Gemcitabine sensitization by checkpoint kinase 1 inhibition correlates with inhibition of a Rad51 DNA damage response in pancreatic cancer cells

Leslie A. Parsels,1 Meredith A. Morgan,2 Daria M. Tanska,1 Joshua D. Parsels,1 Brian D. Palmer,3 R. John Booth,4 William A. Denny,3 Christine E. Canman,1 Alan J. Kraker,5 Theodore S. Lawrence,2 and Jonathan Maybaum1

Abstract
The protein kinase checkpoint kinase 1 (Chk1) has been implicated as a key regulator of cell cycle progression and DNA repair, and inhibitors of Chk1 (e.g., UCN-01 and EXEL-9844) potentiate the cytotoxic actions of chemotherapeutic drugs in tumor cells. We have examined the ability of PD-321852, a small-molecule Chk1 inhibitor, to potentiate gemcitabine-induced clonogenic death in a panel of pancreatic cancer cell lines and evaluated the relationship between endpoints associated with Chk1 inhibition and chemosensitization. Gemcitabine chemosensitization by minimally toxic concentrations of PD-321852 ranged from minimal (<3-fold change in survival) in Panc1 cells to >30-fold in MiaPaCa2 cells. PD-321852 inhibited Chk1 in all cell lines as evidenced by stabilization of Cdc25A; in combination with gemcitabine, a synergistic loss of Chk1 protein was observed in the more sensitized cell lines. Gemcitabine chemosensitization, however, did not correlate with abrogation of the S-M or G2-M checkpoint; PD-321852 did not induce premature mitotic entry in gemcitabine-treated BxPC3 or M-Panc96 cells, which were sensitized to gemcitabine 6.2- and 4.6-fold, respectively. In the more sensitized cell lines, PD-321852 not only inhibited gemcitabine-induced Rad51 focus formation and the recovery from gemcitabine-induced replication stress, as evidenced by persistence of γ-H2AX, but also depleted these cells of Rad51 protein. Our data suggest the inhibition of this Chk1-mediated Rad51 response to gemcitabine-induced replication stress is an important factor in determining gemcitabine chemosensitization by Chk1 inhibition in pancreatic cancer cells. [Mol Cancer Ther 2009;8(1):45–54]

Introduction
Most tumor cells exhibit abnormalities in one or more aspects of cell cycle regulation (checkpoints), and a consensus view has emerged that these abnormalities present an attractive target for therapeutic intervention (reviewed in refs. 1–4). One member of the checkpoint system that is currently generating intense interest as a therapeutic target is checkpoint kinase 1 (Chk1), a protein that functions in multiple pathways in response to genotoxic stress. Although interfering with Chk1 function can produce substantial increases in the sensitivity of tumor cells to a variety of chemotherapeutic agents, this effect varies markedly among human tumor cell lines. A better understanding of the mechanistic basis for differential responses to Chk1 inhibitors is therefore necessary to rationally translate these agents to the clinic.

One of the best characterized functions of activated Chk1 is its ability to phosphorylate and inactivate Cdc25 phosphatases in response to replication stress (5). Many chemotherapeutic agents that target DNA synthesis and integrity, including camptothecin (6), 5-fluorodeoxyuridine (7), gemcitabine (8–10), and doxorubicin (6), have been shown to activate Chk1 as indicated by phosphorylation at Ser317 and/or Ser345. This activation is followed by destabilization of Cdc25A, via phosphorylation by Chk1 at multiple sites (reviewed in ref. 11), which in turn results in inactivation of cyclin-dependent kinase 1 complexes and G2 arrest (the G2-M checkpoint; refs. 6, 12) and/or inactivation of cyclin-dependent kinase 2 complexes and intra-S-phase arrest (13). Inhibition of Chk1 with either siRNA (6, 10) or chemical inhibitors (8, 14, 15) prevents drug-induced Cdc25A degradation, leading to abrogation of the intra-S and/or G2-M checkpoint and inappropriate entry into mitosis with either a 4N or a sub-4N DNA content (premature mitotic entry; ref. 16). Abrogation of these checkpoints and premature mitotic entry have been correlated previously with drug sensitization and increased cell death (8, 15, 17).
In addition to its effects on Cdc25A and cell cycle checkpoints, Chk1 inhibition has been proposed to sensitize cells to DNA-damaging agents by destabilizing chemotherapy-induced stalled replication forks and/or inhibiting DNA repair. This hypothesis is supported by studies using histone H2AX phosphorylation at Ser139 (γ-H2AX) as a marker for DNA damage or replication stress, in which inhibition of Chk1 caused an increase in γ-H2AX staining after treatment with gemcitabine (8, 18). Chk1 also functions directly in the homologous recombination repair of chemotherapy-induced DNA damage in part by regulating Rad51, a key protein in homologous recombination repair (19, 20). Hydroxyurea-induced replication stress induces nuclear accumulation of Rad51 foci in an ATR- and Chk1-dependent manner, and inhibition of Chk1 prevents localization of Rad51 to sites of homologous recombination repair (19, 20).

In preliminary studies using the Chk1 antagonist PD-321852, we observed that catalytic inhibition of Chk1, stabilization of Cdc25A, and premature mitotic entry were discordant with the degree of gemcitabine chemosensitization in a panel of pancreatic cancer cell lines. These observations suggested to us that other molecular events might better account for potentiation of gemcitabine-induced clonogenic death. In our present study, we determined that molecular events related to replication stress and/or DNA damage and repair, such as inhibition of Rad51 focus formation and persistence of γ-H2AX staining, might be more informative markers for gemcitabine chemosensitization by Chk1 inhibitors in pancreatic cancer cells.

Materials and Methods

Cell Culture and Drug Solutions

MiaPaCa2, BxPC3, Panc1, and M-Panc96 cells were grown in DMEM (MiaPaCa2 and Panc1) or RPMI 1640 (BxPC3 and M-Panc96) supplemented with 10% fetal bovine serum (Life Technologies) and 2 mmol/L L-glutamine (Sigma). Gemcitabine (Eli Lilly) was dissolved by Pfizer Global Research and Development and Dr. W.A. Denny University of Auckland (Supplementary Fig. S1). Gemcitabine Sensitization by Chk1 Inhibition

7 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Cell Culture and Drug Solutions

MiaPaCa2, BxPC3, Panc1, and M-Panc96 cells were grown in DMEM (MiaPaCa2 and Panc1) or RPMI 1640 (BxPC3 and M-Panc96) supplemented with 10% fetal bovine serum (Life Technologies) and 2 mmol/L L-glutamine (Sigma). Gemcitabine (Eli Lilly) was dissolved by Pfizer Global Research and Development and Dr. W.A. Denny University of Auckland (Supplementary Fig. S1).

The IC50 of PD-321852 for inhibition of Chk1 in a cell-free assay system is 5 nmol/L (21). PD-321852 was dissolved in DMSO (Sigma) and stored in aliquots at −20°C.

Clonogenic Assay

Cells treated with PD-321852 alone, gemcitabine alone, or gemcitabine plus PD-321852 for 24 h were trypsinized and processed for clonogenic survival as described previously (22). Surviving fractions (SF) for samples treated with a single agent represent the plating efficiency for a given drug-treated sample divided by the plating efficiency for the corresponding control. For samples treated with both gemcitabine and PD-321852, the SF was calculated as the plating efficiency for a given sample treated with both agents divided by the plating efficiency for the corresponding sample treated with PD-321852 alone. Unless noted, cells were treated with a moderately toxic concentration of gemcitabine for 24 h. This concentration was 100 nmol/L in MiaPaCa2 (SF = 0.36 ± 0.04) and BxPC3 (SF = 0.32 ± 0.04) cells, 300 nmol/L in M-Panc96 cells (SF = 0.24 ± 0.03), and 1 μmol/L in Panc1 cells (SF = 0.45 ± 0.04).

Western Blot Analysis

Whole-cell lysates were prepared in buffer containing 10 mmol/L Tris (pH 7.4), 2% SDS, 1× Complete Protease Inhibitor Cocktail (Roche), 1 mmol/L sodium fluoride, 2 mmol/L sodium orthovanadate, and 1 mmol/L sodium pyrophosphate, diluted in 2× loading buffer [0.32 mol/L Tris-HCl, 10% glycerol, 2% SDS, 0.2% bromophenol blue, and 4% 2-mercaptoethanol (pH 6.8)] and resolved on 10% polyacrylamide gels. Separated proteins were transferred to Immobilon polyvinylidene difluoride membranes (Millipore) and incubated overnight at 4°C with one the following antibodies: anti-Chk1 (G-4), anti-Cdc25A (F-6), and anti-Rad51 (H-92; Santa Cruz Biotechnology); anti-Chk2 (clone 7; Upstate Biotechnology); or anti-β-actin (Sigma). For Chk1 quantitation, autoradiograms were scanned for densitometric analysis and normalized first to actin loading control and then to the effects of gemcitabine alone. Data were analyzed with AlphaEase FC software (Alpha Innotech). For experiments with the proteasome inhibitor calpain inhibitor 1 N-acetyl-l-leucinyl-l-leucinyl-l-norleucinol (Sigma), 25 μg/mL N-acetyl-l-leucinyl-l-leucinyl-l-norleucinol or vehicle control (0.1% DMSO) was added to the drug medium for the last 3 h of treatment.

Flow Cytometry

Exponentially growing cells were treated as indicated, trypsinized, washed with ice-cold PBS, and fixed at a concentration of 2 × 109/mL in ice-cold 70% ethanol. For phospho-histone H3 analysis, washed, fixed cells were incubated with a rabbit anti-phospho-histone H3-specific antibody (Upstate Cell Signaling Solutions) followed by a FITC-conjugated anti-rabbit secondary antibody (F-0382; Sigma) as described previously (7). Trout erythrocyte nuclei (BioSure) were included as internal standards.

Samples were then stained with propidium iodide to measure total DNA content and analyzed on a FACScan flow cytometer (Becton Dickinson) with FlowJo software (Tree Star). Normal and premature mitoses were separated by first defining normal mitosis under control conditions as phospho-histone H3-positive cells with 4N DNA content and then applying those variables to treated samples. Premature mitosis was defined as phospho-histone H3-positive cells with sub-4N DNA content.


9 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
Supplementary Fig. S2A). We next assessed the ability of PD-321852 to sensitize these cell lines to gemcitabine (Fig. 1; Supplementary Fig. S2B). MiaPaCa2, M-Panc96, and BxPC3 cells were each sensitized to gemcitabine by PD-321852, with a 13-, 17-, or 6-fold shift in the gemcitabine IC_{50} respectively (Table 1), and a significant increase in gemcitabine-induced clonogenic death. In each of these sensitized cell lines, the effects of PD-321852 on gemcitabine-induced clonogenic death were most pronounced at minimally or, in MiaPaCa2 cells, even nontoxic concentrations of gemcitabine. Although PD-321852 also caused a significant 4-fold shift in the gemcitabine IC_{50} in Panc1 cells, the differences in clonogenic survival between Panc1 cells treated with gemcitabine alone compared with gemcitabine plus PD-321852 were minimal (<3-fold) compared with the other cell lines.

Catalytic Inhibition and Degradation of Chk1 Induced by PD-321852

To test the hypothesis that differences in the ability of PD-321852 to sensitize different pancreatic cancer cell lines to gemcitabine-induced clonogenic death result from differences in Chk1 inhibition, we examined Cdc25A protein levels in cells treated with PD-321852 alone, gemcitabine alone, or gemcitabine and PD-321852 combined. The generally accepted model of Cdc25A regulation by Chk1 predicts that inhibition of Chk1 should be reflected by abrogation of gemcitabine-induced Cdc25A degradation (17, 25). Although each of the cell lines examined expressed similar levels of Chk1, and PD-321852 stabilized Cdc25A protein levels in each, both basal Cdc25A levels and the effects of PD-321852 on gemcitabine-induced Cdc25A degradation varied (Fig. 2A and B). Although MiaPaCa2 and BxPC3 cells were sensitized to gemcitabine by 0.03 μmol/L PD-321852 (Supplementary Fig. S2B), there was no measurable effect on Cdc25A protein levels until higher concentrations of PD-321852 were used (0.1-0.3 μmol/L) and the effects of PD-321852 on Cdc25A stability were minimal in BxPC3 cells. Inhibition of gemcitabine-induced Cdc25A degradation was most effective in the Panc1 cells, the cell line least sensitized to gemcitabine by PD-321852. Finally, although Cdc25A levels were stabilized by PD-321852 in M-Panc96 cells, these cells did not exhibit gemcitabine-induced Cdc25A degradation. Collectively, these results suggest that, although PD-321852 inhibits Chk1 in each of these cell lines, as indicated by attenuated Cdc25A degradation under basal conditions, gemcitabine sensitization by PD-321852 did not require abrogation of gemcitabine-induced Cdc25A degradation.

Surprisingly, PD-321852 caused a synergistic loss of Chk1 protein in gemcitabine-treated cells. This effect was most pronounced in the three more sensitized cell lines, MiaPaCa2, BxPC3, and M-Panc96 (Fig. 2C), and may be attributed to the activation-mediated degradation of Chk1 (26). To test whether PD-321852 accelerated Chk1 degradation in gemcitabine-treated cells, we added the

8 We described previously a similar phenotype in HT29 cells, where, despite activation of both Chk1 and Chk2, Cdc25A was induced, rather than degraded, after treatment with the antimetabolite 5-fluorodeoxyuridine (7). HT29 cells, like M-Panc96 cells, express relatively low levels of Cdc25A (L.A. Parsels and J. Maybaum, unpublished results).
proteasome inhibitor, N-acetyl-L-leucinyl-L-leucinyl-L-norleucinol, during the last 3 h of drug treatment and measured Chk1 protein levels by Western blot. As shown in Fig. 2D, Chk1 protein levels were restored by N-acetyl-L-leucinyl-L-leucinyl-L-norleucinol, suggesting that the loss of Chk1 in cells treated with both gemcitabine and PD-321852 is at least partly due to proteasome-mediated degradation.

Relationship between the Effects of PD-321852 on Cell Cycle Progression and Chemosensitization

Previous studies have linked aberrant Cdc25A expression to premature induction of mitosis (in cells with <4N DNA content) and cell death (7, 8, 17). Based on these observations, we examined whether PD-321852 would promote premature mitotic entry, or S-M checkpoint abrogation, in gemcitabine-treated cells and whether these cell cycle effects were associated with chemosensitization. Cells treated with PD-321852, gemcitabine, or gemcitabine and PD-321852 were analyzed by flow cytometry for expression of the mitotic marker, phospho-histone H3 (Fig. 3; ref. 27). Despite the elevated levels of Cdc25A, Chk1 inhibition with PD-321852 alone (0.3 μmol/L) did not cause premature mitotic entry. It did, however, lead to an enrichment of cells in late S and G2-M (see also Supplementary Fig. S3). In each of the cell lines examined, gemcitabine alone caused an early S-phase arrest, which is accompanied by activation of Chk1 (10). Consistent with our previous Chk1 siRNA data, Chk1 inhibition with PD-321852 partially abrogated the G2-M and/or S-M checkpoints in gemcitabine-treated Panc1 cells, with phospho-histone H3-positive cells equally distributed between normal mitosis (4.8 ± 0.6%) and premature mitosis (4.4 ± 0.7%; Fig. 3B; ref. 17). Despite this checkpoint abrogation, Panc1 cells were minimally sensitized to gemcitabine under these conditions (Fig. 1D). Surprisingly, M-Panc96 cells appeared to be resistant to PD-321852-induced premature mitotic entry, or abrogation of the gemcitabine-induced S-M checkpoint, although PD-321852 attenuated Cdc25A degradation in these cells (Fig. 2B). It should also be noted that, despite a failure to degrade Cdc25A, each of these cell lines still arrested in late G1 or early S phase when treated with gemcitabine and PD-321852 likely as a consequence of gemcitabine-induced nucleotide pool depletion. Only in MiaPaCa2 cells did the effects of Chk1 inhibition on Cdc25A correlate with premature mitotic entry, S-M checkpoint abrogation, and sensitization to gemcitabine. These data suggest that aberrant Cdc25A regulation and premature mitotic entry or abrogation of the S-M checkpoint are neither required nor sufficient for sensitization of pancreatic cancer cells to gemcitabine.

Table 1. Gemcitabine IC50 values for pancreatic cancer cells treated with gemcitabine ± 0.3 μmol/L PD-321852 for 24 h and assayed for clonogenic survival

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Gemcitabine IC50 (nmol/L) (gemcitabine alone)</th>
<th>Gemcitabine IC50 (nmol/L) (gemcitabine + PD-321852)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MiaPaCa2</td>
<td>160 ± 19</td>
<td>12 ± 4.6*</td>
</tr>
<tr>
<td>M-Panc96</td>
<td>150 ± 21</td>
<td>9.2 ± 0.16*</td>
</tr>
<tr>
<td>BxPC3</td>
<td>50 ± 8.5</td>
<td>8.0 ± 0.05*</td>
</tr>
<tr>
<td>Panc1</td>
<td>480 ± 63</td>
<td>110 ± 29*</td>
</tr>
</tbody>
</table>

NOTE: Mean ± SE (n = 3-7).

*P < 0.05, one-way ANOVA.
Relationship between the Effects of PD-321852 on Gemcitabine-Induced Rad51 Focus Formation, γ-H2AX Expression, and Chemosensitization

We next considered that the ability of PD-321852 to sensitize pancreatic cancer cells to gemcitabine may correlate with inhibition of the DNA damage response and recovery from gemcitabine-induced replication stress rather than S-M or G2-M checkpoint abrogation. Chk1 participates in the cell’s response to DNA damage in part through regulation of Rad51, a critical protein that functions in homologous recombination repair (19). To assess the effects of Chk1 inhibition on the DNA damage response, we tested the ability of PD-321852 to abrogate gemcitabine-induced Rad51 focus formation (Fig. 4). Although PD-321852 alone (0.3 μmol/L) did not significantly alter the percentage of BxPC3 or Panc1 cells staining positive for Rad51 foci (10 or more foci/cell), it did attenuate Rad51 focus formation in MiaPaCa2 and M-Panc96 cells (Fig. 4C). There were also differences in PD-321852-induced γ-H2AX staining between the cell lines; 0.3 μmol/L PD-321852 had minimal effects on γ-H2AX in Panc1 cells but induced strong, pan-nuclear γ-H2AX staining in the other cell lines (see also Supplementary Fig. S4). In most cases, gemcitabine alone induced Rad51 focus formation and γ-H2AX staining, and in each cell line, PD-321852 significantly blocked formation of Rad51 foci in gemcitabine-treated cells. The magnitude of this effect, however, varied. Whereas PD-321852 caused a >10-fold decrease in the percentage of gemcitabine-treated BxPC3, MiaPaCa2, and M-Panc96 cells staining positive for Rad51 foci, the percentage of Panc1 cells staining positive only decreased 2.4-fold from 46.4 ± 2.57% to 19.6 ± 5.5%. Surprisingly, PD-321852 not only blocked Rad51 focus formation in the sensitized cell lines but also caused a decrease in Rad51 protein levels, a result confirmed by Western blot (Fig. 4D). Therefore, as with Chk1 protein depletion, drug-induced loss of Rad51 protein is associated with gemcitabine sensitization in these cell lines.

To further test the hypothesis that sensitization to gemcitabine correlates with inhibition of a Chk1-mediated DNA damage response, BxPC3 and Panc1 cells treated with PD-321852, gemcitabine alone, or gemcitabine and

Figure 2. Effects of PD-321852, gemcitabine, or gemcitabine plus PD-321852 on checkpoint protein levels. A, basal levels of Cdc25A, Chk1, Chk2, and Rad51 in pancreatic cancer cell lines. Representative of two independent experiments. B, cells treated with gemcitabine alone (100 nmol/L for MiaPaCa2 and BxPC3, 300 nmol/L for M-Panc96, and 1 μmol/L for Panc1), 0.3 μmol/L PD-321852, or gemcitabine plus PD-321852 for 24 h were assayed for Cdc25A, Chk1, and Chk2 protein levels. Breaks indicate noncontiguous lanes from a single membrane. Representative of three to five independent experiments. C, for quantitation of Chk1 depletion in cells treated with gemcitabine plus PD-321852, autoradiograms were scanned for densitometric analysis and normalized first to actin loading control and then to the effects of gemcitabine alone. Mean ± SE (n = 3-5). *, P < 0.05; **, P < 0.01, one-way ANOVA. D, MiaPaCa2 and BxPC3 cells were treated with gemcitabine plus 0.3 μmol/L PD-321852 for 24 h, with no other addition, or with 25 μg/mL N-acetyl-l-leucinyl-l-leucinyl-l-norleucinol added for the final 3 h of drug treatment. Representative of two independent experiments.

Mol Cancer Ther 2009;8(1). January 2009
PD-321852 were assayed for γ-H2AX by flow cytometry (Fig. 5). γ-H2AX is a commonly used marker for DNA damage (28) and/or replication stress (29) that has been used previously to assay for gemcitabine-induced stalled replication forks (9, 18). In these experiments, we used 0.1 μmol/L PD-321852 as this concentration did not induce γ-H2AX staining. In BxPC3 cells, treatment with 100 nmol/L gemcitabine for 24 h induced γ-H2AX in 34.4 ± 5.4% of cells (Fig. 5C). After removal of gemcitabine, cells resumed progression through S phase over the next 48 h, and γ-H2AX staining decreased to near control levels, suggestive of either DNA repair or recovery from gemcitabine-induced replication arrest. Seventy-two hours after the removal of gemcitabine, we observed an increase in γ-H2AX-positive cells that was accompanied by the accumulation of cells with sub-G1 DNA content. Although PD-321852 alone (0.1 μmol/L) had minimal effects on γ-H2AX staining in BxPC3 cells, it enhanced induction of γ-H2AX when combined with gemcitabine and significantly attenuated the loss of γ-H2AX after removal of both drugs (*, P < 0.05, one-way ANOVA). This persistence of γ-H2AX suggests that PD-321852 inhibits recovery from gemcitabine-induced replication stress in BxPC3 cells. Similar results were observed in the other cell lines sensitized to gemcitabine by Chk1 inhibition, MiaPaCa2 and M-Panc96 (data not shown). In Panc1 cells, a similarly toxic concentration of gemcitabine (1 μmol/L; SF = 0.45 ± 0.04) induced γ-H2AX in 74.9 ± 3.8% of cells, and this level was maintained for 72 h after removal of drug (Fig. 5D). This persistence of γ-H2AX is consistent with the persistence of the gemcitabine-induced S-phase arrest (18). Panc1 cells treated with a lower, minimally toxic concentration of gemcitabine (100 nmol/L; SF = 0.76 ± 0.09) also retained a high level of γ-H2AX staining after drug removal (Fig. 5D). In contrast to BxPC3 cells, PD-321852 did not enhance the induction of γ-H2AX in Panc1 cells. Due to the result that
Panc1 cells accumulated in S phase, and γ-H2AX intensity did not decrease over the course of this experiment, we were unable to determine the effects of Chk1 inhibition on recovery from gemcitabine-induced replication arrest in Panc1 cells with this assay. Collectively, the Rad51 and γ-H2AX results suggest that Rad51 plays a significant role in the recovery from gemcitabine-induced replication stress in BxPC3 cells but not in Panc1 cells and that inhibition of this response contributes to gemcitabine chemosensitization.

Figure 4. Immunofluorescent analysis of gemcitabine-induced Rad51 and γ-H2AX focus formation. BxPC3 (A) and Panc1 (B) cells were either irradiated with 7.5 Gy and fixed 5 h post-irradiation or treated with gemcitabine (Gem) 0.3 μmol/L PD-321852 for 24 h and stained for both Rad51 (green) and γ-H2AX (red). Representative of three independent experiments. C, percentage of cells with ≥10 Rad51 foci after treatment with 100 nmol/L (MiaPaCa2), 300 nmol/L (BxPC3 and M-Panc96), or 3 μmol/L gemcitabine (Panc1) ± 0.3 μmol/L PD-321852 for 24 h. Mean ± SE. *, P < 0.01, compared with control; †, P < 0.01, compared with gemcitabine plus PD-321852, one-way ANOVA. D, whole-cell lysates from cells treated with gemcitabine alone, PD-321852, or gemcitabine plus PD-321852 for 24 h were assayed for Chk1, Chk2, and Rad51 protein levels. Representative of two to three independent experiments.
Discussion

In this study of pancreatic cancer cells, we found evidence supporting the hypothesis that gemcitabine chemosensitization by Chk1 inhibition correlates well with inhibition of the Rad51 DNA damage response. Our data further suggest that the significance of the replication stress or DNA damage marker γ-H2AX depends on the cell line examined and may not always be a reliable indicator of gemcitabine-induced DNA damage and/or repair.

It has been reported previously that Chk1 directly activates Rad51 and homologous recombination repair in response to replication stress (19). Consistent with previous reports (30), we found that gemcitabine treatment caused the accumulation of Rad51 nuclear foci. It is unclear whether these foci result from gemcitabine-induced stalled replication forks (replication stress) and/or gemcitabine-induced accumulation of cells in S phase. This distinction may be important in some cell lines, as Tarsounas et al. have suggested that the mechanism for DNA damage-induced Rad51 focus formation, which is BRCA2-dependent, differs from the BRCA2-independent formation of Rad51 foci in unperturbed S-phase cells (31).

It has been reported that Rad51 overexpression may be a common event in pancreatic cancer that contributes to tumor resistance to DNA-damaging agents (32). PD-321852 may therefore sensitize cells to gemcitabine by inhibiting Rad51 localization in response to gemcitabine-induced replication arrest. Indeed, we found that PD-321852 attenuated gemcitabine-induced Rad51 focus formation in each cell line examined. The magnitude of this effect, however, was much greater in the more sensitized cell lines where PD-321852 not only inhibited gemcitabine-induced

Figure 5. Phosphorylation of γ-H2AX in BxPC3 and Panc1 cells following treatment with gemcitabine ± PD-321852. BxPC3 cells were treated with 100 nmol/L gemcitabine ± 0.1 μmol/L PD-321852 for 24 h. The agents were then washed out (C, arrows), and cells were collected either immediately or after incubation in control medium for an additional 6, 24, 48, or 72 h. Cells were analyzed by flow cytometry for the presence of γ-H2AX staining as a function of DNA content (A and C). The number inside the gate is the percentage of the population staining positive for γ-H2AX. Panc1 cells were treated with either 1 μmol/L gemcitabine ± 0.1 μmol/L PD-321852 or 100 nmol/L gemcitabine ± 0.1 μmol/L PD-321852 (B and D) for 24 h and analyzed as above. C and D, mean ± SE of three to five independent experiments. *, P < 0.05; one-way ANOVA.
Rad51 focus formation but also was associated with depletion of Rad51 protein from whole-cell lysates. This result is consistent with previous reports from Yao et al., who reported that depletion of Chk1 with siRNA leads to a loss of Rad51 protein in human leukemia cells (33), and Bahassi et al., who reported that Chk1 siRNA treatment prevented UV-induced Rad51 focus formation (20). The ability of PD-321852 to block induction of gemcitabine-induced Rad51 foci is further evidence that Chk1 is inhibited by this compound.

Recent studies have shown that inhibition of Chk1 sensitizes tumor cells to a variety of chemotherapeutic agents (8, 9, 15, 17). As a result, this protein has become a promising target for therapeutic intervention, with at least three Chk1 inhibitors entering clinical trials recently and others poised to follow (34). A major issue in clinical development of molecularly targeted agents, including Chk1 inhibitors, is how to assess pharmacodynamic endpoints that will reflect the variation in efficacy among individual patients. It has been widely assumed that accelerated cell cycle progression resulting from increased Cdc25A activity is pivotal in mediating the cytotoxic consequences of Chk1 inhibition and that the molecular markers for these events should be useful correlates for therapeutic effectiveness. Perez et al. observed G2-M checkpoint abrogation in tumor biopsy specimens from patients treated with a Chk1 inhibitor (UCN-01) in combination with cis-platinum, showing this effect in tumor tissue for the first time in a clinical trial (35).

Our results indicate, however, that Cdc25A stabilization and S-M or G2-M checkpoint abrogation are not necessarily reliable correlates of the chemosensitizing effects of a Chk1 inhibitor. For example, PD-321852 did not cause premature mitotic entry in either gemcitabine-treated BxPC3 or M-Panc96 cells, both of which were chemosensitized to gemcitabine by this compound. This result was unexpected, as we and others have previously found sensitization to gemcitabine by Chk1 inhibition with either siRNA (17) or the checkpoint inhibitor EXEL-9844, which inhibits both Chk1 and Chk2 (8), was associated with premature mitotic entry. Only in MiaPaCa2 cells did our results fit the prevailing model: inhibition of Chk1 led to abrogation of gemcitabine-induced Cdc25A degradation, premature mitotic entry, and sensitization to gemcitabine. Although these results do not mean that Cdc25A up-regulation and S-M or G2-M checkpoint abrogation are not important players in mediating the sensitizing effects of Chk1 inhibitors, they indicate that endpoints reflecting other molecular events need to be considered to assess the efficacy of treatment with a Chk1 antagonist.

Although catalytic inhibition of Chk1, as indicated by Cdc25A stabilization, was not strictly associated with gemcitabine chemosensitization, depletion of Chk1 protein occurred in each of the sensitized cell lines after drug treatment. This result is consistent with our previous siRNA studies, where we found that depletion of Chk1 with siRNA resulted in modest chemosensitization of Panc1 cells (17). Zhang et al. have suggested that, in response to replication stress, the proteasome-mediated degradation of activated Chk1 functions to terminate this cell cycle checkpoint and that the loss of Chk1 protein in chronically stressed tumor cells may be a determinant of sensitivity to topoisomerase inhibitors such as camptothecin (26). Our data suggest there may be an added benefit, in terms of chemosensitization, with stimulation of the Chk1 degradation pathway in addition to catalytic inhibition of Chk1 activity.

Although our original intent was to use γ-H2AX as a marker of gemcitabine-induced DNA damage and repair, it has become increasing clear that γ-H2AX also accumulates in response to drug-induced stalled replication forks in S-phase cells (18, 29) and, in our model, most likely represents an inability to recover from gemcitabine-induced replication stress. This interpretation is consistent with the recovery from gemcitabine-induced S-phase arrest and accompanied loss of γ-H2AX we observed in BxPC3 cells, the persistence of γ-H2AX in gemcitabine-treated Panc1 cells, which remained S phase arrested 72 h after removal of drug, and the ability of PD-321852 to inhibit Rad51 focus formation, recovery of replication stress, and loss of γ-H2AX in BxPC3 cells. Another possible interpretation of our results is that the high level of γ-H2AX induction in Panc1 cells, at relative nontoxic drug concentrations, reflects a more robust response to gemcitabine-induced replication stress that is actually protective in terms of drug-induced loss of clonogenicity.

One limitation of the current work is that, although PD-321852 is reasonably selective for Chk1 (20), it may inhibit other kinases as well, and these activities may contribute to the chemosensitization patterns observed. Furthermore, each of the three Chk1 inhibitors that are now in clinical trials has a distinctive selectivity profile (e.g., two of the three also inhibit Chk2), and results obtained with PD-321852, or any particular Chk1 inhibitor, may not necessarily extrapolate to others.

Finally, although the four cell lines used in this study clearly represent some extent of the biological diversity of human pancreatic cancer, it is unclear whether any of the phenotypes observed are more representative of a “typical” tumor than the others. A limitation shared by all in vitro studies is that established cell lines are frequently seen to have diverged substantially from the tumors from which they were derived. It would therefore be useful to interrogate the endpoints used in this study with a model system that is closer to primary tumors, such as early-passage xenografts (36).

The results of our studies suggest that, although stabilization of Cdc25A and S-M or G2-M checkpoint abrogation may be good surrogate markers for validating catalytic inhibition of Chk1, abrogation of drug-induced Rad51 focus formation and depletion of Rad51 protein, accompanied by inhibitor-induced persistence of γ-H2AX, may be more reliable markers of the chemosensitizing effects of a Chk1 inhibitor in pancreatic cancer cells.
Disclosure of Potential Conflicts of Interest

J. Maybaum: grant recipient, AstraZeneca. No other potential conflicts of interest were disclosed.

Acknowledgments

We thank Drs. Mary A. Davis and Sheryl A. Flanagan for critical reviews of this article.

References

Molecular Cancer Therapeutics

Gemcitabine sensitization by checkpoint kinase 1 inhibition correlates with inhibition of a Rad51 DNA damage response in pancreatic cancer cells

Leslie A. Parsels, Meredith A. Morgan, Daria M. Tanska, et al.

Mol Cancer Ther 2009;8:45-54.

Updated version
Access the most recent version of this article at:
http://mct.aacrjournals.org/content/8/1/45

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2008/12/29/8.1.45.DC1

Cited articles
This article cites 35 articles, 19 of which you can access for free at:
http://mct.aacrjournals.org/content/8/1/45.full.html#ref-list-1

Citing articles
This article has been cited by 20 HighWire-hosted articles. Access the articles at:
/content/8/1/45.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.