Sensitization of Pancreatic Cancers to Gemcitabine Chemoradiation by WEE1 Kinase Inhibition Depends on Homologous Recombination Repair[1,2]

Tasneem Kausar[*], Jason S. Schreiber[*], David Karnak[*], Leslie A. Parsels[†], Joshua D. Parsels[†], Mary A. Davis[*], Lili Zhao[‡], Jonathan Maybaum[†], Theodore S. Lawrence[*] and Meredith A. Morgan[*]

[*]Department of Radiation Oncology, University of Michigan Medical School, Ann Arbor, MI 48109; [†]Department of Pharmacology, University of Michigan Medical School, Ann Arbor, MI 48109; [‡]Biostatistics Unit, University of Michigan Comprehensive Cancer Center, Ann Arbor, MI 48109

Abstract

To improve the efficacy of chemoradiation therapy for locally advanced pancreatic cancer and begin to establish patient selection criteria, we investigated the combination of the WEE1 inhibitor AZD1775 with gemcitabine-radiation in homologous recombination (HR) repair proficient and deficient pancreatic cancers. Sensitization to gemcitabine-radiation by AZD1775 was assessed in pancreatic cancer cells by clonogenic survival and in patient-derived xenografts by tumor growth. The contributions of HR repair inhibition and G2 checkpoint abrogation to sensitization were assessed by γH2AX, BRCA2 manipulation, and RAD51 focus formation and pHistone H3 flow cytometry, respectively. We found that AZD1775 sensitized to gemcitabine-radiation in BRCA2 wild-type but not BRCA2 mutant pancreatic cancer cells. In all cells, AZD1775 caused inhibition of CDK1 phosphorylation and G2 checkpoint abrogation. However, sensitization by AZD1775 was associated with persistent γH2AX and inhibition of RAD51 focus formation. In HR-proficient (BRCA2 wild-type) or -deficient (BRAC2 null) isogenic cells, AZD1775 sensitized to gemcitabine-radiation in BRCA2 wild-type, but not in BRCA2 null cells, despite significant G2 checkpoint abrogation. In patient-derived pancreatic tumor xenografts, AZD1775 significantly inhibited tumor growth and impaired RAD51 focus formation in response to gemcitabine-radiation. In conclusion, WEE1 inhibition by AZD1775 is an effective strategy for sensitizing pancreatic cancers to gemcitabine chemoradiation. Although this sensitization is accompanied by inhibition of CDK1 phosphorylation and G2 checkpoint abrogation, this mechanism is not sufficient for sensitization. Our findings demonstrate that sensitization to chemoradiation by WEE1 inhibition results from inhibition of HR repair and suggest that patient tumors without underlying HR defects would benefit most from this therapy.

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Introduction

Radiation in combination with concurrent gemcitabine or 5-fluorouracil is the standard of care for locally advanced pancreatic cancer. In combination with gemcitabine, radiation significantly improves survival compared with gemcitabine treatment alone [1]. Furthermore, recent studies from our group and others suggest that intensification of highly conformal radiation may extend survival in locally advanced patients beyond the approximate 1-year survival associated with standard chemoradiation therapies [2,3]. Although recent advancements in systemic therapy for pancreatic cancer, such as FOLFIRINOX and nab-paclitaxel with gemcitabine, have improved survival in metastatic pancreatic cancer [4,5], these therapies have not yet been effectively

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combined with radiation for locally advanced pancreatic cancer. Because approximately 30% of pancreatic cancer patients die from local disease progression [6] and radiation is the only therapy which provides local tumor control in surgically unresectable patients, strategies for improving both local and systemic disease therapy are critical for improving overall survival in pancreatic cancer patients.

One promising strategy for improving both local and systemic disease control is by selective exploitation of DNA damage response pathways in cancer cells [7]. In response to DNA damage, cells activate both cell cycle checkpoint and DNA repair pathways which function cooperatively to prevent the propagation of cells with damaged DNA [8]. The WEE1 kinase is an important regulator of the cellular response to DNA damage, as it catalyzes phosphorylation of CDK1, resulting in CDK1 inhibition and arrest of cells in the G2 phase of the cell cycle [9]. WEE1 also negatively regulates CDK1-dependent replication origin firing and thus acts to prevent aberrant origin firing leading to nucleotide pool depletion and replication stress [10]. In addition, we and others have shown that WEE1 regulates homologous recombination (HR) repair, although the mechanisms have not been fully elucidated [11,12].

Given the critical role of WEE1 in regulating the cellular response to DNA damage, WEE1 is a promising target for cancer therapy [13]. The small-molecule WEE1 inhibitor AZD1775 is a first-in-class agent that has shown anticancer activity both alone and in combination with radiation and chemotherapy in preclinical studies [14–17]. Many clinical trials (phase 1 and 2) are under way combining AZD1775 with radiation, chemotherapy, and chemoradiation for several disease sites including cancers of the head and neck, lung, ovary, cervix, and pancreas as well as diffuse intrinsic pontine glioma and glioblastoma. As a single agent, AZD1775 exhibits cytotoxicity across various cancer cell types with mechanisms that have been attributed to regulation of both initiation and progression of DNA replication, DNA damage during DNA replication, as well as unscheduled mitotic entry [10,14,18,19]. AZD1775 is also efficacious in combination with radiation [15,20–22] or chemotherapies such as gemcitabine, 5-fluorouracil, cytarabine, temozolomide, and cisplatin [16,23–27]. The vast majority of these studies demonstrating sensitization to radiation or chemotherapy by AZD1775 have implicated abrogation of the G2 checkpoint and/or unscheduled mitotic entry as the mechanism. Recently, however, it was suggested that regulation of DNA double-strand break (DSB) repair by WEE1 inhibition may be a mechanism of radiosensitization [21]. Consistent with this, our prior study indicated that inhibition of HR repair by AZD1775 is also a possible mechanism for radiosensitization [11].

To determine the efficacy of WEE1 inhibitors in combination with chemoradiation in pancreatic cancer and to begin to identify mechanisms that might inform patient selection, in this study, we investigated AZD1775 as a sensitizer to gemcitabine-based chemoradiation in HR-proficient (BRCA2 wild-type) and HR-deficient (BRCA2 mutant) pancreatic cancers. When we found that sensitization to chemoradiation by AZD1775 occurred in BRCA2 wild-type (MiaPaCa-2, Panc-1) but not BRCA2 mutant (Capan-1) pancreatic cancer cells, we went on to hypothesize that inhibition of HR repair by WEE1 inhibition is a critical mechanism of sensitization by AZD1775. To test this hypothesis, we assessed sensitization to chemoradiation by AZD1775 in BRCA2 isogenic cell lines. In addition, we tested the ability of AZD1775 to sensitize and inhibit HR repair *in vivo* in patient-derived pancreatic tumor xenografts. The findings of this study provide a preclinical rationale for the

development of AZD1775 as a sensitizer to gemcitabine-radiation in selected HR-proficient locally advanced pancreatic cancers.

Materials and Methods

Cell Culture and Drug Solutions

MiaPaCa-2 and Panc-1 cells were obtained from and authenticated (via short tandem repeat profiling) by the American Type Culture Collection (2009 and 2006, respectively). Cells were cryopreserved within 6 months of authentication. Capan-1.NEO is a clonal cell line expressing the neomycin resistance gene obtained from S. Powell (Memorial Sloan Kettering Cancer Center, New York, NY) [28]. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (MiaPaCa-2), RPMI 1640 (Panc-1), or Iscove's modified Dulbecco's medium (Capan-1) supplemented with 10% fetal bovine serum (Life Technologies), 2 mM L-Glutamine (Sigma), penicillin, and streptomycin (Sigma). Isogenic DLD1 cells were obtained from Horizon Discovery (2014) and grown in DMEM or McCoy's as described above except that DLD1 BRCA2 null cells were maintained under selection in 0.1 mg/ml of hygromycin (Invitrogen). For *in vitro* experiments, AZD1775 (AstraZeneca) was dissolved in dimethyl sulfoxide (Sigma) and stored in aliquots at – 20°C. For *in vivo* experiments, AZD1775 was suspended in 0.5% methylcellulose (Sigma) and stored for a maximum of 5 days at room temperature with constant stirring. Gemcitabine (Eli Lily) was dissolved in either PBS or saline for *in vitro* or *in vivo* use, respectively.

Clonogenic Survival Assays

Cells treated with drugs or radiation were processed for clonogenic survival as previously described [29,30]. Radiation survival curves were normalized for drug toxicity, and the radiation enhancement ratio was calculated as the ratio of the mean inactivation dose (area under the cell survival curve) under control conditions divided by the mean inactivation dose after drug exposure [31]. A value significantly greater than 1 indicates radiosensitization. Cytotoxicity in the absence of radiation treatment was calculated by normalizing the plating efficiencies of drug-treated cells to non–drug-treated cells.

Flow Cytometry

Cells were trypsinized, washed with ice-cold PBS, and fixed at a concentration of 2×10^6 cells/ml in ice-cold 70% ethanol. For pHistone H3 analysis, cells were incubated with a rabbit anti-pHistone H3 (S10) antibody (Millipore) as previously described [32]. For γH2AX analysis, samples were incubated with a mouse anti–γH2AX-specific antibody (clone JBW301; Millipore) overnight at 4°C followed by incubation with a fluorescein isothiocyanate–conjugated secondary antibody (Sigma) as previously described [33]. For quantification of γH2AX positivity, a gate was arbitrarily set on the control, untreated sample to define a region of positive staining for γH2AX of approximately 5%. This gate was then overlaid on the treated samples. Samples for both analyses were stained with propidium iodide to measure total DNA content and analyzed on a FACScan flow cytometer (Becton Dickinson) with FlowJo software (Tree Star).

Immunoblotting

Whole cell lysates were prepared in cold SDS lysis buffer (10 mM Tris pH 7.4, 2% SDS) supplemented with PhosSTOP phosphatase inhibitor and Complete protease inhibitor tablets (Roche) as previously described [29]. The following antibodies were used: CDK1, pCDK1 (Y15), CHK1, pCHK1 (S345), GAPDH (Cell Signaling Technology),

and RAD51 (SantaCruz). Immunoblots were quantitated using ImageJ software (NIH).

Immunofluorescence

For *in vitro* immunofluorescence experiments, cells were grown and treated on cover slips in 12-well dishes. Following treatment, cells were fixed and stained as previously described [34] with a mouse monoclonal RAD51 antibody (GeneTex) and 4′,6-diamidino-2-phenylindole (DAPI). For *in vivo* immunofluorescence experiments, RAD51 foci in tumor xenografts were assessed based on a previously published protocol [35] with the following modifications. Cryosections were fixed with 4% para-formaldehyde in PBS for 5 minutes at room temperature. After permeabilization in ice-cold methanol for 5 minutes, slides were washed with PBS and blocked with 5% goat serum for 1 hour at room temp. Samples were probed with anti-RAD51 primary antibody (1:300, Calbiochem) in 3% goat serum/PBS for 1 hour in a humidified chamber. Slides were washed with PBS and stained with Alexafluor-488 secondary antibody (1:1000 in 3% goat serum/PBS; EMD Millipore) for 1 hour. After washing with PBS, samples were stained with 1 mg/ml of DAPI and mounted with Prolong Gold antifade mounting medium. Samples were imaged with an Olympus IX71 FluoView confocal microscope (Olympus America) with a 60× oil objective. Fields were chosen at random based on DAPI staining. For quantitation of RAD51 foci, at least 100 cells from each of three independent experiments were visually scored for each condition. Cells with five or more RAD51 foci were scored as positive.

Irradiation

Irradiations were performed using a Philips RT250 (Kimtron Medical) at a dose rate of ~2 Gy/min in the University of Michigan Comprehensive Cancer Center Experimental Irradiation Core. Dosimetry was performed using an ionization chamber connected to an electrometer system that is directly traceable to a National Institute of Standards and Technology calibration. For tumor irradiation, animals were anesthetized with isoflurane and positioned such that the apex of each flank tumor was at the center of a 2.4-cm aperture in the secondary collimator, with the rest of the mouse shielded from radiation.

Tumor Growth Studies

Animals were handled in accordance with protocols approved by the University of Michigan Committee for Use and Care of Animals. Patient-derived xenografts were provided by Dr. Diane Simeone (University of Michigan). Single-cell suspensions of patient-derived xenografts were handled as described previously [36]. Five million tumor cells were suspended in a 1:1 mixture of 10% FBS-DMEM/Matrigel (BD Biosciences) and injected subcutaneously, bilaterally into the flanks of 3- to 5-week-old female athymic nude mice (Harlan). Treatment was initiated when the average tumor volume (TV) reached 100 mm^3 and consisted of gemcitabine (60 mg/kg; days 0, 7), AZD1775 (30 mg/kg; QD; 2 h and 24 h postgemcitabine and 1 h pre-radiation; days 0, 1 and 7, 8), and radiation (1.8 Gy/fraction; days 0 to 4 and 7 to 11). Gemcitabine was administered via intraperitoneal injection, and AZD1775 was administered via oral gavage. Tumor size was measured two times per week. TV was calculated according to the following equation: TV = $\pi/6$ (ab^2), where *a* and *b* are the longer and shorter dimensions of the tumor, respectively. Measurements were made until day 50 or until the TV increased by approximately a factor of 5.

Statistics

Statistically significant differences for the clonogenic survival, flow cytometry, and immunofluorescence assays were determined by one-way ANOVA with the Tukey postcomparison test in GraphPad PRISM version 5 (GraphPad software). For tumor growth experiments, the time required for TV doubling was determined for each xenograft by identifying the earliest day on which it was at least twice as large as on the first day of treatment. The Kaplan-Meier method was used to analyze the doubling times. Log-rank test (PROC LIFETEST in SAS) was used to compare the doubling times between any two treatment groups. In addition, the Bayesian hierarchical changepoint model [37] was used to compare tumor regression rates, regression periods, and regrowth rates between any two treatment groups.

Results

To investigate whether inhibition of the WEE1 kinase is an effective strategy for sensitization of pancreatic cancers to chemoradiation, we began by assessing radiation survival in a panel of pancreatic cancer cell lines. MiaPaCa-2, Panc-1, and Capan-1 pancreatic cancer cells were treated with minimally toxic concentrations of gemcitabine and the WEE1 inhibitor AZD1775, followed by radiation (Figure 1A). AZD1775 produced significant sensitization to gemcitabine-radiation as evidenced by radiation enhancement ratios of 2.1 ± 0.2 and 1.5 ± 0.2 in MiaPaCa-2 and Panc-1 cells, respectively (Figure 1, B and C; Suppl. Table 1). In contrast, Capan-1 cells were sensitized to neither radiation nor gemcitabine-radiation by AZD1775, despite being radiosensitized by gemcitabine (Figure 1D). These findings suggest that pancreatic cancers may be differentially sensitized by WEE1 inhibition and provide a rationale for investigation of the mechanisms of sensitization to chemoradiation by WEE1 inhibition.

One possible explanation for differences in the ability of AZD1775 to sensitize different cells to chemoradiation is that AZD1775 does not inhibit WEE1 effectively in all cell lines. As the primary activity of WEE1 is phosphorylation of CDK1, which is associated with inhibition of CDK1 activity and initiation of the G2 checkpoint [9], we analyzed pCDK1 (Y15) protein levels by immunoblotting to verify WEE1 inhibition (Figure 2, A and B, Suppl. Figure 1). Although AZD1775 inhibited WEE1-mediated CDK1 phosphorylation in all three cell lines, we found that higher concentrations of AZD1775 were required to achieve similar inhibition of pCDK1 protein levels in MiaPaCa-2 and Panc-1 relative to Capan-1 cells. This result suggests that Capan-1 cells are not resistant to AZD1775-mediated radiosensitization as a result of persistent WEE1 kinase activity.

As radiosensitization by AZD1775 has previously been attributed to abrogation of the G2 checkpoint [15,20,22], we next evaluated the ability of AZD1775 to inhibit radiation-induced G2 arrest in both MiaPaCa-2 and Capan-1 cells. Flow cytometric analysis of the mitotic marker pHistone H3 confirmed that AZD1775 abrogated the G2 checkpoint not only in cells sensitized to gemcitabine-radiation by WEE1 inhibition (MiaPaCa-2; Figure 2C; Suppl. Figure 2) but also in Capan-1 cells, which were not radiosensitized by AZD1775 (Figure 2D; Suppl. Figure 3). This finding suggests that AZD1775-mediated G2 checkpoint abrogation is not sufficient for sensitization to gemcitabine-radiation.

To begin to determine whether sensitization to chemoradiation by AZD1775 is associated with inhibition of DNA DSB repair, we assessed the resolution of γH2AX over time following radiation. In MiaPaCa-2 cells, treatment with radiation or gemcitabine-radiation caused a robust induction of γH2AX by 2 hours that was resolved to
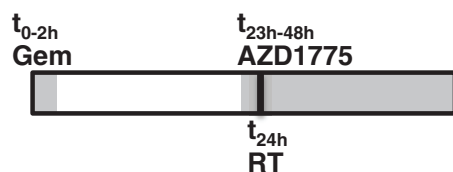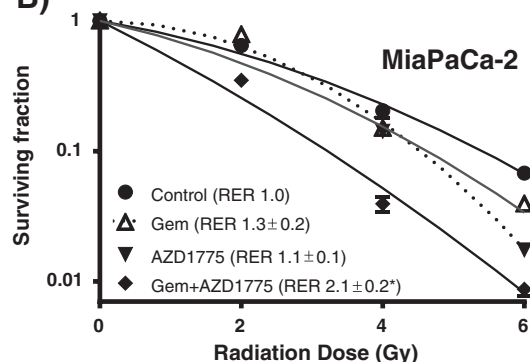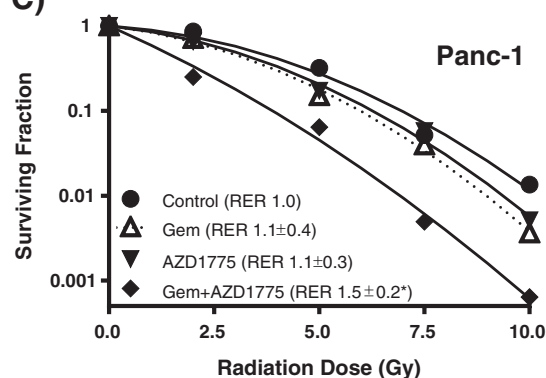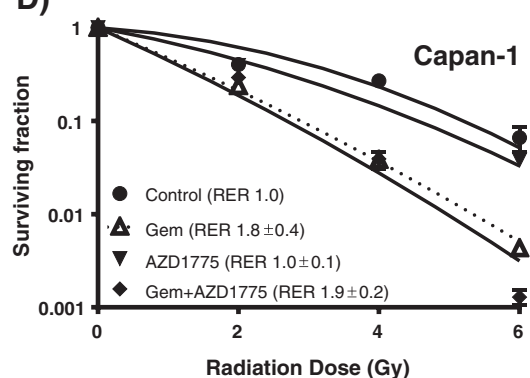
Figure 1. Sensitization to gemcitabine-radiation by AZD1775 in pancreatic cancer cells. Pancreatic cancer cells were treated with radiation (RT) and equitoxic concentrations of gemcitabine (MiaPaCa-2, 100 nM; Panc-1, 50 nM; Capan-1, 50 nM) and/or AZD1775 (MiaPaCa-2 and Panc-1, 200 nM; Capan-1, 50 nM) as illustrated (A). Radiation survival was assessed by clonogenic survival assay 24 hours post-RT in MiaPaCa-2 (B), Panc-1 (C), and Capan-1 (D) cells. Plots are from single representative experiments. Radiation enhancement ratios (RERs; inset) are the mean RER ± standard error of four to five independent experiments with statistical significance indicated versus GemRT* ($P < .05$). Additional cytotoxicity data are shown in Suppl. Table 1.

baseline by 24 hours postradiation (Figure 3A). Treatment with AZD1775 significantly delayed the resolution of γH2AX evidenced by the significantly higher levels of γH2AX 24 hours postradiation relative to either radiation or gemcitabine-radiation, consistent with the presence of unrepaired DNA DSBs (Figure 3A, Suppl. Figure 4). In contrast, Capan-1 cells treated with radiation or gemcitabine-radiation exhibited a smaller initial induction of γH2AX relative to MiaPaCa-2 cells, which persisted through the time course of the experiment and is consistent with the intrinsic repair deficiency in Capan-1 cells (Figure 3B). Importantly, AZD1775 did not further delay the resolution of γH2AX in response to either radiation or gemcitabine-radiation in these cells. The prolonged γH2AX DNA damage response observed in MiaPaCa-2 cells treated with AZD1775 was also accompanied by an AZD1775-induced increase is pCHK1 (S345), another marker for sustained DNA damage that has previously been associated with sensitization by CHK1 inhibition (Figure 2A, Suppl. Figure 1B) [38,39]. In contrast, AZD1775 did not increase pCHK1 (S345) levels in irradiated Capan-1 cells, although AZD1775 alone induced CHK1 phosphorylation (Figure 2B). Taken together, these findings are consistent with the differential effects of AZD1775 on radiation survival in MiaPaCa-2 and Capan-1 cells and suggest that inhibition of DNA DSB repair is an important mechanism.

We and others have previously shown that WEE1 inhibition by AZD1775 also inhibits HR [11,12]. Given the finding that HR-proficient pancreatic cancer cells (MiaPaCa-2 and Panc-1) were

sensitized to chemoradiation by WEE1 kinase inhibition whereas BRCA2 mutant pancreatic cancer cells deficient in HR (Capan-1) were not, we hypothesized that inhibition of HR may contribute to AZD1775-mediated sensitization to chemoradiation. To test this hypothesis, we assessed the ability of AZD1775 to sensitize an isogenic pair of cell lines differing only in their BRCA2 status (wild-type versus null; Suppl. Figure 5) to gemcitabine-radiation. Radiation survival was assessed in BRCA2 wild-type and null DLD1 cells treated with equitoxic concentrations of gemcitabine and AZD1775, as described in Figure 1. We found that BRCA2 wild-type cells were significantly sensitized to gemcitabine-radiation by AZD1775 (Figure 4A, Suppl. Table 2). In contrast, although BRCA2 null cells were intrinsically more radiosensitive than BRCA2 wild-type cells (Figure 4B, Suppl. Table 2), they were not further sensitized to gemcitabine-radiation by AZD1775. The ability of HR inhibition to sensitize to gemcitabine-radiation is further supported by the finding that RAD51 depletion by siRNA also sensitized MiaPaCa-2 cells to gemcitabine-radiation (Suppl. Figure 6).

As data from previous studies suggested that AZD1775-mediated G2 checkpoint abrogation is the primary mechanism of radiosensitization by WEE1 inhibition [15,20,22], we next assessed the ability of AZD1775 to abrogate the G2 checkpoint in either BRCA2 wild-type or null cells treated with gemcitabine-radiation. Consistent with these studies, we found that AZD1775 caused significant G2 checkpoint abrogation in response to gemcitabine-radiation in BRCA2 wild-type
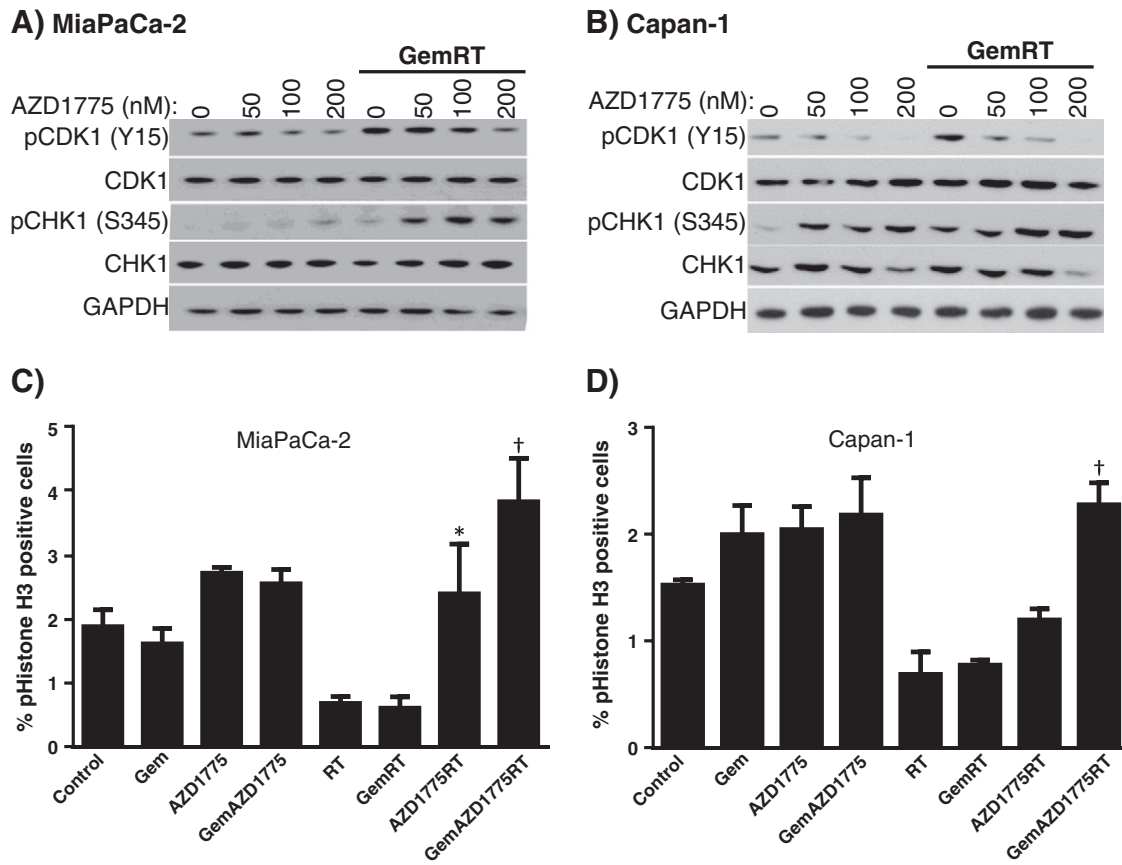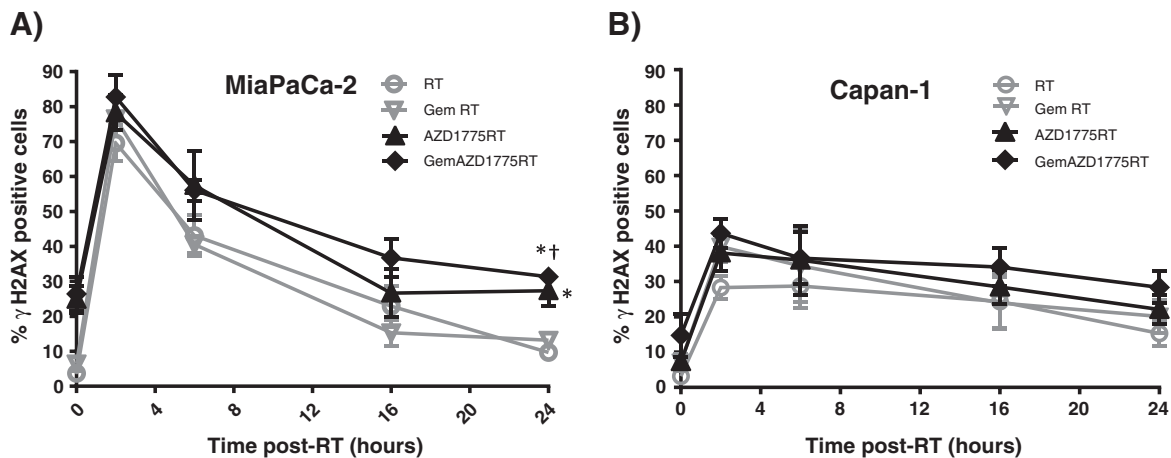
Figure 2. The effects of AZD1775 and gemcitabine-radiation on DNA damage checkpoint signaling and abrogation of the G2 checkpoint. MiaPaCa-2 (A, C) and Capan-1 (B, D) cells were treated with gemcitabine, AZD1775, and radiation as described in Figure 1. Six hours post-RT (6 Gy), cells were harvested for immunoblotting (A, B). Images are from single representative experiments. Quantitation of immunoblots is shown in Suppl. Figure 1. Alternatively, samples were analyzed for pHistone H3 (Ser10) and DNA content by flow cytometry (C, D). Data are the mean percentage ± standard error of pHistone H3–positive mitotic cells from three independent experiments. Statistical significance is indicated versus RT* or GemRT[†] ($P < .05$).

cells (Figure 4C). Interestingly, however, AZD1775 also abrogated the G2 checkpoint in the BRCA2 null cells, which were not sensitized to chemoradiation by AZD1775 (Figure 4D). Thus, although 200 nM AZD1775 was sufficient to inhibit WEE1 in the BRCA2 null DLD1

cells, the consequent G2 checkpoint abrogation was not sufficient to sensitize these HR-deficient cells to chemoradiation.

We further tested the hypothesis that sensitization by AZD1775 in this model is associated with inhibition of HR by assessing the effect

Figure 3. The effects of AZD1775 on DNA damage signaling in response to gemcitabine-radiation. MiaPaCa-2 (A) and Capan-1 (B) cells were treated with gemcitabine, AZD1775, and/or radiation as described in Figure 1. At the indicated times post-RT (6 Gy), cells were analyzed for γH2AX by flow cytometry. Data are the mean percentage ± standard error of γH2AX-positive cells from three independent experiments. Statistical significance is indicated versus RT* or GemRT[†] ($P < .05$).
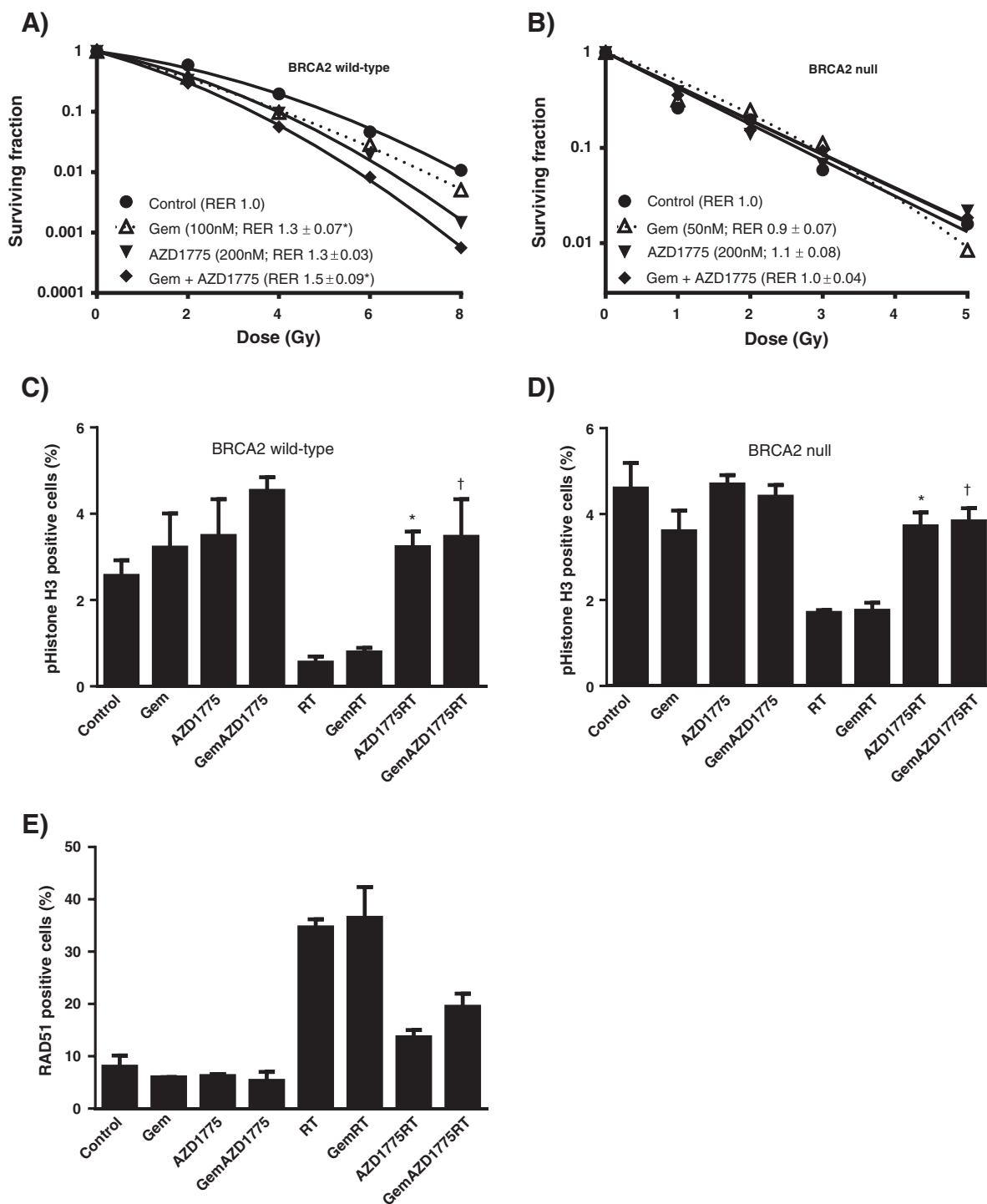
Figure 4. Sensitization to gemcitabine-radiation by AZD1775 involves inhibition of HR. Radiation survival was assessed in BRCA2 wild-type and BRCA2 null isogenic DLD1 cells treated with equitoxic concentrations of gemcitabine (BRCA2 wild-type, 100 nM; BRCA2 null, 50 nM) and AZD1775 (200 nM) as illustrated in Figure 1. Plots are from single representative experiments (A, B). RERs (inset) are the mean RER $\pm$ standard error of three to four independent experiments with statistical significance indicated versus control* ($P < .05$). Additional cytotoxicity data are given in Suppl. Table 2. BRCA2 isogenic DLD1 cells were analyzed for pHistone H3 (Ser10) and DNA content 6 hours post-RT (C, D). Data presented are the mean percentage $\pm$ standard error of pHistone H3–positive mitotic cells from three independent experiments with statistical significance indicated versus RT* or GemRT[†] ($P < .05$). RAD51 foci were analyzed at 24 hours post-RT (6 Gy). Cells with five or more foci were scored as RAD51 positive (E). Data presented are the mean percentage $\pm$ standard deviation of RAD51-positive cells from two independent experiments. Images of RAD51 foci are presented in Suppl. Figure 7.

of AZD1775 on gemcitabine-radiation–induced RAD51 focus formation. Consistent with the HR-proficient and -deficient status of these cells, BRCA2 wild-type cells formed RAD51 foci in response to gemcitabine-radiation, whereas BRCA2 null cells did not (Suppl. Figure 7). Furthermore, AZD1775 inhibited RAD51 focus formation by gemcitabine-radiation in the BRCA2 wild-type cells (Figure 4E). Taken together, these results demonstrate that AZD1775 preferentially sensitizes BRCA2 wild-type, HR-proficient cells to gemcitabine chemoradiation and suggest that inhibition of HR, in contrast to G2 checkpoint abrogation, is an important mechanism of action for sensitization to chemoradiation.

Having demonstrated that AZD1775 sensitizes HR-proficient pancreatic cancer cells to gemcitabine chemoradiation *in vitro*, we next assessed the ability of AZD1775 to sensitize a patient-derived pancreatic tumor xenograft, designated 08-444T, to chemoradiation *in vivo*. Tumor-bearing mice were treated for two cycles with gemcitabine, AZD1775, and fractionated radiation (Figure 5A). In the absence of radiation, treatment with gemcitabine and/or AZD1775 had minimal effect on tumor growth (Figure 5B). In contrast, treatment with radiation, alone or in combination with gemcitabine and/or AZD1775, significantly inhibited tumor growth rates. AZD1775 did not significantly sensitize tumors to radiation alone, a result consistent with the modest sensitization by AZD1775 to radiation alone observed *in vitro* (Figure 1). AZD1775 did, however, sensitize patient-derived pancreatic tumor xenografts to gemcitabine-radiation as evidenced by significantly longer times required for tumor volume doubling in response to AZD1775 in combination with gemcitabine-radiation (34

days) compared with gemcitabine-radiation (21.5 days) (Figure 5C). Treatment with the combination of AZD1775 and gemcitabine-radiation was tolerable as evidenced by 10% or less weight loss during treatment with no other obvious adverse events (data not shown). Taken together, these results demonstrate that AZD1775 is an effective strategy for sensitizing patient-derived pancreatic tumor xenografts to gemcitabine chemoradiation.

Finally, to better understand the mechanism(s) by which AZD1775-mediated WEE1 inhibition sensitizes tumors to gemcitabine-radiation *in vivo*, we assessed the DNA damage response in tumors from animals bearing patient-derived pancreatic tumor xenografts that were treated acutely with gemcitabine, radiation, and/or AZD1775 (Figure 5A). pCDK1 (Y15) protein levels were reduced in response to AZD1775 alone or in combination with gemcitabine-radiation, consistent with inhibition of WEE1 kinase activity (Figure 6A; Suppl. Figure 8). Furthermore, although pCHK1 (S345), a marker of DNA damage [38] and replication stress [40], was elevated in response to either gemcitabine or AZD1775 alone, the greatest effect on pCHK1 (S345) was seen in response to the triple combination of gemcitabine, radiation, and AZD1775. Finally, to determine whether inhibition of HR is a mechanism of AZD1775-mediated sensitization to chemoradiation *in vivo*, RAD51 foci were measured in tumor xenografts from mice treated with radiation, gemcitabine, and/or AZD1775. Treatment with gemcitabine or gemcitabine-radiation resulted in the most RAD51 focus formation with 6.0% or 8.6% of cells staining positive for RAD51 foci, respectively (Figure 6, B and C; Suppl. Figure 9). Importantly,
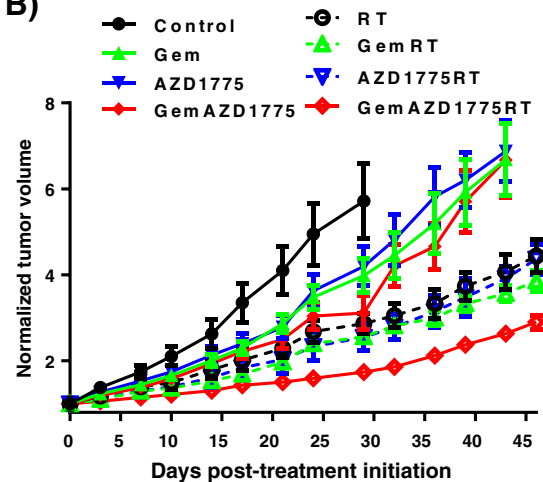

A) Treatment schedule

Day 0	Day 1	Day 2	Day 3	Day 4
Gem AZD1775 RT	AZD1775 RT	RT	RT	RT

2 cycles

IB/IF
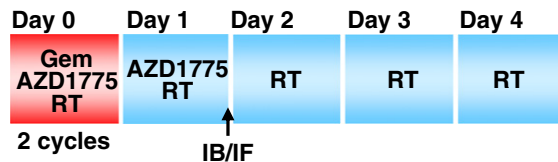
C)

| Median tumor volume doubling time (days)	
Control	11 (6, 12)
Gem	16 (9,18)
AZD1775	13 (7, 19)
GemAZD1775	14.5 (11,22)
RT	17 (8, 25)
GemRT	21.5 (18, 23)
AZD1775RT	21 (16,26)
GemAZD1775RT	34 (30, 38)*†‡

P<0.05 vs con*, RT†, GemRT‡
*Growth rates were significantly reduced in all RT groups vs. control. GemAZD1775RT showed a trend for reduced growth rate vs. GemRT (P=0.07).

Figure 5. Sensitization of patient-derived pancreatic tumor xenografts to gemcitabine-radiation by AZD1775. *In vivo* treatment schedule (A; IB, immunblotting; IF, immunofluorescence) Athymic nude mice bearing subcutaneous, patient-derived pancreatic tumor xenografts, designated 08-444T, were randomized to the indicated treatment groups. Treatments consisted of 60 mg/kg gemcitabine (days 0 and 7), AZD1775 (50 mg/kg QD days 0 to 1 and 7 to 8) and RT (1.8 Gy/fraction; days 0 to 4 and 7 to 11). Data are the mean tumor volume normalized to the tumor volume on the first day of treatment (day 0) $\pm$ standard error (B) or are the median time required for tumor volume doubling with lower and upper limits in parentheses (C). Data are from 7 to 10 tumors per treatment group. Statistical significance is indicated versus control*, RT† and GemRT‡ ($P < .05$).
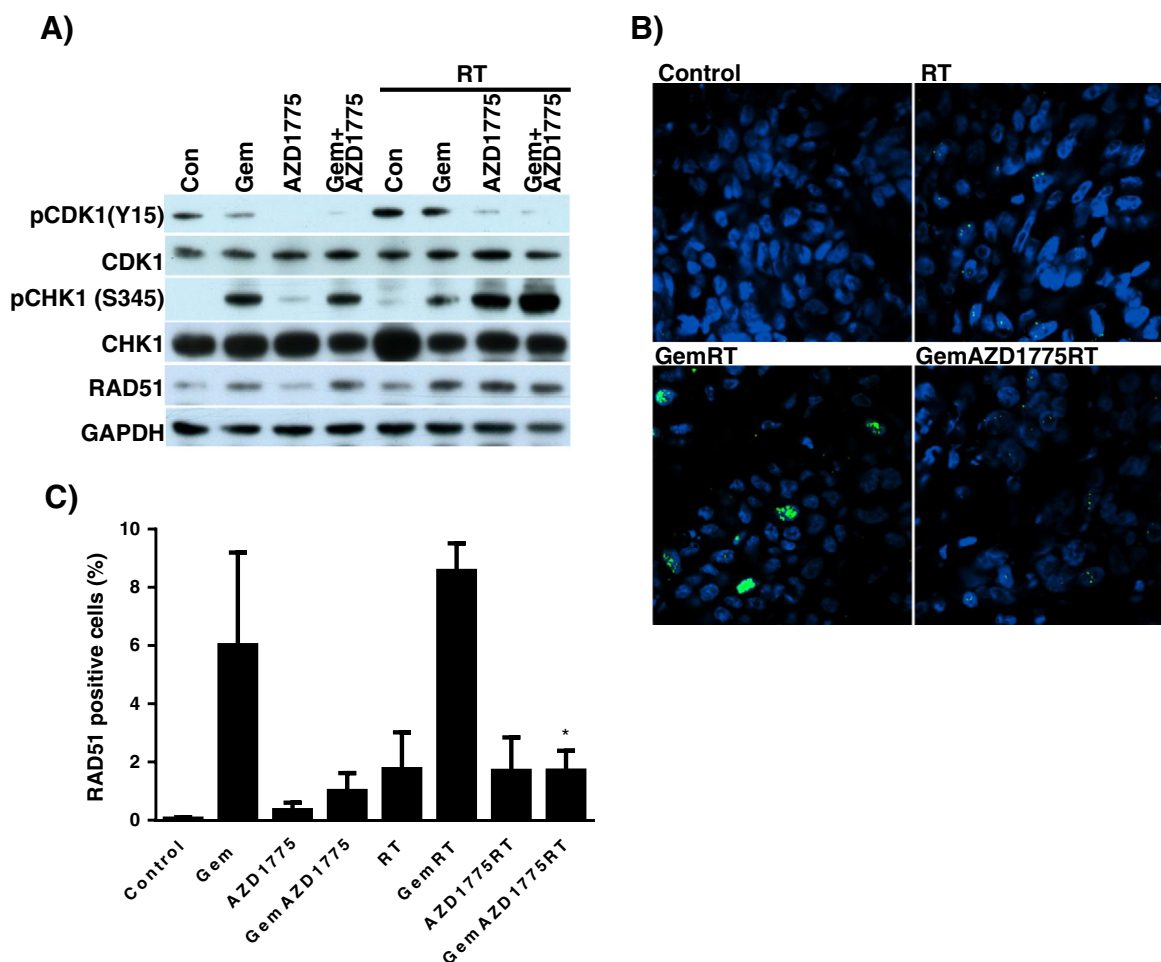
Figure 6. Sensitization of patient-derived tumor xenografts to gemcitabine-radiation by AZD1775 involves inhibition of HR. Athymic nude mice bearing 08-444T subcutaneous, patient-derived pancreatic tumor xenografts were treated as described in Figure 5, except that, on day 1 (2 hours post-RT), tumors were harvested for immunoblotting (A) and immunofluorescence (B, C). Immunoblot images are from a single representative experiment. Additional independent experiments are shown in Suppl. Figure 8. Tumors were stained with anti-RAD51 antibody (green) and DAPI (blue). Cells with five or more RAD51 foci were scored as positive. Data are the mean ± standard error of RAD51 positive cells from three to four tumors per treatment condition. Statistical significance versus GemRT* is indicated ($P < .05$). Additional RAD51 images are provided in Suppl. Figure 9.

AZD1775 treatment significantly inhibited RAD51 focus formation induced by gemcitabine-radiation under conditions which did not alter cellular RAD51 protein levels (Figure 6A). These results support the hypothesis that inhibition of HR repair is a significant mechanism of sensitization to chemoradiation by WEE1 inhibitors in patient-derived pancreatic tumors.

Discussion

In this study, we found that WEE1 inhibition by AZD1775 is an effective strategy for sensitizing HR-competent pancreatic cancers to gemcitabine chemoradiation. Our data show that although WEE1 inhibition is associated with abrogation of the G2 checkpoint, this mechanism may not be sufficient for sensitization. Rather, our findings support the hypothesis that sensitization to chemoradiation by WEE1 inhibition results from inhibition of HR, leading to persistent radiation-induced DNA damage. Taken together, these data suggest that inhibition of HR repair is an important mechanism of action for the therapeutic activity of WEE1 inhibitors and, furthermore, that the combined treatment of a WEE1 inhibitor with chemoradiation may be most effective in pancreatic cancers without

underlying HR defects. Given that 5% of pancreatic cancers harbor BRCA1/2 mutations [41], the findings of this study have important clinical implications.

The finding that AZD1775-mediated WEE1 inhibition causes impaired HR is consistent with previous studies from our group and others [12,42]. CDK1 activation in response to WEE1 inhibition represents a plausible mechanism of HR regulation by WEE1 which is further suggested by the finding that several different agents which result in CDK1 activation, including PP2A and CHK1 inhibitors, also inhibit HR [39,43,44]. CDK1 and CDK2 play a critical role in the cell cycle–dependent regulation of HR, limiting HR activity only to S or G2 phases of the cell cycle. The role of CDK1/2 in HR is complex because it both positively and negatively regulates HR. For example, CDK1/2-mediated phosphorylation of RPA, CtIP, and NBS1 positively regulates HR [45]. In contrast, CDK1-mediated phosphorylation of BRCA2 (S3291) negatively regulates HR by disrupting the interaction between BRCA2 and RAD51 [46]. AZD1775 has previously been shown to increase phosphorylation of BRCA2 (S3291), suggesting that this is a mechanism for inhibition of HR activity by WEE1 inhibitor [12]. Here we demonstrate for the

first time that inhibition of HR is required for sensitization to chemoradiation by WEE1 inhibition.

Although we have focused on understanding the therapeutic significance of the ability of WEE1 to regulate both the G2 checkpoint and HR in response to chemoradiation-induced DNA damage, studies suggest that WEE1 also regulates DNA replication in unstressed cells. According to this model, the hyperactivation of CDK1 caused by WEE1 inhibition leads to futile cycles of increased replication origin firing, nucleotide depletion, and decreased replication fork progression [10,18]. This replication stress activates the MUS81-EME1 endonuclease which catalyzes DNA DSBs at the sites of aberrant replication structures [18,47]. Although the early increase in γH2AX levels in MiaPaCa-2 cells treated with AZD1775 alone is consistent with this model (Figure 3C; t = 0 hour), the relative contribution of replication stress to radiosensitization by WEE1 inhibition is currently unknown.

Although our study suggests that sensitization to chemoradiation by WEE1 inhibition occurs preferentially in HR-proficient cells, the single-agent activity of WEE1 inhibition is greatest in HR-deficient cells [48], a finding that is supported by the activity of AZD1775 in patients carrying BRCA2 mutations [49]. These seemingly contradictory findings suggest that different mechanisms are responsible for the single- versus combined-agent activity of WEE1 inhibitors. It is plausible that in the absence of exogenous DNA damage, DNA replication–associated mechanisms have a greater contribution, whereas in the presence of exogenous DNA damage, DNA damage response–associated mechanisms (cell cycle checkpoints and DSB repair) are of increased importance. Furthermore, in combinatorial approaches, the key mechanisms contributing to cytotoxicity are likely dependent on the type of agent being combined with the WEE1 inhibitor. For example, in combination with chemotherapies such as antimetabolites, which function primarily via perturbing DNA replication, the ability of WEE1 to regulate DNA replication may be of increased importance. In contrast, for agents such as ionizing radiation, which directly induce DNA DSBs, the ability of WEE1 to regulate cell cycle checkpoints and DNA DSB repair is likely of greater relative importance. In addition, it is likely that these pathways exhibit differential concentration responsiveness to WEE1 inhibition which may also influence their contributions to the single- versus combined-agent activity of WEE1 inhibitors. Future studies will be required to define the relative contributions of cell cycle checkpoints, DNA DSB repair, and DNA replication to the therapeutic activity of WEE1 inhibitors alone or in combination with other agents.

There is substantial evidence to support the hypothesis that tumor cells expressing mutant P53 are more sensitive than cells expressing wild-type P53 to the therapeutic effects of checkpoint inhibitors such as AZD1775 on radiosensitivity [16,50]. This selectivity is generally attributed to the combined effects of G1 checkpoint loss in P53 mutant cancer cells and G2 checkpoint loss following WEE1 or CHK1 inhibition, which together promote cellular proliferation despite the presence of unrepaired DNA damage. The ability of AZD1775 to inhibit HR, however, suggests an alternative model. Because P53 mutant cancer cells treated with ionizing radiation do not arrest in G1, where NHEJ is the dominant DNA DSB repair pathway, these cells likely have an increased dependency on other DNA DSB repair pathways, such as HR [51]. Thus, the relatively selective radiosensitization by WEE1 inhibitors such as AZD1775 in cancer cells which lack functional p53 may result from a shift in dependence from NHEJ to HR for repair of ionizing radiation-induced DNA DSBs combined with inhibition of HR activity.

The findings of this study demonstrating the efficacy of WEE1 inhibition for sensitizing pancreatic cancers to chemoradiation represent a promising strategy for improving therapy in locally advanced pancreatic cancer patients. Furthermore, these findings suggest that patients selected on the basis of having an intact HR repair pathway may benefit most from this therapeutic strategy. The results of this study are the foundation of our current clinical trial combining AZD1775 with gemcitabine-radiation in locally advanced pancreatic cancer patients (NCT02037230) and provide a rationale for patient selection on the basis of HR status for future clinical trials.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.neo.2015.09.006.

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