

In vitro and *In vivo* Radiation Sensitization of Human Tumor Cells by a Novel Checkpoint Kinase Inhibitor, AZD7762

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Abstract

Purpose: Inhibition of checkpoint kinase 1 has been shown to enhance the cytotoxicity of DNA-damaging targeted chemotherapy through cell cycle checkpoint abrogation and impaired DNA damage repair. A novel checkpoint kinase 1/2 inhibitor, AZD7762, was evaluated for potential enhancement of radiosensitivity for human tumor cells *in vitro* and *in vivo* xenografts.

Experimental Design: Survival of both p53 wild-type and mutant human cell lines was evaluated by clonogenic assay. Dose modification factors (DMF) were determined from survival curves (ratio of radiation doses for control versus drug treated at 10% survival). Flow cytometry, Western blot, and radiation-induced tumor regrowth delay assays were conducted.

Results: AZD7762 treatment enhanced the radiosensitivity of p53-mutated tumor cell lines (DMFs ranging from 1.6-1.7) to a greater extent than for p53 wild-type tumor lines (DMFs ranging from 1.1-1.2). AZD7762 treatment alone exhibited little cytotoxicity to any of the cell lines and did not enhance the radiosensitivity of normal human fibroblasts (1522). AZD7762 treatment abrogated radiation-induced G₂ delay, inhibited radiation damage repair (assessed by γ -H2AX), and suppressed radiation-induced cyclin B expression. HT29 xenografts exposed to five daily radiation fractions and to two daily AZD7762 doses exhibited significant radiation enhancement compared with radiation alone.

Conclusions: AZD7762 effectively enhanced the radiosensitivity of mutated p53 tumor cell lines and HT29 xenografts and was without untoward toxicity when administered alone or in combination with radiation. The results of this study support combining AZD7762 with radiation in clinical trials. *Clin Cancer Res*; 16(7); 2076-84. ©2010 AACR.

Actively proliferating cells experience blocks in the cell cycle after exposure to ionizing radiation. Blocks that occur in G₁ and G₂ following treatment with radiation and DNA-damaging drugs have been called checkpoints and are presumed to allow DNA damage repair before further cell cycle progression (1). There has been considerable interest in targeting molecular pathways involved with these checkpoints to inhibit repair, particularly in cancer cells (2, 3). Because nearly half of all human tumors have abnormal p53 and thus are unable to arrest in G₁ following DNA damage, attention has primarily focused on the G₂ checkpoint (4). There are several lines of evidence suggesting that the G₂ checkpoint can be exploited to enhance radiosensitivity. The marked radiosensitivity of Ataxia telangiectasia fi-

broblasts is related to the lack of G₂ arrest (5). Caffeine enhances the radiosensitivity of cells primarily through abrogation of the G₂ checkpoint (6). 7-Hydroxystaurosporine has been shown to radiosensitize human tumor cells by abrogation of the G₂ checkpoint (7); however, 7-hydroxystaurosporine can target multiple pathways and has been difficult to develop due to its poor drug-like properties (8).

Both the G₁ and G₂ checkpoints are controlled by the ataxia telangiectasia mutated (ATM)/ataxia telangiectasia and Rad3 related (ATR) signaling pathway (2). Critical downstream molecules in these pathways are the checkpoint kinase (Chk)1 and Chk2 threonine kinases, which facilitate both the G₁ and G₂ checkpoints. Inhibition of these kinases (particularly Chk1) can result in the abrogation of cell cycle progression, premature entry into the cell cycle following DNA damage, and insufficient DNA repair (8). Recently, a novel Chk inhibitor [3-(carbamoylamino)-5-(3-fluorophenyl)-N-[(3S)-3-piperidyl]thiophene-2 carboxamide, AZD7762] was shown to enhance the cytotoxicity of DNA-damaging chemotherapy agents by abrogation of the cell cycle arrest (9). The current study shows that AZD7762 is also a potent radiation sensitizer of p53-compromised cells both *in vitro* and *in vivo*. The mechanism of AZD7762 radiosensitization involved inhibition of radiation-induced DNA damage repair; however, the

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Translational Relevance

This study reports the evaluation of a novel checkpoint kinase inhibitor (AZD7762) as a radiation sensitizer. AZD7762 inhibits downstream checkpoint kinases 1 and 2 of the ATM/ATR DNA damage response pathway. AZD7762 is currently being evaluated in clinical trials to enhance the cytotoxicity of DNA-damaging chemotherapy agents. In the current study, tumor cell lines and xenografts with p53 mutations were shown to be significantly radiosensitized with AZD7762 treatment, whereas normal fibroblasts were not. These data suggest that AZD7762 could also be evaluated in clinical trials as a radiation sensitizer in patients with tumors harboring p53 mutations.

abrogation of the G₂ checkpoint was not an absolute requirement for AZD7762-mediated radiosensitization. Collectively, the preclinical data presented in this study support the evaluation of AZD7762 in human trials as a radiation sensitizer.

Materials and Methods

AZD7762 was obtained from AstraZeneca. Stock solutions of AZD7762 (500 μ mol/L) in DMSO/PBS were diluted with cell culture medium with a final DMSO concentration of <0.1%. For *in vivo* studies, AZD7762 was dissolved in 11.3% 2-hydroxypropyl- β -cyclodextrin (Sigma-Aldrich) in 0.9% sterile saline at a final concentration of 2.75 mg/mL. Mouse monoclonal anti-phospho histone H2AX (Ser139), clone JBW301, rabbit antiserum to histone H2A (acidic patch), and mouse monoclonal β -actin were purchased from Millipore. Rabbit polyclonal pChk1(Ser296) and pChk1(Ser345) antibodies were purchased from Cell Signaling. Mouse monoclonal Chk1 antibody was purchased from Santa Cruz Biotechnology and mouse monoclonal cyclin B antibody was purchased from BD Transduction Laboratories.

Cell survival studies. The following cell lines were purchased from American Type Culture Collection: A549 (human lung adenocarcinoma), H460 (human large cell lung carcinoma), PC3 and DU145 (human prostate carcinoma), MiaPaca2 (human pancreatic carcinoma), and HT29 (human colon carcinoma). Normal human fibroblasts (1522) were purchased from the Coriell Institute for Medical Research. PC-Sw (human pancreatic adenocarcinoma) cells were obtained from Dr. William Sindelar (Towson, MD) (10). The glioblastoma brain tumor cell line (SF-295) was kindly supplied by Dr. Kevin Camphausen (National Cancer Institute, Bethesda, MD). H460-DNp53 cells were constructed by retroviral infection of a dominant-negative p53 construct as previously described (9). All cell lines were grown in RPMI 1640 supplemented with 10% FCS and antibiotics. For cell survival studies, cells were plated (5×10^5 cells/100-mm culture dishes) and incubated for 16 h at 37°C. AZD7762 was added to the exponentially growing cells

1 h before radiation. A range of radiation doses was delivered to cell samples using an Eldorado 8 cobalt-60 teletherapy unit (Theratronics International Ltd.) at dose rates of 2.0 to 2.5 Gy/min. Vehicle control radiation survival curves were conducted in parallel. Twenty-four hours after radiation and drug treatment, cells were trypsinized, counted, plated, and incubated for 10 to 14 d. Colonies were fixed with methanol/acetic acid (3:1) and stained with crystal violet. Colonies with >50 cells were scored and cell survival was determined after correcting for the plating efficiency and for AZD7762 cytotoxicity alone. Survival curve data were fit using a linear quadratic model according to Albright (11). Survival curves for each cell line were repeated two to three times. The dose modification factor (DMF) was determined by taking the ratio of radiation doses at the 10% survival level (control radiation dose divided by the AZD7762-treated radiation dose). DMF values > 1.0 indicate enhancement of radiosensitivity. For plateau phase studies, cells were grown to confluence and maintained without medium change for 3 d after which they were treated with AZD7762/radiation as described above. Flow cytometry analysis confirmed that these cell cultures were enriched in G₁ phase.

Flow cytometry studies. Abrogation of cell cycle checkpoints was evaluated by flow cytometry. Exponentially growing cells were exposed to a single dose of radiation without or with AZD7762 as described above. Cells were collected as a function of time following radiation. Cells were washed with PBS, trypsinized, fixed in cold 70% ethanol in HBSS, centrifuged at 1,000 rpm for 5 min, and the supernatant discarded. The pellet was washed in cold PBS and suspended in 1 mL of 20 μ g/mL of propidium iodide solution containing 0.1% Triton X-100 and 500 ng of DNase-free RNase. Cell cycle distribution was immediately analyzed using a BD FACSCalibur (BD Biosciences).

Western blot analysis. Exponentially growing cells were exposed to a single dose of radiation without or with AZD7762 as described above. As a function of time after treatment, cell samples were rinsed with PBS, lysed with radioimmunoprecipitation assay buffer (Santa Cruz Biotechnology) in the presence of sodium orthovanadate and protease inhibitors (Sigma-Aldrich), incubated for 30 min on ice and centrifuged at $14,000 \times g$, supernatant removed, protein concentration determined (DC Protein Assay, Bio-Rad), aliquoted, and stored at -70°C . For xenograft protein analysis studies, tumors were snap frozen in liquid nitrogen and stored at -70°C . Tumor pieces were homogenized in ice-cold radioimmunoprecipitation assay buffer with protease inhibitors, incubated on ice for 30 min, centrifuged at $10,000 \times g$ for 10 min at 4°C , and the supernatant was removed and recentrifuged at $10,000 \times g$ for 30 min. Supernatant was removed, aliquoted, and stored at -70°C . Protein samples of equal amounts were subjected to SDS PAGE on 4% to 20% Tris-glycine acrylamide gels (Novex-Invitrogen). Following transfer to nitrocellulose, samples were probed with primary antibodies (1:200-2,000), followed by the appropriate secondary antibody diluted to 1:2,000, and were visualized by chemiluminescence (Perkin-Elmer). To confirm equal protein loading and transfer,

Table 1. AZD7762 cytotoxicity alone and radiation DMFs

Cell line	Histology	p53 status	AZD7762 survival alone (%)	DMF*
HT29 (log)	Colon carcinoma	Mut	0.83 ± 0.062	1.7 ± 0.23
HT29 (plateau)	Colon carcinoma	Mut	0.92	0.91
DU145	Prostate carcinoma	Mut	0.82 ± 0.170	1.60 ± 0.17
A549	Lung adenocarcinoma	WT	0.88 ± 0.059	1.20 ± 0.08
1522	Normal skin fibroblast	WT	0.71 ± 0.16	1.05 ± 0.098
H460	Large cell lung carcinoma	WT	0.93 ± 0.08	1.11 ± 0.02
H460 d/n p53	Large cell lung carcinoma	Mut	0.82 ± 0.08	1.58 ± 0.16
MiaPaca2	Pancreatic carcinoma	Mut	0.65	1.70
PC-Sw	Pancreatic adenocarcinoma	Mut	0.99	1.21
SF-295	Brain glioblastoma	Mut	0.57	1.30

Abbreviations: Mut, mutated, WT, wild-type.

*Control radiation dose at 10% survival divided by the AZD7762-treated radiation dose at 10% survival ± SEM.

membranes were stripped by ReBlot Plus (Chemicon) and probed using anti-actin antibody (or other control protein antibody). Densitometric analysis was accomplished with image analyzer software coupled with the Fluorchem FC800 system (Alpha Innotech). Density values for each protein were normalized to actin or other control protein values.

Mitotic catastrophe. Mitotic catastrophe was assessed using a modified procedure (12). Briefly, H460 and 460DNp53 cells were seeded (60,000 cells/chamber) in four-well chamber slides and incubated overnight at 37°C. Cells were exposed to AZD7762 (100 nmol/L) for 1 h and then exposed to 2 Gy. After 24 h, the cell monolayer was rinsed and fresh medium was added. At 24, 48, and 72 h, the medium was removed from the slides and the cells were fixed with cold methanol for 15 min at -20°C. After a PBS wash, slides were blocked with 1% bovine serum albumin/5% goat serum/PBS for 1 h at room temperature followed by incubation with anti- α -tubulin antibody (1:1,000, Sigma) in 1% bovine serum albumin/PBS overnight at 4°C. Texas red-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) was added at a concentration of 1:200 in 1% bovine serum albumin/PBS and incubated at room temperature for 1 h followed by PBS washes. Chambers were removed from the slides and 8 μ L of 4',6-diamidino-2-phenylindole mounting medium (Vector Laboratories) was added. Nuclear fragmentation was defined as the presence of more than two distinct nuclear lobes within a single cell. Two separate experiments were done, each with 300 cells per sample scored on a Zeiss Axiolmager.A1 upright fluorescent microscope using the Axiovision 4.7.2 software.

Xenograft studies. Female athymic nude mice, 5 to 6 wk of age, bred in the National Cancer Institute Animal Production Area, were used for this study. All experiments were carried out under a protocol approved by the National Cancer Institute Animal Care and Use Committee and were in compliance with the Guide for the Care and Use of Laboratory Animal Resource, (1996) National Research Council. For radiation regrowth delay studies, 1.0×10^6

HT-29 cells were injected into the s.c. space of the right hind leg. Mice were ear tagged to monitor tumor volume measurements in individual mice. Tumor growth was followed until the diameter of tumor reached 0.6 to 0.8 mm as measured by a caliper. At this point, the animals were randomized into four groups (eight mice per group): control, fractionated radiation, AZD7762 control, and AZD7762 + fractionated radiation. Fractionated radiation treatment consisted of five daily 2-Gy fractions (Monday to Friday, total radiation dose 10 of Gy). AZD7762 (25 mg/kg) was administered by i.p. injection immediately after each radiation fraction in one study and immediately after each radiation fraction and again 8 h later in a second study. Tumor volume as a function of time is plotted for the various treatments and represents an average tumor volume for each group. Documenting the tumor volume of each individual mouse enabled the determination of the time required (days) for a tumor to reach thrice the starting tumor volume. All tumor growth data were fit using an exponential growth equation; the tumor growth time (days) for control animals was calculated and then subtracted from all treated groups. SDs of the derived values (treated and control) were obtained using the propagation of error formula (13) and then the SDs were used to calculate the Student's *t* test and *P* values for the differences between the various groups (14).

Results

AZD7762-mediated enhancement of radiosensitivity. The activation of pChk1 by radiation was rapid and persisted for several hours postradiation as shown in Supplementary Fig. S1 for DU145 and HT29 cells. Consistent with this activation profile, pilot studies showed that AZD7762 treatment postradiation was more effective than pretreatment protocols and that an AZD7762 concentration of 100 nmol/L yielded maximal radiation enhancement with minimal cytotoxicity alone (data not shown). Subsequently for all *in vitro* studies, AZD7762 (100 nmol/L final concentration) was added to cells 1 hour before radiation

(to ensure pChk1 inhibition at the time of radiation) and left on for 24 hours after radiation followed by clonogenic survival assessment. AZD7762 enhanced the radiosensitivity of multiple cancer cell lines (Supplementary Fig. S2; Table 1; Fig. 1). AZD7762 cytotoxicity alone was minimal for all cell lines studied. Radiation DMFs for AZD7762 were substantially greater for cell lines with p53 mutations (HT29, DU145, and MiaPaca2). Normal p53 wild-type (WT) human fibroblasts (Fig. 1B) showed no radiosensitization with AZD7762. To further test the dependency of AZD7762-mediated radiation sensitization on p53 status, two H460 cell lines were compared that differed only in their p53 status. As shown in Fig. 1C and D, AZD7762 radiosensitized H460 DN p53 cells to a greater extent than H460 WT cells (DMF, 1.58 versus 1.11, respectively). The radiosensitivity of two human pancreatic and one glioblastoma cell lines was also enhanced by AZD7762 (Table 1). All of the studies described above used exponentially growing cell cultures. When confluent (plateau phase) cultures of HT29 cells

were used, no radiosensitization by AZD7762 was observed (see Supplementary Fig. S3; Table 1). Compared with exponentially growing HT29 cells, the plateau phase HT29 cells were enriched in the G₁ cell cycle phase (45% versus 86%, respectively). Thus, active movement through the cell cycle is necessary for maximal AZD7762 radiation sensitization.

AZD7762 abrogates radiation-induced G₂ arrest. Chk1 inhibition has been shown to result in an abrogation of the G₂ checkpoint following treatment with DNA-damaging cytotoxic drugs (9). To determine if AZD7762 might similarly abrogate radiation-induced G₂ arrest, flow cytometry studies were conducted for irradiated cells treated with or without AZD7762. A series of flow profiles were generated for several cell lines as a function of time after treatment (Supplementary Figs. S4-6) and the effects of AZD7762 treatment on the radiation-induced G₂ arrest are summarized in Fig. 2 and Supplementary Fig. S6A and B. Regardless of the p53 status, all cell lines evaluated exhibited a G₂ arrest following radiation treatment. Likewise, AZD7762 abrogated the

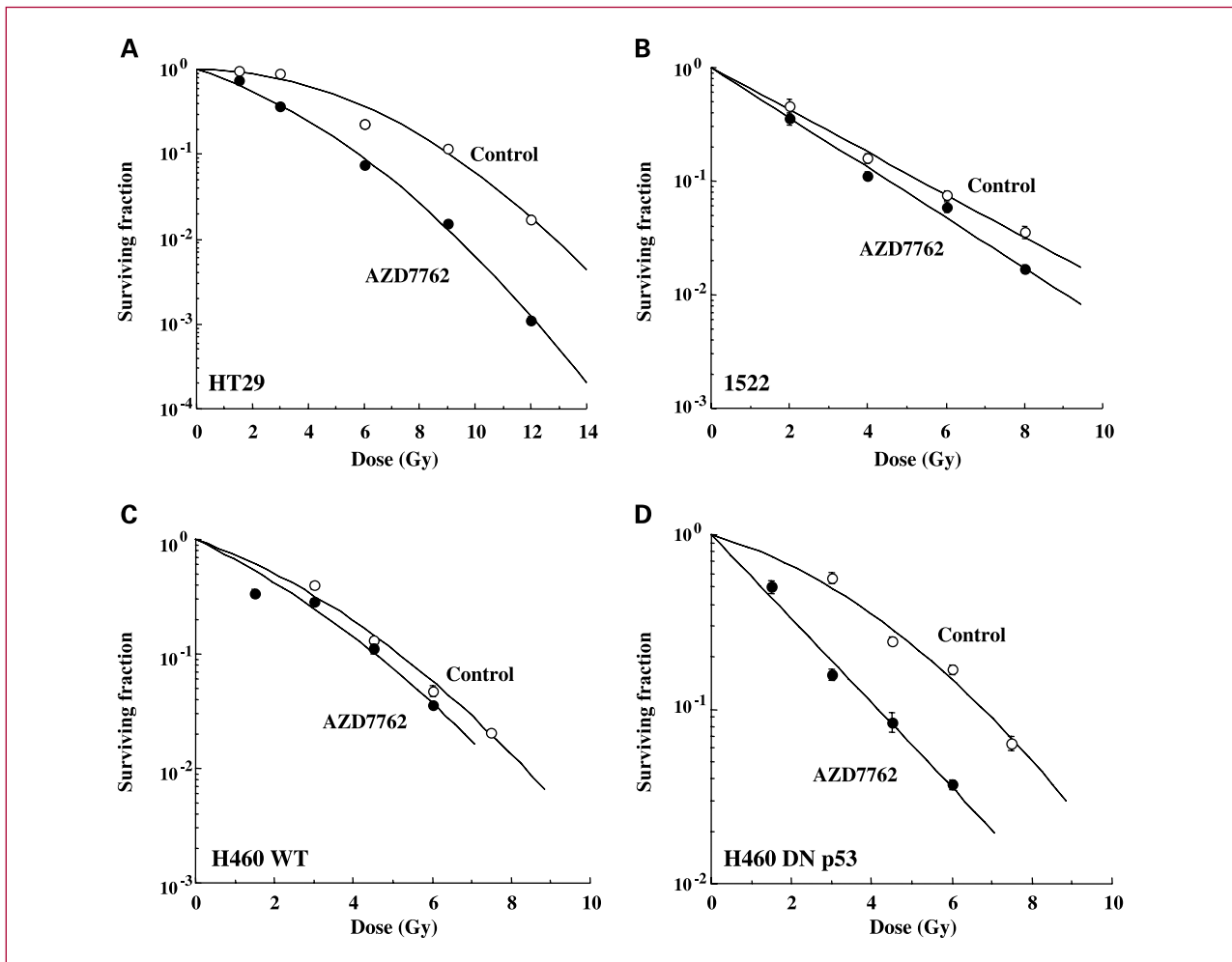


Fig. 1. Radiation survival curves for cell lines treated with AZD7762 (100 nmol/L) 1 h before and 24 h postirradiation (●) or without AZD7762 treatment (○). A, HT29; B, 1522; C, H460; and D, H460 DN p53. DMFs and AZD7762 survival alone are shown in Table 1. Survival curve data from two to three independent experiments were fitted using a linear quadratic model (11).

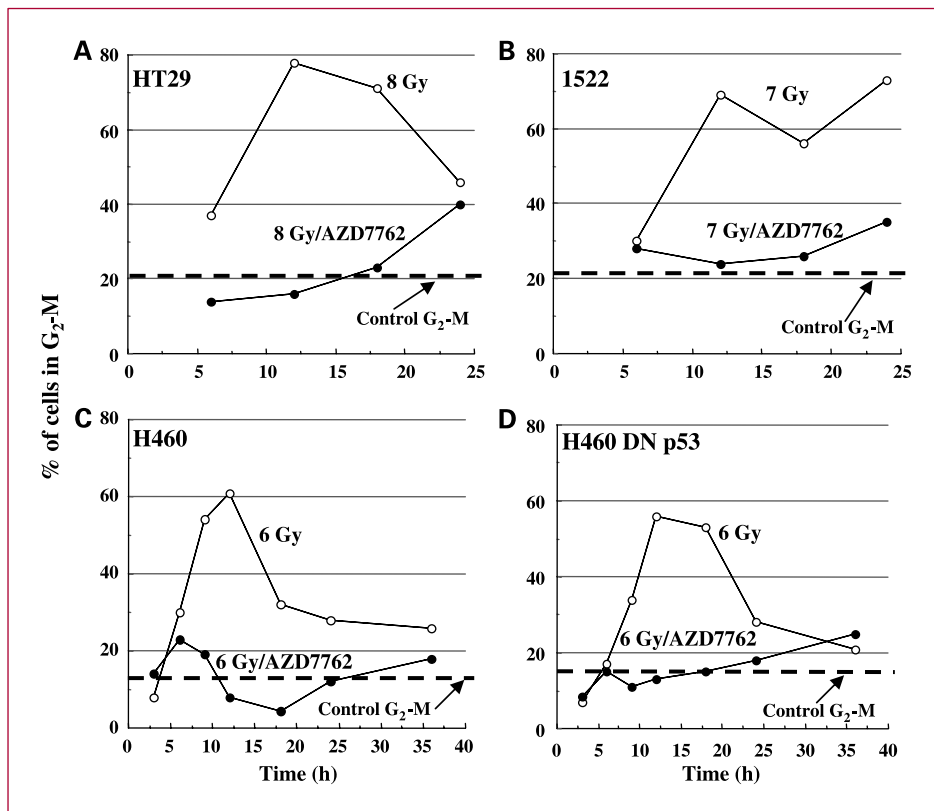


Fig. 2. Percentage of cells in G₂-M assessed by flow cytometry for cells treated with radiation (○) or radiation plus AZD7762 (●) 100 nmol/L, added 1 h before and 24 h postradiation) as a function of time. A, HT29; B, 1522; C, H460; and D, H460 DN p53. Dashed lines, nontreated control G₂-M percentages. Data were derived from flow cytometry profiles (Supplementary Fig. S4-5) and were in qualitative agreement with replicate experiments.

radiation-induced G₂ arrest for all cell lines. Thus, there was no relationship between the abrogation of the G₂ arrest and AZD7762-mediated radiation sensitization.

AZD7762 inhibits radiation-induced DNA damage repair and enhances radiation-induced mitotic catastrophe.

To determine the influence of AZD7762 on radiation-induced DNA damage repair and direct DNA damage, phosphorylated γ H2AX induction (15) and mitotic catastrophe were evaluated, respectively. Figure 3A and B and Supplementary Fig. S7A and B show the effects of AZD7762 on radiation-induced γ H2AX induction for four cell lines. In response to radiation alone, phosphorylated γ H2AX levels rapidly increased following radiation (0.5 hour), but with time returned to near control values by 24 hours, indicating the repair of DNA double-strand breaks. For HT29, DU145, and A549 cells, AZD7762 inhibited repair at 8 and 24 hours postradiation with the most inhibition noted in HT29 and DU145 cells, a small amount repair inhibition in A549 cells, and very little inhibition observed for 1522 cells. AZD7762 treatment alone resulted in elevated levels of phosphorylated γ -H2AX at 24 hours in HT29, DU145, and A549 cells (Supplementary Fig. S7A and B; Fig. 3A), suggesting that AZD7762 mediated a DNA damage/repair response. This was not observed in 1522 cells (Fig. 3B). To determine if DNA damage as manifested by nuclear fragmentation was increased by combination of radiation and AZD7762 treatment, mitotic catastrophe studies were conducted as shown in Fig. 3C and D and Supplementary Fig. S8. For H460 DN p53 cells, radiation treat-

ment alone increased mitotic catastrophe at 24 hours, but returned to near control values at 48 and 72 hours. The combination of AZD7762 and radiation significantly elevated mitotic catastrophe at all time points for H460 DN p53 (Fig. 3C) and HT29 cells (Supplementary Fig. S8). In contrast, there were no significant differences in mitotic catastrophe among the various groups for H460 WT p53 cells across the time course. These data clearly show a correlation between elevated mitotic catastrophe and radiosensitization (see Table 1).

AZD7762 enhances radiation-induced tumor growth delay in HT29 xenografts. HT29 tumor xenografts were next evaluated to determine if AZD7762 would enhance the radiation response *in vivo*. As shown in Fig. 4A, compared with vehicle control, five daily injections of AZD7762 had no effect on tumor growth. Fractionated radiation (2 Gy \times 5) delayed tumor growth and the combination of AZD7762 and fractionated radiation further enhanced the tumor delay; however, the enhancement was not significant ($P = 0.37$, compared with fractionated radiation). Because only one dose of AZD7762 was given after each daily radiation dose, it was questioned whether adequate drug levels were present to inhibit activated Chk1 between the radiation fractions. An *in vivo* study was conducted to determine the duration of pChk1 activation following a single dose of radiation in HT29 xenografts (see Fig. 4B). Radiation treatment activated pChk1 beginning at 3 hours and persisting to 30 hours compared with unirradiated controls. Based on the first xenograft study and the reported half-life

of AZD7762 in mice of 1 to 2 hours (16), another study was conducted in which following each daily radiation fraction, two injections of AZD7762 were given immediately after radiation treatment and 8 hours later as shown in Fig. 4C. As was seen in Fig. 4A, AZD7762 treatment alone had little effect on tumor growth, whereas fractionated radiation

delayed tumor growth similar to that observed in Fig. 4A. The time for tumors to reach thrice the initially measured tumor volume relative to the control for AZD7762 alone, fractionated radiation, and AZD7762 plus fractionated radiation was 2.3 ($P < 0.53$), 7.4 ($P < 0.07$), and 18.7 ($P < 0.00014$) days, respectively. Relative to fractionated

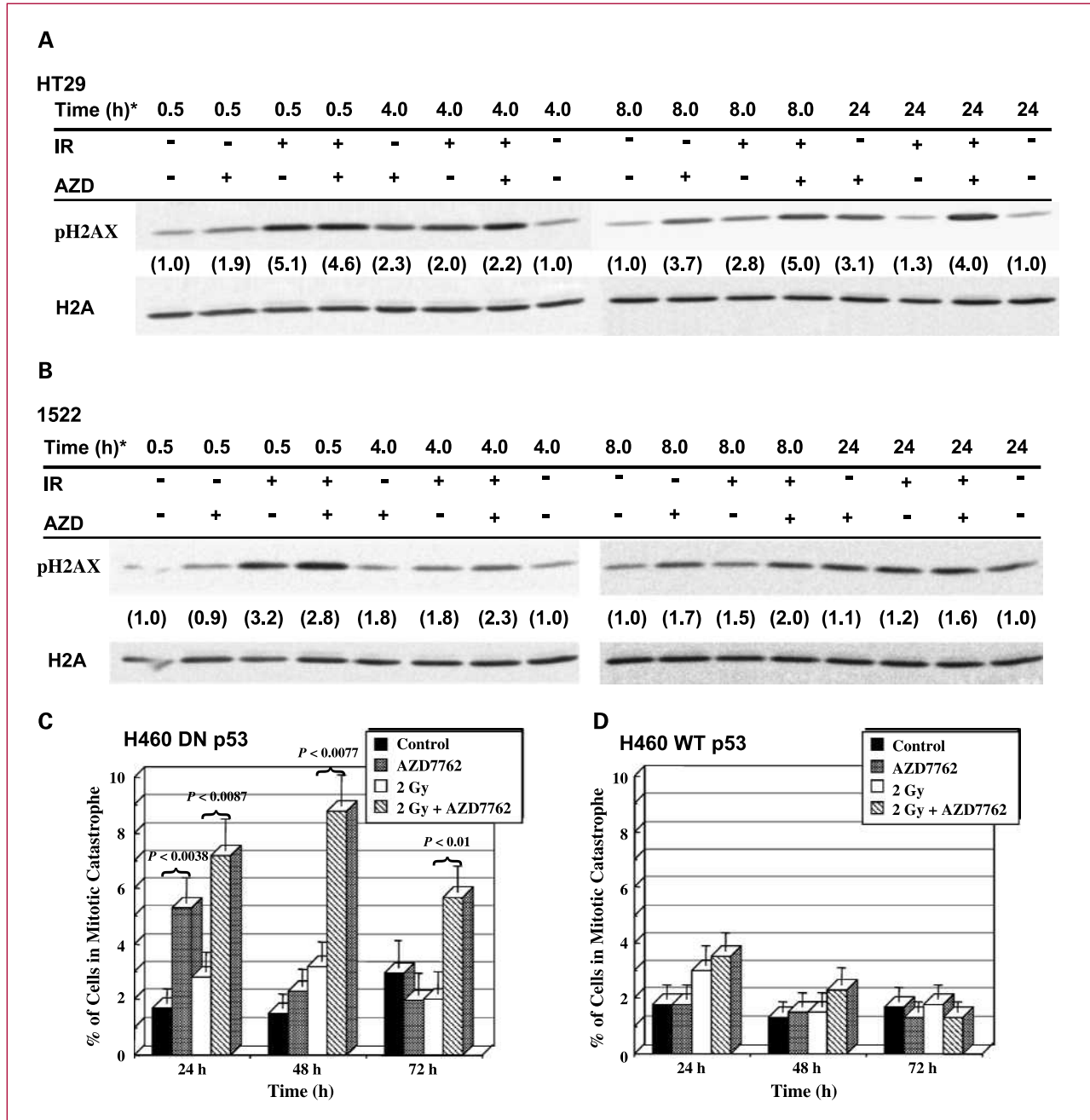


Fig. 3. The effects of radiation with and without AZD7762 treatment (100 nmol/L, added 1 h before and 24 h postradiation) on radiation-induced damage repair and mitotic catastrophe. Western analysis of phosphorylated γ H2AX from HT29 (A) and 1522 (B) cells as a function of time following treatment with AZD7762 and radiation (8 Gy, HT29; 7 Gy, 1522). Normalized density levels of protein bands from Western blots using H2A as the loading control are indicated by values in parenthesis. Additional data are provided for DU145 and A549 cells in Supplementary Fig. S7. Mitotic catastrophe as a function of time following treatment with radiation (2 Gy) or radiation with AZD7762 (100 nmol/L, 1 h before and 24 h postradiation) in H460 DN p53 cells (C) or H460 WT p53 cells (D); P values shown according to Student's t test; MC, mitotic catastrophe.

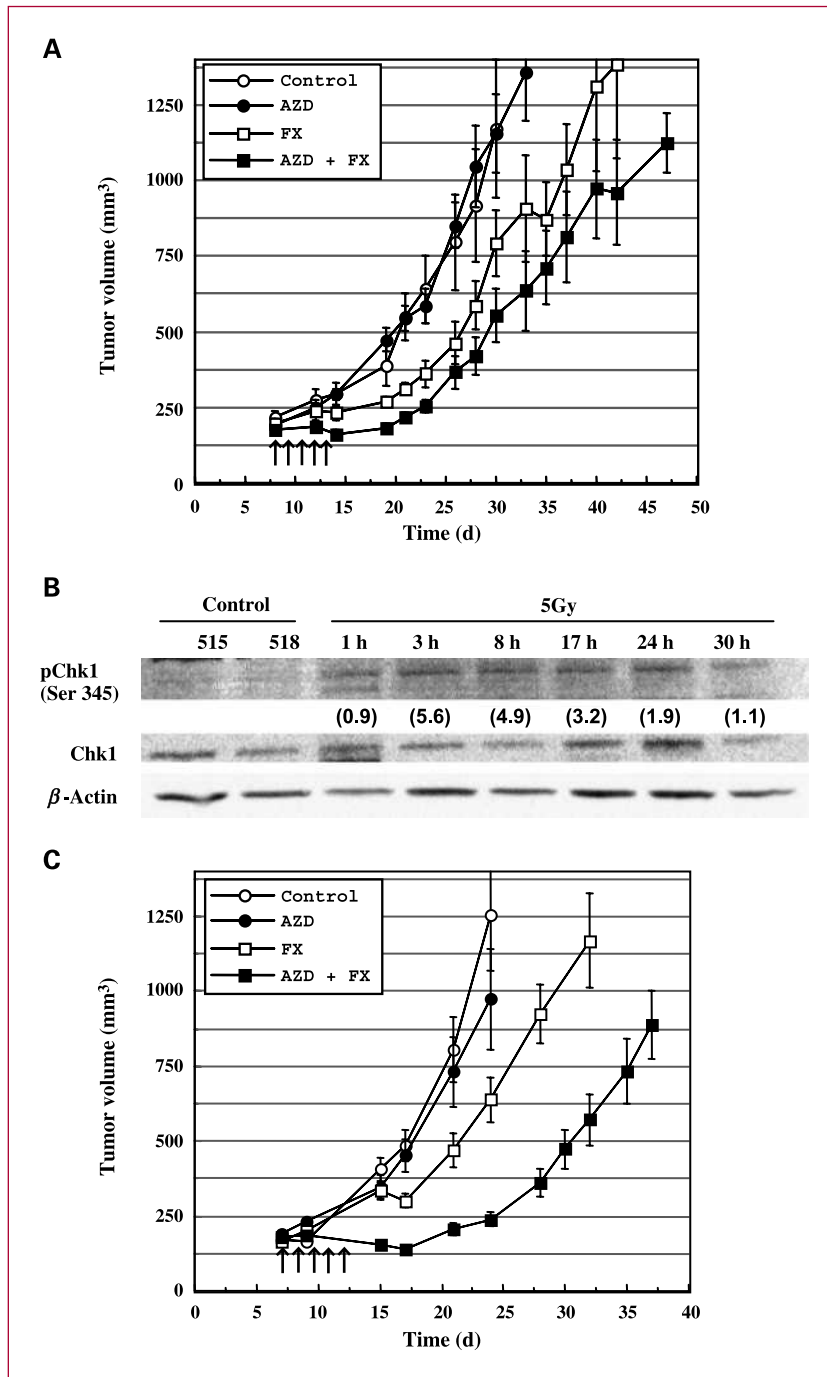


Fig. 4. A, Fractionated radiation (daily 2 Gy fractions $\times 5$) with and without AZD7762 treatment (25 mg/kg, one i.p. injection immediately after each radiation fraction) on radiation-induced tumor regrowth for HT29 xenografts. B, Western analysis of pChk1 from HT29 tumors as a function of time following treatment with radiation (5 Gy). Numbers in parenthesis indicate normalized densities of pChk1 bands. C, fractionated radiation (daily 2 Gy fractions $\times 5$) with and without AZD7762 treatment (25 mg/kg, two i.p. injections immediately and 8 h later for each radiation fraction) on radiation-induced tumor regrowth for HT29 xenografts. *, $P < 0.0038$, relative to fractionated radiation alone.

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radiation alone, the combination of AZD7762 and fractionated radiation was also highly significant ($P < 0.0038$). Thus, the combination of AZD7762 and fractionated radiation showed a greater tumor growth delay than the sum of the individual treatments alone. From this study, it was concluded that two injections of AZD7762 given immediately after each radiation treatment and again 8 hours later provided longer systemic drug levels for pChk1 inhibition. Lastly, AZD7762 treatment alone or in combination with radiation resulted

in no untoward toxicity. Animal weights throughout the study for both groups were similar to the nontreated controls.

Protein biomarkers responsive to AZD7762 and radiation in HT29 xenografts. To identify specific proteins that might be useful in guiding future clinical trials combining radiation with AZD7762, a HT29 xenograft study was conducted. The three proteins evaluated were as follows: γ -H2AX, pChk1, and cyclin B as shown in Fig. 5. As was seen for *in vitro* studies (Supplementary Fig. S7A-B; Fig. 3A-B),

radiation treatment induced γ -H2AX in a time-dependent manner returning to near control levels by 24 hours. AZD7762 plus radiation inhibited the return of γ -H2AX levels at 24 hours, consistent with radiation repair inhibition. Interestingly, AZD7762 alone induced γ -H2AX at all time points evaluated. Both radiation and AZD7762 activated pChk1. In response to radiation treatment, cyclin B was upregulated and AZD7762 when combined with radiation clearly decreased this induction across all time points.

Discussion

Successful cancer treatment with radiation depends heavily on whether a therapeutic gain can be achieved. Sophisticated radiation delivery instrumentation can minimize the normal tissue included in the radiation field; however, invariably normal tissues are included, necessitating a need to identify agents that might differentially radiosensitize tumor as opposed to normal tissues. Cytotoxic chemotherapy combined with radiation is currently used to enhance local tumor control at the expense of increasing normal tissue toxicity (17). Ideally, what is needed are approaches that result in selective tumor radiosensitization.

The current findings suggest that AZD7762-mediated Chk1/2 inhibition may offer considerable selective tumor radiosensitization. AZD7762 did not exert appreciable cytotoxicity alone both *in vitro* and *in vivo*. In addition, the normal human fibroblast cell line 1522 was not radiosensitized by AZD7762, suggesting that other normal tissues would

not be radiosensitized by AZD7762. In general, there was a relationship between AZD7762-mediated radiation sensitization and the p53 status of the cell line. Cell lines that carried p53 mutations were enhanced to a greater extent than p53 WT lines. This was particularly apparent in the H460 cell line pair, in which the only difference between the cell lines was the p53 status. Consistent with the *in vitro* data for HT29 cells, when AZD7762 and fractionated radiation treatment were evaluated in a HT29 xenograft tumor model, significant enhancement in radiation-induced tumor regrowth delay was observed. It should be noted that AZD7762-mediated enhancement of tumor regrowth delay required two daily doses of AZD7762 separated by 8 hours after each radiation fraction, consistent with the prolonged radiation-induced activation of pChk1 (Fig. 4B).

Inhibition of Chk1/2 by AZD7762 has been shown to enhance the cytotoxicity of DNA-damaging chemotherapy drugs in part by abrogation of the G₂ checkpoint. The enhancement was greater in cell lines with compromised p53 status (9). In the current study, AZD7762 treatment resulted in the abrogation of the radiation-induced G₂ delay for every cell line tested (Supplementary Fig. S6A-B; Fig. 2), yet normal 1522 cells were not radiosensitized by AZD7762. Thus, abrogation of the radiation-induced G₂ checkpoint by AZD7762 was insufficient to explain the mechanism of radiosensitization. Like AZD7762, the mechanism for caffeine-mediated radiosensitization has been largely attributed to abrogation of the G₂ checkpoint (18). However, there are reports that show no relationship

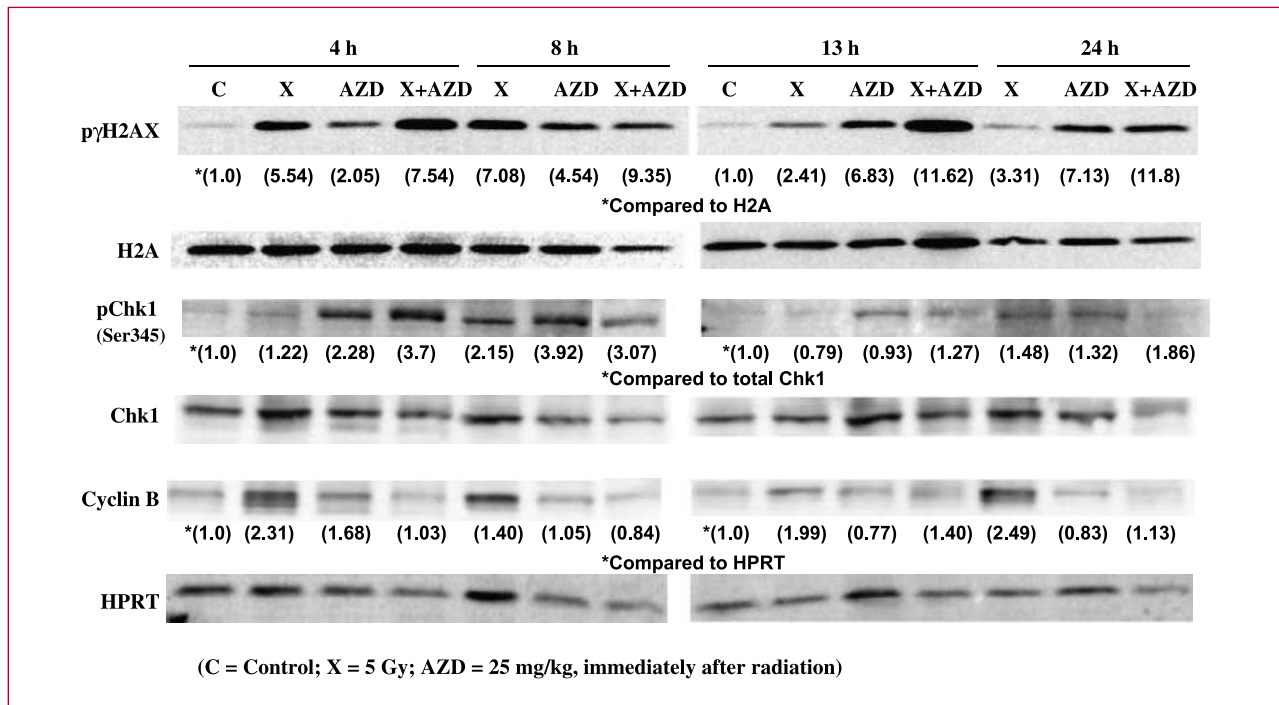


Fig. 5. Western analysis of γ -H2AX, pChk1, and cyclin B proteins taken from HT29 tumors as a function of time following treatment with radiation (5 Gy) with or without AZD7762 (25 mg/kg administered immediately after radiation). Numbers in parenthesis beneath each row indicate fold increase/decrease of proteins relative to respective loading controls.

between radiation-induced G_2 abrogation with caffeine and radiosensitization (19). Other mechanisms identified in the current study that may be more pertinent include the effects of AZD7762 on radiation-induced repair. It has been proposed that Chk1 is required for homologous recombination repair (HRR; ref. 20), which normally occurs in the S and G_2 phase (21). Likewise, another major repair pathway is the nonhomologous end joining (NHEJ), which predominantly occurs in G_1 (22). Because p53-mutated cells lack a G_1 checkpoint, they may be more dependent on HRR as opposed to nonhomologous end joining. WT p53 cells, expressing both a G_1 and G_2 checkpoint following radiation treatment, should be capable of utilizing both types of repair. Thus, it would be anticipated that Chk1/2 inhibition would predominantly affect HRR in p53-mutated cells (20). Consistent with this was our findings that AZD7762 inhibited the repair of radiation-induced damage (γ -H2AX) and enhanced mitotic catastrophe, which led to greater radiosensitization in p53-mutated cells. Further support for the inhibition of HRR by Chk1/2 inhibition comes from plateau phase HT29 cells, which were not radiosensitized by AZD7762 (Supplementary Fig. S3). Plateau phase HT29 cells were predominantly in G_1 (86%) during the radiation and AZD7762 treatment. It is interesting to speculate that repair of radiation damage in plateau phase cells would be through NHEJ and not affected by Chk1/2 inhibition. Studies are ongoing to test this hypothesis.

Several protein biomarkers from xenograft studies were identified as potential surrogates to guide clinical trials

with AZD7762 and radiation. As was seen for *in vitro* studies, AZD7762 alone and radiation activated γ -H2AX levels. AZD7762 combined with radiation inhibited the return of γ -H2AX to normal levels. The reason for the AZD7762 induction of γ -H2AX is not clear; however, it may be activated as a result of replication stress (23, 24). Interestingly, pChk1 was activated by both radiation and AZD7762 alone. The latter might be indicative of a DNA damage response associated with γ -H2AX activation. Lastly, radiation was shown to induce cyclin B and AZD7762 markedly inhibited its induction, consistent with the radiation-induced G_2 abrogation observed in *in vitro* studies. Collectively, various combinations of these markers may give indication that AZD7762 is targeting necessary pathways to elicit tumor radiosensitization in clinical trials.

Disclosure of Potential Conflicts of Interest

S. Zabludoff, employed by AstraZeneca; all other authors are employed by the National Cancer Institute.

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