gemcitabine and PF-00477736. The rationale for this sequence considers time post-DNA damage to be important to allow Chk1-mediated accumulation of S-G2–M-arrested cells rendering them vulnerable to S-G2–M checkpoint inhibition while also allowing normal cells time to repair damaged DNA. PF-00477736 showed no single-agent antitumor activity in the Colo205 xenograft (Fig. 6A). PF-00477736 dose-dependently enhanced the antitumor activity of a MTD of gemcitabine with no apparent exacerbation of systemic toxicity as assessed by monitoring body weight. No formal necropsies were done (Fig. 6A). The highest tolerated single dose of PF-00477736 (40 mg/kg) enhanced TGI by 75% with a time-to-progression enhancement ratio of 50% (Fig. 6B). Log cell kill was increased from 0.5 to 1.25 and hazard ratio analysis indicated the time-to-progression endpoint was improved 3.6-fold compared with gemcitabine treatment alone. Chemopotentiation of PF-00477736 shows dependence on schedule of administration rather than depending exclusively on total dose. A 15 mg/kg dose of PF-00477736 administered as a single dose or as 7.5 mg/kg b.i.d. showed comparable TGI; however, tumor growth delay, time-to-progression enhancement ratio, and log cell kill were superior with 15 mg/kg administered according to a 7.5 mg/kg b.i.d. (Fig. 6B).

PF-00477736 Induces Histone H3 Phosphorylation and DNA Damage and Increases Apoptosis In vivo

Immunohistochemical analyses were done on Colo205 tumor samples harvested from in vivo studies with gemcitabine and PF-00477736. A dose-dependent induction of caspase-3 at 15 and 30 mg/kg PF-00477736 was observed indicating increased apoptosis (Fig. 6C). Furthermore, an increase in the phosphorylation of histone H3 (Ser10) and of phospho-histone H2AX was induced by PF-00477736 compared with gemcitabine-only treated tumors, confirming the abrogation of the gemcitabine-induced checkpoint, as well as a greater degree of DNA damage due to lack of DNA repair after checkpoint abrogation (Fig. 6C). Collectively, increased pH3, H2AX, and apoptosis show a dose-dependent correlation to antitumor efficacy and support the concept that enhanced antitumor efficacy of gemcitabine by PF-00477736 may be mediated by checkpoint inhibition and consequential mitotic catastrophe leading to apoptotic cell death thereby potentiating cytotoxicity.

Discussion

Chk1 inhibitors represent a new class of chemopotentiators that act by modulating DNA damage responses. The essential and indispensable role of Chk1 in initiating the S- and G2-phase checkpoints has been shown through biochemical and genetic studies (6). Although Chk1 homozygous knockout is lethal in embryonic stem cells (6), adult somatic cells with Chk1 knockout are still viable and their cell cycle profile remains mostly normal (21, 22). This suggests that the extent of cellular requirement for Chk1 may vary depending on the different stages of development. Caution must be exercised when comparing biological systems with genetic manipulations of Chk1 to pharmacologic inhibition of Chk1 in an adjunct setting to chemotherapy. Inhibition of Chk1 represents a molecularly targeted approach to selectively enhance the cytotoxicity of DNA-damaging agents in tumor cells with intrinsic checkpoint defects (mutated p53) while sparing normal cells that have a different molecular phenotype (wild-type p53). Many conventional agents used to treat cancer impart damage to the genome and activate cell cycle checkpoints. One of the major limitations of many current cancer therapies is a narrow therapeutic index due to the lack of selectivity for tumor cells. We have shown that combining PF-00477736, a small-molecule inhibitor of Chk1, with a
variety of DNA-directed cytotoxic agents abrogates cell cycle arrest and results in enhanced cytotoxicity in p53 mutated cells and xenograft models.

Evaluation of PF-00477736 across a panel of >100 diverse receptor tyrosine and serine/threonine kinases indicated that it exhibited a high degree of selectivity at pharmaceutically relevant concentrations. For PF-00477736, kinases that are most pharmaceutically relevant for selectivity considerations are those for which transient intermittent inhibition would influence cell cycle progression (e.g., CDKs and mitotic kinases) or checkpoint control (e.g., Chk2, ATM, and ATR) or act on apoptotic pathways (e.g., AKT and p38). PF-00477736 showed activity at VEGFR2, CSF1R, FGFR2, Flt3, and Ret, but these are not considered to be pharmaceutically relevant because sustained inhibition is required to evoke observable pharmacology from these receptor tyrosine kinases and the intended use of PF-00477736 would not produce sustained inhibition. Similarly, no effect is expected from transient inhibition of Yes kinase; the Yes knockout mouse exhibits no significant phenotype (23).

Figure 6. A, PF-00477736 potentiation of gemcitabine in human colon Colo205 xenograft model. B, summary of PF-00477736 potentiation of gemcitabine in human colon xenograft models. *, P < 0.05 versus vehicle (one-way ANOVA with Dunnett’s post-test); **, P < 0.05 versus cytotoxic alone (one-way ANOVA with Dunnett’s post-test); ***, P < 0.05 versus cytotoxic alone (Mantel-Haenszel log-rank test). Differences are considered significant if P < 0.05. MTD of PF-00477736 was determined to be 40 mg/kg considering the severity of the behavioral response on i.p. administration and body weight loss 5% to 10%. For cytotoxic agents, MTD is the occurrence of mean bodyweight loss of 5% to 10%. a, gemcitabine administered i.p. according to q3d/C24, PF-00477736 administered i.p. according to q3d × 4 beginning 24 h after gemcitabine. b, %TGI was calculated as 100 × [1 - (TVf - Tvi)treated / (TVf - Tvi)vehicle], where TVf and Tvi are the final dose + 2 d and initial average tumor volume of a group, respectively. c, %PTGI (potentiated TGI) was calculated as 100 × [1 - (TVf - Tvi)combination / (TVf - Tvi)cytotoxic alone], where TVf and Tvi are the final dose + 2 d and initial average tumor volume of a group respectively. d, growth delay was calculated as treatment-vehicle (T-C) for median days to reach 2 doublings (800 mm3). e, % TTP ER (time-to-progression enhancement ratio) was calculated as delay[combination / delay(cytotoxic alone) × 100 - 100]. f, gross log cell kill was calculated as (T - C) / (3.32 / Td) where T - C is growth delay divided by log210 and Td is doubling time in days. The Td in each study was estimated from a log-linear growth plot of the control group tumors in exponential growth (100-800 mm3) period. C, in vivo chemopotentiation mechanism of action of PF-00477736 in Colo205 human colon xenograft models. PF-00477736 induces caspase-3 activation in gemcitabine-treated Colo205 xenografts in a dose-dependent manner. PF-00477736 increases the phosphorylation of histone H3 and H2AX compared with gemcitabine-only-treated tumors. Colo205 tumors were harvested and sectioned for immunohistochemical detection of caspase-3, phospho-histone H3, and phospho-H2AX.
Aurora-A is a relevant kinase, but it has been found that the enzyme assay does not correlate well with cell activity. The selectivity ratio of PF-00477736 for Chk1 versus Chk2 is essentially equal to 100-fold.

Many classes of chemotherapeutic agents impart damage to DNA and consequently activate cell cycle checkpoints resulting in cell cycle arrest. Checkpoint abrogating activity was shown in a dose-dependent manner as measured by the increase in phospho-histone H3, a marker of mitosis. PF-00477736 showed checkpoint abrogating activity when combined with either camptothecin, a topoisomerase I poison known to elicit cell cycle arrest in G2, or gemcitabine, an antimetabolite, known to induce S-phase arrest. Cell cycle progression into mitosis with damaged DNA results in increased cell death as indicated by a time-dependent increase in apoptosis.

We did a detailed analysis of the Chk1 signaling pathway in response to treatment with PF-00477736 in combination with gemcitabine. The cell cycle arrest induced by gemcitabine caused elevated levels of CDK1 phosphorylation at Tyr15 compared with untreated or PF-00477736-only-treated cells. The combination of gemcitabine and PF-00477736 caused a significant, time-dependent decrease in the phosphorylation of CDK1, consistent with checkpoint abrogation and cell entry into mitosis. The presence of the mitotic form of Cdc25C at 10 h after combination treatment further confirms mitotic progression. The significant increase in cyclin B levels followed by its complete disappearance at 24 h provided further evidence of checkpoint abrogation, mitotic entry, and cell cycle progression through metaphase of mitosis. Addition of PF-00477736 to gemcitabine-arrested cells induced a dramatic increase in the intensity of H2AX phosphorylation, reflecting a greater number of γ-H2AX molecules near sites of DNA damage. These results suggest a greater degree of DNA damage due to checkpoint abrogation, lack of DNA repair, and subsequent mitotic catastrophe.

Cell survival and MTT assays were done in a panel of p53-defective human cancer cell lines to characterize the activity of PF-00477736 in enhancing the cytotoxic effect of clinically important chemotherapeutic agents. PF-00477736 alone caused no significant effect on cell viability. In combination with gemcitabine, PF-00477736 caused significant potentiation (89%) of gemcitabine cytotoxicity compared with gemcitabine alone. PF-00477736 induced robust and consistent potentiation with most agents with some variability observed between cell lines. The results obtained in both cell survival and MTT assays provide strong evidence for the in vitro efficacy of PF-00477736 in enhancing the cytotoxicity of DNA-damaging agents in p53-defective cancer cells with minimal single-agent activity attributed to PF-00477736 alone.

DNA damage–induced cell cycle arrest depends on a signal transduction network in which the kinase Chk1 plays a critical role. In mammalian cells, the p53 pathway further augments the cell cycle arrest response to prevent mitotic catastrophe. The expectation to achieve a therapeutic window with a Chk1 inhibitor is based on the fact that most tumor cells are p53 deficient, defective in the G1 checkpoint, and therefore can only arrest at S or G2 in response to DNA damage. Because normal cells will largely arrest at G1 for DNA repair, they are expected to be less responsive to a S or G2 checkpoint abrogation induced by Chk1 inhibition. Therefore, the effect of DNA damage should be preferentially enhanced in p53-deficient tumor cells compared with p53-proficient tumor cells or normal cells. Our studies support this hypothesis: the checkpoint abrogating activity and cytotoxic activity attributed to PF-00477736 in combination with chemotherapy agents show selectivity for tumor cells over normal cells.

A series of experiments were done to evaluate PF-00477736 chemopotentiation of the antitumor activity of cytotoxic agents in mouse xenograft models. Colo205 human colon carcinoma xenograft was selected as a model for combination studies based on its p53-deficient status and chemosensitivity profile to gemcitabine. Gemcitabine was selected for combination studies based on its ability to induce checkpoint activation and subsequent S-G2-M-phase arrest. The optimal dosing schedule for PF-00477736 was determined to be sequential administration with a 24-h interval between gemcitabine and PF-00477736. The rationale for this sequence considers time to be important to allow Chk1-mediated accumulation of S-G2-M-arrested cells rendering them vulnerable to S-G2-M checkpoint inhibition while also allowing normal cells time to repair damaged DNA. PF-00477736 showed no single-agent antitumor activity in the Colo205 xenografts. These data show that PF-00477736 potentiates the antitumor activity of gemcitabine with no exacerbation of side effects commonly associated with cytotoxic agents, suggesting selectivity toward cancer cells over normal proliferating cells.

In summary, these studies describe the pharmacologic effects of PF-00477736 in combination with common DNA-directed agents on a variety of tumor cell lines and in the Colo205 tumor model. The significant enhancement of antitumor efficacy, flexible dosing regimens, and favorable kinase selectivity profile of this molecule suggests its potential as a novel agent in combination with standard of care agents for the treatment of a variety of cancers. A key issue in the clinical development of Chk1 inhibitors lies in defining the optimal combinations and dosing regimens for achieving maximal therapeutic window. Another important aspect will be to stratify patients according to p53 status thereby maximizing benefits of combination therapy with Chk1 inhibitors.

Disclosure of Potential Conflicts of Interest
All authors are present or former employees of Pfizer Global Research & Development.

References
Breaching the DNA damage checkpoint via PF-00477736, a novel small-molecule inhibitor of checkpoint kinase 1

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