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Sensitization of Pancreatic Cancer Stem Cells to Gemcitabine by Chk1 Inhibition[1]

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Abstract

Checkpoint kinase 1 (Chk1) inhibition sensitizes pancreatic cancer cells and tumors to gemcitabine. We hypothesized that Chk1 inhibition would sensitize pancreatic cancer stem cells to gemcitabine. We tested this hypothesis by using two patient-derived xenograft models (designated J and F) and the pancreatic cancer stem cell markers CD24, CD44, and ESA. We determined the percentage of marker-positive cells and their tumor-initiating capacity (by limiting dilution assays) after treatment with gemcitabine and the Chk1 inhibitor, AZD7762. We found that marker-positive cells were significantly reduced by the combination of gemcitabine and AZD7762. In addition, secondary tumor initiation was significantly delayed in response to primary tumor treatment with gemcitabine + AZD7762 compared with control, gemcitabine, or AZD7762 alone. Furthermore, for the same number of stem cells implanted from gemcitabine– *versus* gemcitabine + AZD7762-treated primary tumors, secondary tumor initiation at 10 weeks was 83% *versus* 43%, respectively. We also found that pS345 Chk1, which is a measure of DNA damage, was induced in marker-positive cells but not in the marker-negative cells. These data demonstrate that Chk1 inhibition in combination with gemcitabine reduces both the percentage and the tumor-initiating capacity of pancreatic cancer stem cells. Furthermore, the finding that the Chk1-mediated DNA damage response was greater in stem cells than in non–stem cells suggests that Chk1 inhibition may selectively sensitize pancreatic cancer stem cells to gemcitabine, thus making Chk1 a potential therapeutic target for improving pancreatic cancer therapy.

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Introduction

Pancreatic cancer is the fourth leading cause of cancer-related death and remains one of the least curable cancers with an overall 5-year survival rate of less than 5% [1]. Although a recent study has shown that the combination of 5-fluorouracil, leucovorin, irinotecan, and oxaliplatin (FOLFIRINOX) is superior to gemcitabine for high-performance status patients with metastatic pancreatic cancer, gemcitabine still plays a key role in the management of metastatic and nonmetastatic, locally advanced disease [2,3]. A number

Abbreviations: Chk1, checkpoint kinase 1

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of clinical trials have attempted to improve gemcitabine-based chemotherapy, but very few have produced clinically significant survival advantages [4–6].

Inhibition of the DNA damage response is a promising strategy for sensitizing tumor cells to therapy. Under the regulation of ATR/ATM, checkpoint kinase 1 (Chk1) functions as an integral component of the DNA damage response by mediating cell cycle arrest and DNA damage repair. A number of small-molecule Chk1 inhibitors have been recently developed including, AZD7762, SCH900776, LY2606368, and LY2603618 [7–9]. We have previously demonstrated that inhibition of Chk1 sensitizes pancreatic cancer cells and tumors to gemcitabine by mechanisms including G_2 checkpoint abrogation and homologous recombination repair (HRR) inhibition [10,11]. In addition, tumor cells that harbor aberrations in other DNA damage response machinery (i.e., p53, p16, Rb) and thus do not arrest at G_1 in response to DNA damage will be selectively affected by Chk1 inhibition. Conversely, normal cells will be protected from Chk1 inhibition by their other intact checkpoints (i.e., p53-mediated G_1 arrest) [12–15].

Although the role of Chk1 inhibition in sensitizing pancreatic tumor cells to gemcitabine has been extensively explored [11,16], the potentially crucial role of cancer stem cells has not been investigated. Especially in the context of pancreatic cancer, where even complete surgical resection is often quickly followed by disease progression, therapies targeted to tumor initiation (versus tumor growth) are necessary. Pancreatic cancer stem cells were recently identified by expression of the cell surface markers CD24, CD44, and ESA (epithelial-specific antigen) [17]. Because pancreatic cancer stem cells are refractory to conventional chemotherapy and radiotherapy [18,19], approaches to target both bulk cancer cells and cancer stem cells are important in improving the efficacy of current therapies.

The concept of selectively sensitizing cancer stem cells to chemotherapy and/or radiotherapy has generated great enthusiasm in the oncology community but thus far has yielded only a few successes [20,21]. Chk1/2 inhibition sensitizes human glioma cancer stem cells to radiation [22]. This study demonstrated that cancer stem cells possess enhanced cell cycle checkpoint activity in response to radiation and more effectively repair radiation-induced DNA damage than non–stem cells. In addition, ATR or Chk1 inhibition sensitizes colon cancer stem cells to cisplatin [23]. This study and others suggest that the resistance of cancer stem cells to therapy is mediated by more robust DNA damage response and repair pathways [21,24,25]; hence, it seems logical to target these pathways to overcome therapy resistance. Because Chk1 inhibition has been shown to inhibit both the DNA damage–induced cell cycle checkpoint response and homologous recombination repair [16,26], it is a promising target for sensitizing cancer stem cells to DNA-damaging agents.

Thus, the goal of the present study was to determine whether Chk1 inhibition could sensitize pancreatic cancer stem cells to gemcitabine. We used two low-passage primary patient xenograft models to determine the effects of gemcitabine and the Chk1 inhibitor, AZD7762, on the percentage of CD24, CD44, ESA-positive cells and their functionality. We found that the combination of gemcitabine and AZD7762 significantly reduced the percentage of marker-positive cells and decreased the tumor-initiating capacity of cancer stem cells using a limiting dilution assay. Further, we determined that Chk1 inhibition played a specific role in the DNA damage response, overall highlighting the potential efficacy of this approach to target pancreatic cancer stem cells.

Materials and Methods

Pancreatic Tumor Xenografts

Samples of human pancreatic adenocarcinomas were obtained within 30 minutes after surgical resection according to institutional review board–approved guidelines as previously described [17]. Briefly, tumors were mechanically dissociated into 2 × 2-mm pieces and implanted surgically into the mid abdomen of NOD/SCID mice. After engraftment into mice, single-cell suspensions were created from surgically resected xenografts by mechanical mincing in RPMI containing penicillin/streptomycin followed by a 1-hour incubation at 37°C with 1 U/μl of collagenase type IV (Worthington Biochemical Corp, Lakewood, NJ) with constant agitation. Cells were filtered consecutively through 100- and 40-μm polypropylene cell strainers (BD, Franklin Lakes, NJ). Tumor cells (1×10^6) were suspended in a 1:1 mixture of 10% fetal bovine serum-RPMI–Matrigel (BD Biosciences, San Jose, CA) and injected subcutaneously into the right and left flanks of NOD/SCID mice. Animals were handled according to a protocol approved by the University of Michigan Committee for Use and Care of Animals.

In Vivo *Treatment*

AZD7762 was dissolved in 11.3% 2-hydroxyproply-β-cyclodextrin (Sigma, St Louis, MO) and sterile saline, and the pH was adjusted to 4.0. Gemcitabine was dissolved in sterile saline. Treatment was initiated when the average tumor volume reached approximately 200 mm^3. Mice were administered gemcitabine (90 mg/kg, intraperitoneally, Mon and Thu) and/or AZD7762 (20 mg/kg, intraperitoneally, Mon, Tue, Thu, and Fri) for three cycles. 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.

FACS Analyses

Tumor cell suspensions were washed twice with Hank's balanced salt solution (HBSS) containing 2% fetal bovine serum, penicillin, and streptomycin and resuspended at a cell concentration of 1×10^6 cells per 100 μl of HBSS. Cells were stained with the following antibodies at a 1:50 dilution: CD24-FITC, CD44-PE (BD Biosciences), and ESA/CD326 (EpCAM)-APC (Miltenyi Biotec, Cambridge, MA) as well as a 1:100 dilution of H2k-d-PE-Cy5 antibody (BD Biosciences) for 20 to 30 minutes on ice, protected from light. Dead cells were excluded by DAPI staining (0.75 μg/ml). Stained cells were again washed twice with HBSS before FACS analyses/sorting in MoFlo (Becton and Dickenson) with SUMMIT software (version 5.3.0; Dako). Unstained samples were used to set gates. Side scatter and forward scatter profiles were used to eliminate cell doublets.

Immunoblot Analysis

Single cells from untreated tumor xenografts were stained for CD24, CD44, ESA and FACS sorted for DAPI– (viable), H2k-d– (human), CD24+, CD44+, ESA+ cells or only for DAPI–, H2k-d– cells. The sorted cells were immediately treated *ex vivo* with gemcitabine (250 nM, t = 0-2 hours) and/or AZD7762 (100 nM, t = 24-30 hours). At the end of treatment, cells were lysed in SDS lysis buffer containing protease (Roche, Indianapolis, IN) and phosphatase inhibitors (Sigma). Cellular protein levels in the extracts were measured using a BCA kit (Thermo Scientific, Rockford, IL). Fifteen micrograms of total cellular protein was separated on NuPAGE Novex 4% to 12% Bis-Tris

Midi gel and electroblotted onto a polyvinylidene difluoride membrane. Blots were incubated with primary antibodies to phosphorylated Chk1 (S345), Chk1 (total), and GAPDH (Cell Signaling, Danvers, MA) and followed by secondary horseradish peroxidase–conjugated anti-rabbit IgG antibody (Cell Signaling). Immunoreactive bands were visualized by SuperSignal WestDura chemiluminescence (Pierce).

Statistical Analysis

The percentages of CD24, CD44, ESA-positive cells were compared between treatments using ANOVA models with Tukey *post hoc* test in GraphPad Prism, version 5.0c (GraphPad Software, Inc, La Jolla, CA). Tumor initiation was determined for each xenograft by identifying the earliest day on which a tumor volume was larger than 20 mm^3. The Kaplan-Meier method was used to calculate the median initiation times. Cox proportional hazards regression was used to model tumor initiation as a function of treatments, the number of viable human cells, or the number of marker-positive cells. The mixed linear model with random slopes and intercepts was applied to the log-transformed tumor volumes to compare tumor growth rates (after initiation) between treatment arms. Analyses on tumor initiation and tumor growth were conducted using SAS (version 9.3; SAS Institute, Cary, NC). Results are presented as mean ± SE unless otherwise stated. Data with $P < .05$ were considered significant.

Results

To begin to determine whether inhibition of Chk1 might sensitize pancreatic cancer stem cells to gemcitabine, we treated mice bearing subcutaneous p53 mutant, pancreatic tumor xenografts designated "patient J" and "patient F" for three cycles with the Chk1 inhibitor, AZD7762 (Mon, Tue, Thu, Fri) and gemcitabine (Mon and Thu), as illustrated in Figure 1*A*. During treatment, we found that AZD7762, in combination with gemcitabine, produced significant tumor growth inhibition (Figure 1, *B* and *C*), which is consistent with our previous tumor growth experiments demonstrating that AZD7762 sensitizes these patient-derived pancreatic tumor xenografts to gemcitabine [11]. On the last day of treatment, the percentage of CD24, CD44, ESA-positive cells (stem cell markers previously identified by our group in patient-derived pancreatic tumor xenografts [17]) was analyzed. We found in patient J–derived xenografts that neither gemcitabine nor AZD7762 significantly reduced the percentage of marker-positive cells (Figure 1*D*). However, the combination of gemcitabine with AZD7762 led to a significant depletion of the marker-positive cells, suggesting that AZD7762 may sensitize pancreatic cancer stem cells to gemcitabine. We went on to determine the effects of gemcitabine and AZD7762 on CD24, CD44, ESA-positive cells in an independent model, patient F, and found that gemcitabine or AZD7762 alone reduced the percentage of marker-positive cells (Figure 1*E*). However,

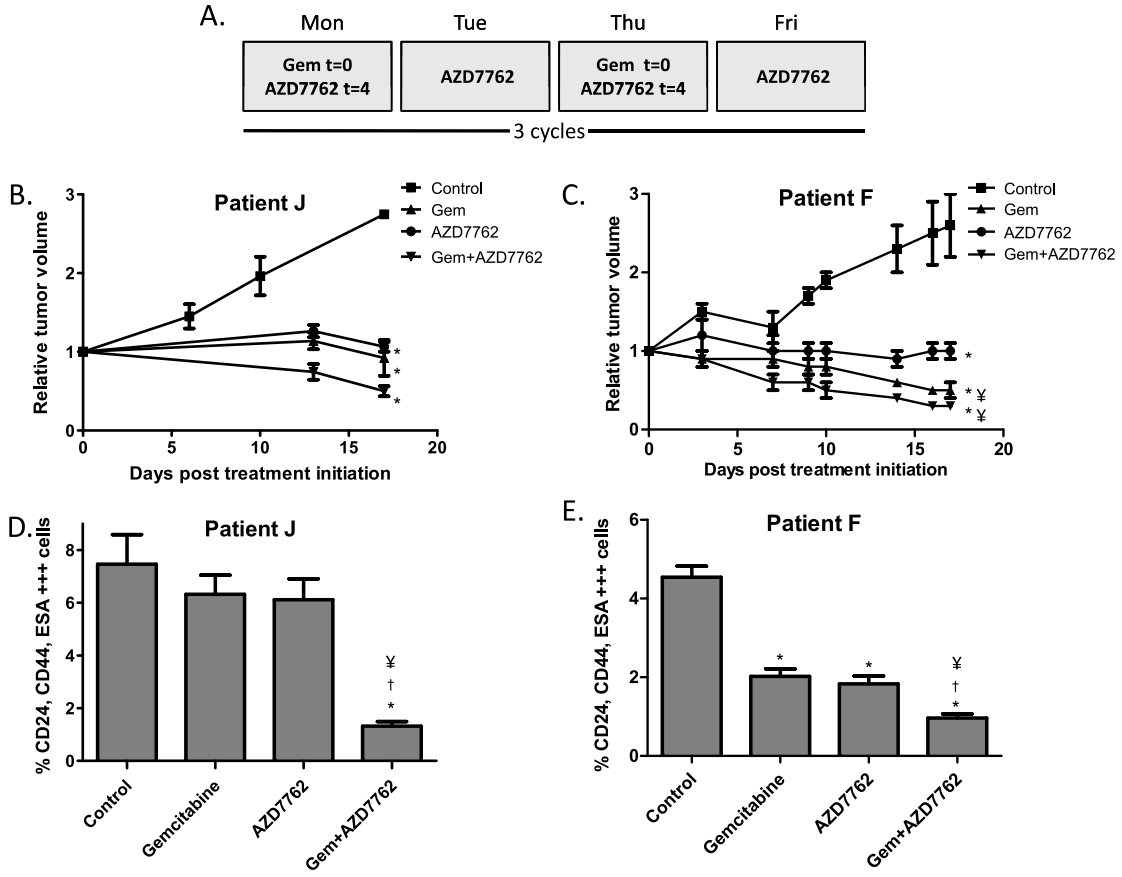
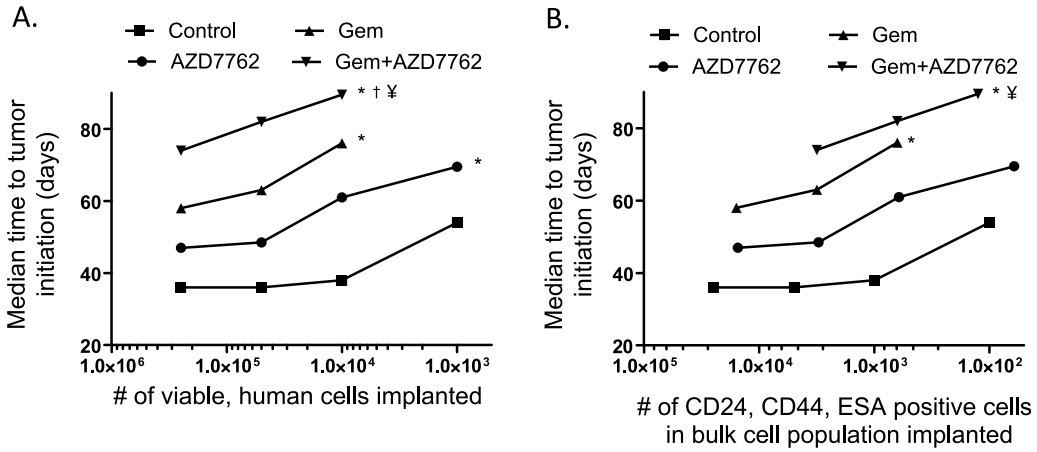


Figure 1. Reduction in the percentage of CD24, CD44, and ESA-positive cells by gemcitabine and AZD7762. NOD/SCID mice bearing patient J– (B) or patient F– (C) derived early-passage xenografts were treated with gemcitabine (90 mg/kg) and/or AZD7762 (20 mg/kg) as illustrated (A). Tumor volumes as a function of increasing time from treatment initiation are shown. On the last day of treatment, tumors were harvested, and the percentages of viable, human cells positive for the indicated markers (CD44-PE, ESA-APC, and CD24-FITC) were analyzed by FACS in patient J– (D) and patient F– (E) derived xenografts. Data are the mean of $n = 3$ to 8 tumors per treatment condition ± SE. Statistically significant differences ($P < .05$) are indicated *versus* *control, †gemcitabine, and ¥AZD7762.

the combination of gemcitabine with AZD7762 produced maximal depletion of marker-positive cells, which was significant, compared with control-, gemcitabine-, or AZD7762-alone treatment conditions. These data support the hypothesis that Chk1 inhibition sensitizes pancreatic cancer stem cells to gemcitabine.

To further test the hypothesis that Chk1 inhibition sensitizes pancreatic cancer stem cells to gemcitabine, we conducted a secondary tumor initiation assay with primary tumor cells that had previously been treated with gemcitabine and AZD7762 as described in Figure 1. After three cycles of treatment with gemcitabine and/or AZD7762, primary tumor cells were analyzed for their percentage of CD24, CD44, ESA-positive cells (Figure 1*D*) and then reimplanted at limiting dilutions into secondary mice. The time required for initiation of secondary tumors, after implantation with an equal number of viable tumor cells (i.e., containing both stem cells and non–stem cells), was monitored for each of the treatment groups. We found that treatment with gemcitabine or AZD7762 alone significantly delayed secondary tumor initiation (Figure 2*A*). More importantly, tumor initiation was significantly delayed in response to the combination of gemcitabine with AZD7762 compared with control, gemcitabine, or AZD7762 alone ($P < .05$). We further analyzed these data by the number of marker-positive stem cells implanted. We found that, for a similar number of marker-positive stem cells implanted, treatment with gemcitabine and AZD7762 led to delayed tumor initiation relative to control or AZD7762 alone ($P < .05$; Figure 2*B*). This difference in tumor initiation in response to gemcitabine + AZD7762 is further illustrated by a comparison of the tumor initiation at 10 weeks after implantation for a given number of stem cells ($n = 3150$; Figure 2*C*). In response to primary tumor treatment with gemcitabine, 10 (83%) of 12 secondary tumors had initiated by 10 weeks, whereas in response to gemcitabine + AZD7762, only 3 (43%) of 7 tumors had initiated. These data demonstrate that treatment of primary tumors with gemcitabine in combination with AZD7762 reduces both functional stem cells (i.e., the ability to generate a tumor) and the apparent viability of marker-positive stem cells.

Having shown that the combination of gemcitabine and AZD7762 decreased the fraction of surviving functional stem cells and the viability of marker-positive stem cells, we wished to determine whether the drug combination affected the ability of the marker-positive cells that did survive to promote tumor growth. Therefore, we monitored the growth rate of secondary tumors as well as their percentage of CD24, CD44, ESA-positive cells (Figure 3). Despite the ability of gemcitabine and AZD7762 to reduce the percentage CD24, CD44, ESA-positive cells as well as their tumor-initiating capacity (Figures 1 and 2), secondary tumor growth rates were similar across all treatment groups. To determine whether the reduction in CD24, CD44, ESA-positive cells observed in primary tumors was maintained, secondary tumors were assessed for their percentage of CD24, CD44, ESA-positive cells. We found that secondary tumors originating from gemcitabine- and gemcitabine + AZD7762–treated primary tumors maintained significantly



C.

Tumor initiation at 10 weeks post-implantation

Number of bulk cells implanted:	250,000	50,000	10,000	1000
Control	8/8 (24,900)	10/10 (4,980)	12/12 (996)	12/12 (100)
Gem	10/10 (15,750)	10/12 (3,150)	6/12 (630)	0/12 (63)
AZD7762	10/10 (15,275)	9/10 (3,055)	12/12 (611)	8/12 (61)
Gem AZD7762	3/7 (3,150)	2/9 (630)	1/12 (126)	0/12 (13)

The calculated number of stem cells is shown in parenthesis.

Figure 2. Inhibition of secondary tumor initiation in response to primary tumor treatment with gemcitabine and AZD7762 treatment. Single cells derived from the patient J xenografts (from Figure 1*A*) were reimplanted into secondary mice at 250, 50, 10, and 1×10^3 human viable cells. Animals were monitored twice weekly for tumor initiation for up to 5 months. Tumors ≥ 20 mm^3 were scored as "initiated." The median time for tumor initiation in days is shown as a function of the number of bulk cells reimplanted (A) or the number of marker-positive stem cells reimplanted (B). A summary of the actual number of CD24, CD44, ESA-positive cells implanted (in parentheses) and the corresponding tumor initiation at 10 weeks after implantation is shown (C). Data are from 8 to 12 tumors per treatment condition per cell concentration. Statistically significant differences ($P < .05$) are indicated *versus* *control, †gemcitabine, and ¥AZD7762.

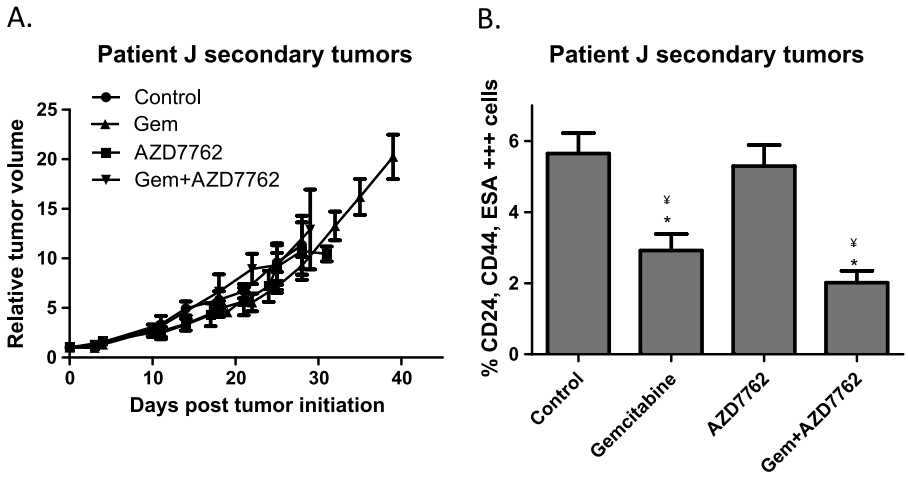


Figure 3. Secondary tumors display similar growth rates but altered percentages of marker positive in response to primary gemcitabine and AZD7762 treatments. (A) The tumor growth kinetics of secondary xenografts implanted from gemcitabine- and/or AZD7762-treated primary tumors are illustrated. Data are the mean ± SE of 9 to 12 tumors per treatment condition and are expressed relative to the day of initiation (defined by a tumor ≥ 20 mm^3). (B) Secondary tumors reaching a volume of approximately 400 mm^3 were harvested and analyzed for CD24, CD44, and ESA. Data are the mean ± SE of 14 to 28 tumors per condition. Statistically significant differences ($P < .05$) are indicated *versus* *control and ¥AZD7762.

reduced percentages of marker-positive cells. Taken together, these results demonstrate that treatment with gemcitabine and AZD7762 produces lasting inhibitory effects on both the percentage and the tumor-initiating capacity of CD24, CD44, ESA-positive cells. However, once a tumor is able to initiate, previous combination treatment does not affect the tumor growth rates.

To begin to determine the mechanisms and selectivity of Chk1 inhibition for sensitizing pancreatic cancer stem cells to gemcitabine, we assessed the Chk1-mediated DNA damage response on the cancer stem cell and non–cancer stem cell populations within the tumor (Figure 4). After sorting these two cell populations, cells were *ex vivo* treated with gemcitabine and AZD7762 according to previously established concentrations and treatment schedules and using S345 Chk1 phosphorylation as a pharmacodynamic biomarker of AZD7762-mediated DNA damage [11]. Consistent with our previous observations in bulk

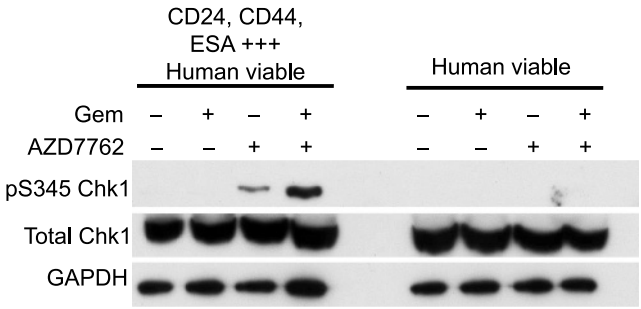


Figure 4. Enhanced Chk1-mediated DNA damage response in pancreatic cancer stem cells. Single cells derived from patient J early-passage xenografts were FACS sorted to obtain human (H2kD−), viable (DAPI−), CD24, CD44, ESA-positive cells, or human viable cells. After FACS sorting, cells were treated *ex vivo* with gemcitabine (250 nM, t = 0-2 hours) and/or AZD7762 (100 nM, t = 24-30 hours) and then prepared for immunoblot analysis for the indicated proteins. Data are from a single representative experiment.

pancreatic cancer cells and tumors [11], we found in response to AZD7762 alone or in combination with gemcitabine that phosphorylation of Chk1 (S345) was induced. Whereas phosphorylation was maximal in response to gemcitabine and AZD7762, this occurred only in the marker-positive cancer stem cells and not in the human, viable bulk cancer cell population. These data suggest that CD24, CD44, ESA-positive pancreatic cancer stem cells encounter a greater degree of DNA damage in response to Chk1 inhibition and thus elicit a more robust DNA damage response and are consistent with the observed sensitization to gemcitabine by Chk1 inhibition in our *in vivo* studies.

Discussion

In this study, we have found that the combination of gemcitabine with a Chk1 inhibitor (AZD7762) decreases both the number of pancreatic cancer stem cells and the tumor-initiating capacity of the cancer stem cell population. These findings suggest that Chk1 inhibition may be a useful strategy for overcoming therapy resistance in pancreatic cancer and for preventing tumor recurrence. In addition, this study demonstrates a heightened DNA damage response in stem cells (*vs* non–stem cells) after Chk1 inhibition, suggesting a greater extent of DNA damage in the stem cells, which is consistent with their sensitization to gemcitabine by Chk1 inhibition.

In our previous work, we have extensively explored the use of Chk1 inhibition as a means of sensitizing bulk pancreatic tumor cells to gemcitabine and radiation therapy primarily through mechanisms involving G_2 checkpoint abrogation and inhibition of HRR and, more specifically, demonstrated that AZD7762 significantly inhibits tumor growth in response to gemcitabine in the patient-derived tumors used in this study [11,16]. This sensitizing effect of AZD7762 to gemcitabine on bulk tumor growth was not apparent in this study owing to the short tumor monitoring period before tumor harvesting for stem cell analysis. We and others have found sensitization in response to Chk1 inhibition to occur selectively in tumor cells that is in part attributable to the presence of mutant p53 [27,28]. Although

AZD7762 inhibits both Chk1 and Chk2, previous studies from our group and others suggest that sensitization is attributable to Chk1 but not Chk2 inhibition [10,16,29–33]. In the current study, we present an additional mechanism of tumor sensitization to gemcitabine by Chk1 inhibition through selective targeting of pancreatic cancer stem cells. Given the important roles these DNA damage responses play in stem cells, targeting them represents a potentially highly effective treatment strategy. Our data suggest that Chk1 inhibition may preferentially target these overactivated DNA damage response pathways in cancer stem cells, thereby sensitizing them to therapy.

While CD24, CD44, and ESA positivity was the first reported set of markers to enrich for pancreatic cancer stem cells [17], several other markers have been found to identify pancreatic cancer stem cells since then. CD133 defines cancer stem cells from patient-derived pancreatic tumors [18]. In addition, high ALDH activity has been shown by several laboratories to enrich for pancreatic cancer stem cells [34,35] and, in one case, to be superior to CD133. More recently, some of these markers have been identified as potential therapeutic targets. Notably, inhibition of ALDH has been shown to sensitize $ALDH^{high}$ cancer cells (breast and ovarian) to chemotherapy and radiotherapy [36,37], which may be through mechanisms involving an increase in reactive oxygen species (ROS) [38]. Taken together, these results suggest that there are multiple markers that define pancreatic cancer stem cells. It will be important to understand how these different markers relate to each other and to the cancer stem cell phenotype.

Previous studies have reported that cancer stem cells are resistant to chemotherapies [39] such as gemcitabine in the case of pancreatic cancer stem cells [40,41]. In particular, these studies demonstrated that pancreatic cancer stem cell markers were either enriched or unaffected by gemcitabine treatment. Our present findings demonstrate that gemcitabine does not significantly increase the percentage of CD24, CD44, ESA-positive cells. Furthermore, pancreatic cancer stem cells are sensitive to gemcitabine because we found that secondary tumor initiation is delayed in response to gemcitabine. Thus, although many studies have found that cancer stem cells are relatively resistant to therapy, the current study and our previous findings regarding the sensitivity of some breast cancer stem cells to radiation [42] suggest that it is premature to conclude that cancer stem cell resistance is universal.

Although the combination of gemcitabine and AZD7762 reduced both the percentage of marker-positive cells and their tumor-initiating capacity, after tumor initiation, tumor growth rates were similar across treatment groups (Figure 3*A*). These data are consistent with the hypothesis that, after tumor initiation, tumor growth is independent of the initial number and viability of the stem cells. Progenitor cells that are produced by asymmetrical division with stem cells retain proliferative capacity for a limited number of generations, and it seems possible that these cells could support tumor growth during our observation period. Given that none of the treatments used in this study completely eradicated the pancreatic cancer stem cells or their tumor-initiating capacity, it is conceivable that, in the absence of any treatment on the secondary tumors, the cancer stem cells may recover to a threshold level that is adequate to support tumor growth. We must also entertain the possibility that there are additional stem cells that are not detected by CD24, CD44, ESA positivity, although the correlation between marker-positive cells and primary tumor initiation would suggest that maker positivity does, in general, correlate with a stem-like property.

Other studies have shown that cancer stem cells elicit a more robust Chk1-mediated DNA damage response than non–stem cells [22,23,25]. Consistent with these observations, we found that Chk1 inhibition had a more profound effect on cancer stem cells as evidenced by greater pChk1 (S345) accumulation in the marker-positive *versus* the human viable cells. We have previously shown that pChk1 (S345) is a pharmacodynamic biomarker of gemcitabine-AZD7762 activity, which is associated with elevated DNA damage in response to this therapy [11]. Thus, our data suggest that inhibition of Chk1 produces more DNA damage that is marked by a more robust DNA damage response in cancer stem cells. It will be important in future studies to better understand the mechanisms contributing to selective cancer stem cell sensitization through the incorporation of *in vitro* pancreatic cancer stem cell models.

Given that cancer stem cells are generally thought to be resistant to chemotherapy and radiotherapy, many strategies for sensitizing cancer stem cells are emerging. Consistent with our study, small-molecule inhibitors of ATR or Chk1 sensitized colon cancer stem cells to cisplatin [23], establishing Chk1 as a promising target. Another potential mechanism driving the resistance of cancer stem cells to therapy is through increased DNA damage responses including an increased capacity for minimizing ROS [43]. Thus, strategies to increase ROS (by increasing ROS or blocking scavenging) are a promising approach for sensitizing to radiation. Other studies have shown that the AKT inhibitor, perifosine, sensitizes breast cancer stem cells to radiation [21], whereas targeting DR5, SHH, or mTOR in combination with gemcitabine reduces pancreatic cancer stem cells [41,44]. Planned and ongoing trials should soon reveal whether the promising preclinical results of targeting cancer stem cells could translate into improved patient outcome.

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