Radiosensitization of Pancreatic Cancer Cells In Vitro and In Vivo through Poly (ADP-ribose) Polymerase Inhibition with ABT-888

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OBJECTIVES: To determine whether poly (ADP-ribose) polymerase-1/2 (PARP-1/2) inhibition enhances radiation-induced cytotoxicity of pancreatic adenocarcinoma in vitro and in vivo, and the mechanism by which this occurs. Methods: Pancreatic carcinoma cells were treated with ABT-888, radiation, or both. In vitro cell viability, apoptosis, and PARP activity were measured. Orthotopic xenografts were generated in athymic mice and treated with ABT-888 (25 mg/kg), radiation (5 Gy), both, or no treatment. Mice were monitored with bioluminescence imaging. RESULTS: In vitro, treatment with ABT-888 and radiation led to higher rates of cell death after 8 days ($P < .01$). Co-treatment with 5 Gy and 1, 10 or 100 μmol/l of ABT-888 led to dose enhancement factors of 1.29, 1.41 and 2.36, respectively. Caspase activity was not significantly increased when treated with ABT-888 (10 μmol/l) alone (1.28-fold, $P = .08$), but became significant when radiation was added (2.03-fold, $P < .01$). PARP activity increased post-radiation and was abrogated following co-treatment with ABT-888. In vivo, treatment with ABT-888, radiation or both led to tumor growth inhibition (TGI) of 8, 30 and 39 days, and survival at 60 days of 0%, 0% and 40%, respectively. CONCLUSIONS: ABT-888 with radiation significantly enhanced tumor response in vitro and in vivo. ABT-888 inhibited PAR protein polymerization resulting in dose-dependent feedback up-regulation of PARP and p-ATM suggesting increased DNA damage. This translated into enhancement in TGI and survival with radiation in vivo. In vitro PAR levels correlated with levels of tumor apoptosis suggesting potential as a predictive biomarker. These data are being used to support a Phase I study in locally advanced pancreatic cancer.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a devastating disease with a cumulative 5 year overall survival of 4% for all stages [1]. Current treatment of non-metastatic, unresectable disease similarly results in dismal median survival rates of 11 to 12 months, nearly uniform local persistence of disease and poor local control [2,3]. Indeed, recent data suggests that failure to control the primary tumor results in complications that contribute to mortality in approximately 30% of patients [4]. To date, no treatment has had a truly significant impact on improving outcomes in unresectable PDAC. The pivotal trial validating gemcitabine as first-line chemotherapy for pancreatic cancer showed a modest improvement in median survival of 6 months compared to 4 months with 5-fluorouracil [5]. Gemcitabine has also been shown to enhance radiosensitivity of pancreatic cancer cells in laboratory and clinical studies [6]. A Phase I study evaluated radiotherapy dose escalation using three-dimensional conformal techniques with full-dose gemcitabine (1000 mg/m^2),

yet it was not possible to escalate the dose beyond 36 Gray (Gy; 2.4 Gy daily fractions) secondary to gastrointestinal (GI) toxicities [7]. In an attempt to minimize dose-limiting toxicities to organs-at-risk and simultaneously allow an increase in tumor dose, Ben Josef et al. recently reported excellent outcomes (response rate of 52%, median overall survival 23 months) using dose-escalated IMRT combined with full-dose gemcitabine [8]. A potential mechanism to

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further exploit this synergy is through identification of targeted agents with chemo- and radiosensitizing properties that have minimal intrinsic cytotoxicity.

Targeting of the poly (ADP-ribose) polymerase-1/2 (PARP-1/2) proteins is one such strategy with immense potential. PARP activation and poly (ADP-Ribose) polymerization represent one of the first in a coordinated series of events following single- and double-strand DNA damage repair, through the base excision repair (BER) and non-homologous end-joining (NHEJ) pathways, respectively [9–11]. Based on conserved genetic sequences, encoded for by 18 different genes, 18 nuclear proteins have been classified as members of the PARP superfamily. The superfamily is further subdivided into three branches, the PARP-1 group, the tankyrase group, and other PARP enyzmes. The PARP-1 group of NAD+-dependent enzymes has been extensively studied, and its members PARP-1 and PARP-2 are generally considered as the primary enzymes involved in DNA repair [12]. Enhanced PARP-1 expression and activity has been demonstrated to be higher in tumor cells, such as lymphoma, hepatocellular carcinoma, and cervical carcinoma, as compared to normal cells, which in turn is felt to confer increased drug resistance [13]. However, when PARP is impaired, cells are noted to become exquisitely sensitive to DNA damaging agents such as radiotherapy [14,15]. As a result, the clinical development of PARP inhibitors has followed two approaches: 1) combining PARP1/2 inhibition with DNA-damaging agents, such as radiation, to derive additional therapeutic benefit; and 2) targeting tumor cells with pre-existing defects in double-strand DNA break repair, such as Brease Cancer (BRCA)-deficient cells, which are genetically predisposed to die when PARP activity is lost [16].

ABT-888 is an orally available, small molecule inhibitor of PARP which has been shown to potentiate the effects of alkylators and radiotherapy in xenograft tumor models [17]. Recognizing the therapeutic potential of PARP-1/2 inhibition in PDAC, we have investigated the addition of veliparib to focused radiation in vitro and in vivo using a novel preclinical pancreatic cancer radiation research model [18,19].

Materials and Methods

Cell Culture and In Vitro Assays

The PDAC cell line, MiaPaCa-2, stably transfected with the luciferase-aminoglycoside phosphotransferase fusion gene under the control of the elongation factor-1α promoter, was kindly provided by Dr. Ralph Graeser, ProQinase GMBH, Freiburg, Germany. Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, and 100 units/mL penicillin/streptomycin. Subconfluent cell monolayers were removed using 0.25% trypsin containing 1 mmol/L EDTA (Invitrogen) and passaged at a ratio of 1:3 or utilized for study. Cells were seeded in triplicate monolayer and treated with varying doses of ionizing radiation using a ^{137}Cs irradiator (5 Gy/min; Mark I, Shepherd and Associates), ABT-888 (Selleck Chemicals, Houston, TX), or a combination of the two. All in vitro studies were performed in triplicate. When cells were co-treated, ABT-888 was added to the cell suspension 30 minutes prior to irradiation and left until routine media change at 48 hours. Cell viability was determined by the ability to convert a redox dye (resazurin) into a fluorescent end product (resorufin) using the Cell Titer-Blue® Assay (Promega Corporation, Madison, WI) at varying time points after treatment. Treatment

doses resulting in 10% (IC10), 20% (IC20) and 50% (IC50) cell death were calculated for ABT-888 and irradiation, respectively. ABT-888 dose enhancement factors were determined after co-treatment with varying irradiation doses. Levels of apoptosis were determined using a chemiluminescent caspase 3/7 assay (G8091, Promega Corporation, Madison, WI) 48 hours after treatment with ABT-888, radiation, or a combination thereof. PARP-1/2 inhibition was quantitated using an enzyme-linked immunosorbent assay for PAR protein (Trevigen, Gaithersburg, MD) after treatment with ABT-888, radiation, ABT-888 plus radiation, or no treatment. Total protein extracts were harvested 6 hours after treatment and PAR levels were determined by chemiluminescence.

Immunoblotting

MiaPaCa-2 nuclear extracts were isolated after treatment with ABT-888, radiation, or combinations thereof. Cytosolic extracts were harvested following addition of a buffer (50 mmol/L Tris-HCl, pH 7.4, 0.14 M NaCl, 1.5 mmol/L MgCl$_2$, protease and phosphatase inhibitors, PMSF, 1 mmol/L DTT). Nuclear pellets were then suspended in RIPA buffer and nuclear proteins were harvested. Protein quantification was performed with the Bradford DC assay (BioRad, Hercules, CA). Immunoblotting of nuclear lysates was performed with the following monoclonal mouse antibodies: PARP-1 (NB100-111; Novus Biologicals, Littleton, CO) and phosphorylated ATM (p-ATM; Ser1981, 10H11.E12, Mouse mAb #4526 Cell Signaling, Danvers, MA) and the following polyclonal rabbit antibodies: PAR (4336-BPC-10; Trevigen, Gaithersburg, MD) and Lamin-A (sc-20680, Santa Cruz Biotechnology, Santa Cruz, CA). Infrared dye-conjugated secondary antibodies were used and imaged using the Odyssey® imaging system (Li-Cor Biotechnology, Lincoln, Nebraska).

Pancreatic Tumor Xenografts and Imaging

Six-week old female athymic nude mice (Harlan Sprague Dawley, Madison, WI) were used in accordance with institutional Animal Care and Use Committee guidelines under an approved protocol. Mice were anesthetized by intraperitoneal injection of 10:1 ketamine/xylazine and 2×10^6 cells in a 1:1 mixture with Matrigel (356235, BD Matrigel™ Basement Membrane Matrix; Becton Dickinson, Franklin Lakes, NJ) were injected into the tail of the pancreas per previously established protocols [19]. Two-dimensional bioluminescence imaging (BLI) was performed with the IVIS® Spectrum (Caliper Life Sciences, Hopkinton, MA) to allow image-guided delivery of radiation and longitudinal assessment of treatment response. Prior to imaging, mice were anesthetized and injected intraperitoneally with 150 mg/kg D-luciferin (Catalog No. LUCNA, Gold Biotechnology, St. Louis, MO) in sterile PBS. After a 10 second exposure and image acquisition, the coronal optical pseudocolor image was overlaid upon a corresponding grayscale photographic image of the animal and a region of interest was created around the optical tumor image so that the luminescence at the edge of the circle was 5% of the peak intensity of that region [18–20]. Signal intensity was quantified within an identified region of interest in photons per second per squared centimeter per steradian (p/s/cm^2/sr) using Living Image software (Caliper Life Sciences, Hopkinton, MA). Treatment-related fold-tumor change was determined longitudinally as a function of time by normalizing signal intensity to that obtained on day 0, as previously described [19]. All mice in each treatment cohort were imaged simultaneously with BLI five minutes post injection of substrate.

In Vivo Treatment

Three days after surgery, all mice were imaged for development of solitary pancreatic tumors using BLI. Tumor bearing mice were randomized to receive one of four treatments (n = 7 per group): vehicle alone (i.p. PBS), a single dose of ABT-888 (i.p. 25 mg/kg), a single fraction of radiation (5 Gy), or the combination of radiation and ABT-888. Sham treatment or ABT-888 was administered 30 minutes prior to irradiation. Anesthetized mice were imaged with BLI and subsequently transported to the small animal radiation research platform (SARRP). Using the guidance software utility of the SARRP, bioluminescent images were co-registered by manual fusion with CBCT images and the isocenter of the tumor was identified and aligned with the central axis of the beam, as previously described [20]. Mice were irradiated with the SARRP using 225 kVp x-ray beams at a dose rate of 2.5 Gy/minute using varying collimator widths adapted to the optical image of the tumor (gross tumor volume) plus a 5 mm radial margin for set up error (planning target volume). Mice underwent BLI twice per week until day 9 and weekly thereafter to assess tumor response and were humanely euthanized when moribund, if they experienced weight gain or loss in excess of 20% of pre-treatment weight, or if tumor burden increased more than 10-fold as determined by BLI.

Statistical Analysis

Two-tailed Student's t test was utilized to assess statistically significant differences between groups ($P < .05$). Kaplan-Meier curve was constructed for survival analysis with log-rank test.

Results

Effects of Radiation and ABT-888 on Cell Viability

The effects of increasing doses of radiation and ABT-888, individually and concurrently, on cell viability were assessed to determine levels of radiation dose-enhancement (Figure 1). Significant reductions in cell viability were seen with single-fraction radiation doses exceeding 2 Gy at 2, 4, 6 and 8 days post-treatment. The IC10, IC20 and IC50 of radiation were calculated to be 0.5 Gy, 2 Gy and 5 Gy, respectively (Figure 1A, 6 days post-treatment). Increasing doses of ABT-888 had little effect on cell viability until doses exceeding 5 μmol/l were used. The IC10 for treatment with ABT-888 alone was calculated to be 10 μmol/l and this dose was utilized for subsequent in vitro studies (Figure 1B). Significant radiosensitization was noted when ABT-888 was added to cells irradiated with vehicle alone. Co-treatment with 1 μmol/l, 10 μmol/l and 100 μmol/l of ABT-888 led to radiation dose enhancement factors of 1.29, 1.41 and 2.36 ($P < .05$), respectively (Figure 1C). Minimal intrinsic cytotoxicity was noted when cells were treated with ABT-888 alone at these same doses.

DNA damage response following treatment with radiation and ABT-888

Radiation-induced DNA damage results in relatively immediate activation of PARP and accumulation of ribosylated protein products, such as PAR, primarily through single-strand breaks and BER. Therefore, PARP and PAR protein levels were measured as a function of time to assess the impact of treatment with radiation. An immediate and significant increase was noted in PAR levels following treatment with 10 Gy consistent with single-strand DNA damage, which persisted through the 30 minute time point before returning to control levels (Figure 2A). Conversely, PARP levels were reduced
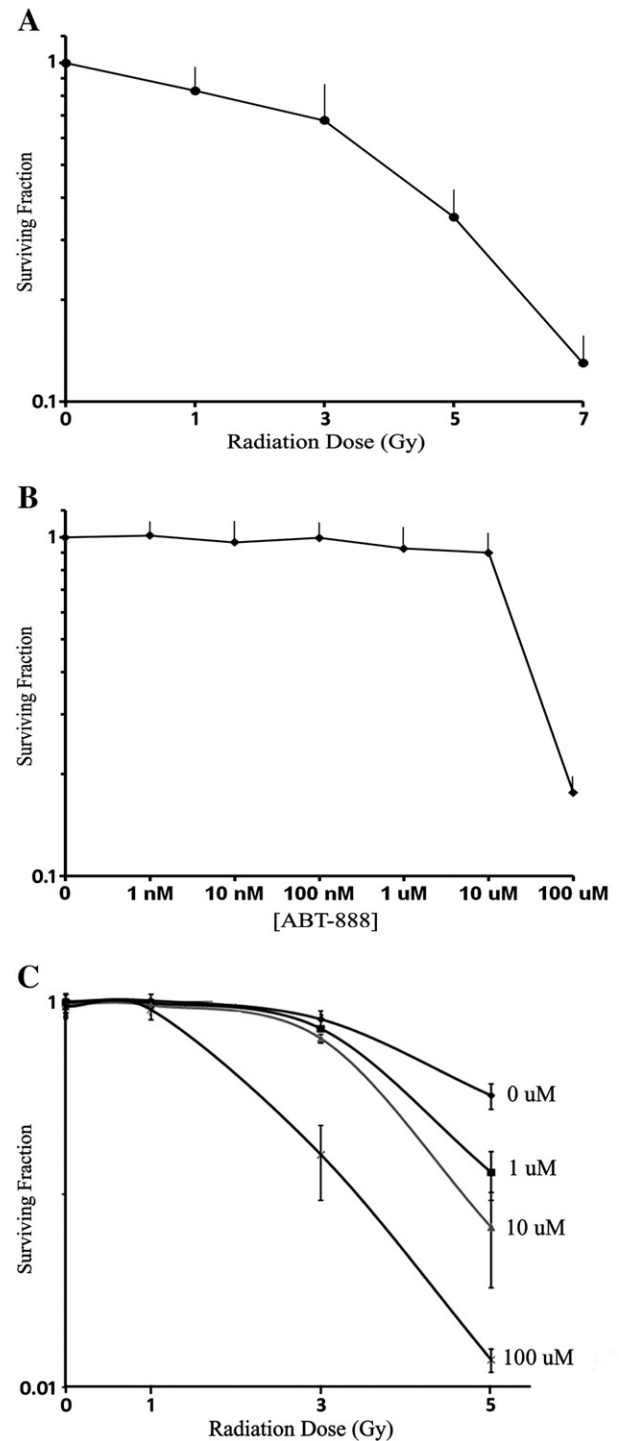

Figure 1. In vitro survival curves of MiaPaCa-2 cells treated with varying doses of RT (A), ABT-888 (B) or both (C). Half-maximal half inhibitory concentration (IC50) of RT was noted to be 5 Gy and IC10 for ABT-888 was 10 μmol/l. Co-treatment with 5 Gy and 1, 10 or 100 μmol/l of ABT-888 led to dose enhancement factors of 1.29, 1.41 and 2.36, respectively. Means and SEM are plotted.

following irradiation relative to controls over the same period and also returned to baseline levels 60 minutes post-treatment. Additionally, cells were treated with increasing doses of ABT-888 to assess the level of PARP-1/2 inhibition and resulting PAR protein formation. A clear dose dependent reduction in PAR levels was noted with complete abrogation with doses of 100 μmol/l and above at both 15 and
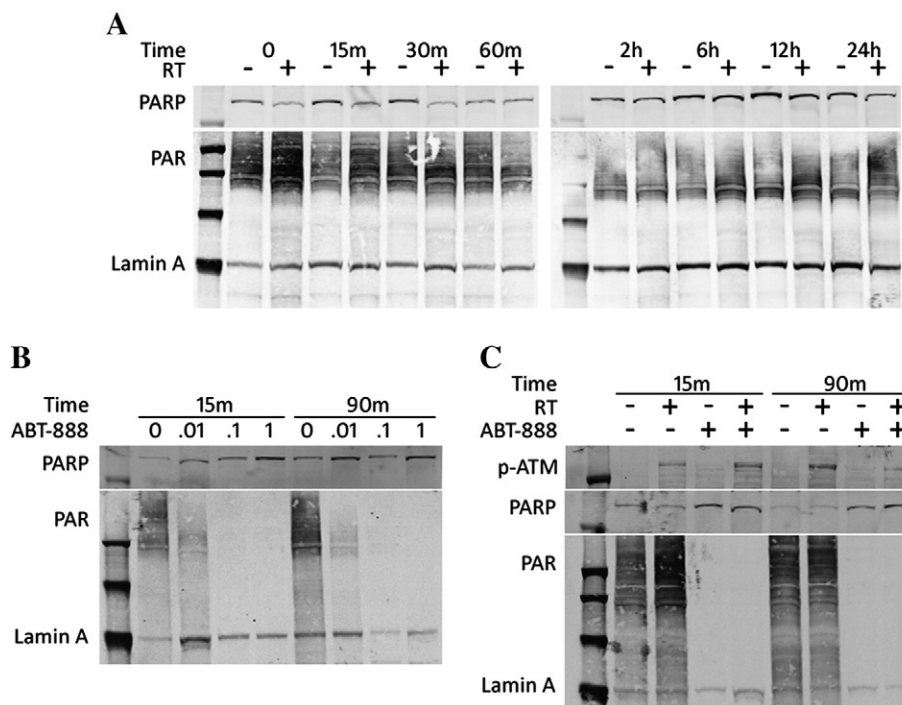
Figure 2. Immunoblot of MiaPaCa-2 cells treated with RT (*A*; +/− 10 Gy), ABT-888 (*B*; 0-1 mmol/L) or both (*C*; 10 Gy, 100 μmol/l ABT-888). Extracts were probed for PAR, PARP, p-ATM and Lamin A.

90 minute post-treatment. As a result, 100 μmol/l ABT-888 was selected for co-treatment with radiation (Figure 2B). A corresponding dose dependent increase in PARP protein was noted as early as 15 minutes following treatment with ABT-888 alone, and PARP levels remained elevated as a function of time in the presence of the treatment drug (Figure 2B). Interestingly, ABT-888 (100 μmol/l) completely abrogated radiation-induced PAR formation to undetectable levels at both early time points (Figure 2C). PARP protein levels were again noted to be inversely proportional to PAR protein formation with significant up-regulation following treatment with ABT-888 likely as a result of feedback inhibition. Phosphorylated-ATM levels were up-regulated after radiation treatment relative to controls and further induced following co-treatment with ABT-888.

Inhibition of PARP Activity and Enhanced Apoptosis with Concurrent ABT-888

A PAR ELISA was utilized to assess the effect of radiation with and without ABT-888 on PARP activity and to provide a quantitative means of assessing PARP-inhibition. Six-hours post-treatment with 2 Gy (IC20), led to significant 23% increase in PARP activity relative to untreated controls ($P < .05$; Figure 3A). This was further reduced by 41% following co-treatment with 10 μmol/l ABT-888 (IC10; $P < .05$) and similar to immunoblot data, this level of abrogated activity was not significantly different when compared to cells treated with ABT-888 (10 μmol/l; $P < .32$) alone, suggesting maximal inhibition was occurring independent of treatment with radiation. To help determine the mechanism of cytotoxicity, caspase 3/7 levels were
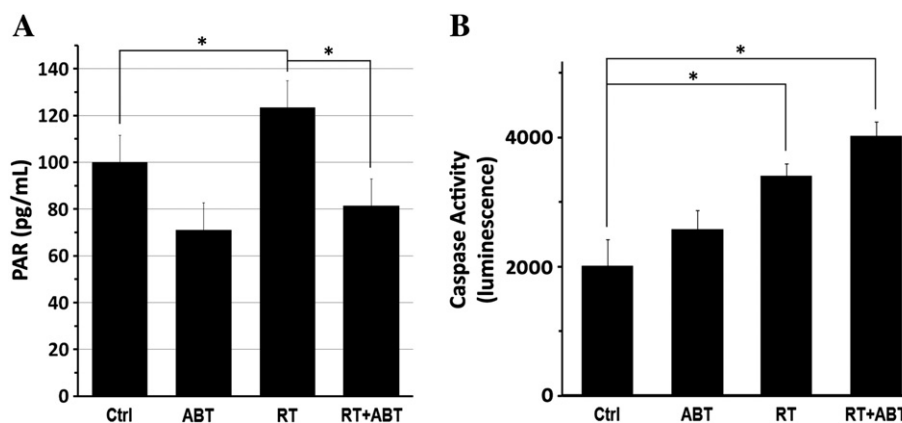

Figure 3. In vitro ELISA-based quantitation of PAR levels 6 hours post-treatment with ABT-888 (10 μmol/l), RT (2 Gy), or both (A). Levels of apoptosis were determined using a chemiluminescent caspase 3/7 assay 48 hours following treatment of MiaPaCa-2 with ABT-888 (10 μmol/l), RT (2 Gy), or both (B). Means and standard error of the mean are plotted. Asterisk represents statistical significance.

assessed 48 hours after treatment with radiation (2 Gy), ABT-888 (10 μmol/l), or a combination of the two (Figure 3B). Whereas treatment with ABT-888 alone failed to induce significant caspase-3/7 activity, treatment with radiation led to a 1.69-fold increase ($P < .05$) in levels relative to untreated controls and these were further enhanced to 1.99 ($P < .05$) following the addition of ABT-888 suggesting increased apoptotic cell death.

Enhanced Radiation-Induced Tumor-Growth Inhibition and Survival with ABT-888

Utilizing a previously reported small animal pancreatic cancer radiation research model, MiaPaCa-2-derived orthotopic tumors were treated with BLI-guided, focused radiation (5 Gy), ABT-888 (25 mg/kg), or a combination of the two [19]. Co-treatment with ABT-888 resulted in significant tumor growth inhibition of 36 days relative to controls treated with saline sham injection (Figure 4). This was significantly greater than tumors treated with either radiation (28 days) or ABT-888 (10 days) alone. The addition of ABT-888 to radiation also translated into a significant overall survival benefit compared to either treatment alone (Figure 5). Survival at 30 days for mice treated with ABT-888, radiation or a combination of the two was 63%, 75% and 100%, respectively, while at 60 days, it was 0%, 0% and 29% ($P < .05$), respectively. Of note, mice in the combination treatment group died more often as a result of metastatic disease, ascites, excessive weight loss or failure to thrive, as compared to mice treated with either modality alone, which died more frequently from growth of the primary tumor as assessed by BLI (data not shown).

Discussion

Herein, we demonstrated that the addition of ABT-888 to radiation significantly enhanced tumor response of pancreatic cancer cells in
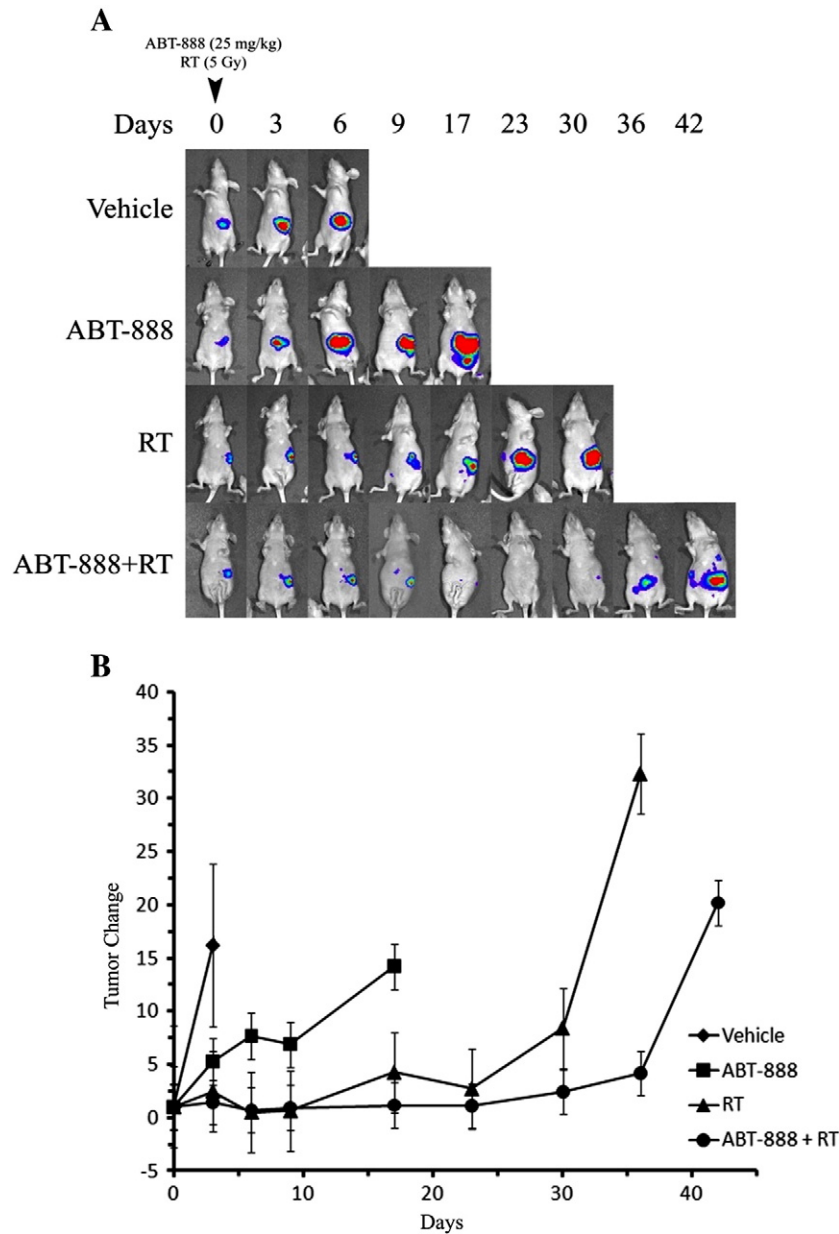
Figure 4. Orthotopic MiaPaCa-2 tumors were treated with saline sham, ABT-888 (25 mg/kg), RT (5 Gy), or both and followed longitudinally using bioluminescence imaging (A). Tumor growth inhibition of 8, 30 and 39 days was noted compared to treatment with saline sham, respectively (B).
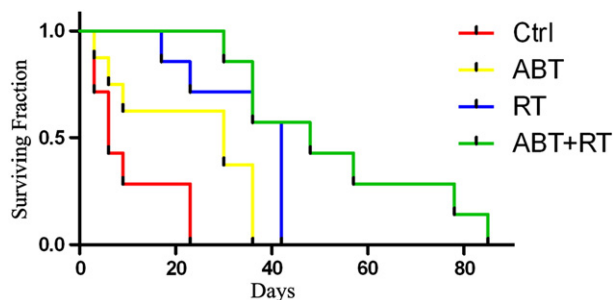
Figure 5. Orthotopic MiaPaCa-2 tumors were treated with saline sham, ABT-888 (25 mg/kg), RT (5 Gy), or both. Survival at 30 days for mice treated with ABT-888, RT or a combination of the two was 63%, 75% and 100%, respectively, while at 60 days, it was 0%, 0% and 29% ($P < .05$, ABT + RT vs. all other treatments).

vitro and in vivo. ABT-888 inhibited PAR protein polymerization resulting in dose-dependent feedback up-regulation of PARP enzyme, as well as p-ATM suggesting increased DNA damage and potential repair by mechanisms such as homologous recombination (HR). This translated into significant enhancement in tumor growth inhibition and survival when combined with focused image-guided radiation of orthotopic pancreatic xenografts.

Several studies have examined the mechanism of cell-death induced following PARP inhibition. Similar to our study, Horton et al. have suggested that inhibition of PARP activity results in a caspase-dependent apoptotic programmed cell death, as inhibition of caspase and Chk1 resulted in increased necrotic cell death as well as percentage of viable cells, respectively [21]. Interestingly, other studies have suggested no difference in the percentage of apoptotic cells following PARP inhibition, or mechanisms independent from apoptosis, such as mitotic catastrophe [22,23]. Liu et al. suggest this may be a cell-line dependent phenomenon; irrespective, we noted that the increased cytotoxicity seen following the addition of ABT-888 to radiation was at least in part mediated through increased caspase activity and programmed cell death [24].

Significant and immediate induction of PAR protein was noted following radiation, as previously reported, with dose-dependent attenuation following PARP-inhibition in the pancreatic cancer cells. Following PARP inhibition, we identified a coincident up-regulation of radiation-induced p-ATM, which is a key regulator of homologous recombination following double-strand DNA breaks. Similar to our study, Metzger et al. recently reported a 1.7-fold increase in the rate of nick-induced HR following PARP inhibition without affecting DSB-induced HR utilizing an integrated reporter system in human cells to measure HR and non-homologous end-joining [25]. These findings further confirm PARP-1 as a primary mediator in single-strand DNA repair and further allude to the significance of interplay with BRCA1/2-mediated DSB repair and the potential clinical significance of synthetic lethality. In addition to inhibiting the catalytic activity of PARP, Murai et al. recently reported on a novel secondary mechanism of action of PARP inhibitors [26]. This involves binding of the PARP inhibitor to the NAD$^+$ site on the PARP enzyme, thereby enhancing the allosteric DNA binding domain of the protein and essentially trapping the PARP1- and PARP2-DNA complexes. The authors concluded that this secondary trapping effect was significantly more cytotoxic than catalytic inhibition based on the observation that olaparib-treated wild type DT40 cells were

significantly more sensitive to the alkylating agent, methylmethane sulfonate (MMS), compared to PARP1$^{-/-}$ DT40 cells treated with MMS alone, thereby suggesting a secondary mechanism of action responsible for the enhanced sensitivity.

To our knowledge, this study is the first to report on ABT-888-mediated radiosensitization of pancreatic cancer in vivo. Similar to preclinical studies in other disease sites, we noted limited clinical benefit of ABT-888 when used as a single-agent. However, in combination with radiation, we saw at least an additive effect of treatment on survival. Whereas these findings were consistent with in vitro results, the benefit was not as robust, and may be attributable to differences in treatment dose(s) and method of treatment delivery among other factors. We believe, however, that these clinical findings are appropriately representative of what might be expected in the clinical setting given the novel preclinical platform (SARRP) used to deliver radiation [19]. Similar to clinical studies, the potential therapeutic benefit of PARP-inhibition with ABT-888 may be further potentiated when used in combination with radiosensitizing chemotherapeutic agents. Jacob et al. have reported on the gemcitabine-sensitizing effects of the PARP-inhibitor, 3-aminobenzamide [27]. Co-treatment of heterotopic Capan-1 pancreatic tumors in mice with both agents resulted in a significant synergistic improvement in survival relative to either treatment alone. As a fluorine-substituted analog of cytarabine, the primary mechanism of gemcitabine cytotoxicity is due to impairment of DNA synthesis through inhibition of DNA polymerase and ribonucleoside reductase by gemcitabine diphosphate and triphosphate with subsequent depletion of deoxyribonucleotide pools necessary for DNA synthesis [28]. As these mechanisms seem independent of PARP-regulated SSB DNA repair, the mechanism of potential synergism with gemcitabine remains unclear. Consistent with the findings of Jacob et al, however, we have noted similar dose enhancement and cytotoxicity following co-treatment of MiaPaCa-2 cells with radiation, gemcitabine and ABT-888 further suggesting that ABT-888 acts as both a radiation- and chemo-sensitizer. A recent clinical study compared full dose gemcitabine (1000 mg/m^2) to a lower dose of gemcitabine (600 mg/m^2) combined with standard fractionated radiation (50.4 Gy over 5.5 weeks) in patients with locally advanced PDAC [29]. Although the study was closed prior to reaching its planned accrual, there was a significant improvement in survival with combined gemcitabine and radiation compared to gemcitabine alone. Recognizing the potential benefits of combining gemcitabine with radiotherapy for patients with locally advanced pancreatic cancer, future treatment strategies could aim to further exploit this synergy through PARP-inhibition.

The concept of synthetic lethality applies to cells with impaired HR, which are further subjected to PARP-1/2 inhibition. The resulting single-stranded DNA breaks ultimately lead to an accumulation of double-strand breaks that cannot be effectively repaired, culminating in complex chromosomal alterations and increased levels of apoptosis [30]. Indeed, Cass et al. have reported on the improvement in survival in patients with BRCA-associated ovarian carcinoma treated with cisplatin, and more recently, Fogelman et al. reported a case of a pancreatic adenocarcinoma patient with germline BRCA-2 mutation who demonstrated complete pathologic response to the PARP-inhibitor, BSI-201 [31,32]. Henessy et al. recently investigated the frequency of somatic and germline BRCA-1/2 mutations in ovarian cancer and attempted to correlated these findings to progression-free survival after treatment with cisplatin [33]. Interestingly, 30% of all patients had either germline or somatic BRCA-1/2 mutations and

these patients had a concordant significant improvement in clinical outcomes relative to patients not harboring these mutations. These data suggest that PARP-inhibitor therapy may be most appropriate not only for the 17% of pancreatic cancer patients who harbor BRCA-1/2 germline mutations, but also those harboring somatic mutations in other proteins involved in HR repair, such as mutated partner and ligand of BRCA2 (PALB2) and phosphatase and tensin homolog deleted on chromosome 10 (PTEN) [34,35]. Based on the results presented herein, as well as the clinical success of PARP-inhibitors to date, we have initiated a Phase 1 study investigating the maximum tolerated dose, safety and toxicity of ABT-888 with full dose gemcitabine and intensity modulated radiotherapy in patients with locally advanced PDAC.

Acknowledgements

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