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Small-molecule inhibition of Wee1 kinase by MK-1775 selectively sensitizes p53-deficient tumor cells to DNA-damaging agents

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Abstract

Wee1 is a tyrosine kinase that phosphorylates and inactivates CDC2 and is involved in G_2 checkpoint signaling. Because p53 is a key regulator in the G_1 checkpoint, p53-deficient tumors rely only on the G_2 checkpoint after DNA damage. Hence, such tumors are selectively sensitized to DNA-damaging agents by Wee1 inhibition. Here, we report the discovery of a potent and selective small-molecule inhibitor of Wee1 kinase, MK-1775. This compound inhibits phosphorylation of CDC2 at Tyr15 (CDC2Y15), a direct substrate of Wee1 kinase in cells. MK-1775 abrogates G_2 DNA damage checkpoint, leading to apoptosis in combination with DNA-damaging chemotherapeutic agents such as gemcitabine, carboplatin, and cisplatin selectively in p53-deficient cells. *In vivo*, MK-1775 potentiates tumor growth inhibition by these agents, and cotreatment does not significantly increase toxicity. The enhancement of antitumor effect by MK-1775 was well correlated with inhibition of CDC2Y15 phosphorylation in tumor tissue and skin hair follicles. Our data indicate that Wee1 inhibition provides a new approach for treatment of multiple human malignancies. [Mol Cancer Ther 2009; 8(11):2992–3000]

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Introduction

Many of the conventional anticancer treatments, including ionizing radiation, antimetabolites, alkylating agents, DNA topoisomerase inhibitors, and platinum compounds, damage DNA in cells (1, 2). Although these DNA-damaging agents are among the most effective anticancer agents, their clinical use has many limitations. Their therapeutic potentials are not sufficient because of poor patient responses and because of side effects due to their lack of tumor selectivity. When cellular DNA is damaged, cells can arrest the cell cycle temporally to allow for the damaged DNA to be repaired (3, 4). This cell cycle checkpoint could protect normal cells or tissues from damage and promote their survival, but it may reduce the effectiveness of chemotherapy on tumor cells. Thus, if one can selectively reduce the checkpoint activity in tumor cells, treatment with DNA-damaging agents could be much more effective (5–7).

p53 is a key regulator of the G_1 checkpoint and is one of the most frequently mutated genes in cancer (4). Therefore, a majority of human cancers lack G_1 checkpoint but retain the S- and G_2-phase checkpoints. As a result, p53-deficient cells are predicted to be more dependent on S or G_2 checkpoint. Hence, p53-deficient tumors treated with G_2 checkpoint abrogator may be particularly susceptible to DNA damage (5, 6). Nontumor tissue will retain G_1 checkpoint activity due to its normal p53 pathway function. Thus, checkpoint escape induced by G_2-checkpoint abrogator may selectively sensitize p53-deficient cells to DNA-damaging anticancer agents while sparing normal tissues from toxicity.

Wee1 is a tyrosine kinase that selectively phosphorylates the Tyr15 residue of cyclin-dependent kinase 1 (also known as CDC2) and inactivates its activity (8–11). As CDC2Y15 phosphorylation is involved in G_2-M checkpoint regulation by DNA damage (12, 13), Wee1 is an interesting target for development of a G_2 checkpoint abrogator. Consistent with this hypothesis, Wee1 silencing with siRNA or inhibition of Wee1 by small molecular inhibitor compounds was reported to sensitize cells toward DNA damage (14, 15). In this article, we describe the profile of MK-1775, a potent and selective small-molecule inhibitor of Wee1 that abolishes CDC2Y15 phosphorylation in cells. MK-1775 abrogates DNA damage checkpoint, leading to apoptosis in combination with several DNA-damaging agents selectively in p53-deficient tumor cell lines.

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Materials and Methods

In vitro Kinase Assays

Recombinant human Wee1 was purchased from Carna Biosciences. Kinase reaction was conducted with 10 μmol/L ATP, 1.0 μCi of [γ-^{33}P]ATP, and 2.5 μg of poly(Lys, Tyr) as a substrate at 30°C for 30 min. Radioactivity incorporated into the substrate was trapped on MultiScreen-PH plates and was counted on a liquid scintillation counter.

Cell Culture

WiDr and NCI-H1299 cell lines were obtained from the American Type Culture Collection and were cultured according to the supplier's instructions. TOV21G p53-isogenic matched-pair cell lines were provided from Rosetta Inpharmatics (16). HeLa-luc cells were obtained from Caliper Life Sciences. These cells were cultured with DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Moregate BioTech).

Cell Viability Assay

Cells were seeded in 96-well plates and treated with gemcitabine for 24 h, then with MK-1775 for an additional 24 h. Cell viability was determined with a WST-8 kit (Kishida Reagents Chemicals) using SpectraMax (Molecular Devices).

p-CDC2 and pHH3 Assays

Tumor cells were cultured in 96-well plates and incubated with DNA-damaging agents for 24 h, then with MK-1775 and nocodazole for additional 8 h. For p-CDC2Y15 assay, cells were lysed and subjected in a colorimetric ELISA to determine the amounts of p-CDC2Y15 (1:100; Cell Signaling Technologies) and total CDC2 (1:200; Santa Cruz Biotechnology). For phospho-histone H3 (pHH3), cells were fixed with methanol, stained with anti-pHH3 specific antibody (Millipore) and bound antibody was stained with Alexa Fluor 488 goat anti-rabbit antibody. Images were acquired with an INCell Analyzer 1000.

Flow Cytometric Analysis

Cells were first treated with DNA-damaging agents for 24 h, followed by addition of MK-1775 for 8 h. Trypsinized cells were stained with propidium iodide with the CycleTEST plus DNA reagent kit (BD Biosciences) and were analyzed in a FACSCalibur (Becton Dickinson) apparatus and with CellQuest Pro (Becton Dickinson) software.

Caspase-3/7 Induction Assay

Cells were seeded in black-walled 96-well plates and treated with gemcitabine for 24 h, then with MK-1775 for a further 24 h. Cellular caspase-3/7 activities were determined with a Caspase-3/7 Glo kit (Promega).

Colony Formation Assay

NCI-H1299 cells were seeded in six-well plates at a density of 150 cells/well. After treatment with DNA-damaging agents and Wee1 inhibitors, cells were cultured for a total of 7 d. Colonies were fixed with methanol and stained with Giemsa stain, modified (Sigma-Aldrich). Colonies with more than 50 cells were scored using an inverted microscope.

Animal Experiments

All animal studies were carried out in accordance with good animal practice as defined by the Institutional Animal Care and Use Committee. Subcutaneous xenograft tumors were formed by injection of the human cancer cell lines in the hind flank of immunodeficient nude rats (F344/NJcl-rnu, CLEA Japan). To facilitate tumor formation, cells were injected in medium containing Matrigel (BD Biosciences), a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm mouse sarcoma. Gemcitabine (Gemzer, Eli Lilly), carboplatin (Paraplatin Injection, Bristol-Myers Squibb), and cisplatin (Randa Inj., Nippon Kayaku) were dissolved or diluted in saline and were dosed i.v. MK-1775 was prepared in a vehicle of 0.5% methylcellulose solution and was dosed p.o. 24 h after dosing DNA-damaging agents. For efficacy studies, tumor volumes were measured with a caliper every 3 d and body weights were determined each weekday. Statistical analysis was done using repeated-measure ANOVA followed by Dunnett's test for relative tumor volume. T/C (%) was calculated as $(\Delta T/\Delta C) \times 100$ if $\Delta T > 0$ or $(\Delta T/T_I) \times 100$ if $\Delta T < 0$. ΔT was the change in mean tumor volume to the initial tumor volume for the treatment group, and ΔC was the change in mean tumor volume to the initial tumor volume for the vehicle control group. T_i was the initial tumor volume of the treatment group.

For all biomarker assays, tumors were isolated 8 h after MK-1775 administration. The CDC2 protein was solubilized by homogenizing cells in a buffer containing 1% NP40 and 0.1% Triton X-100 and was detected by Western blotting with an anti–p-CDC2Y15 specific antibody (Cell Signaling). For pHH3 immunohistochemistry, tumors were fixed in 10% formalin, paraffin embedded, and sectioned. Sections were incubated with rabbit polyclonal anti-pHH3 Ser10 antibody (1:400 dilution; Millipore) followed by incubation with biotinylated goat anti-rabbit IgG (H + L) antibody (1:100 dilution; Chemicon, Millipore) and then with streptavidin/horseradish peroxidase (Dako). Signal was detected by development with peroxidase substrate (diaminobenzidine reagent kit, Dako). Immunostained area was quantified using Image Pro Plus software. Necrotic regions of the tumor were excluded from the analysis. The percentage of area positively immunostained in each tumor was calculated as the percentage of the total field area. For p-CDC2Y15 measurements in skin, tissue was fixed and sectioned as described above for tumor tissue. Skin tissue sections were probed with the same antibody used for Western blots. Detection of captured antibodies was done as with pHH3 immunohistochemistry.

Results

MK-1775 Inhibits Phosphorylation of CDC2 at Tyr15 and Abrogates the G_2 DNA Damage Checkpoint in a Dose-Dependent Manner

A high-throughput screening was done with a small chemical compound library to find potent inhibitors of Wee1 kinase in enzymatic assay. Modification of the initial hit compounds by leveraging the information on structure-activity relationships led to the identification of a potent and selective small-molecule inhibitor of Wee1 kinase, MK-1775 (Fig. 1A), with an IC_{50} value of 5.2 nmol/L in *in vitro* kinase

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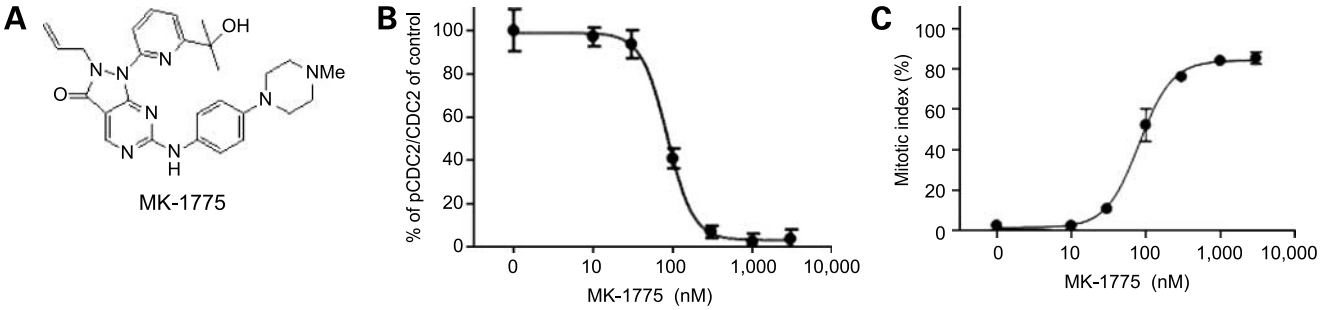

Figure 1. MK-1775 is a potent and selective Wee1 inhibitor that abrogates the G_2 checkpoint and sensitizes cells to DNA-damaging agents *in vitro*. **A,** chemical structure of MK-1775. **B,** MK-1775 inhibits Wee1 activity. DNA damage was induced in WiDr cells by treatment with gemcitabine (100 nmol/L) for 24 h, followed by the addition of MK-1775 for a further 8 h. The amount of p-CDC2Y15 was determined with a colorimetric ELISA. **C,** MK-1775 abrogates the G_2 checkpoint induced by gemcitabine. Cells were treated as in **B** and were stained with the anti-pHH3 specific antibody. Total cell numbers in each image were counted following 4′,6-diamidino-2-phenylindole staining, and the percentage of pHH3-positive cells was plotted as the mitotic index.

assays. An increasing linear relationship was observed between the IC_{50} value of MK-1775 and ATP concentration in an enzyme assay, suggesting that MK-1775 inhibited Wee1 kinase in an ATP-competitive manner. MK-1775 is highly selective against other serine/threonine or tyrosine kinases. Among 223 kinases in the Upstate Kinase Profiler panel, only 8 kinases were inhibited by >80% with 1 μmol/L MK-1775. The IC_{50} values determined for these eight kinases indicate that MK-1775 is 10-fold less potent against seven of these kinases relative to Wee1 and 2- to 3-fold less potent against Yes (IC_{50} was 14 nmol/L). MK-1775 shows >100-fold selectivity over human Myt 1, another kinase that suppresses CDC2 by a phosphorylation at an alternative site (Thr14; ref. 17).

Cellular activity of the Wee1 inhibitor was determined in two different cell-based assays in WiDr, a human colorectal cancer cell line with mutated p53. MK-1775 inhibited phosphorylation of CDC2 at Tyr15 with an EC_{50} value of 85 nmol/L in cells pretreated with gemcitabine (Fig. 1B). Abrogation of the gemcitabine-induced cell cycle arrest by MK-1775 was determined by the induction of pHH3, which reflects premature mitotic entry. MK-1775 treatment induced pHH3 in a dose-dependent manner with an EC_{50} value of 81 nmol/L (Fig. 1C). Similar results were obtained in the same cell line in combination with platinum agents; EC_{50} values of p-CDC2Y15 inhibition and mitotic entry (induction of pHH3) were 180 and 163 nmol/L in carboplatin-treated and 159 and 160 nmol/L in cisplatin-treated cells, respectively. These results suggest that MK-1775 inhibited Wee1 activity and abrogated the DNA damage checkpoint in cells in combination with chemotherapy. These effects were repeated in additional human tumor cell lines with inactive p53 (H1299 and TOV21G, constitutively expressing short hairpin RNA for p53 and MIA-PaCa-2), suggesting that MK-1775 abrogates the DNA damage checkpoint generally in p53-deficient cells.

MK-1775 inhibited p-CDC2 with and without chemotherapy. The EC_{50} without chemotherapy was 49 nmol/L in WiDr cells.

Premature Mitotic Entry Caused by MK-1775 Induces Cell Death in WiDr Cells and Acts Synergistically with Gemcitabine

To show sensitization of tumor cells to chemotherapy by MK-1775, human tumor cells were treated with gemcitabine and MK-1775 in a sequential manner. In WiDr cells, the IC_{50} value of gemcitabine alone in cell viability assay was >100.0 nmol/L (Fig. 2A). In contrast, MK-1775 cotreatment remarkably enhanced the antigrowth effect of gemcitabine. The cotreatment with 30 and 100 nmol/L of MK-1775 reduced the IC_{50} to 21.5 and 7.1 nmol/L, respectively. Similar potentiation of gemcitabine was observed in another p53-deficient lung cancer cell line, H1299 (Fig. 2A). Flow cytometric analysis showed that treatment with gemcitabine (100 nmol/L) or MK-1775 (300 nmol/L) induced only a minimal sub-G_1 population (2.9% and 5.2%, respectively), whereas combination treatment drastically induced sub-G_1 population in a MK-1775 dose–dependent manner (55% at 100 nmol/L and 59% at 300 nmol/L of MK-1775; Fig. 2B and C). Consistently, caspase-3/7 activation was induced only by combination treatment (Fig. 2D). Cell death was induced and reached the plateau at 100 nmol/L of MK-1775, which was close to the EC_{50} of MK-1775 to inhibit p-CDC2Y15 in the same cell line. Thus, treatment of cells with MK-1775 at higher EC_{50} values might be important for the enhancement of chemotherapy. Similar results were obtained in other p53-negative cell lines (H1299, TOV21G-shp53, and MIA-PaCa-2), which is consistent with p-CDC2Y15 inhibition and escape from the DNA damage–dependent checkpoint (data not shown).

The single-agent effects of MK-1775 on WiDr and H1299 cells were moderate. No significant antiproliferative effect was observed at 30 to 100 nmol/L (Fig. 2A; see viability percent at 0 nmol/L gemcitabine). At 300 nmol/L, at which concentration sufficient inhibition of Wee1 (>80%) was observed in cells when examined for p-CDC2 phosphorylation level (Fig. 1B and C), antiproliferative effects were 34.1% in WiDr and 28.4% in H1299 cells.

MK-1775 Selectively Sensitizes p53-Deficient Tumor Cells to Various Antitumor Agents

To show selective enhancement in p53-deficient cells, we used an isogenic matched pair of TOV21G cell lines. TOV21G is a human ovarian adenocarcinoma cell line that has wild-type p53 function. We used TOV21G that constitutively expresses a short hairpin RNA that significantly

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reduces p53 mRNA and p53 function (TOV21G-shp53; ref. 16). Silencing of p53 mRNA was confirmed by PCR, and functional loss of p53 was confirmed by exposing cells to DNA-damaging agent doxorubicin and monitoring cell cycle progression. In addition, down-regulation of p53 target gene, such as *CDKNA1* (p21), was reported (16). In our experiments, we confirmed that cisplatin treatment arrested TOV21G-vec cells at both the G_1 and G_2 phases, whereas it arrested TOV21G-shp53 cells mainly at the late S and G_2 phases (Fig. 3A). MK-1775 inhibited CDC2Y15 phosphorylation in both cell lines (Fig. 3B), but G_2 checkpoint escape determined by pHH3 was much efficient in p53-negative cells (Fig. 3C). Consistent with this result, MK-1775 cotreatment dramatically sensitized TOV21G-shp53 cells to gemcitabine, carboplatin, or cisplatin. The sensitization of TOV21G-vec cells to these agents was only marginal (Fig. 3D). These results support our hypotheses that p53 silencing compromises the G_1 checkpoint in TOV21G cells, that p53-deficient cells rely solely on G_2 checkpoint after DNA damage, and that G_2 checkpoint abrogation by Wee1 inhibition sensitizes p53-deficient cells to DNA-damaging agents.

Identification of Optimal Dosing Regimen for Wee1 Inhibitor and DNA-Damaging Agent in Combination

Optimization of treatment schedule is important for combination therapy. We explored this by using W1, which was obtained at early stage during development of MK-1775. The chemical name of W1 is 6-{[3-(hydroxymethyl)-4-(4-methylpiperazin-1-yl)phenyl]amino}-2-(prop-2-en-1-yl)-1-(thiophen-3-yl)-1,2-dihydro-3*H*-pyrazolo[3,4-*d*]

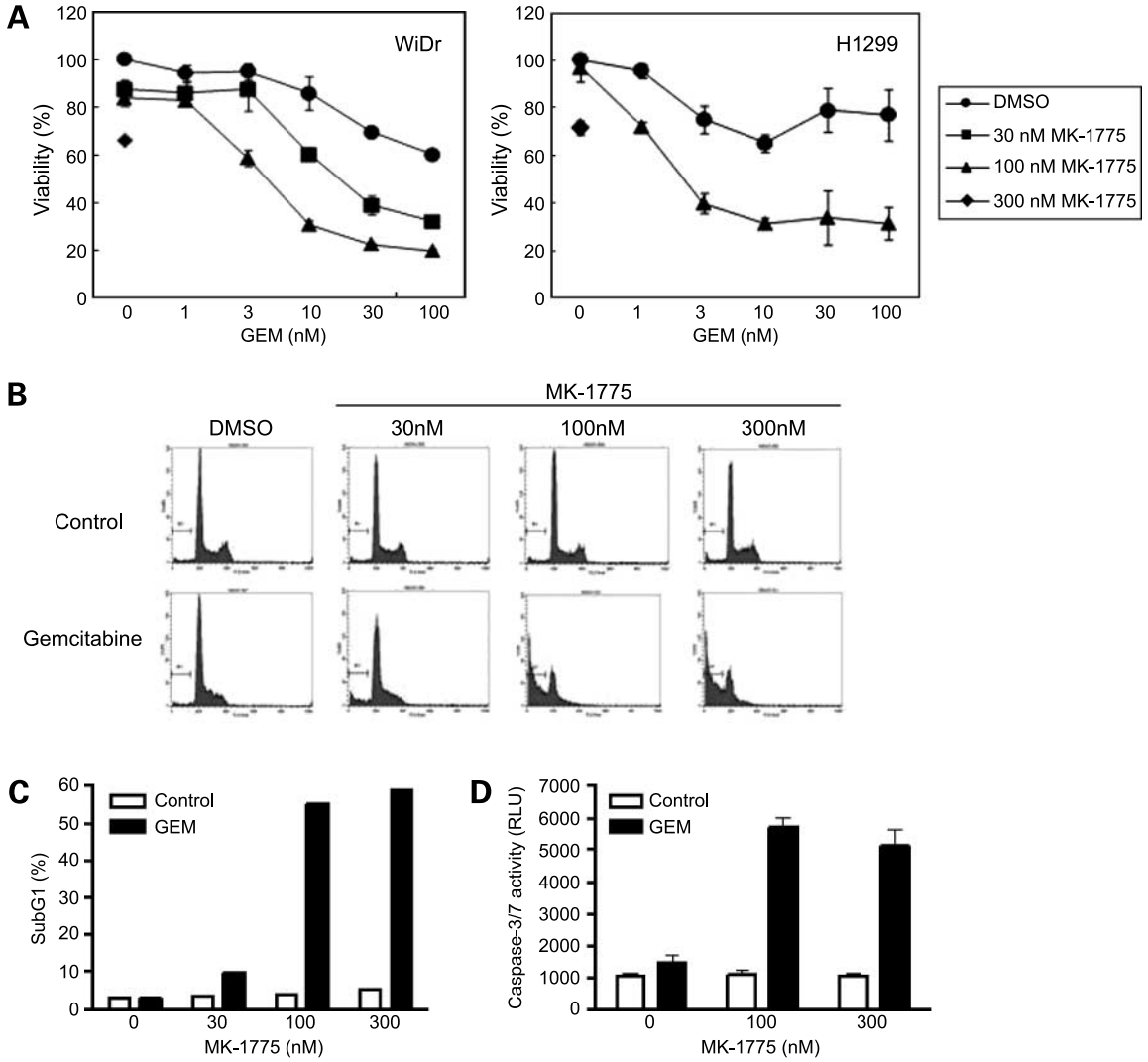

Figure 2. **A** and **B,** dose-response curve of gemcitabine in cell viability assay with or without MK-1775. Cells were treated with gemcitabine for 24 h and then with MK-1775. Cell viability by MK-1775 alone at 300 nmol/L was also included. **B** and **C,** cell death assay analyzed by fluorescence-activated cell sorting (FACS). Cells were treated as in Fig. 1B, and the DNA content of the cells was analyzed by FACS (**B**). Percentage of sub-G_1 was determined and shown as bar graph (**C**). **D,** cell death assay analyzed by caspase-3/7 activation. Cells were treated as in Fig. 1B, except that MK-1775 treatment was for 24 h. Cell death was induced only in the presence of gemcitabine and MK-1775.

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pyrimidine-3-one (the chemical structure is given in Supplementary Fig. S1). W1 inhibited human Wee1 enzyme with an IC_{50} of 8.7 nmol/L. In the cell death assay, a 24-hour treatment with gemcitabine or with W1 alone induced only minimum sub-G_1 fraction. A stepwise treatment (gemcitabine first then W1) greatly enhanced cell death induction compared with gemcitabine treatment alone (45% compared with 12%, respectively; Fig. 4A). However, simultaneous or reverse sequential treatment (W1 first then gemcitabine) induced only moderate cell death (19% and 6%, respectively). These results suggest that sequential treatment with the DNA-damaging agent followed by Wee1 inhibitor is the optimum schedule to induce the maximum cell death–enhancing effect of the Wee1 inhibitor.

We next explored the effect of W1 treatment over time in a colony formation assay (Fig. 4B). An 8-hour treatment with W1 in combination with 3 nmol/L gemcitabine enhanced the suppression of colony formation by gemcitabine (47% inhibition compared with 2% inhibition, respectively), and this enhancement was consistent up to 144 hours. Similarly, almost all colonies disappeared following the combination treatment of W1 and 10 nmol/L gemcitabine at all treatment time points, indicating that a short treatment period of ~8 hours might be sufficient for induction of optimal sensitization by the Wee1 inhibitor. Although detailed dosing optimization experiments were done with W1, we confirmed that MK-1775 requires the stepwise treatment to obtain substantial chemosensitizing effect by Wee1 inhibition.

MK-1775 Potentiates the Antitumor Efficacies by Gemcitabine, Carboplatin, or Cisplatin at Tolerated Doses *In vivo*

To evaluate the effects of Wee1 inhibitor *in vivo*, gemcitabine was administered to nude rats bearing WiDr (human colorectal) tumors at a dose of 50 mg/kg (i.v., bolus). Twenty-four hours later, MK-1775 was p.o. administered at a dose of

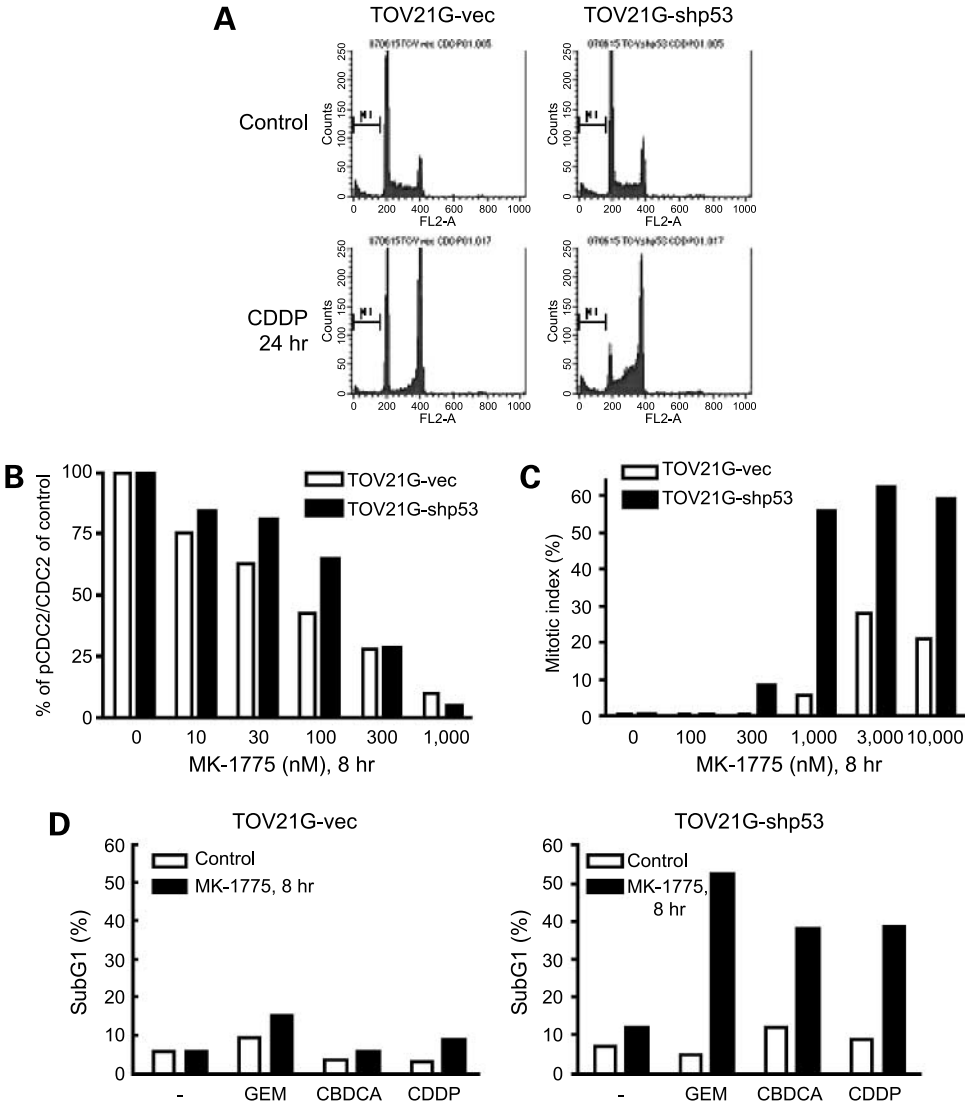

Figure 3. **A,** the G_1 checkpoint was compromised in shp53 cells treated with cisplatin (*CDDP*). The DNA content of control and shp53 cells was analyzed by FACS after treatment with 2 μmol/L cisplatin. **B** and **C,** p-CDC2Y15 inhibition (**B**) and G_2 checkpoint escape (**C**) by MK-1775 in TOV21G p53 matched-pair cells. Both cells were treated as in Fig. 1B. The level of p-CDC2Y15 was determined by Western blot and quantified by densitometric scanning. **D,** cell death induction by MK-1775 in combination with three different DNA-damaging agents was determined by FACS. The concentrations of MK-1775, gemcitabine, carboplatin, and cisplatin were 300 nmol/L, 30 nmol/L, 30 μmol/L, and 2 μmol/L, respectively.

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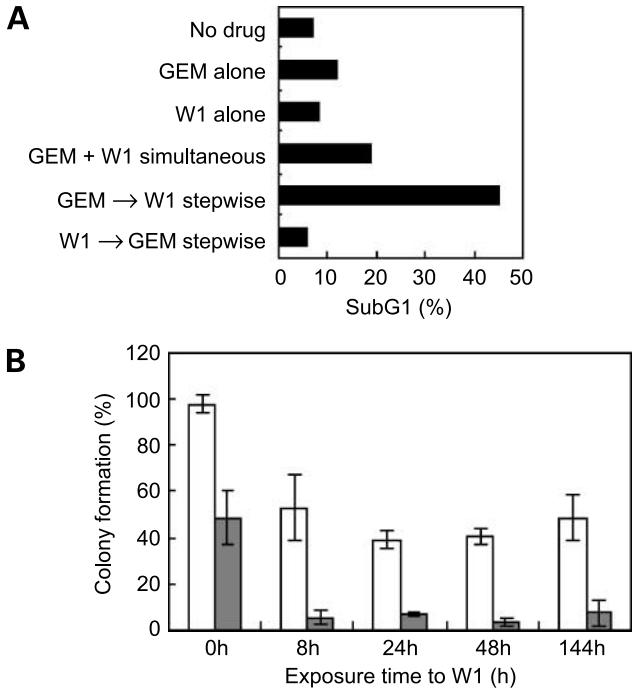

Figure 4. Optimization of the combination treatment schedule *in vitro*. **A,** sequential treatment greatly enhanced cell death induction by gemcitabine. TOV21G-shp53 cells were treated with 30 nmol/L gemcitabine (24 h) and/or 300 nmol/L of the compound W1 (24 h) in a stepwise or a simultaneous treatment schedule. Cell death induction was assessed by sub-G_1 FACS analysis. **B,** time course of W1 treatment in the colony formation assay. H1299 cells were treated with 3 nmol/L (*white columns*) or 10 nmol/L (*gray columns*) of gemcitabine (24 h), followed by treatment with W1 at 300 nmol/L for the indicated time periods. The colony formation percentage was determined by calculation of the proportion of colonies in gemcitabine + W1–treated cells relative to that in untreated cells.

5, 10, or 20 mg/kg. Gemcitabine alone only moderately inhibited tumor growth (T/C = 35% at day 3; $P < 0.05$). Cotreatment with MK-1775 significantly enhanced the antitumor effects in a dose-dependent manner (T/C of 1% and −25% at doses of 10 and 20 mg/kg, respectively; $P < 0.05$; Fig. 5A) and was well tolerated. Cotreatment did not significantly increase toxicity as measured by body weight (>15%; Supplementary Fig. S2), WBC levels, and platelet counts. In contrast, antitumor effects following MK-1775 monotherapy were minimal (T/C = 69% at day 3).

In vivo enhancements of the antitumor effects of carboplatin and cisplatin by MK-1775 were tested in the nude rat HeLa-luc (cervical cancer; Fig. 5B) and TOV21G-shp53 (ovarian cancer; Fig. 5C) xenograft models, respectively. HeLa cells are p53 deficient because the cells express papilloma viral E6 oncoprotein. *In vitro* cell death assay using HeLa cells confirmed that MK-1775 enhanced cell death induction by carboplatin (data not shown). MK-1775 significantly enhanced the antitumor effects of these agents under tolerated doses ($P < 0.05$). Antitumor efficacy by MK-1775 alone in these models was also moderate.

We then tested whether cotreatment of MK-1775 could reduce the dose of chemotherapy required to achieve antitumor effects (Fig. 5D). Gemcitabine was administered at a dose of 2.5, 5 or 10 mg/kg in a once-a-week for 3 weeks schedule. When MK-1775 was cotreated with 5 mg/kg gemcitabine, it enhanced the efficacy by gemcitabine alone. This efficacy of the combined treatment greatly exceeded that by gemcitabine alone at a higher dose, 10 mg/kg ($P < 0.05$), which was the maximum tolerated dose of gemcitabine in this model. This result suggests that cotreatment with MK-1775 could reduce the dose of chemotherapy required to achieve a similar or better antitumor efficacy in preclinical models.

Inhibition of CDC2 Phosphorylation and Induction of pHH3 Phosphorylation Correlate with Antitumor Efficacy *In vivo*

To investigate whether pharmacodynamic changes were accompanied with the enhancement of antitumor efficacy by MK-1775, p-CDC2Y15 and pHH3 were evaluated in the nude rat WiDr xenograft model. MK-1775 was administrated 24 hours after chemotherapy, and WiDr tumors were analyzed 8 hours after MK-1775 administration. MK-1775 inhibited p-CDC2Y15 and induced pHH3 in WiDr xenograft tumor in a dose-dependent manner (Fig. 6A and B). MK-1775 inhibited p-CDC2Y15 (77%) in tumor at a dose of 20 mg/kg and induced pHH3 (~8-fold), and caused tumor regression in combination with gemcitabine. Similarly, a correlation between pharmacodynamic marker change and enhanced antitumor effects was observed in the combination with carboplatin (Fig. 6D). MK-1775 inhibited p-CDC2Y15 (42%) in tumor at a dose of 20 mg/kg and enhanced the antitumor efficacy by carboplatin. These results support that MK-1775 inhibits Wee1 activity and abrogates the G_2 checkpoint, leading to chemosensitized antitumor efficacy, and indicate that inhibition of p-CDC2Y15 and phosphorylation of histone H3 can predict the enhancement of antitumor effect by MK-1775.

We next examined p-CDC2Y15 in skin hair follicles, which include proliferating cells. Phosphorylation of CDC2 at Tyr15 was undetectable following administration of MK-1775 (Fig. 6C). Thus, p-CDC2Y15 inhibition in skin hair follicle is a promising surrogate marker for pharmacodynamic effects in tumor tissue and antitumor effects of MK-1775 treatment.

Discussion

MK-1775 is the first reported Wee1 inhibitor compound with high potency, selectivity, and oral bioavailability in preclinical animal models. It selectively enhanced cytotoxic activities of gemcitabine, carboplatin, and cisplatin in p53-inactivated human tumor cell lines *in vitro* and *in vivo*. These agents are frequently used to treat cancer patients (18–20). Our data suggest that adding MK-1775 to these standard treatments may provide therapeutic benefits to patients with tumors that are deficient for p53 function. It may increase responses to these agents or achieve similar antitumor effects with reduced adverse events. This compound provides a clinical opportunity to test a Wee1 inhibitor as a context-specific sensitizer of DNA-damaging agents.

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Currently, MK-1775 is under phase I clinical trial in combination with gemcitabine, cisplatin, and carboplatin in patients with advanced solid tumors.

Our results showed that MK-1775 possesses preferential killing effect in p53-deficient tumors by using p53 matched-pair cell lines. The selective antitumor effect of MK-1775 on p53-deficient cells was shown in combination with DNA-damaging agents with different modes of action, gemcitabine and platinum compounds. The p53 context specificity of Wee1 inhibition was carefully investigated by another modality, Wee1 siRNA, in the additional comparative study with H1299 (p53-deficient) cancer cells and human normal renal epithelial cells (p53 wild-type) as shown in Supplementary Fig. S3. These studies also confirmed that Wee1 silencing is effective only in cells with dysfunctional p53.

Wee1 inhibition alone did not kill human tumor cells *in vitro*, and antitumor efficacy following MK-1775 monotherapy was limited in animal xenograft models. This is contrast to previous results with another Wee1 inhibitor, PD0166285 (21). One possible explanation would be selectivity of MK-1775 against Myt1, another kinase that phosphorylates and inactivates CDC2 to prevent the premature entry of mitosis. Thus, MK-1775 was not effective in monotherapy and should be used only in combination with DNA-damaging agents that induce the G_2 cell cycle checkpoint. In contrast to DNA-damaging agents, MK-1775 did not enhance the cytotoxic effects of docetaxel or paclitaxel *in vitro* (Supplementary Fig. S4). This is reasonable because these agents target microtubules and do not induce the G_2 checkpoint. Importantly, MK-1775 enhanced cell death by platinum compounds even in the presence of

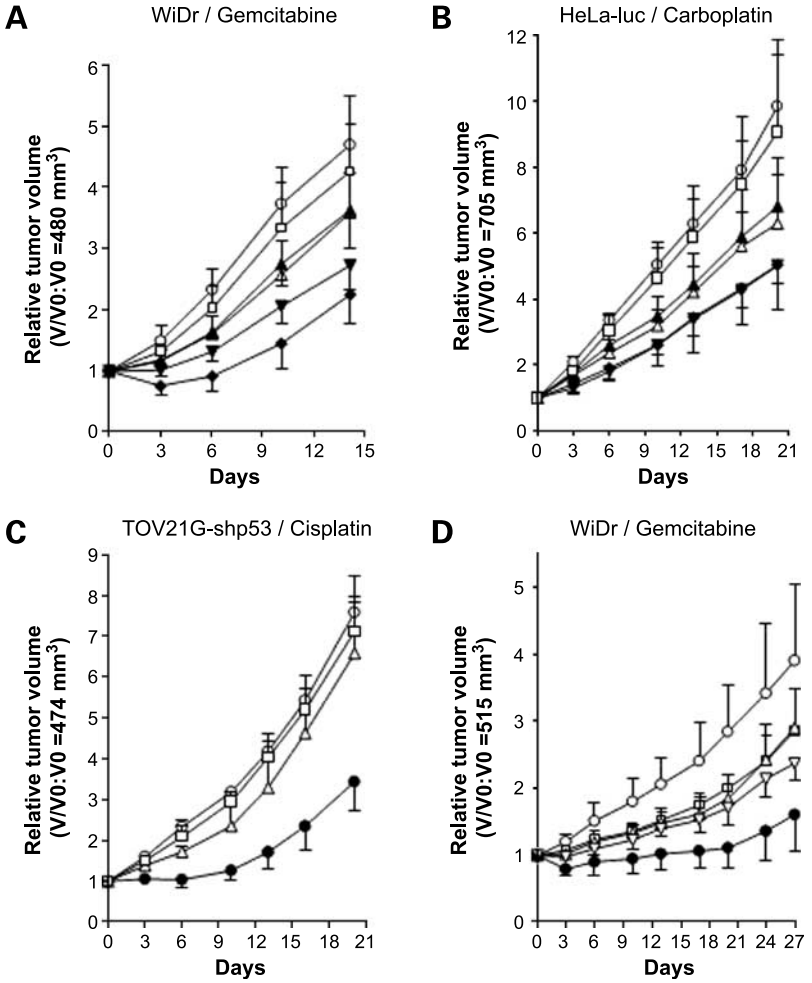

Figure 5. MK-1775 potentiated the antitumor efficacies of gemcitabine, carboplatin, or cisplatin *in vivo*. $n = 5$ per arm. *Points,* mean tumor volumes; *bars,* SE. **A,** nude rats bearing WiDr tumors were dosed with vehicle (○), gemcitabine (50 mg/kg, i.v.; △), MK-1775 (20 mg/kg, p.o.; □), or gemcitabine plus MK-1775. In the combination arms, MK-1775 was dosed after 24-h gemcitabine treatment at 5 mg/kg (▲), 10 mg/kg (▼), or 20 mg/kg (♦). **B,** nude rats bearing HeLa-luc tumors were dosed with vehicle (○), carboplatin (50 mg/kg, i.v.; △), MK-1775 (30 mg/kg, p.o.; □), or carboplatin plus MK-1775 at 10 mg/kg (▲), 20 mg/kg (♦), and 30 mg/kg (▼). **C,** nude rats bearing TOV21G-shp53 tumors were dosed with vehicle (○), cisplatin (1 mg/kg, i.v.; △), MK-1775 (30 mg/kg, p.o.; □), or cisplatin plus MK-1775 at 30 mg/kg (●). **D,** nude rats bearing WiDr tumors were dosed with vehicle (○), with gemcitabine once a week for 3 wk at 2.5 mg/kg (i.v.; □), 5 mg/kg (i.v.; △), or 10 mg/kg (i.v.; ▽), or with gemcitabine (5 mg/kg, i.v.) plus MK-1775 (10 mg/kg, p.o.; ●).

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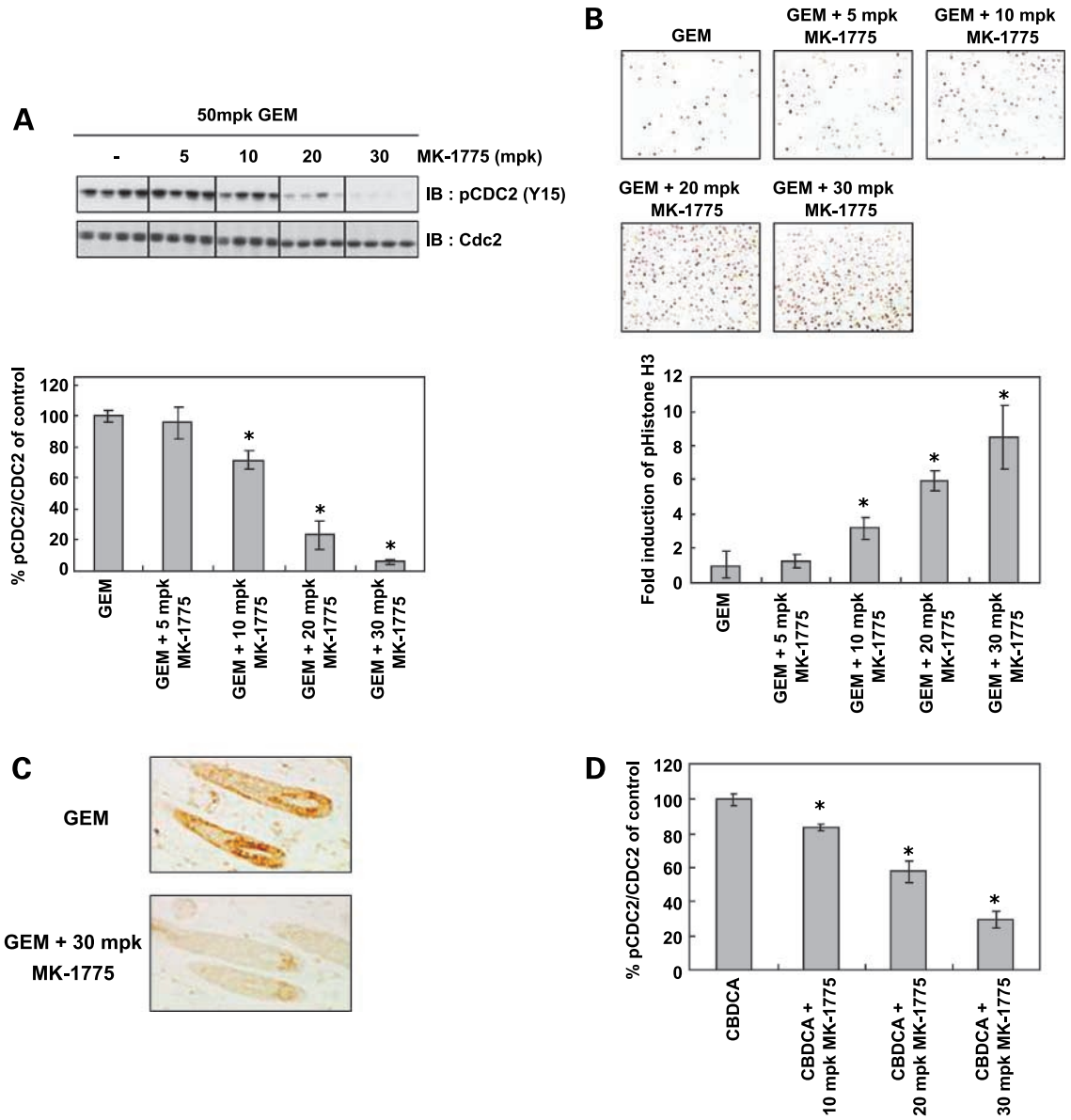

Figure 6. Oral dosing of MK-1775 inhibits p-CDC2Y15 and induces pHH3 in tumors *in vivo*. Nude rats bearing WiDr or HeLa tumors were treated with gemcitabine or carboplatin and/or MK-1775. **A,** p-CDC2Y15 inhibition in the WiDr tumor was detected by Western blot and was quantitated by densitometric scanning. **B,** induction of histone H3 phosphorylation was assayed by immunohistochemistry and quantitated by image analysis. **C,** skin from the same rats was isolated and p-CDC2Y15 in hair follicles was detected by immunohistochemistry. **D,** inhibition of p-CDC2Y15 in HeLa-luc tumors in combination with carboplatin was determined as in **A**.

taxanes *in vitro*. Gemcitabine or platinum agents are often used in combination with taxanes to treat cancer patients. These data suggest that MK-1775 should be beneficial for DNA damages + taxane combination treatment. It would be also interesting to find additional DNA-damaging agents or molecular-targeted drugs that are beneficial with MK-1775. As previously reported with PD0166285, radiotherapy that causes DNA damages is another promising combination partner with MK-1775 (15).

The stepwise treatment, DNA damage first and then Wee1 inhibitor, was most effective in *in vitro* schedule optimization experiments. DNA-damaging agents activate the G_2 checkpoint and accumulate cells in S or G_2 phase of the cell cycle. These arrested cells might be susceptible to a Wee1 inhibitor. We treated cells with Wee1 inhibitor 24 hours after chemotherapy, as it took ~24 hours to activate the DNA damage–dependent G_2 checkpoint after chemotherapy, which was determined by DNA contents and induction of CDC2Y15 phosphorylation in cells (data not shown). Moreover, this stepwise protocol might enable to shorten the MK-1775 exposure period. Indeed, 8-hour treatment with a Wee1 inhibitor was sufficient to enhance the

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cytotoxic effects of DNA-damaging agents. The time course of DNA damage–dependent checkpoint activation following chemotherapy in human subjects may differ from that observed in our *in vitro* and *in vivo* preclinical models (22, 23). A clinical dosing regimen for Wee1 inhibition must be verified in carefully designed clinical trials.

Development of biomarkers to monitor target engagement in tumor tissue and measure subsequent biological effects will be very important during clinical development. This will allow investigators to identify early signs of efficacy, which facilitate to make a go/no go decision during the early phase of clinical trial. We have developed two assays: a p-CDC2Y15 assay for target engagement and a pHH3 assay that monitors M-phase entry due to abrogation of the G_2 checkpoint. *In vitro* and *in vivo* data showed good correlation between reduction of CDC2 phosphorylation on Tyr15 (reduction by at least 50%) and antitumor efficacy. The presence of Tyr15 phosphorylated CDC2 and Ser10 or Ser28 phosphorylated histone H3 has been reported in clinical tumor samples from various tumor types (24–27). Thus, these biomarkers may be useful in a clinical setting. Moreover, we found p-CDC2Y15 in hair bulb in skin, and it was inhibited by MK-1775 with good correlation to the inhibition observed in tumor tissue. Such biomarkers in surrogate tissues will be very important, given that accesses to tumor biopsies in patients are limited in some types of tumors. Recently, we identified genes that were modified by treatment with gemcitabine and MK-1775 commonly in tumor and skin (28). This Wee1 inhibitor regulatory gene set is available for additional pharmacodynamic biomarkers in both tumors and surrogate tissues. In addition, a biomarker that reflects p53 deficiency in tumor is important as a predictive biomarker for Wee1 inhibitor.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Small-molecule inhibition of Wee1 kinase by MK-1775 selectively sensitizes p53-deficient tumor cells to DNA-damaging agents

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