AZD7762, a novel checkpoint kinase inhibitor, drives checkpoint abrogation and potentiates DNA-targeted therapies

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Abstract

Insights from cell cycle research have led to the hypothesis that tumors may be selectively sensitized to DNA-damaging agents resulting in improved antitumor activity and a wider therapeutic margin. The theory relies on the observation that the majority of tumors are deficient in the G1-DNA damage checkpoint pathway resulting in reliance on S and G2 checkpoints for DNA repair and cell survival. The S and G2 checkpoints are regulated by checkpoint kinase 1, a serine/threonine kinase that is activated in response to DNA damage; thus, inhibition of checkpoint kinase 1 signaling impairs DNA repair and increases tumor cell death. Normal tissues, however, have a functioning G1 checkpoint signaling pathway allowing for DNA repair and cell survival. Here, we describe the preclinical profile of AZD7762, a potent ATP-competitive checkpoint kinase inhibitor in clinical trials. AZD7762 has been profiled extensively in vitro and in vivo in combination with DNA-damaging agents and has been shown to potentiate response in several different settings where inhibition of checkpoint kinase results in the abrogation of DNA damage-induced cell cycle arrest. Dose-dependent potentiation of antitumor activity, when

AZD7762 is administered in combination with DNA-damaging agents, has been observed in multiple xenograft models with several DNA-damaging agents, further supporting the potential of checkpoint kinase inhibitors to enhance the efficacy of both conventional chemotherapy and radiotherapy and increase patient response rates in a variety of settings. [Mol Cancer Ther 2008;7(9):2955–66]

Introduction

Conventional DNA-targeting therapies such as chemotherapy and radiation are among the most common cancer treatments and have produced significant increases in patient survival, particularly when used in combination with drugs having different mechanisms of action. Due to their efficacy, DNA-targeting agents are likely to remain a standard of care for the treatment of many cancers for the foreseeable future. Although effective, this mechanism also leads to significant side effects as the majority of these agents are used at the maximum tolerated dose (MTD). Toxicities to the hematologic, gastrointestinal, and other organ systems are commonly observed and limit the degree of tumor control that can be achieved. Another limitation of DNA-damaging agents is that many patients develop resistance and therefore become refractory to treatment. Resistance can arise from multiple mechanisms including modulation of cellular levels of the drug, defects in apoptosis, or DNA repair.

DNA damage induced by chemotherapy or radiation therapy occurs through multiple mechanisms (e.g., alkylating agents, topoisomerase poisons, and antimetabolites). Although different mechanisms are required to repair each type of DNA damage, common to all is the cellular response of cell cycle arrest regulated by signaling cascades that are generally referred to as cell cycle checkpoint pathways.

Checkpoint signaling is initiated following genotoxic insult by the proximal kinases, ATR and ATM, two phosphatidylinositol 3-kinase family members. Activation of these kinases leads to activation of the effector kinases, checkpoint kinases 1 and 2 (Chk1 and Chk2; serine/threonine kinases). The activated effector kinases are then able to transiently delay cell cycle progression through the G1, S, or the G2 phases so that DNA can be efficiently repaired. The ATM/Chk2 pathway predominantly regulates the G1 checkpoint and the ATR/Chk1 pathway the S and G2 checkpoints. However, there is cross-talk between the pathways implying a role for both ATR and ATM pathways in all cell cycle checkpoints. In addition to directly regulating the cell cycle, the pathways also affect DNA repair, transcription, chromatin regulation, and cell death.
The hypothesis that tumors may be selectivity sensitized to DNA-damaging agents through abrogation of the G2-DNA damage checkpoint was first proposed in the 1980s followed by further support in the 1990s (1–3). The theory relies on the observation that the majority of tumors are deficient in the G1-DNA damage checkpoint pathway or other components of checkpoint signaling and response. For example, high p53 mutation rates result in reliance on S and G2 checkpoints to repair DNA damage and promote cell survival. Therefore, abrogation of these remaining intact checkpoints should lead to enhanced tumor cell death compared with normal tissue. Inhibition of Chk1 signaling using small-molecule inhibitors, dominant-negative enzymes, interference RNA, and ribozymes leads to abrogation of the S and G2 checkpoints, impaired DNA repair, and increased tumor cell death (4–12).

The present studies describe the identification and characterization of AZD7762, a novel, ATP-competitive and selective checkpoint kinase inhibitor in clinical trials. AZD7762 potently inhibits Chk1 and Chk2, abrogates DNA damage-induced S and G2 checkpoints, enhances the efficacy of gemcitabine and topotecan, and modulates downstream checkpoint pathway proteins as predicted. Furthermore, AZD7762 potently abrogates these same checkpoints in vivo and significantly enhances the anti-tumor efficacy of gemcitabine and irinotecan in rodent tumor models at well-tolerated doses.

Materials and Methods

Compounds
AZD7762 [3-(carbamoylamino)-5-(3-fluorophenyl)-N-[35]-3-piperidyl]thiophene-2-carboxamide (Fig. 1) was synthesized at AstraZeneca R&D. Gemcitabine, irinotecan, and SN-38 were obtained from IC International.

High-Throughput Screen and Chk1 Kinase Assay
Recombinant human Chk1 was expressed as a glutathione S-transferase fusion in insect cells using a baculovirus vector and purified by glutathione affinity chromatography. A synthetic peptide substrate for Chk1 was synthesized by Bachem (N-biotinylaminohexanoyl-KKVSRSGLYRSPMPENLNRPR). Compounds were screened at a single concentration (10 μmol/L) for the primary assay using a standard scintillation proximity assay protocol. For follow-up, a dose response was determined (IC50). Final assay concentrations of peptide and ATP (cold + 40 nCi [33P]ATP) were 0.8 and 1 μmol/L, respectively. In brief, compound, buffer containing peptide and kinase and ATP, were added sequentially to a 384-well assay plate. The plate was incubated for 2 h, reaction was stopped by the addition of buffer containing EDTA and scintillation proximity assay beads (Amersham), and plates were read using a TopCount reader (Packard). Data analysis was carried out using proprietary software.

For kinetic analysis, a filter binding assay was used. The assay reaction contained the reagents listed above, but with the following concentrations of ATP [0-600 μmol/L (cold + Ci [33P]ATP), determined Km], Chk1 (0.5 nmol/L), and AZD7762 (0, 1.5, 5, 10 nmol/L). The reaction was incubated at room temperature for 20 min, stopped by the addition of EDTA, transferred to Streptavidin Flashplate (Perkin-Elmer), incubated at room temperature for 1 h, aspirated out, and wells washed with PBS. Plates were counted using a TopCount reader and data were analyzed using proprietary software.

Cells
Cells were obtained from the American Type Culture Collection unless otherwise indicated. HT29 and HCT116 cells were cultured in McCoy’s medium (Invitrogen) containing 10% FCS; SW620, H460-DNp53, HCT116 (p53+/+ and p53−/−), a generous gift from Dr. Bert Vogelstein, Johns Hopkins University, to G.K. Schwartz) and MDA-MB-231 cells were cultured in RPMI containing 10% FCS. H460-DNp53 was made by retroviral infection of a dominant-negative p53 construct. Individual clones were selected and characterized by evaluating endogenous p53 up-regulation, lack of p21 up-regulation, and G1 arrest following treatment with actinomycin D.

Antibodies
Anti-cdc25A was obtained from Santa Cruz; anti-cdc2 and anti-phospho-cdc2(T15) were from Cell Signaling Technology; anti-phospho-histone H3 (phH3; S10) and anti-cyclin B were from Upstate Biotechnology; and anti-cyclin A was from Neomarkers.

Western Blot Analysis
SW620 cells (3 × 105 per 10 cm plate) were seeded, incubated overnight, and treated with compounds solubilized in DMSO or vehicle (0.01% DMSO). Protein lysates were prepared in Phospho Safe buffer (Novagen) and protein concentrations were determined by BCA protein assay kit (Pierce). Standard Western blot analyses were conducted using the antibodies listed above.

Checkpoint Abrogation Assay
HT29 cells (3 × 105) were seeded in 96-well plates and incubated overnight. Cells were treated for 2 h with camptotheacin (topoisomerase I inhibitor; 0.07 μg/mL) to induce the G2 checkpoint. Cells were then treated for 20 h with vehicle (0.5% DMSO) or caffeine (4 mmol/L; positive control) plus nocodazole (1 μg/mL) or a 12-point titration of AZD7762 (12.5 μmol/L to 6 nmol/L) plus nocodazole. Nocodazole alone-treated cells with no camptothecin pretreatment were used to determine the maximum mitotic index. Cells were fixed with 3.7% formaldehyde for 1 h, permeabilized with PBS containing 0.05% Triton X, and incubated with anti-phH3 antibody for 1 h followed by Alexa Fluor 488 anti-rabbit (Molecular Probes) and Hoechst stain for 1 h. Mitotic index was determined on the ArrayScan and expressed as the percentage of cells undergoing mitosis. The EC50 was calculated by concentration-response curve fitting using three-variable logistical equations within XLfit (model 205) with the curve bottom constrained to 0 and the top constrained to 100 by nocodazole alone treatment.

Potentiation Assays
SW620 (5.5 × 103 per well) or MDA-MB-231 (5 × 103 per well) cells were seeded in 96-well plates and incubated...
overnight. Cells were dosed for 24 h with a 9-point titration of gemcitabine ranging from 0.01 to 100 nmol/L with or without a constant dose of AZD7762 (300 nmol/L). Control wells were dosed with vehicle alone (0.1% DMSO) or 300 nmol/L AZD7762. After 24 h, medium was removed and AZD7762 alone was added back to the wells treated previously with AZD7762 for an additional 24 h. Cells were then incubated in drug-free medium for an additional 72 h. The effect on cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethophenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay as recommended by the supplier (Promega). The same experimental procedure was used for topotecan combinations (topoisomerase I inhibitor, analogue of camptothecin) except an 11-point titration of topotecan ranging from 0.1 nmol/L to 30 μmol/L was used. Net growth was calculated \( \frac{A_{T120} - A_{T0}}{A_{T0}} \times 100 \) and plotted versus concentration of chemotherapy in the presence and absence of AZD7762. IC\(_{50}\) values were calculated by concentration-response fitting using four-variable logistical equations (Sigmoidal fit) within Origin Pro.

**Clonogenicity Assays**

Log-phase parental and p53-null HCT116 cells were plated, in triplicate, onto 100-mm dishes at 1,000 to 5,000
per dish. Cells were treated with increasing concentrations of gemcitabine alone for 2 h, gemcitabine followed immediately by 100 nmol/L AZD7762 for 24 h, or gemcitabine followed by drug-free medium for 24 h before AZD7762. At the end of treatment, cells were cultured in drug-free medium for 10 to 14 days. The resulting colonies were scored after staining with 0.01% crystal violet.

**In vivo Studies**

**Animals.** Male NCr mice were purchased from Taconic Laboratories and male mnu rats were purchased from Charles River. All procedures were conducted in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and within the protocols approved by the Institute of Animal Care and Use Committee at AstraZeneca.

**Hollow Fiber Assay.** Fibers made from polyvinylidene difluoride (molecular weight cutoff, 500 kDa; Spectrum) were immersed in 70% ethanol for 72 h, flushed with distilled water, and autoclaved. HCT116 cells were suspended in McCoy’s medium with 20% fetal bovine serum and loaded into the fibers at 3 × 10⁶/mL. HCT116 cells have a strong G2 arrest and thus it is an ideal cell line to measure abrogation of G2 arrest. Following overnight incubation in McCoy’s medium, topotecan (30 nmol/L) was added to the appropriate groups and allowed to incubate for 18 h before fibers were rinsed and implanted into the mice. Animals were dosed i.v. with AZD7762. The fibers were recovered 30 h later; cells were flushed out, fixed in 70% ethanol, and stained with propidium iodide. Samples were analyzed by flow cytometry. Modfit was used to calculate detailed cell cycle profiles and results were reported as odds ratio values. The odds ratio is defined as the ratio of G2-to-G1 populations for topotecan alone versus in combination with AZD7762.

**Xenograft Models in Mice.** Tumor cells were harvested, pelleted by centrifugation for 5 min, and resuspended in sterile PBS. Cells (3 × 10⁶ × 6 × 10⁵) were implanted s.c. into the right flank of the mice in a volume of 0.1 to 0.2 mL using a 25-gauge needle. Tumors were allowed to grow to the designated size of ~100 to 200 mm³ before the administration of compound.

**Xenograft Models in Rats.** Cells were harvested, pelleted by centrifugation for 5 min, and resuspended in 50% sterile PBS and 50% Matrigel. Rats received a 5 Gy whole-body radiation dose 5 days before cell implantation to improve tumor growth. H460-DNp53 cells (1 × 10⁶) were implanted s.c., under anesthesia with isoflurane, into the right flank of the rats in a volume of 0.2 mL using a 25-gauge needle. Tumors were allowed to grow to the designated size of ~100 to 200 mm³ before the administration of compound.

**Efficacy studies.** Compounds were administered by i.v. injection via the tail vein. AZD7762 was formulated in 11.3% hydroxypropyl-β-cyclodextrin. Cyclic schedules were used and treatment ranged from three to five cycles. Each cycle included administration of a standard agent [gemcitabine or irinotecan (topoisomerase I inhibitor, camptothecin analogue)] every 3 days followed by delivery of AZD7762 as described in the figure legends. Tumor volumes were measured with electronic calipers and calculated using the formula [length × (width × width)] × 0.5. log cell kill = GD / (3.32 × TD), where GD is the growth delay and TD is the tumor doubling time of the control.

**Results**

**High-Throughput Screening Led to the Identification of Thiophene Carboxamide Ureas as Potent Chk1 Inhibitors**

The AstraZeneca compound library was screened using the Chk1 enzyme assay with an overall hit rate of 2.2%. Six thousand compounds were profiled in dose response and ~30% of these had IC₅₀ < 30 µmol/L. The screen performance was robust and reproducible with consistent Z’ values between 0.7 and 0.8. The flow diagram in Fig. 1 shows the high-throughput screen process, statistics for the screen, and early follow-up.

Cluster analysis revealed several structural series, three of which were selected for further optimization. From these studies, the thiophene carboxamide urea series emerged as the most promising as this structural class provided not only potent Chk1 inhibitors but also excellent solubility and drug metabolism and pharmacokinetic properties.

**AZD7762 Is a Potent and Selective Inhibitor of Chk1 Kinase**

AZD7762 was discovered as a potent and selective Chk1 kinase inhibitor through multiple rounds of structure-activity relationship-driven chemistry (Fig. 1). The activity of AZD7762 was evaluated against human recombinant Chk1 and a panel of protein kinases in vitro. AZD7762 potently inhibited Chk1 phosphorylation of a cdc25C peptide with an IC₅₀ of 5 nmol/L as measured by a scintillation proximity assay. Due to the determined high Kₘ (80 µmol/L) of Chk1 for ATP, the scintillation proximity assay could not be run at Kₘ. However, data generated by a filter capture assay, which contained ATP concentrations at Kₘ, yielded an IC₅₀ of 5 nmol/L. The Kᵢ for AZD7762 was determined to be 3.6 nmol/L. Kinetic characterization suggested that AZD7762 binds in the ATP-binding site of Chk1 and is thought to compete directly for ATP binding in a reversible manner.

In selectivity screens, AZD7762 showed significantly less inhibition against unrelated protein kinases. In general, kinases with less than 10-fold selectivity were from the same family of kinases as Chk1, CAM kinases, or Src-like kinases from the tyrosine kinase family although not Src itself (Yes, Fyn, Lyn, Hck, and Lck). Following preliminary profiling, expanded screening was biased toward CAM and tyrosine kinase family members. Data generated from in-house and Upstate screening indicated good selectivity (>10-fold) of Chk1 kinase versus 164 kinases (Supplementary Tables S1 and S2). Importantly, selectivity was shown

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4 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
in the cyclin-dependent kinase (cdk) 1/cyclin B1 scintillation proximity assay (>1,000-fold) and other cdks tested. Greater than 100-fold selectivity against multiple protein kinase C isoforms, cdks, p38 and MAPKAP kinase 2 clearly differentiates the activity profile of AZD7762 from UCN-01 (13–15). AZD7762 is equally potent against Chk2. Low-fold selectivity was observed against phosphoinositide-dependent kinase (isolated enzyme), but in cellular assays AZD7762 did not affect the phosphoinositide-dependent kinase pathway (data not shown).

AZD7762 Abrogated Checkpoint Cell Cycle Arrest Mediated by DNA-Damaging Agents

The ability of AZD7762 to abrogate the G2 checkpoint was determined in the checkpoint abrogation assay. Cells were pretreated with camptothecin for 2 h followed by AZD7762 or vehicle treatment and nocodazole for 20 h. Cells were then analyzed for pH3. Cells treated with camptothecin arrest in G2 and did not stain for pH3. Cells treated with camptothecin and AZD7762 did not arrest in G2, moved into mitosis, and were trapped by nocodazole. These cells stained for pH3 indicating abrogation of the G2 checkpoint. AZD7762 was shown to abrogate the G2 arrest induced by camptothecin with an average EC50 of 10 nmol/L (n = 12) and maximal abrogation in the range of 100 nmol/L. In the absence of camptothecin, AZD7762 alone induced cell arrest with an EC50 0.620 μmol/L, >50-fold higher than the concentrations required to abrogate the damage induced phenotype.

Combination Treatments Led to Anticipated Effects on Cell Cycle Proteins

Gemcitabine and SN-38 (active metabolite of irinotecan) lead to cell cycle arrest at the S and G2 checkpoints, respectively. Therefore, different downstream cell cycle proteins and checkpoint kinase substrates must be evaluated for each combination. The effect on cell cycle proteins was evaluated at 8 h (continuous exposure to agents) to evaluate the more immediate effects and 30 h (8 h drug treatment followed by 22 h washout) to evaluate the cell cycle proteins following the release from checkpoint kinase inhibition (Fig. 2A and B). Treatment with gemcitabine and
alone led to the predicted destabilization of cdc25A, a direct substrate of Chk1, which on Chk1 activation is phosphorylated and consequently degraded. AZD7762 treatment stabilized cdc25A. Importantly, the combination led to a dose-dependent increase in cdc25A. At 30 h, the significant effect of Chk1 inhibition on the stabilization of cdc25A was no longer observed due to the reversibility of AZD7762. Cyclin A levels, in contrast, were decreased for the combination, consistent with the abrogation of the S-phase checkpoint and the effect on the cell cycle (Fig. 2A and B).

On activation of the G2 checkpoint by SN-38, cdc25C, a checkpoint kinase substrate, is phosphorylated and inactivated. Subsequently, inactivation of cdc25C leads to an...
increase in phosphorylation of cdc2 (Tyr15). A high-quality cdc25C antibody was not identified, so the effect on cdc25C could not be evaluated directly. However, consistent with checkpoint kinase inhibition, combination treatment with SN-38 decreased phospho-cdc2 (Tyr15) at 8 and 30 h. Cyclin B levels also decreased, which is consistent with abrogation of the G2 checkpoint. phH3, a marker for mitotic cells, decreased on treatment with irinotecan, indicating blocked mitotic entry. In combination, phH3 increased, again consistent with abrogation of the G2 checkpoint (Fig. 2C and D).

AZD7762 Enhanced the Activity of DNA-Damaging Agents

AZD7762 showed robust gemcitabine potentiation and enhanced activity of topotecan-mediated inhibition of cell growth. Potentiation was observed in SW620 colon cancer cells (p53 mutant) with gemcitabine (Fig. 3A). It is interesting to note that stasis was observed only in the SW620 cells at the highest dose of gemcitabine tested, whereas cell death occurred in the combination treatment because the curves dropped below 0% net growth. Additional cell lines were tested with the most consistent and profound potentiation observed in combination with gemcitabine (data not shown). Enhanced activity was observed in MDA-MB-231 breast carcinoma cells (p53 mutant; Fig. 3B) with topotecan. MDA-MB231 cells compared with SW620 cells showed a slight reduction in percent net growth from AZD7762 alone.

Enhanced Cytotoxicity with AZD7762 Is Observed in p53-Mutant Tumor Cell Lines

To examine the effect of p53 loss on tumor sensitivity to combined treatment with chemotherapy and checkpoint inhibitor, we studied clonogenic inhibition by gemcitabine and AZD7762 in an isogenic pair of colon cancer cell lines. Parental and p53-null HCT116 cells were equally sensitive to a 2-h treatment of single-agent gemcitabine (Fig. 3C). Sequential treatment with gemcitabine followed immediately by 100 nmol/L AZD7762 for 24 h (Gem→AZD; Fig. 3C) or by drug-free medium for 24 h before AZD7762 (Gem→ND→AZD; Fig. 3C) resulted in a marked potentiation of clonogenic loss, more selectively in cells that lack p53. These data are consistent with the overall hypothesis that checkpoint inhibitors specifically enhanced the cytotoxicity of DNA-damaging agents in checkpoint-defective p53-mutant tumors.

AZD7762 Abrogated the G2 Checkpoint In vivo

A hollow fiber pharmacodynamic model was developed as shown in Fig. 4. The assay was used to measure the in vivo pharmacodynamic activity of checkpoint kinase inhibitors in nude mice and rats for both compound selection and to determine the pharmacokinetic/pharmacodynamic relationship. As shown in Fig. 4B, topotecan

Figure 4. AZD7762 abrogated the G2 checkpoint in a novel hollow fiber assay. A, schematic diagram illustrating the hollow fiber assay. HCT116 cells were loaded into prepared fibers, incubated in medium, and then incubated in topotecan-containing medium for 18 h. Fibers were implanted into mice and AZD7762 was delivered i.v. Thirty hours later, fibers were removed from the mice and cells from the fibers were fixed with ethanol and stained with propidium iodide. Cell cycle profiles were determined by fluorescence-activated cell sorting analysis. B, fluorescence-activated cell sorting profiles of cells treated with vehicle control, topotecan alone, or topotecan followed by AZD7762. The percentage of cells in the G1 and G2 phase and calculated odds ratio values are listed. C, dose-dependent increase in the odds ratio following treatment with AZD7762.
treatment typically arrested 70% to 90% of cells in the G2 phase, and this arrest was relatively stable for up to 30 h post-implantation. Checkpoint kinase inhibition abrogated the G2 checkpoint causing a reduction in the number of cells in G2, with a concomitant increase in the number of cells reentering the G1 phase (Fig. 4B). The odds ratio is defined as the ratio of G2-to-G1 populations for topotecan alone versus in combination with AZD7762. Thus, the greater the odds ratio, the higher the degree of checkpoint abrogation. This analysis allows pharmacodynamic activity to be quantitatively assessed and statistical significance to be determined. As seen in Fig. 4C, AZD7762 treatment resulted in a dose-dependent increase in abrogation of the G2 checkpoint.

AZD7762 Potentiated the Tumor Efficacy of DNA-Damaging Chemotherapy

The ability of AZD7762 to potentiate gemcitabine and irinotecan was evaluated in mouse and rat xenograft models. Mouse xenograft studies were done using either the H460-DNp53 mutant human lung cancer cell line or the SW620 colorectal tumor cell line. The H460-DNp53 cell line is relatively resistant to gemcitabine single-agent treatment, whereas SW620 cells are more sensitive. Established tumors were treated with vehicle, gemcitabine alone, AZD7762 alone, or gemcitabine in combination with AZD7762 (Fig. 5). In the H460-DNp53 xenograft study, neither gemcitabine nor AZD7762 alone showed significant antitumor activity (Fig. 5A). In contrast, AZD7762 significantly potentiated gemcitabine in the combination group resulting in a log cell kill of 0.9 or percent treated/control (%T/C) of 26. There was a slight but not statistically significant body weight loss (7%) in the combination group compared with the gemcitabine alone control group (no loss). In the mouse SW620 xenograft study, AZD7762 in combination with gemcitabine showed significant...
antitumor activity compared with either agent alone. Similar efficacy was achieved within the low- and high-dose combination group (Fig. 5A). No significant increase in body weight loss was observed in the combination group over the single-agent gemcitabine treatment group. In summary, AZD7762 significantly enhanced the activity of gemcitabine without significantly affecting the gross toxicology associated with gemcitabine therapy in the nude mouse.

In the rat H460-DNp53 xenograft study, AZD7762 potentiated the antitumor activity of gemcitabine in a dose-dependent manner (Fig. 5B) as evidenced by a decrease in %T/C with increasing dose (48% and 32%, 10 and 20 mg/kg AZD7762, respectively; Fig. 5B). In the rat model (unlike the mouse), gemcitabine was delivered at the MTD (10 mg/kg), and AZD7762 was still able to potentiate gemcitabine. In this xenograft study, gemcitabine alone caused severe toxicity (deaths or moribund) in 4 of 10 rats a few days after the third dose. Additional single-agent gemcitabine-related toxicities at this dose included skin rash and hemorrhage. In contrast, none of the rats in the low-dose combination group exhibited severe toxicity, and only one rat was sacrificed in the high-dose combination group due to body weight loss. No skin rash was observed in any of the rats in the combination groups. These data indicate that the addition of AZD7762 does not lead to significant toxicity beyond that induced by gemcitabine and that the combination can be delivered at the MTD of gemcitabine.

In the mouse xenograft study in combination with irinotecan, SW620 established tumors were treated with vehicle, irinotecan alone, AZD7762 alone, or AZD7762 in combination with irinotecan. AZD7762 dosed alone showed insignificant antitumor activity, whereas irinotecan alone displayed striking and significant activity (%T/C with increasing dose was 9 and 1, respectively; Fig. 6). In Figure 6. AZD7762 treatment resulted in tumor-free survival in combination with irinotecan in the mouse xenograft efficacy model. A, athymic mice bearing established SW620 tumors were treated with four cycles of therapy every 3 d (arrows). Each cycle consisted of irinotecan alone (25 or 50 mg/kg) or irinotecan (25 or 50 mg/kg) followed by two doses of AZD7762 (2 and 14 h after irinotecan dose). Tumor volume was measured using calipers on the indicated days with the median ± SE tumor volumes indicated for groups of 10 animals. B, %T/C was calculated as (T2 - T1) / (C2 - C1) where T is the treated group, C is the vehicle group, 1 is the start date, and 2 is the end day as defined as the day the control group is euthanized. Percentage inhibition was calculated as (geometric means of control - geometric means of treatment) / (geometric means of control - 1) × 100. Complete regression is defined as tumors measuring <63 mm3. Tumor-free survival is defined as complete regressions for a minimum of 14 d.
combination with AZD7762, %T/C increased significantly to -66% and -67%, respectively. Because tumors were not detected, animals were observed for an extended period and tumors and body weights measured for a total of 94 days post-tumor implantation to monitor tumor regrowth. At the end of the study, tumor-free survival was observed in the low- and high-dose irinotecan combinations (5/9 and 8/9 tumor-free survival, respectively). No statistically significant body weight loss was observed in any treatment group.

Discussion
In the present studies, we describe AZD7762, a novel ATP-competitive inhibitor of checkpoint kinases and the consequences of inhibiting checkpoint pathways in tumor cell lines and models. AZD7762 is greater than 100-fold selective for checkpoint kinases over the majority of a panel of protein tyrosine and serine/threonine kinases. Because greater than 100-fold selectivity was observed against cdk, protein kinase C isoforms, and MAPKAP kinase 2, AZD7762 is clearly differentiated from the UCN-01 kinase selectivity profile (13–15).

AZD7762 has been shown in multiple assays to abrogate DNA damage-induced cell cycle checkpoints. Additionally, fluorescence-activated cell sorting analysis has shown abrogation of the S checkpoints induced by gemcitabine. Profiling of cell cycle proteins gave results that are consistent with mechanism of action and checkpoint abrogation. Gemcitabine treatment of cells leads to the phosphorylation and activation of Chk1 and Chk2 and predominantly S-phase arrest (16). Activated Chk1 then directly phosphorylates cdc25A leading to proteolytic degradation (11, 17–22) and downstream to increased phosphorylation of cdk2 (Tyr15). As a consequence, cyclin E/cdk2 and cyclin A/cdk2 complexes are inactivated, and cells arrest in the S phase. Concurrent treatment of AZD7762 and gemcitabine led to a dose-dependent increase in cdc25A levels at early time points when compound was still available to inhibit Chk1 and a delayed decrease in cyclin A levels as predicted by cell cycle progression.

SN-38 treatment of cells leads to phosphorylation of Chk1 and Chk2 within 1 h of treatment (23) and eventually results in G2 arrest. Activated checkpoint kinase proteins phosphorylate cdc25C, which is relocated by 14-3-3 to the cytoplasm. Relocalization results in inactivation of cdc25c and leads to increased phosphorylation of cdc2 (Tyr15), which remains inactive and prevents progress through the G2 checkpoint (18, 24, 25). As expected, combination treatment leads to dose-dependent decreases in phospho-cdc2 and cyclin B1. phH3 increases, showing abrogation of the G2 checkpoint as cells have moved into mitosis.

AZD7762 has been profiled extensively (in vitro) and has been shown to increase the response to multiple DNA-damaging agents in several different cancer cell lines. As described above, gemcitabine and SN-38 have very different mechanisms of action with gemcitabine arresting cells predominantly in S phase and irinotecan in G2. Nevertheless, enhanced efficacy of both agents is clearly achieved in combination with AZD7762 although the most striking seen with gemcitabine. This might in part be due to the role of Chk1 in maintenance of replication forks, which are stalled by gemcitabine through multiple mechanisms (26, 27). Alternatively, better combination efficacy in vitro could be related to the ability of gemcitabine to activate both ATM- and ATR-driven checkpoints (16) compared with SN-38, which primarily activates ATR-driven checkpoints (23).

Given that AZD7762 is an equally potent inhibitor of Chk1 and Chk2 in vitro, the relative contributions of Chk1 and Chk2 inhibition to the observed phenotypic outcome cannot be conclusively determined from the present studies. However, there is strong literature precedent indicating a major role for Chk1 versus Chk2 when both kinases were assessed simultaneously. For example, gemcitabine treatment activates both Chk1 and Chk2, but Chk1 to a greater extent as measured by phosphospecific antibodies (16, 17). Additionally, both Chk1 and Chk2 can phosphorylate cdc25A, but in gemcitabine-treated cells only Chk1 siRNA knockdown resulted in stabilization of cdc25A. Chk2 depletion in gemcitabine-treated cells had no effect on entry into mitosis, in contrast to the premature entry into mitosis observed with Chk1 depletion (17). Finally, siRNA knockdown of ATR, ATM, or Chk1 led to sensitization of gemcitabine-treated cells, whereas Chk2 depletion had no effect (16).

In studies evaluating topoisomerase I poisons such as camptothecin (28), SN-38 (23), and BNP1350 (29), activation of both Chk1 and Chk2 was observed, but the Chk1/ATR pathway was again determined to play a more prominent role in enhancing cytotoxicity. Interestingly, simultaneous knockdown of Chk1 and Chk2 does not improve efficacy over Chk1 alone (28). A similarly prominent role of Chk1 has been shown for other chemotherapies including cytosine arabinoside (30) and platins (31). Finally, VRX046617, a Chk2 inhibitor with no Chk1 activity, did not potentiate doxorubicin or cisplatin (32).

Other recently described checkpoint kinase inhibitors such as XL-844 and PF-477736 are potent inhibitors of Chk1 with differential activities against Chk2. Whereas XL-844 is equally potent against Chk2 (33), PF-477736 is ~100-fold selective for Chk1 (34). Both inhibitors have been shown to potentiate the effects of DNA-damaging agents such as gemcitabine. Because Chk2 does have a role in checkpoint signaling especially in response to radiation, a potential role of Chk2 inhibition in driving the cellular response to AZD7762, XL-844, or PF-477736 cannot be formally dismissed. Furthermore, it is currently unclear whether the differential profiles against Chk1 and Chk2 of these agents will lead to different outcomes or what balance of Chk1 and/or Chk2 inhibition will result in the maximum clinical response.

5 Tse and Schwartz, in preparation.
AZD7762 has also been profiled in vivo by monitoring abrogation of the G2 checkpoint and efficacy in combination with gemcitabine and irinotecan. Using a novel pharmacodynamic model, a dose-dependent increase in G2 abrogation was observed following i.v. administration of AZD7762. This model was extremely useful because it allowed for potency ranking of multiple compounds in vivo in a highly efficient manner (35–37). Reflecting our in vitro findings, dose-dependent potentiation of antitumor activity in combination with DNA-damaging agents was shown in several in vivo models. Potentiation was determined not to be due to increased exposures to chemotherapy in the combination setting (data not shown). Efficacy is enhanced in gemcitabine-sensitive (SW620) and relatively insensitive (H460-DNp53) human tumor models in combination with AZD7762. To test the ability of AZD7762 to potentiate gemcitabine delivered at MTD, a rat efficacy model was used and showed that increased efficacy was achieved without affecting the tolerability or MTD of gemcitabine. In combination with irinotecan delivered at MTD, AZD7762 was able to convert the growth stasis observed with irinotecan alone to sustainable tumor-free survival in eight of nine mice. This study was compelling not only because the combination treatment converted stasis to regression but also because tumors were unable to regrow in contrast to the rapid regrowth observed in the irinotecan-treated group.

The present preclinical studies show the ability of AZD7762 to potentiate gemcitabine and irinotecan in a tolerated regimen. AZD7762 and other checkpoint kinase inhibitors have the potential not only to potentiate these agents but also to enhance additional therapies that induce DNA damage. Clearly, there are many therapeutic opportunities for a checkpoint kinase inhibitor such as AZD7762. Current and future clinical trials with AZD7762 and other checkpoint kinase inhibitors will reveal which combinations provide the most effective and safe cancer treatment.

### Disclosure of Potential Conflicts of Interest


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