Inhibition of Protein Phosphatase 2A Radiosensitizes Pancreatic Cancers by Modulating CDC25C/CDK1 and Homologous Recombination Repair

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Abstract

Purpose: To identify targets whose inhibition may enhance the efficacy of chemoradiation in pancreatic cancer and thus improve survival, we conducted an siRNA library screen in pancreatic cancer cells. We investigated PPP2R1A, a scaffolding subunit of protein phosphatase 2A (PP2A) as a lead radiosensitizing target.

Experimental Design: We determined the effect of PP2A inhibition by genetic (PPP2R1A siRNA) and pharmacologic (LB100, a small molecule entering phase I clinical trials) approaches on radiosensitization of Panc-1 and MiaPaCa-2 pancreatic cancer cells both in vitro and in vivo.

Results: PPP2R1A depletion by siRNA radiosensitized Panc-1 and MiaPaCa-2 cells, with radiation enhancement ratios of 1.4 ($P < 0.05$). Likewise, LB100 produced similar radiosensitization in pancreatic cancer cells, but minimal radiosensitization in normal small intestinal cells. Mechanistically, PPP2R1A siRNA or LB100 caused aberrant CDK1 activation, likely resulting from accumulation of the active forms of PLK1 (pPLK1 T210) and CDC25C (pCDC25C T130). Furthermore, LB100 inhibited radiation-induced Rad51 focus formation and homologous recombination repair (HRR), ultimately leading to persistent radiation-induced DNA damage, as reflected by γ-H2AX expression. Finally, we identified CDC25C as a key PP2A substrate involved in LB100-mediated radiosensitization as depletion of CDC25C partially reversed LB100-mediated radiosensitization. In a mouse xenograft model of human pancreatic cancer, LB100 produced significant radiosensitization with minimal weight loss.

Conclusions: Collectively, our data show that PP2A inhibition radiosensitizes pancreatic cancer both in vitro and in vivo via activation of CDC25C/CDK1 and inhibition of HRR, and provide proof-of-concept evidence that PP2A is a promising target for the improvement of local therapy in pancreatic cancer.

Introduction

Pancreatic cancer remains one of the most difficult-to-treat cancers with a 5-year survival of less than 6%, representing the most lethal type of cancer in the United States (1). Although gemcitabine has been the standard systemic therapy in this malignancy, new therapies for metastatic disease, such as FOLFIRINOX and Abraxane plus gemcitabine are emerging (2, 3). However, for approximately 30% of patients with pancreatic cancer, local disease progression is the cause of mortality (4), highlighting the importance of local disease control. Intensification of therapy for locally advanced disease with concurrent radiation and gemcitabine results in improved survival (5–7). Despite these advancements, the median survival for patients with locally advanced disease treated with chemoradiation ranges from 11 to 19 months, underscoring the need for further improvement of local disease therapy.

Previously, we used a library of siRNA against protein kinases and E3 ubiquitin ligases to identify potential radiosensitizing targets in glioblastoma cells (8). Using an expanded version of this platform designed to target the druggable genome, we conducted a high-throughput siRNA library screen in human pancreatic cancer cells in an attempt to identify novel molecular targets whose silencing caused sensitization to the combination of gemcitabine and radiation. We identified PPP2R1A, a structural subunit of protein phosphatase 2A (PP2A), as a lead target that when depleted by siRNA enhanced the cytotoxicity of chemoradiation treatment (Supplementary Fig. S1).
**Translational Relevance**

Intensification of local therapy through the use of radiation (in combination with chemotherapy) improves survival in patients with locally advanced pancreatic cancer. To further improve the efficacy of chemoradiation therapy in pancreatic cancer, we sought to identify novel targets whose inhibition would sensitize pancreatic cancer cells to therapy. Identification and confirmation of PPP2R1A, a subunit of PP2A, as a radiosensitizing target led us to combine the small-molecule PP2A inhibitor, LB100, currently entering phase I clinical trials with radiation in pancreatic cancer. We found that LB100 sensitized pancreatic cancer cells and tumors to radiation, but produced only minimal sensitization of normal small intestinal cells with no observable toxicity in animals. Our study provides proof-of-concept evidence that PP2A inhibition is a promising strategy for selectively improving local therapy and thus survival in pancreatic cancer.

PP2A is a ubiquitous, multifunctional serine/threonine phosphatase that typically contains a catalytic (C), structural (A), and variable regulatory subunit (B). In humans, the C and A subunits are each encoded by 2 distinct genes (PPP2CA/Cα and PPP2CB/Cβ for C subunit; PPP2R1A and PPP2R1B for A subunit). The B subunit is divided into 4 unrelated families (B/B55/PR55, B′/B56/PR61/PPP2R5, B''/PR72/PPP2R3 and B''''/PR93/PR110) with at least 26 isoforms that control the substrate specificity of the PP2A heterotrimeric complex (9–11). The complexity of the PP2A complex within cells allows the enzyme to mediate diverse physiologic functions. Its aberrant activity has been linked with several pathologic conditions, notably cancer (9). On the basis of the role of PP2A in mediating the DNA damage response (12) and the prevalence of aberrations in the DNA damage response in cancer, inhibition of PP2A may further exacerbate DNA damage in cancer cells.

Pharmacologic inhibition of PP2A produces antitumor activity against pancreatic cancer and other human cancer types highlighting PP2A as an attractive target for the development of novel anticancer drugs with an emphasis on cantharidin and norcantharidin analogs (13–17). Inhibition of PP2A sensitizes cancer cells to radiation and chemotherapy by mechanisms including sustained phosphorylation of p53, γ-H2AX, PLK1, AKT, Ku, and DNA-PKcs leading to apoptosis, cell-cycle deregulation, and inhibition of DNA repair (13, 14, 18–21). PP2A also regulates CDC25C although it is not known what effect this imparts on sensitization to radiation or chemotherapy (22). On the basis of these mechanisms of action, PP2A is potentially a useful target for sensitization to radiation.

Given that previous studies investigating PP2A inhibition as a radiosensitizing strategy were conducted with siRNA or toxic small-molecule inhibitors (e.g., okadaic acid), in the present study, we investigated the novel pharmacologic PP2A inhibitor, LB100, a clinical candidate agent currently entering phase I clinical trials (13). While LB100 (and its homolog LB1.2) has been shown to sensitize to temozolomide and doxorubicin (14, 23), it remains to be determined whether it has radiosensitizing activity. As we identified PPP2R1A as a sensitizing target in our siRNA library screen, we compared radiosensitization by LB100 to PPP2R1A siRNA. We found that both LB100 and PPP2R1A siRNA produced similar and significant radiosensitization in pancreatic cancer cells. To determine the mechanism(s) of radiosensitization by PP2A inhibition, we assessed known PP2A substrates such as CDC25C, as well as DNA response and repair via analysis of γ-H2AX, RAD51, and an HRR reporter assay. Finally, we tested the radiosensitizing efficacy and tolerability of LB100 in in vivo pancreatic tumor models.

**Materials and Methods**

**Cell culture, siRNA, and drug solutions**

Human pancreatic cancer cell lines MiaPaCa-2 and Panc-1 and the normal human small intestine epithelial cell line CCL-241 were obtained from American Type Culture Collection (ATCC) and grown in either Dulbecco's modified Eagle medium (DMEM) (MiaPaCa-2 and Panc-1) with 10% FBS or HybricCare medium (ATCC) supplemented with 30 ng/mL EGF and 10% FBS (CCL-241). Cell cultures were maintained in an atmosphere of 5% CO₂/95% air at 37°C and tested free of Mycoplasma contamination. Nonspecific, PPP2R1A, and CDC25C SMARTpool siRNAs (100 nmol/L; Dharmacon) were delivered using X-tremeGENE transfection reagent (Roche) as per the manufacturer's protocol. LB100 (for structure, see Supplementary Fig. S3A), a water-soluble homolog of LB102 that is a specific competitive small-molecule inhibitor of PP2A (versus PP1; refs. 13, 24), was provided by Lixte Biotechnology Holdings, Inc.

**siRNA screen**

Primary and confirmatory siRNA screens were conducted with the Dharmacon siRNA library. This library includes siRNA oligonucleotides against 8800 “druggable” genes with 4 siRNAs per gene that have each been validated to silence their target mRNA up to 75%. MiaPaCa-2 cells plated in a 96-well format at 400 cells per well were transfected with the siRNA library (40 nmol/L) using X-tremeGENE transfection reagent (Roche) and tested free of contamination. Nonspecific, CHK1- and PLK1-targeting SMARTpool siRNAs (Dharmacon) were included as negative and positive controls, respectively. Twenty-four hours after transfection, either gemcitabine (50 nmol/L) or vehicle (serum-free medium) was added to the wells for 24 hours, after which the medium was replaced with fresh growth medium with antibiotics. After additional incubation for 24 hours, the gemcitabine-treated cells were treated with 4 Gy ionizing radiation (IR). Cell viability was determined 72 to 96 hours post-IR with the ATPlite Kit (Perkin Elmer) according to the manufacturer’s instructions. Radiation enhancement ratios were calculated by dividing the viability of si-NS+gemcitabine+IR-treated wells (normalized for si-NS toxicity) by the viability of specific...
siRNA + gemcitabine + IR-treated wells (normalized for specific siRNA toxicity). Using similar methodology, a secondary, confirmatory siRNA screen was conducted; ultimately yielding a total of 69 identified and confirmed hits. Supplementary Figure S1 illustrates the siRNA screening methodology and the top 15 hits.

**Clonogenic survival assay**

Cells were seeded in 60 mm dishes at cloning densities in duplicate or triplicate, treated with siRNA or LB100, and irradiated, followed by incubation at 37°C for 7 to 12 days. After fixation with 0.2% crystal violet, colonies containing more than 50 individual cells were counted. Survival curves were fitted using the linear-quadratic equation and mean inactivation dose was calculated as previously described (25). Radiation enhancement ratios (RER) were calculated as the ratio of the mean inactivation dose under control conditions divided by the mean inactivation dose after either siRNA or LB100 treatment (26).

**Immunoblotting**

Whole cell and homogenized tissue lysates were prepared in cold lysis buffer (50 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 2 mmol/L EDTA, 1% SDS, 0.2% Triton X-100, and 0.3% NP-40) supplemented with phosphatase (Roche) and protease inhibitors (Roche) as previously described (27). The following antibodies were used: PPP2R1A (Abcam), phospho-protease inhibitors (Roche) as previously described (27). The following antibodies were used: PPP2R1A (Abcam), phospho-CDK1 (Y15), phospho-PLK1 (T120) (Cell Signaling Technology), γ-H2AX (Millipore), WEE1, CDK1, CDC25C (Santa Cruz Biotechnology), β-actin (Sigma), and phospho-CDC25 (T130; a gift from S. Kornbluth, Duke University, Durham, NC).

**Flow cytometry**

Cells were trypsinized, washed with PBS, and fixed in ice-cold 70% ethanol. For γ-H2AX analysis, cells were incubated overnight at 4°C with γ-H2AX antibody (Millipore) diluted in PBS containing 1% FBS and 0.2% Triton X-100 (PBT). After centrifugation, cells were incubated for 1 hour with fluorescein isothiocyanate–conjugated anti-mouse antibody (Sigma) diluted 1:100 in PBT. After centrifugation, cells were incubated for 1 hour with fluorescein isothiocyanate–conjugated anti-mouse antibody (Sigma) diluted 1:100 in PBT. Samples were then rinsed with PBT buffer and stained with propidium iodide solution (BD) for analysis. In untreated, control samples a gate was arbitrarily set to define a region of positive staining for γ-H2AX of approximately 5%. This gate was then overlayed on the treated samples. For phospho-histone H3 (S10) analysis, samples were processed as previously described (28). Samples were then permeabilized with PBS containing 0.5% Triton X-100, blocked with 15% FBS in PBS, and incubated in blocking buffer with primary antibody against either RAD51 (GeneTex), γ-H2AX (Millipore), or 53BP1 (Novus Biologicals), followed by incubation with Alexa Fluor 594 goat anti-mouse IgG or Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen). Samples were then rinsed with Prolong Gold Antifade Reagent containing DAPI (Invitrogen). RAD51 foci were imaged with an Olympus FV500 confocal microscope (Olympus America; × 60 objective), whereas γ-H2AX and 53BP1 foci were imaged with a fluorescent microscope (IX71, Olympus America; × 40 objective) and a CoolSnapEZ camera (Photometrics).

**Homologous recombination repair analysis**

MiaPaCa2 cells were transfected with a construct encoding the DR-GFP recombination substrate (29, 30) using SuperFect transfection reagent (Qiagen) according to the manufacturer’s protocol. Stable clones were selected with puromycin. To measure HRR, double-strand breaks were induced by adenoviral-mediated expression of the restriction enzyme I-SceI, which cleaves the DR-GFP gene. HRR of this break restores wild-type GFP expression. The model was validated with Rad51 siRNA, which inhibited restoration of the GFP signal. Experiments were carried out in a single clone selected for consistent GFP expression following adenoviral transfection. Cells were collected 46 hours post-I-SceI adenoviral infection, and the extent of double-strand break repair by HRR was quantified by flow cytometric analysis of GFP expression.

**PP2A phosphatase activity assay**

Pancreatic cancer cells grown to 70% confluence in 100 mm dishes were treated with LB100 as indicated. After treatment for 2 hours, cells were washed twice with cold PBS (pH 7.4) and then lysed in extraction buffer (20 mmol/L imidazole-HCl, 2 mmol/L EDTA, 2 mmol/L EGTA, pH 7.0) supplemented with protease inhibitors (Roche) for 30 minutes on ice. Cell lysates were sonicated for 10 seconds and centrifuged at 2,000 × g for 5 min. Supernatants containing 400 μg of protein were assayed with the PP2A Immunoprecipitation Phosphatase Assay Kit (Millipore) according to the manufacturer’s protocol. PP2A activity in MiaPaCa-2 xenografts was determined using the conditions described above.

**Animal experiments**

All animal experiments were carried out according to a protocol approved by the University Committee for Use and Care of Animals. Five million MiaPaCa-2 cells in a 1:1 mixture of 10% FBS-DMEM/Matrigel (BD Biosciences) were injected subcutaneously into both flanks of nude mice. When the average tumor volume reached the size of approximately 120 mm³, the mice were randomized and the treatment was initiated. LB100 (1.5 mg/kg, i.p.) and radiation (1.2 Gy/fraction) were given once a day, 5 days a week for 2 weeks. Radiation was delivered directly to the tumor with the rest of the animal shielded. For combination treatment, LB100 was given 2 hours before radiation. The growth of tumors (8 for control group, 12–16 for the other groups) was measured twice a week and the average tumor...
volume (TV) was calculated according to the equation: $\text{TV} = \frac{(L \times W^2)}{2}$, where $L$ and $W$ are the longer and shorter dimensions of the tumor, respectively.

**Irradiation**

Irradiations were carried out using a Philips RT250 (Kimtron Medical) at a dose rate of approximately 2 Gy/minute in the University of Michigan Comprehensive Cancer Center Experimental Irradiation Core (Ann Arbor, MI). Dosimetry was carried out using an ionization chamber connected to an electrometer system that is directly traceable to a National Institute of Standards and Technology calibration. For tumor irradiation, animals were anesthetized with isoflurane and positioned such that the apex of each flank tumor was at the center of a 2.4 cm aperture in the secondary collimator, with the rest of the mouse shielded from radiation.

**Statistical analysis**

For *in vitro* experiments, one-way ANOVA with a Tukey post-test was used. For *in vivo* tumor growth studies, log-rank tests were conducted to compare tumor volume doubling times between treatment arms. Time to tumor volume doubling is defined as the earliest day on which the tumor volume is at least twice as large as on the first day of treatment. Statistical significance was defined as a two-sided $P$ value < 0.05. A Bayesian hierarchical changepoint (BHC) model was used to estimate tumor growth profiles, characterized by a pre-nadir regression rate, a regression period, a nadir volume, and a post-nadir regrowth rate (31). Tumor cell killing fraction and tumor growth delay were derived from the BHC model and compared between treatment arms as previously described (32). A feature is considered to be statistically significant if the 95% highest probability density (HPD) interval of the difference in that feature does not cover zero.

**Results**

**PP2R1A depletion radiosensitizes pancreatic cancer cells**

To identify novel targets that modulate the sensitivity of pancreatic cancer cells to gemcitabine-based chemoradiation treatment, we conducted an siRNA library screen in MiaPaCa-2 cells (Supplementary Fig. S1). Confirmed “hits” were ranked ordered by their magnitude of sensitization, and we found PP2R1A, a structural subunit of PP2A, among the top 5 targets identified (which also included CHK1, PLA2G4B, GPR2, and ATR). To validate PP2R1A as a novel target for radiosensitization, we conducted clonogenic survival assays with PP2R1A siRNA in MiaPaCa-2 and Panc-1 cells. Depletion of PP2R1A resulted in a significant radiosensitization with enhancement ratios of 1.4 in both MiaPaCa-2 and Panc-1 cells (Fig. 1). Given the necessity of PP2R1A in the assembly of the functional PP2A holoenzyme (9), these data support the candidacy of PP2A as a radiosensitizing target in pancreatic cancer cells.
DNA double-strand breaks. Using flow cytometry, we found that PPP2R1A depletion caused persistent γ-H2AX expression in response to radiation (at 24 hours post-IR) in MiaPaCa-2 cells compared with control cells treated with radiation alone (Fig. 2A and B). Combined treatment with PPP2R1A siRNA and radiation caused persistent γ-H2AX expression in both MiaPaCa-2 and Panc-1 cells, as measured by immunoblotting (Fig. 2C). Given that PP2A functions as a negative regulator of CDK1 via inhibition of CDC25C (21, 34), we hypothesized that PPP2R1A siRNA would decrease pCDK1 (Y15) to increase, a state consistent with inhibition in response to radiation. Indeed, radiation alone caused a decrease in pCDK1 (Y15; an inhibitory phosphorylation site) (21, 34), while radiation plus PPP2R1A siRNA increased pCDK1, consistent with inhibition of CyclinB/CDK1 activity and G2 arrest. PPP2R1A siRNA sensitization in response to radiation (at 24 hours post-IR) in MiaPaCa-2 cells treated with either si-NS or si-PPP2R1A + IR (7.5 Gy) were determined by the gates shown (A). Data presented are the mean percentage of γ-H2AX-positive cells ± SE (n = 3) and statistical significance versus IR (*). Immunoblots are representative of at least 2 independent experiments.

Figure 2. PPP2R1A depletion leads to persistent radiation-induced DNA damage. MiaPaCa-2 (A–C) or Panc-1 (C) cells transfected with either si-NS or si-PPP2R1A were treated with radiation and collected 24 hours later for flow cytometric analysis of γ-H2AX (A and B) and immunoblot analysis of the indicated proteins (C). γ-H2AX–positive cells are defined by the gates shown (A). Data presented are the mean percentage of γ-H2AX-positive cells ± SE (n = 3) and statistical significance versus IR (*). Immunoblots are representative of at least 2 independent experiments.

Figure 3. LB100 sensitizes pancreatic cancer cells to radiation. (A) γ-H2AX-positive cells are defined by the gates shown (A). Data presented are the mean percentage of γ-H2AX-positive cells ± SE (n = 3) and statistical significance versus IR (*). Immunoblots are representative of at least 2 independent experiments. (B) Cells treated with either control siRNA (si-NS) or si-PPP2R1A were treated with radiation and collected 24 hours later for flow cytometric analysis of γ-H2AX (A and B) and immunoblot analysis of the indicated proteins (C). γ-H2AX–positive cells are defined by the gates shown (A). Data presented are the mean percentage of γ-H2AX-positive cells ± SE (n = 3) and statistical significance versus IR (*). Immunoblots are representative of at least 2 independent experiments.
and radiation on mitotic entry (Supplementary Fig. S5).

mitosis, there was no obvious interaction between LB100
caused an increase in the percentage of cells entering
LB100 and radiation. Although LB100 treatment alone
mitotic marker pHistoneH3 (Ser10) in cells treated with
these two possibilities, we first examined levels of the
by LB100 may also inhibit HRR. To distinguish between
HRR (33, 40), we hypothesized that inhibition of PP2A
and CDK1 have recently been implicated in regulation of
entry, LB100 may promote aberrant entry into mitosis
contribute to LB100-mediated radiosensitization. First,
CDK1 suggest at least two possible mechanisms that may
result from a feedback loop between PLK1 and/or CDK1
increase in CDK1 phosphorylation at Y15). Indeed, treat-
ment with LB100 in the presence or absence of radiation led
to a decrease in Y15 phosphorylation on CDK1 (Fig. 4B).
Interestingly, we found that LB100 treatment also led to a
decrease in WEE1 protein levels (Fig. 4B). This change may
result from a feedback loop between PLK1 and/or CDK1
leading to WEE1 phosphorylation and subsequent ubiqui-
tin-mediated proteosomal degradation (38). Taken togeth-
er, these data show a series of molecular changes in response
to inhibition of PP2A by LB100 which likely result in
activation of CDK1.

Inhibition of HRR by LB100 correlates with persistent DNA damage

The observed changes in CDC25C, PLK1, WEE1, and
CDK1 suggest at least two possible mechanisms that may
contribute to LB100-mediated radiosensitization. First,
based on the well-established role of CDK1 in mitotic
entry, LB100 may promote aberrant entry into mitosis
following radiation (39). Second, because PP2A, WEE1,
and CDK1 have recently been implicated in regulation of
HRR (33, 40), we hypothesized that inhibition of PP2A
by LB100 may also inhibit HRR. To distinguish between
these two possibilities, we first examined levels of the
mitotic marker pHistoneH3 (Ser10) in cells treated with
LB100 and radiation. Although LB100 treatment alone
causde an increase in the percentage of cells entering
mitosis, there was no obvious interaction between LB100
and radiation on mitotic entry (Supplementary Fig. S5).

On the other hand, in a previously described reporter
assay for homology-directed repair of DNA double-strand
breaks (29), LB100 significantly inhibited HRR in irradi-
cated cells (Fig. 4C). Furthermore, LB100 significantly
inhibited radiation-induced RAD51 focus formation
(Fig. 4D).

Given our finding that PPP2R1A depletion interferes with
the DNA damage response (Fig. 2), we hypothesized that
HRR suppression by the combination of LB100 and radi-
ation would also correlate with persistent DNA damage. To
test this hypothesis, we measured γ-H2AX levels in cells
treated with radiation, LB100, or the combination, by both
immunoblot and immunofluorescent staining. As shown
in Fig. 4E, γ-H2AX levels were higher in the combination
treatment group than in cells treated with either radiation
or LB100 alone. In addition, more γ-H2AX foci–positive cells
were consistently observed in the combination treatment
group than in cells treated with radiation alone (Supple-
mental Fig. S6). To rule out the possibility that the
observed increase in γ-H2AX was a result of direct modu-
lation of γ-H2AX by PP2A (19), we assessed 53BP1 focus
formation, an independent marker of DNA double-strand
breaks (41). Similar to γ-H2AX, we found that LB100 caused
an increase 53BP1 focus formation in response to radiation
in MiaPaCa-2 and Panc-1 cells (Supplementary Fig. S7) thus
confirming the presence of DNA double-strand breaks.
Taken together, these results suggest that LB100 inhibits
HRR in irradiated cells, ultimately leading to persistent DNA
damage.
CDC25C depletion partially reverses LB100-mediated radiosensitization

The CDC25C phosphatase positively regulates CDK1 activity by removing the inhibitory phosphorylation at Y15. On the basis of our observation that the active form of CDC25C (pCDC25C; T130) increased as a consequence of LB100 treatment, we hypothesized that CDC25C may play a critical role in LB100-mediated radiosensitization. We reasoned that if CDC25C activation is causal, then CDC25C inhibition should attenuate the radiosensitizing effects of LB100. Indeed, in MiaPaCa-2 cells, siRNA-based CDC25C silencing depleted CDC25C protein and restored pCDK1 (Y15) levels in response to LB100 treatment (Fig. 5A). More importantly, CDC25C silencing significantly suppressed LB100-induced radiosensitization (Fig. 5B; *P* < 0.05). This rescue experiment clearly shows that CDC25C plays, at least in part, a causal role in LB100-mediated radiosensitization.

LB100 radiosensitizes pancreatic cancer in an in vivo xenograft tumor model

Finally, we investigated the radiosensitizing activity of LB100 using an *in vivo* MiaPaCa-2 xenograft model. We first defined the time frame in which LB100 caused maximal inhibition of PP2A activity in tumor tissues. As shown in Fig. 6A, the greatest inhibition of PP2A activity (~20%) was achieved 2 hours after administration of LB100 (1.5 mg/kg). We therefore chose an *in vivo* treatment schedule in which LB100 was administered 2 hours post-irradiation. We treated tumor-bearing mice with LB100 and/or radiation for 5 consecutive days per week for 2 weeks in a manner similar to clinical radiation fractionation schedules. We found that treatment with LB100 alone had a...
Radiosensitization by PP2A Inhibition

Figure 5. LB100-mediated radiosensitization is partially rescued by CDC25C depletion. A, MiaPaCa-2 cells were transfected with the indicated siRNA pools. Seventy-two hours after transfection, cells were treated with LB100 for 2 hours pre- and 24 hours post-IR and harvested for immunoblotting for the indicated proteins. B, MiaPaCa-2 cells were transfected and plated for clonogenic survival as illustrated in Fig. 1B with the addition of LB100 (1 µmol/L) 2 hours pre- and 48 hours post-IR. Plots shown are representative of 3 independent experiments. Data in the legend are the mean RER ± SE (n = 3). The mean cytotoxicity of siRNA treatments was 1.0 (mock), 0.9 (si-NS), 0.7 (si-NS + LB100), 0.6 (si-CDC25C), and 0.5 (si-CDC25C + LB100) with less than 15% SE. Statistical significance versus Mock (†) or si-NS + LB100 (‡) is indicated.

modest, yet significant effect on tumor growth, extending the time required for tumor volume doubling from 8.5 days in vehicle-treated animals to 12.5 days (P < 0.05; Fig. 6B and C). Radiation treatment also significantly delayed tumor growth and produced a 15.5-day delay in the time required for tumor volume doubling relative to vehicle-treated animals. More importantly, LB100 in combination with radiation produced the greatest effects on tumor growth resulting in 25- and 9.5-day delays in tumor volume doubling relative to vehicle- or radiation-treated groups, respectively. Tumor radiosensitization by LB100 was also evidenced by in vivo tumor growth delay (95% HPD interval of the difference: 0.1–19.4 days), tumor cell killing (95% HPD interval of the difference: 6%–70%), and tumor growth delay (95% HPD interval of the difference: 4.2–26.3 days). The combination of LB100 and radiation was well tolerated in mice and produced minimal (<10%) weight loss (Supplementary Fig. S8). Thus, LB100 is a potent radiosensitizer in this in vivo pancreatic cancer xenograft model.

To assess the molecular mechanisms associated with LB100 radiosensitization in vivo, we examined pCDC25C (T130) and pCDK1 (Y15) in MiaPaCa-2 xenografts following treatment with a single dose of LB100 and radiation. We found a small increase in pCDC25C levels in MiaPaCa-2 tumors in response to LB100 alone or in combination with radiation, although the magnitude of this response was variable between tumors (Fig. 6D). Consistent with this increase in pCDC25C (T130), we observed a decrease in pCDK1 (Y15) levels and an increase in γ-H2AX levels. Taken together, our results show that LB100 is an effective and tolerable agent for sensitizing pancreatic tumors to radiotherapy and acts, at least in part, by modulating CDC25C and CDK1.

Discussion

Through an unbiased siRNA library screen, we have identified PPP2R1A, a subunit of the PP2A holoenzyme, as a potent sensitizing target in pancreatic cancer cells. We found that inactivation of PP2A via genetic (siRNA silencing) or pharmacologic (LB100) approaches sensitized pancreatic cancer cells to radiation. Our mechanistic studies revealed that PP2A inhibition caused an accumulation of the active forms of PLK1 and CDC25C which corresponded to an increase in the active form of CDK1. We further showed that LB100 inhibits HRR in irradiated cells. Given the excitement in the oncology community toward the development of HRR inhibitors as a novel class of anticancer agents (29, 33, 40, 42–44), our data are of importance in that they show that inhibition of PP2A results in impaired HRR, suggesting that PP2A inhibition may be efficacious not only in combination with radiation but likely with other molecularly targeted or DNA-damaging agents. Several previous studies have used both genetic and pharmacologic approaches to show that PP2A inhibition can sensitize tumor cells to both radiation and chemotherapy (13, 18, 45). The development of pharmacologic inhibitors of PP2A, however, has been limited by the intrinsic toxicity of these agents. For many years, cantharidin, a natural product isolated from blister beetles in Chinese medicine, has been investigated for its anticancer properties as well as its ability to inhibit PP2A (46). Given the high cytotoxicity of cantharidin, extensive effort has been made to develop less toxic derivatives, such as LB100. Thus far, inhibition of PP2A with LB100 has not produced substantial toxicity in animal models (23, 24). As PP2A is a ubiquitous enzyme, understanding how inhibition of this phosphatase may selectively sensitize cancer cells (versus normal cells) to radiation or chemotherapy remains a key issue. Given that cancer cells often contain alterations in their DNA damage response machinery (both DNA damage repair- and cell cycle-related), and that PP2A regulates many of these proteins, it is conceivable that deregulation of the DNA...
damage response by PP2A inhibition would be more detrimental to cancer cells than normal cells (12, 47). In addition, endogenous DNA damage related to oncogene expression likely renders cancer cells more vulnerable than normal cells to inhibition of the DNA damage response (48). Furthermore, because tumor cells are often aberrant in their G1 checkpoint, where DNA repair by nonhomologous end-joining mainly occurs, they have an increased reliance on HRR for repair of DNA double-strand breaks (49). Thus, inhibition of HRR by LB100 may represent an additional tumor cell–selective sensitizing mechanism. Indeed, we found that normal small intestinal cells were less radiosensitized by LB100 than pancreatic cancer cells and that the combination of LB100 with radiation produced a minimal toxicity in mice.

PP2A exerts phosphatase activity on an array of proteins involved in the DNA damage response including DNA-PKcs, CHK1, CHK2, CDC25C, γ-H2AX, PLK1, and p53 (12). When we investigated potential PP2A substrates involved in LB100-mediated radiosensitization, we found that LB100 caused accumulation of the active forms of both CDC25C and PLK1. PP2A negatively regulates CDC25C by dephosphorylation of T130, which facilitates 14-3-3 binding to CDC25C, cytosolic sequestration of CDC25C, and ultimately inactivation of CDK1 (47). PLK1 is also negatively regulated by PP2A which further facilitates CDK1 inactivation via diminished CDC25C T130 phosphorylation and increased WEE1-mediated phosphorylation of CDK1 on Y15 (Fig. 4A; refs. 38, 50). Although CDC25C is a known PP2A substrate, what role CDC25C played in radiosensitization by PP2A inhibition was not known. Thus, we tested whether CDC25C was a key downstream protein, responsible for LB100-mediated radiosensitization, and found in a rescue experiment that LB100 radiosensitization can be partially abrogated in pancreatic cells with CDC25C depletion.
Radiosensitization by PP2A Inhibition

The finding that CDC25C depletion only partially rescues cells from LB100-mediated radiosensitization suggests the involvement of other PP2A substrates, such as γ-H2AX. In a previous study, persistent phosphorylation of H2AX following inhibition of PP2A led to impaired DNA damage repair (19). Thus, one possible interpretation of our data showing persistent γ-H2AX in response to PP2A inhibition and radiation (Figs. 2, 4, and 6) is that it is not only a consequence of DNA damage resulting from CDC25C/CDK1 activation and HRR inhibition, but also a partial cause of the DNA damage, as persistent H2AX phosphorylation itself has been shown to impede DNA double-strand break repair. While we did confirm the presence of persistent DNA double-strand breaks in response to LB100 and radiation by an independent method (53BP1 focus formation), still a causative role for γ-H2AX in the persistent damage cannot be excluded. Nevertheless, our radiosensitization data (Fig. 5) do suggest an important role for CDC25C in LB100-mediated radiosensitization.

Given our observation that G2 checkpoint abrogation was not a major mechanism of radiosensitization by LB100 (Supplementary Fig. S5), we went on to investigate the effects of LB100 on radiation-induced DNA damage repair pathways. On the basis of previous studies implicating forced activation of CDK1 in HRR inhibition (via disruption of BRCA2–RAD51 interaction; refs. 40, 51), as well as those connecting PP2A to HRR via regulation of BRCA1 and RAD51 (33), we hypothesized that LB100 might radiosensitize cells via inhibition of HRR. Indeed, we found that LB100 inhibited HRR as measured by both a GFP-based HRR reporter assay and a RAD51 focus formation assay. To our knowledge, this is the first study showing HRR inhibition as a mechanism of radiosensitization by PP2A inhibition. It is possible that activation of CDK1 caused by PP2A inhibition plays a role in this HRR inhibition and it will be important in future studies to further elucidate the molecular mechanisms by which LB100 inhibits HRR.

The data presented in this study provide the first preclinical evidence that a clinical candidate, small-molecule inhibitor of PP2A is a radiosensitizing agent in pancreatic cancer cells. To further develop PP2A inhibitors such as LB100 it will be imperative to carefully examine the mechanisms of tumor cell selectivity as well as normal tissue toxicities. In addition, the results of recently initiated phase I testing will be instructive in the safety and tolerability of LB100 in humans. In the context of HRR inhibition by LB100, it will also be of interest to explore the efficacy of LB100 in combination with other therapies such as PARP1 inhibitors as well as with chemoradiation regimens. The results of this study lay the preclinical foundation for the future development of PP2A inhibitors as a novel class of radiosensitizing agents.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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