Mechanism of Radiosensitization by the Chk1/2 Inhibitor AZD7762 Involves Abrogation of the G$_2$ Checkpoint and Inhibition of Homologous Recombinational DNA Repair

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Abstract

The median survival for patients with locally advanced pancreatic cancer treated with gemcitabine and radiation is approximately 1 year. To develop improved treatment, we have combined a Chk1/2-targeted agent, AZD7762, currently in phase I clinical trials, with gemcitabine and ionizing radiation in preclinical pancreatic tumor models. We found that *in vitro* AZD7762 alone or in combination with gemcitabine significantly sensitized MiaPaCa-2 cells to radiation. AZD7762 inhibited Chk1 autophosphorylation (S296 Chk1), stabilized Cdc25A, and increased ATR/ATM–mediated Chk1 phosphorylation (S345 Chk1). Radiosensitization by AZD7762 was associated with abrogation of the G$_2$ checkpoint as well as with inhibition of Rad51 focus formation, inhibition of homologous recombination repair, and persistent γ-H2AX expression. AZD7762 was also a radiation sensitizer in multiple tumor xenograft models. In both MiaPaCa-2- and patient-derived xenografts, AZD7762 significantly prolonged the median time required for tumor volume doubling in response to gemcitabine and radiation. Together, our findings suggest that G$_2$ checkpoint abrogation and homologous recombination repair inhibition both contribute to sensitization by Chk1 inhibition. Furthermore, they support the clinical use of AZD7762 in combination with gemcitabine and radiation for patients with locally advanced pancreatic cancer. *Cancer Res; 70(12); 4972–81. ©2010 AACR.*

Introduction

Pancreatic cancer remains among the least curable cancers, with an overall 5-year survival for all patients of ~5% (1). Gemcitabine (2′,2′-difluoro-2′-deoxycytidine, dFdCyd) is the standard chemotherapy for pancreatic cancer, and the combination of radiation with gemcitabine has been shown superior to gemcitabine alone for locally advanced disease (2, 3). Thus, we have sought to improve therapy for locally advanced pancreatic cancer by combining additional agents with gemcitabine and radiation (4, 5).

Gemcitabine requires phosphorylation to produce its active diphosphorylated (dFdCDP) and triphosphorylated (dFdCTP) metabolites. dFdCDP inhibits ribonucleotide re-

ductase, which leads to depletion of deoxynucleotide triphosphate pools, whereas dFdCTP competes with endogenous dCTP, resulting in misincorporation of dFdCTP into DNA. Together, these activities result in replication stress and inhibition of DNA synthesis and the activation of checkpoint kinase 1 (Chk1; see below). As a central mediator of the cellular response to DNA damage, activation of Chk1 in response to DNA damage results in cell cycle arrest (6, 7) as well as promotion of homologous recombination repair (HRR), a process promoted by the binding of the recombinase, Rad51, to sites of DNA double strand breaks (8). Based on data demonstrating that Chk1 is an effective target for sensitization to chemotherapy and radiotherapy (9–11), small-molecule Chk1 inhibitors have been developed for clinical use, principally with the idea that they would be used to enhance killing of tumor cells by cytotoxic drugs or by radiation (12–16). The first Chk1 inhibitor to be tested extensively in humans was UCN-01 (7-hydroxystaurosporine; refs. 17–19). Because UCN-01 is a nonselective Chk1 inhibitor with poor protein binding properties *in vivo*, several other Chk1 antagonists are in development for clinical use, and three of them (SCH900776, AZD7762, and PF-00477736) are currently in phase I clinical trials in combination with gemcitabine or irinotecan, with others due to follow (20–22).

In our previous studies, we showed that gemcitabine activates Chk1 (23, 24) and that inhibition of Chk1 promotes premature mitotic entry and cytotoxicity in response to

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gemcitabine (25). In addition, Chk1 inhibition leads to impaired Rad51 focus formation, a key step in HRR and a prolonged DNA damage response in pancreatic cancer cells treated with gemcitabine (26). The goal of the present study was to determine whether the Chk1/2 inhibitor AZD7762 sensitizes pancreatic cancer cells to radiation as well as gemcitabine-radiation. When we found that AZD7762 sensitized to radiation both in the presence and absence of gemcitabine in our *in vitro* pancreatic cancer model, we then went on to determine the mechanism(s) of sensitization. We hypothesized that inhibition of both cell cycle checkpoints and HRR was involved in AZD7762-mediated radiosensitization. To begin to test this hypothesis, we determined whether AZD7762 interfered with cell cycle checkpoint activation in bromodeoxyuridine (BrdUrd) pulse-chase experiments and HRR-mediated DNA repair by Rad51 focus formation and an HRR activity assay. Finally, we tested the efficacy of AZD7762 as a radiation sensitizer *in vivo* in both cell line– and patient-derived pancreatic tumor xenograft models.

Materials and Methods

Cell culture and drug solutions

MiaPaCa-2 cells were obtained from the American Type Culture Collection and grown in DMEM supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 2 mmol/L L-glutamine (Sigma). Experiments were conducted on exponentially growing cells. Cells were tested for *Mycoplasma* once every 3 months. Gemcitabine (Eli Lilly) was dissolved in PBS. AZD7762 was dissolved in DMSO (*in vitro*) or 11.3% 2-hydroxypropyl-β-cyclodextrin (Sigma) and 0.9% sterile saline (*in vivo*). Clonogenic survival assays were conducted as previously described (5, 27, 28). Nonspecific, Chk1, and Chk2 siRNA were purchased from Dharmacon and used as previously described (23).

Flow cytometry

For γ-H2AX analysis, samples were processed as previously described (29). For BrdUrd pulse-chase experiments, samples were pulsed with 30 μmol/L BrdUrd for 15 minutes, washed with medium containing 10 μmol/L thymidine, irradiated, and processed and analyzed as previously described (30) using anti-BrdUrd (Pharmingen) and FITC-conjugated anti-mouse (Sigma Biochemicals) antibodies. Samples were analyzed on a FACScan flow cytometer (Becton Dickinson) with FlowJo software (Tree Star).

Homologous recombination repair

MiaPaCa-2 cells were transfected with the pDR-GFP plasmid (31) using SuperFect transfection reagent (Qiagen) according to the manufacturer's protocol. Clones containing the DR-GFP reporter integrated chromosomally were isolated following puromycin selection. To measure repair of a DNA double strand break, cells were infected with the adenovirus AdNGUS24i, expressing the I-SceI enzyme. I-SceI–induced homologous recombination was measured

as the percentage of green fluorescent protein (GFP)–positive cells 48 hours later by flow cytometry.

Immunoblotting

Cell pellets or pulverized frozen tumors were lysed and immunoblotted as previously described (5). Proteins were detected with Chk1 (S345), Chk1 (S296), Chk1, Chk2 (T68), GAPDH (Cell Signaling), Chk2 (Millipore), Cdc25A (Santa Cruz), or β-actin (Calbiochem) antibodies.

Immunofluorescence

Cells cultured on coverslips were treated as illustrated in Fig. 1A. At 26 and 30 hours, cells were fixed and processed as previously described (26). Samples were imaged with an Olympus FV500 confocal microscope (Olympus America) with a 60× objective. For quantitation of Rad51 foci, at least 100 cells from each of three independent experiments were visually scored for each condition. Cells with ≥5 Rad51 foci were scored as positive and compared for statistical analyses. Foci-positive cells were binned as having 5 to 9 or 10 or more Rad51 foci.

Immunohistochemistry

Harvested tumors were fixed in 10% neutral buffered formalin for 24 hours, then embedded in paraffin blocks and sectioned at 5 μm onto slides. Histopathology was conducted using hematoxylin and eosin staining and immunohistochemistry using Chk1 (S345; Cell Signaling) antibody, biotinylated rabbit secondary antibody, streptavidin–horseradish peroxidase complex, and 3,3′-diaminobenzidine chromogen kit (Ventana). Positive rodent control slides showed strong nuclear staining, and negative control slides (Rabbit IgG) showed levels of nonspecific staining, if any. Tumors were microscopically evaluated with a 20× objective to assess morphologic changes, and results were reported by a pathologist. Slide images were produced by Aperio Imagescope.

Irradiation

Irradiations were carried out 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 carried out 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 according to a protocol approved by the University of Michigan Committee for Use and Care of Animals. MiaPaCa-2 cells or patient-derived pancreatic tumor cells (5×10^6 or 1×10^6, respectively) were suspended in a 1:1 mixture of 10% FBS-RPMI/Matrigel (BD Biosciences) and injected subcutaneously into the flanks of athymic nude or Nod-scid mice, respectively. Samples of human pancreatic
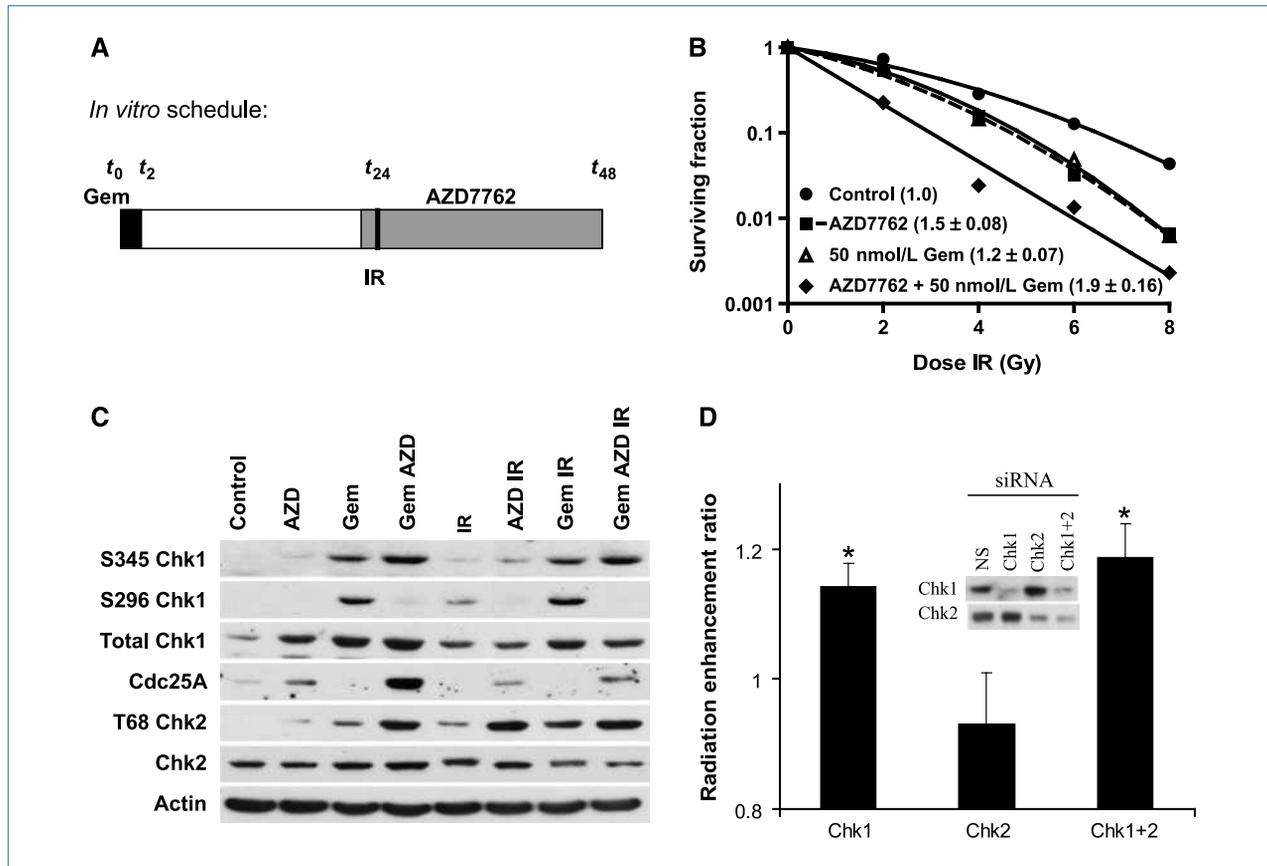
Figure 1. Radiosensitization in response to Chk1 inhibition and gemcitabine. A, schedule of treatments. B, MiaPaCa-2 cells were treated with gemcitabine (Gem; 50 nmol/L), AZD7762 (AZD; 100 nmol/L), and ionizing radiation (IR; 0–8 Gy) as illustrated in A. The plating efficiency of the untreated control cells was 41%. C, MiaPaCa-2 cells were treated as illustrated in A, except with 7.5 Gy. Cells were collected for immunoblotting at $t = 26$ hours. D, alternatively, cells were treated with Chk1 and/or Chk2 siRNA, irradiated 48 hours posttransfection, and then processed for clonogenic survival. The radiation enhancement ratios were normalized to the mean inactivation dose of the nonspecific siRNA (NS)–treated samples. Data are from a single experiment representative of three independent experiments (B, C) or the mean radiation enhancement ratio of four independent experiments ± SEM (D, numeric values in B). For B, error bars are contained within the points. Statistically significant differences are indicated (*, $P < 0.05$).

adenocarcinomas were handled as described previously (32). Treatment was initiated when the average tumor volume reached 100 mm³. For tumor growth delay studies, tumor size was measured two times per week. Tumor volume (TV) was calculated according to the 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 120 or until the tumor volume increased by approximately a factor of 10.

Statistical analysis

For *in vitro* radiation enhancement, drug cytotoxicity, and Rad51 foci, statistically significant differences were determined by one-way ANOVA with the Newman-Keuls' post-comparison test in GraphPad PRISM version 3 (GraphPad software). Additivity was defined by the difference in the area-under-the-curve between the control and gemcitabine-AZD7762 being not significantly different from the sum of the differences between the control and gemcitabine or AZD7762 alone using a two-way ANOVA model with an

interaction term (33). For γ-H2AX, data were analyzed using ANOVA (PROC MIXED in SAS). Estimates of means, differences between means, and statistical significance were all derived from the ANOVA model. For *in vivo* tumor growth, tumor volume 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. A cubic smoothing spline (SMOOTH.SPLINE function in R) was used to obtain the exact time of doubling, and the Kaplan-Meier method was used to analyze the doubling times derived from the smoothed growth curves. Log-rank test (PROC LIFETEST in SAS) was used for comparisons between any two treatment groups.

Results

AZD7762 radiosensitizes pancreatic cancer cells through inhibition of Chk1

To begin to determine if the Chk1/2 inhibitor AZD7762 is a radiation sensitizer, we treated MiaPaCa-2 pancreatic cancer

cells with noncytotoxic concentrations of gemcitabine and AZD7762 according to the schedule illustrated in Fig. 1A and then assessed radiation survival by a clonogenic assay. We found that AZD7762 alone significantly sensitized MiaPaCa-2 cells to radiation, producing a RER (radiation enhancement ratio) of 1.5 ± 0.08 ($P < 0.05$; Fig. 1B; Supplementary Table S1). The combination of AZD7762 with gemcitabine further enhanced radiosensitization beyond that observed with gemcitabine alone (RER 1.9 ± 0.16 and 1.2 ± 0.07, respectively; $P < 0.05$). AZD7762 and gemcitabine produced additive effects on radiosensitization over a range of gemcitabine concentrations (10–50 nmol/L) and under conditions that produced minimal to substantial cytotoxicity (0.9 ± 0.06 to 0.2 ± 0.07; Supplementary Table S1). The cytotoxicity produced by AZD7762 in combination with 50 nmol/L gemcitabine (0.2 ± 0.07) was significantly greater ($P < 0.05$) than that caused by the same concentration of gemcitabine (1.0 ± 0.04) or AZD7762 (0.9 ± 0.05) alone, which is consistent with our previous data demonstrating chemosensitization by

Chk1 inhibition (25). We obtained similar data in MPanc96 cells where AZD7762 produced sensitization to radiation (RER 1.4 ± 0.15) and gemcitabine-radiation (RER gemcitabine 1.2 ± 0.16 versus gemcitabine-AZD7762 1.5 ± 0.20; Supplementary Fig. S1).

To confirm that AZD7762 inhibits Chk1/2 in our models, we analyzed Chk1 and Chk2 signaling. As anticipated, we observed that Chk1 autophosphorylation (S296 Chk1) was inhibited and that Cdc25A was stabilized by AZD7762 in response to gemcitabine, radiation, or gemcitabine-radiation (ref. 34; Fig. 1C). Taken together, these results show that AZD7762 inhibits Chk1. ATR- and ATM-mediated phosphorylation of Chk1 (S345 Chk1) and Chk2 (T68 Chk2) were increased by the addition of AZD7762 to gemcitabine and/or radiation, likely a consequence of the increased level of DNA damage present under these treatment conditions (Fig. 3). To address the relative contributions of inhibition of Chk1 or Chk2 by AZD7762 to radiosensitization, we used siRNA to selectively deplete Chk1 or Chk2 from MiaPaCa-2
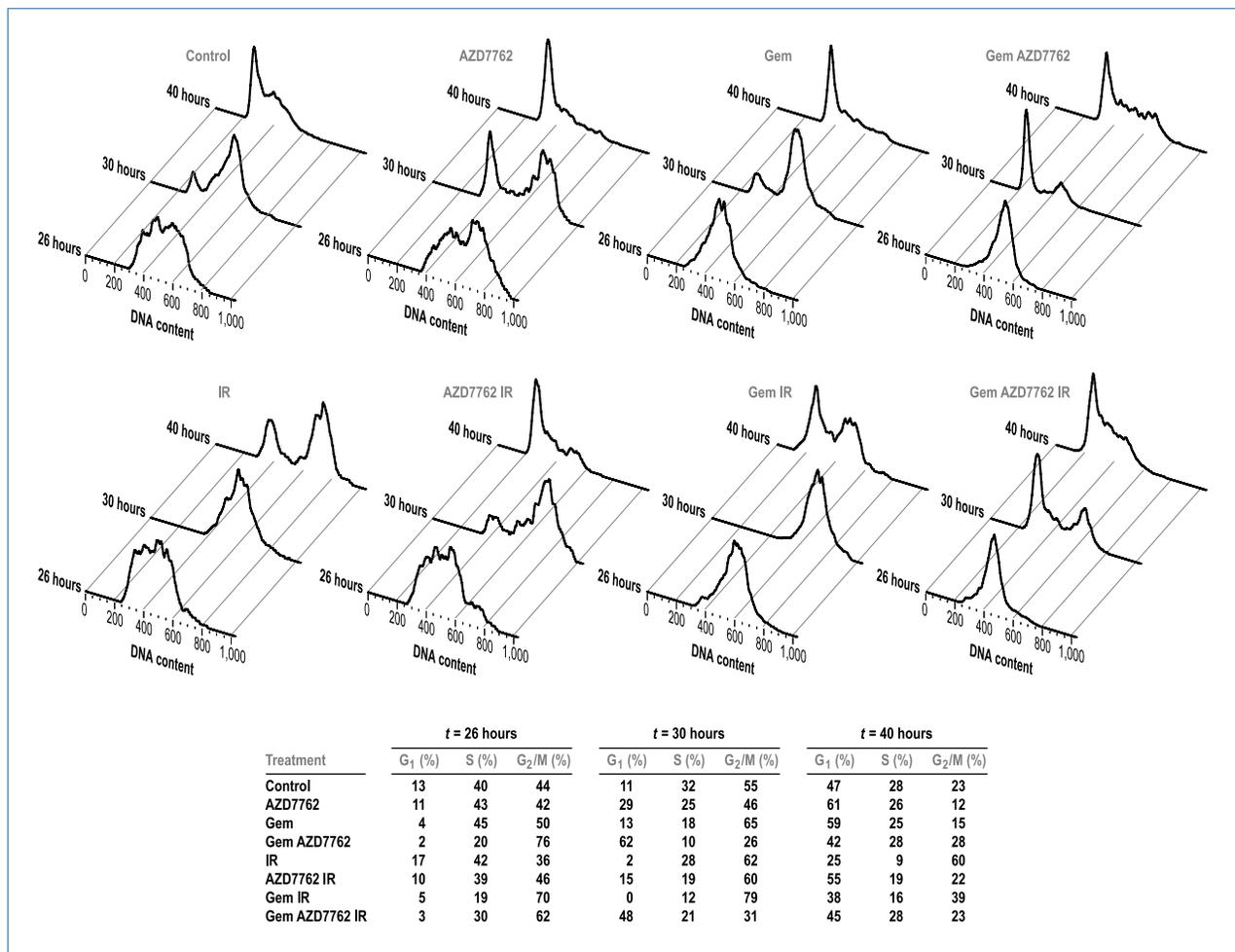
Treatment	$t = 26$ hours			$t = 30$ hours			$t = 40$ hours		
	G_1 (%)	S (%)	G_2/M (%)	G_1 (%)	S (%)	G_2/M (%)	G_1 (%)	S (%)	G_2/M (%)
Control	13	40	44	11	32	55	47	28	23
AZD7762	11	43	42	29	25	46	61	26	12
Gem	4	45	50	13	18	65	59	25	15
Gem AZD7762	2	20	76	62	10	26	42	28	28
IR	17	42	36	2	28	62	25	9	60
AZD7762 IR	10	39	46	15	19	60	55	19	22
Gem IR	5	19	70	0	12	79	38	16	39
Gem AZD7762 IR	3	30	62	48	21	31	45	28	23

Figure 2. The effects of AZD7762 on progression from S-phase in response to gemcitabine and radiation. MiaPaCa-2 cells were treated as illustrated in Fig. 1A with the exception that cells were pulsed with BrdUrd immediately before radiation ($t = 24$ hours; 7.5 Gy). At 26, 30, and 40 hours, cells were collected for flow cytometry. Untreated MiaPaCa-2 cells had a 20-hour cell doubling time. Data in the histograms represent only BrdUrd-positive cells from a single experiment with the percentages of cells in G_1, S, and G_2-M illustrated. Data are representative of 3 independent experiments.
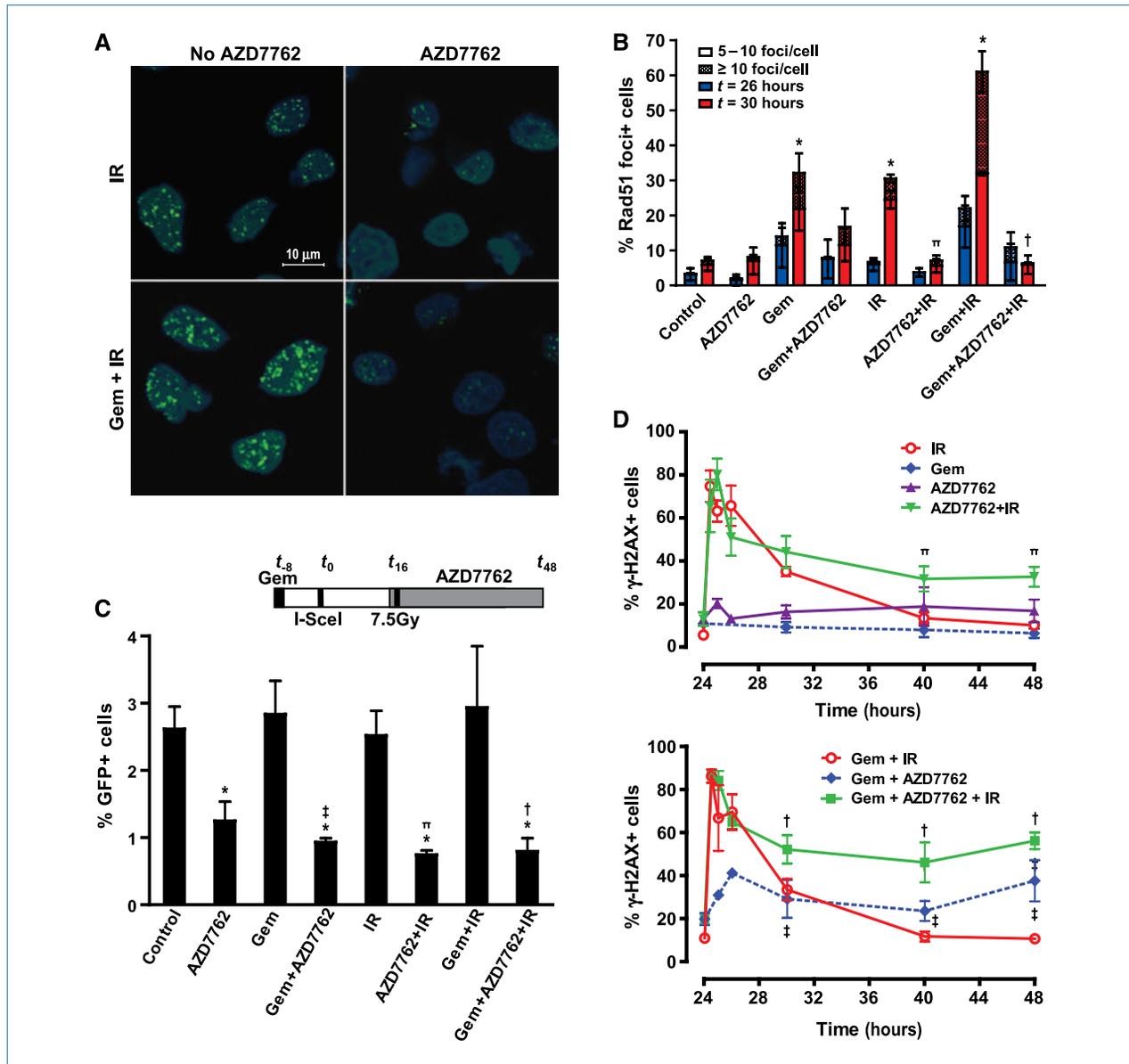
Figure 3. The effects of AZD7762 on Rad51, HRR, and γ-H2AX. A, MiaPaCa-2 cells were treated as illustrated (Fig. 1A) and fixed for immunofluorescence at 30 hours. Cells were stained with 4′,6-diamidino-2-phenylindole (blue) and for Rad51 (green). B, Rad51 foci were analyzed at t = 26 and 30 hours. Data are the mean ± SEM of three independent experiments. C, MiaPaCa-2-DR-GFP cells were treated as illustrated; at t = 48 hours, the percentage of GFP-positive cells was measured by flow cytometry. Data are expressed as the mean percentage of GFP-positive cells ± SEM of n = 6 independent experiments. D, γ-H2AX was assessed by flow cytometry in MiaPaCa-2 cells treated with radiation (7.5 Gy) at t = 24 hours, according to the schedule illustrated in Fig. 1A, and collected at various time points following radiation (t = 24–48 hours). Data are expressed as the percentage of cells staining positive for γ-H2AX and are the mean ± SEM of n = 4 to 6 independent experiments. B to D, statistical significance (P < 0.05) is indicated versus control (*), gemcitabine (‡), radiation (ᴨ), and gemcitabine-radiation (†).

cells. Relative to nonspecific siRNA-treated cells, Chk1-depleted cells were sensitized to radiation similarly, whereas Chk2-depleted cells were not (Fig. 1D). Depletion of Chk2 did not increase the sensitization produced by depletion of Chk1. These data are consistent with our previous observation that Chk1 but not Chk2 siRNA sensitizes pancreatic cancer cells to gemcitabine (25) and suggest that radiosensitization by AZD7762 is mediated by Chk1 inhibition.

Gemcitabine- and radiation-induced cell cycle checkpoints are abrogated by AZD7762

To determine whether AZD7762 would modulate Chk1-mediated cell cycle checkpoints, we labeled S-phase cells with BrdUrd and followed the progression of the cells through the cell cycle over time (t = 26–40 hours). This permitted the observation of effects that were more difficult to distinguish by single parameter flow cytometry (Supplementary

Fig. S2). Treatment with AZD7762 alone resulted in a more rapid progression from S phase into G_2-M, and subsequently G_1, relative to the untreated control cells (Fig. 2; Supplementary Table S2). As anticipated, a noncytotoxic concentration of gemcitabine resulted in temporary S-phase arrest as evidenced by a narrow S-phase distribution ($t = 26$ hours) and delayed reentry into the subsequent S phase ($t = 40$ hours). The addition of AZD7762 to gemcitabine resulted in a more rapid transit of cells from S phase to G_1 and subsequently into a second round of S phase. Radiation induced a G_2 checkpoint, evidenced by G_2-M accumulation at 40 hours that was overcome by AZD7762. Finally, addition of AZD7762 to gemcitabine-radiation resulted in a more rapid transition from G_2-M to G_1. In response to radiation and gemcitabine-radiation, AZD7762 specifically abrogated the G_2 checkpoint, as evidenced by an increase in the percentage of phosphorylated histone H3–positive cells (Supplementary Table S3). Together, these results support the conclusion that AZD7762 accelerates progression through S phase and abrogates the G_2 checkpoint in response to gemcitabine and radiation treatments, likely through inhibition of Chk1.

AZD7762 inhibits HRR leading to increased DNA damage

To further explore the mechanisms of radiosensitization by AZD7762, we investigated the effects of AZD7762 on Rad51 and HRR. In response to gemcitabine and/or radiation, Rad51 formed discrete nuclear foci at the 30-hour time point (Fig. 3A, B). The addition of AZD7762 significantly inhibited the appearance of Rad51 foci in response to gemcitabine or radiation alone, as well as in response to the combination of gemcitabine and radiation. To distinguish whether AZD7762 was attenuating formation versus promoting dissociation of Rad51 foci, we selected two time points for analysis. We found that in response to gemcitabine and/or radiation, Rad51 foci assembly mainly occurred between 26 and 30 hours. The finding that Rad51 foci failed to assemble in the presence of AZD7762 suggests that AZD7762 acts to inhibit Rad51 focus formation, rather than promote Rad51 focus dissociation.

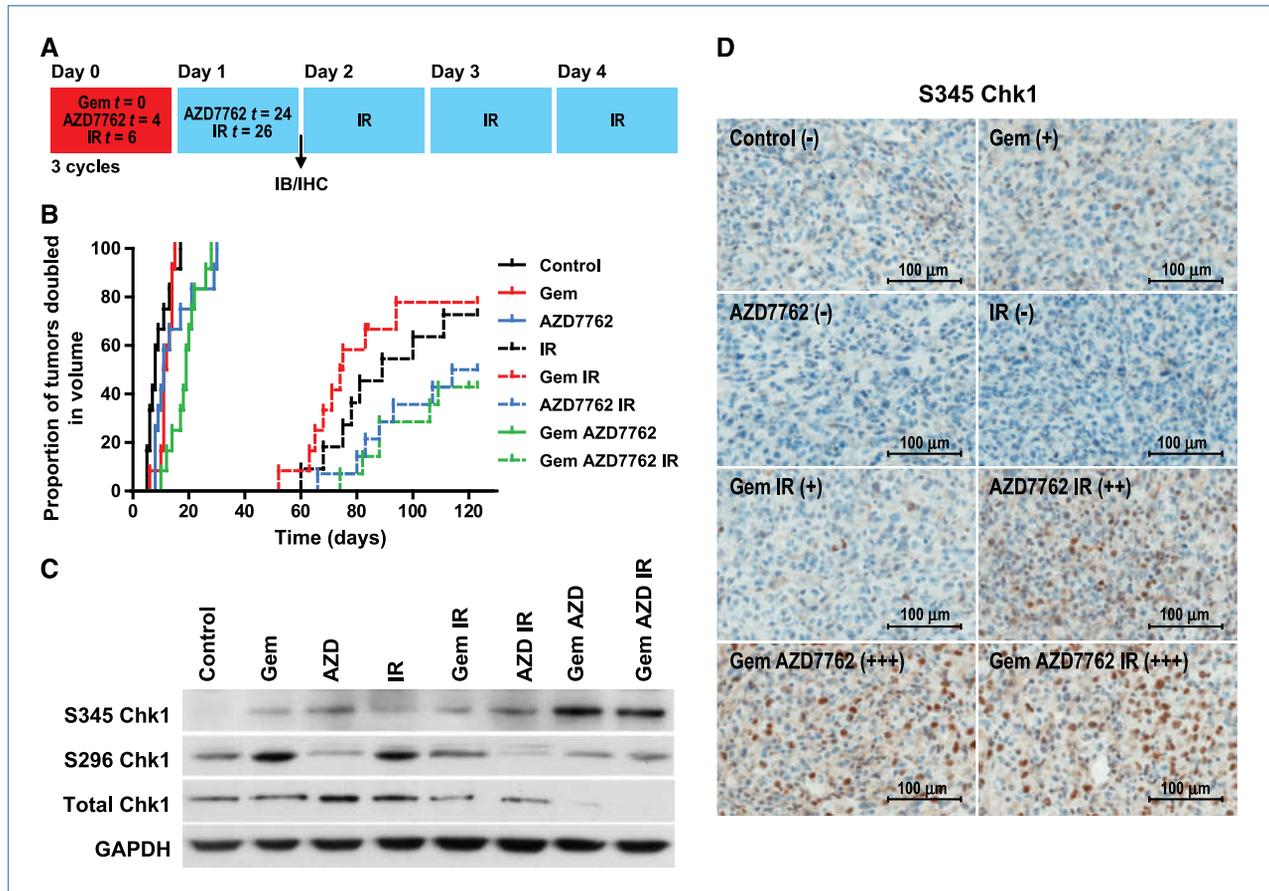To specifically measure whether AZD7762 inhibits HRR, we used the DR-GFP reporter assay, which measures

Figure 4. Effects of AZD7762 on MiaPaCa-2 xenografts in response to gemcitabine and radiation. A, schedule of treatments. B, athymic nude mice bearing bilateral, subcutaneous MiaPaCa-2–derived xenografts were treated with gemcitabine (90 mg/kg), AZD7762 (20 mg/kg), and/or radiation (2 Gy/fraction) for three cycles as illustrated (A). Data are expressed as the proportion of tumors doubled in volume. Each treatment group contained six to seven animals (12–14 tumors). For immunoblotting (C) or immunohistochemistry (D), tumors were harvested on day 1. Immunoblots are from a single experiment representative of three independent experiments. For S345 Chk1 immunohistochemistry, the average score obtained from three to four tumor specimens is illustrated, in which "–" indicates no staining and "+++" indicates maximal staining.

Table 1. Tumor doubling time (days)

Treatment	MiaPaCa-2	Patient F	Patient J
Control	8 (5, 13)	10.0 (7, 10)	6.5 (6, 9)
Gem	12 (10, 14)	33.0* (21, 37)	23.5* (21, 26)
AZD7762	11* (8, 21)	25.5* (20, 30)	26.5* (17, 28)
IR	89* (68, nd)	15.0* (9, 18)	32.5* (16, 42)
Gem IR	75* (63, 94)	49.0*$^{\Pi}$ (32, 54)	49.0*$^{\Pi}$ (45, 51)
AZD7762 IR	>114* (83, nd)	44.0*$^{\Pi}$ (39, 53)	44.5*$^{\Pi}$ (43, 47)
Gem AZD7762	19*‡ (12, 22)	46.5*‡ (42, 52)	41.0*‡ (37, 42)
Gem AZD7762 IR	>109*† (88, nd)	56.0*$^{\Pi}$ (47, 62)	60.5*$^{\dagger\Pi}$ (56, 67)

NOTE: Data are the median time until tumor volume doubling. The upper and lower limits are indicated in parentheses. Statistical differences in the time until tumor volume doubling were determined by the log-rank test and are indicated where $P < 0.05$ is indicated versus control (*), gemcitabine (‡), gemcitabine-radiation (†), or radiation ($^{\Pi}$).
Abbreviations: Gem, gemcitabine; IR, ionizing radiation; nd, the upper limit could not be determined.

homology-directed repair of an I-SceI endonuclease-induced DNA double strand break in an integrated GFP reporter gene (31). Consistent with the ability of AZD7762 to inhibit Rad51 focus formation, AZD7762 significantly inhibited HRR as evidenced by a decrease in the percentage of GFP-positive cells (Fig. 3C). In the presence of gemcitabine, radiation, or gemcitabine-radiation, AZD7762 also produced significant inhibition of HRR activity. As anticipated, neither gemcitabine nor radiation led to an increase in HRR activity, as this model only measures repair of I-SceI endonuclease-induced DNA double strand breaks. We next assessed the presence of unrepaired DNA damage by conducting quantitative flow cytometric studies of γ-H2AX staining. As anticipated, radiation or gemcitabine-radiation produced a γ-H2AX signal as early as 30 minutes postirradiation (t = 24.5 hours), which was resolved to basal levels by 16 hours postirradiation (t = 40 hours; Fig. 3D). The addition of AZD7762 to radiation resulted in a significant prolongation of γ-H2AX signaling for up to 24 hours postirradiation (t = 48 hours; 33 ± 5%) compared with radiation alone (10 ± 3%; P < 0.001; Fig. 3D). Although gemcitabine alone produced Rad51 foci (t = 30 hours), it did not produce a significant increase in γ-H2AX staining, which is likely attributable to the differences in the sensitivity of these two assays. Importantly, treatment with AZD7762 and gemcitabine caused maximal γ-H2AX signaling that persisted throughout the course of this study (t = 48 hours; 56 ± 5%). Together, these results show that AZD7762 inhibits HRR, likely through inhibition of Rad51, in response to gemcitabine and radiation, ultimately resulting in the persistence of unrepaired DNA damage.

Pancreatic tumor xenografts are sensitized to gemcitabine and radiation by AZD7762

Based on the efficacy of AZD7762 as a sensitizer *in vitro*, we hypothesized that AZD7762 would be an effective sensitizer in pancreatic tumor models. We began by testing the effects of AZD7762 on the growth of MiaPaCa-2–derived subcutaneous xenografts in response to gemcitabine and radia-

tion. Tumor-bearing mice were treated with gemcitabine, radiation, and AZD7762 as illustrated (Fig. 4A). AZD7762 alone produced a significant growth delay (Δ = 3 days compared with control; P = 0.04; Fig. 4B; Table 1). More importantly, the combinations of AZD7762 with gemcitabine or gemcitabine-radiation significantly prolonged the time required for tumor volume doubling relative to gemcitabine alone (Δ = 8 days; P < 0.001) or gemcitabine-radiation (Δ > 35 days; P = 0.01). Although there was a trend for AZD7762 to sensitize tumors to radiation (Δ > 25 days), this difference did not reach statistical significance. Therapy with AZD7762, gemcitabine, and radiation was tolerable as the average weight loss for any of the treatment groups in this study was <10%.

To confirm Chk1 inhibition by AZD7762 *in vivo*, we analyzed Chk1/2 signaling in tumors on treatment day 1 (Fig. 4A). Consistent with our *in vitro* findings, S296 Chk1 was inhibited by AZD7762 in the presence of gemcitabine, radiation, and gemcitabine-radiation (Fig. 4C; Supplementary Fig. S3). Also consistent with our *in vitro* data was a trend for S345 Chk1 to be increased in response to any of the treatments; the most prominent increase in S345 Chk1 occurred following treatment with gemcitabine plus AZD7762 (both in the presence and absence of radiation; Fig. 4C–D; Supplementary Fig. S3). Increased phosphorylation of Chk1 (S345), which targets Chk1 for ubiquitin-mediated proteosomal degradation (35), was paralleled by a loss of total Chk1 protein that is consistent with previous data demonstrating Chk1 degradation in response to cytotoxic doses of gemcitabine and Chk1 inhibitor in MiaPaCa-2 cells (26). Although the *in vitro* studies (Fig. 1C) presented in this current work did not show Chk1 degradation in response to gemcitabine and AZD7762, it is likely that this difference is due to the non-cytotoxic dose of gemcitabine used in this study.

We then wished to determine if AZD7762 could sensitize patient-derived pancreatic tumor xenografts. Pancreatic tumor specimens were obtained from two patients (designated patient F and patient J) at the time of surgical resection, then established, expanded, and implanted into

mice for therapeutic studies. In an effort to improve the sensitizing properties of AZD7762 and reduce the effects of radiation alone relative to that observed in the MiaPaCa-2 xenografts (Fig. 4B), we treated mice with AZD7762 five times weekly (versus two times for MiaPaCa-2) and with a total of 18 Gy radiation (versus 30 Gy for MiaPaCa-2) as illustrated (Fig. 5A). For both of the patient-tumor xenografts, treatment with the single agents, gemcitabine, AZD7762, or radiation, produced significant effects on tumor growth (Fig. 5B–C; Table 1). Notably, the addition of AZD7762 to radiation resulted in a significantly prolonged time until tumor volume doubling relative to radiation alone ($\Delta = 29$ days; $P < 0.001$ and 12 days; $P < 0.001$ in patient F and patient J xenografts, respectively). Furthermore, the combination of AZD7762 with gemcitabine or gemcitabine-radiation delayed the tumor volume doubling time relative to gemcitabine ($\Delta = 14$ days; $P < 0.001$ and 18 days; $P < 0.001$ in patient F and patient J xenografts, respectively) as well as gemcitabine-radiation ($\Delta = 7$ days; $P = 0.07$ and 12 days; $P = 0.001$, respectively). Overall, these results show that AZD7762 sensitizes to gemcitabine and radiation in multiple pancreatic cancer model systems.

Discussion

In this study, we have shown that Chk1/2 inhibition by AZD7762 enhances radiation sensitivity and gemcitabine-mediated radiosensitization in pancreatic cancer cells and xenografts. Radiosensitization by AZD7762 is associated with abrogation of the radiation-induced G_2 checkpoint as well as inhibition of HRR. Inhibition of these two processes by AZD7762 results in increased DNA damage, evidenced by

increased ATR-mediated Chk1 phosphorylation (S345 Chk1) and persistent γ-H2AX expression. These data support the clinical investigation of Chk1 inhibitors, specifically AZD7762, in combination with gemcitabine-radiation in patients with locally advanced pancreatic cancer. Furthermore, these data suggest that S345 Chk1 and γ-H2AX might be useful markers for predicting AZD7762 activity in clinical trials.

Although this is the first study demonstrating radiosensitization by a Chk1 inhibitor in clinical development (22), other Chk1-targeted agents are radiosensitizers. Chir-124, a novel Chk1 inhibitor in preclinical development, radiosensitized all HCT116 models but to a greater extent in HCT116 p21−/− cells (14). The Chk1 inhibitor, CEP-3891, although discontinued for clinical development, radiosensitized U2-OS cells (36). Furthermore, the nonselective Chk1 inhibitor UCN-01 induced radiosensitization that was dependent on the presence of mutant p53 (17). These studies have associated radiosensitization induced by Chk1 inhibitors with abrogation of the radiation-induced G_2 checkpoint. Our work now shows that inhibition of Rad51 and HRR is an additional mechanism of sensitization by Chk1 inhibitors in pancreatic cancer models.

Our findings suggest that Chk1 inhibitors may have at least two mechanisms by which they selectively sensitize tumor cells compared with normal cells. Substantial literature supports the model that normal cells (e.g., cells with normal p53 function) should respond to stress by halting at the G_1 checkpoint (through p53 induction) and thus be unaffected by loss of the Chk1-mediated S or G_2 checkpoints. Conversely, tumor cells that harbor p53 mutations should rely exclusively on Chk1/2-mediated pathways for cell cycle arrest in
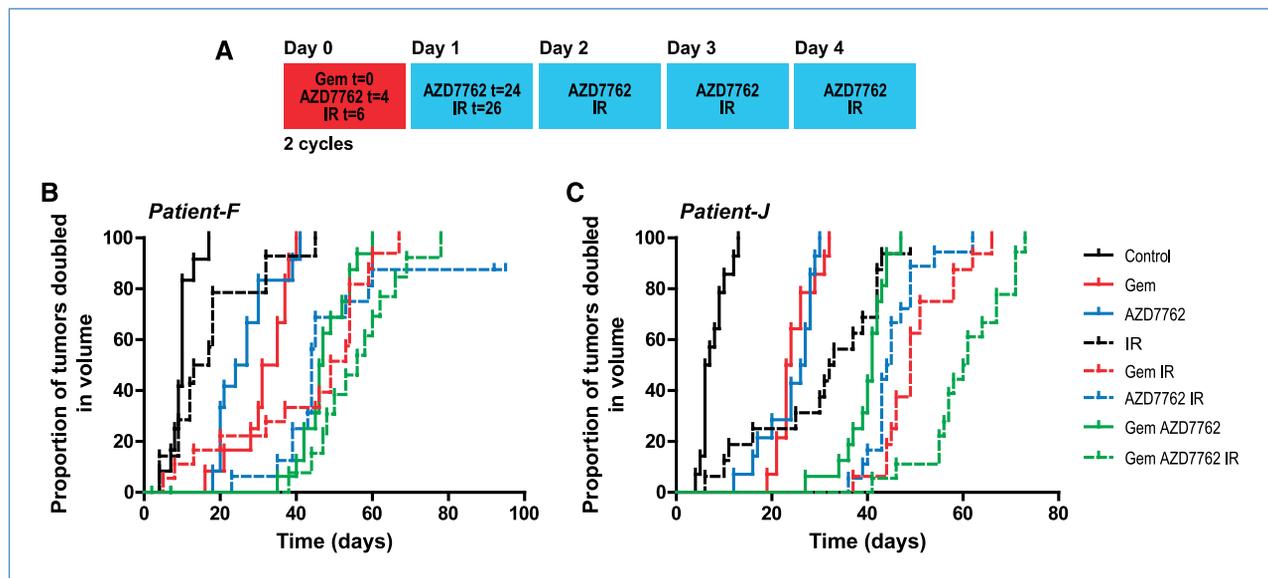

Figure 5. The effects of AZD7762 on patient-derived xenografts in response to gemcitabine and radiation. Nod-scid mice bearing bilateral, subcutaneous xenografts derived from two different patient pancreatic tumors (patients F and J; B and C, respectively) were treated with gemcitabine (90 mg/kg), AZD7762 (20 mg/kg), and/or radiation (1.8 Gy/fraction) for two cycles as illustrated (A). Data are expressed as the proportion of tumors doubled in volume. Each treatment group contained 6 to 10 mice (12–20 tumors).

response to stress. This model is supported by the findings that Chk1 inhibition preferentially sensitizes HCT116 p53−/− cells (versus HCT p53+/+) to gemcitabine (13) and radiation (10) as well as HCT116 p53−/− tumors to 5-fluorouracil (37). In addition to p53, however, our model would predict that tumors that overexpress Rad51, such as pancreatic tumors (38), would rely more heavily on HRR and thus be more sensitive to Chk1 inhibition than their normal cell counterparts. Rad51 overexpression results in increased HRR as well as resistance to radiation (39). Because p53 is mutated and Rad51 is overexpressed in more than half of all pancreatic carcinomas (40, 41), both of these may offer a therapeutic window for selective sensitization of tumor cells to gemcitabine/radiation by Chk1 inhibitors. Thus, it remains possible that p53 wild-type tumors may still be sensitized through HRR inhibition, and it may be premature to restrict Chk1 inhibitor use to p53 mutant tumors.

Although this study shows that both inhibition of the cell cycle checkpoint and HRR are associated with radiosensitization by AZD7762, the relative importance of these effects remains to be determined. HRR plays an important role in radiation-induced DSB repair in S- and G_2-phase cells (42, 43), and HRR deficiency results in radiosensitization relative to matched HRR-proficient cell types (42, 44, 45). Furthermore, the requirement of HRR inhibition in radiosensitization by Chk1 inhibitors is shown by a lack of radiosensitization by checkpoint inhibition in HRR-incompetent cells (44). HRR inhibition by AZD7762 would render gemcitabine-treated cells extremely sensitive to radiation because gemcitabine arrests cells in S phase (23, 46), in which HRR plays a predominant role. It will be important in future studies to establish a causative link between HRR inhibition and radiosensitization by Chk1 inhibitors.

Because AZD7762 is an inhibitor of both Chk1 and Chk2 (IC_{50} 5 and <10 nmol/L, respectively), our studies cannot exclude the possibility that Chk2 inhibition is involved in AZD7762-mediated radiosensitization. The ability of AZD7762 to inhibit Chk2 activity is suggested by the reversal of the radiation-induced Chk2 mobility shift (Fig. 1C; ref. 47). However, several lines of evidence suggest that inhibition of Chk1 and not Chk2 produces sensitization. We found that depletion of Chk1 but not Chk2 with siRNA produced radiosensitization and, furthermore, depletion of Chk2 did not increase the radiosensitization caused by Chk1 depletion. In addition, the Chk1 inhibitors PD-321852 and PF-00477736 (the latter of which is 100-fold more selective for Chk1 than Chk2) have shown *in vitro* radiosensitizing and chemosensitizing properties comparable with AZD7762 (16, 26, 48). Finally, multiple studies using Chk2 siRNA have shown a lack of effect of Chk2 inhibition on sensitization to radiation or gemcitabine (9–11, 25). Taken together, these results suggest that sensitization by AZD7762 is mediated by inhibition of Chk1.

Our finding that AZD7762 in combination with gemcitabine and radiation produced a significant delay in the growth of pancreatic tumor xenografts with tolerable toxicity supports the development of clinical trials in patients with locally advanced disease. In addition, we have found that AZD7762 is a chemosensitizer to gemcitabine (13),[6] suggesting that AZD7762 may also play an important role in improving both adjuvant therapy and the treatment of metastatic disease. It will be important to define the optimal schedule of administration of AZD7762, gemcitabine, and radiation as well as to identify biomarkers of AZD7762 activity in easily attainable surrogate tissues for future clinical trials.

Disclosure of Potential Conflicts of Interest

Acknowledgments

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