Therapeutics, Targets, and Chemical Biology

Smac Mimetic Increases Chemotherapy Response and Improves Survival in Mice with Pancreatic Cancer

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Abstract

Failure of chemotherapy in the treatment of pancreatic cancer is often due to resistance to therapy-induced apoptosis. A major mechanism for such resistance is the expression and activity of inhibitors of apoptosis proteins (IAP). Smac (second mitochondria–derived activator of casapse) is a mitochondrial protein that inhibits IAPs. We show that JP1201, a Smac mimetic, is a potent enhancer of chemotherapy in robust mouse models of pancreatic cancer. Combination of JP1201 with gemcitabine reduced primary and metastatic tumor burden in orthotopic xenograft and syngenic tumor models, induced regression of established tumors, and prolonged survival in xenograft and transgenic models of pancreatic cancer. The effect of JP1201 was necroptosed by XIAP small interfering RNA in vitro and correlated with elevated levels of tumor necrosis factor α protein in vivo. The continued development of JP1201 and other strategies designed to enhance therapy-induced apoptosis in pancreatic cancer is warranted.

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Introduction

Pancreatic cancer is a deadly disease with a dismal 5-year survival (1). Surgery offers a chance for a cure; however, most patients have advanced disease, are not candidates for resection, and must rely on chemotherapy. Resistance to chemotherapy-induced apoptosis is a significant factor contributing to poor prognosis of these patients (2). Tumor cells often circumvent the apoptotic cascade and proliferate in the face of apoptotic stimuli, which facilitates tumor progression and metastasis (3). Thus, restoring apoptotic response in tumor cells is an attractive strategy to improve the prognosis of pancreatic cancer patients (2).

Two major pathways induce apoptosis: the extrinsic or death receptor–mediated pathway and the intrinsic or mitochondrial pathway (4). The extrinsic pathway is activated by the binding of molecules such as tumor necrosis factor α (TNFα)–related apoptosis-inducing ligand (TRAIL) or TNFα to their cognate receptors (3). Members of the TNF receptor superfamily induce apoptosis by recruitment of FADD and formation of the death-inducing signaling complex (5). The intrinsic pathway is initiated when cellular stress causes a change in mitochondrial membrane permeability, which releases proteins such as cytochrome c and second mitochondria–derived activator of casapse (Smac), resulting in the recruitment of Apaf-1 and formation of the apoptosome (4). Executioner caspases are activated by both pathways, resulting in subsequent cell death (5).

Chemotherapy and radiation ultimately cause tumor cell death by inducing apoptosis (5), which is compromised by tumor cell resistance to apoptosis (2). Many cancer cells express elevated levels of inhibitor of apoptosis proteins (IAP) and escape apoptosis through the activity of IAPs (4). IAPs prevent the activation of caspases and, as such, block the extrinsic and intrinsic apoptotic cascades (5). X-linked IAP (XIAP) is one of the best characterized IAPs and is expressed at a higher level in pancreatic cancer cell lines (n = 19; ref. 6) and pancreatic tumors (14 of 18) compared with normal pancreas (7, 8). XIAP is an attractive target for anticancer therapy as it functions as a “gatekeeper” of caspase activation (4). The mitochondrial protein Smac inhibits IAPs, including XIAP, thus promoting caspase activation and subsequent cell death. Smac has been shown to bind to XIAP, cIAP-1, and cIAP-2, and Smac mimetics sensitize tumors to programmed cell death (7, 9–11).

In this series of experiments, we explore the effect of a novel Smac mimetic, JP1201, in combination with chemotherapy. We show that JP1201 enhances the efficacy of chemotherapy and improves survival in multiple animal models of pancreatic cancer. These effects are mediated in part by inhibition of XIAP and induction of TNFα.

Materials and Methods

Cell lines

Human pancreatic cancer cell lines (MIA PaCa-2, PANC-1, BxPC-3, AsPC-1, Capan-1, Capan-2, Hs 766T, and Hs 700T)
were obtained from the American Type Culture Collection. The murine pancreatic cancer cell line Panc02 (also known as Pan02) was obtained from the National Cancer Institute (NCI). Cell lines were confirmed to be pathogen-free and human cell lines were authenticated to confirm origin before use. Cell lines were grown in DMEM (Invitrogen) containing 10% fetal bovine serum (FBS) and maintained at 37°C in a humidified incubator with 5% CO2 and 95% air.

In vitro cytotoxicity and drug response assay

Assays were performed in 96-well format as described (12). Briefly, cells were plated on day 0 and the drug was added on day 1 in 4-fold dilutions. For gemcitabine (Eli Lilly and Company) alone and the gemcitabine-JP1201 (100 nmol/L) combination, the highest dose of gemcitabine given was 2,000 nmol/L. For JP1201 alone, the highest concentration given was 100 μmol/L. Relative cell number was determined by adding MTS (Promega; final concentration, 333 μg/mL), incubating for 1 to 3 h at 37°C, and reading absorbance at a 490-nm plate reader (Spectra Max 190, Molecular Devices). Drug sensitivity curves and IC50 values were calculated using in-house software.

Small interfering RNA and gemcitabine combination therapy

For reverse transfection, 0.25 μL of 20 μmol/L stock of each small interfering RNA (siRNA) in a volume of 19.75 μL of serum-free DMEM was delivered to each well of a 96-well plate. Dharmafect 1 (0.125 μL; Dharmacon) in 9.875 μL of serum-free DMEM was then delivered into each well. RNA-lipid complexes were allowed to form (20–30 min). Following incubation, 8,000 cells were added to each well in DMEM with 5% FBS, with a total volume per well of 100 μL. On day 1, gemcitabine was added to each plate in DMEM with 5% FBS in 4-fold serial dilutions as described above. Plates were read on day 5 using a MTS assay as described.

Animal studies

All animals were housed in a pathogen-free facility with 24-h access to food and water. Experiments were approved by, and performed in accordance with, the Institutional Animal Care and Use Committee at the University of Texas Southwestern. Athymic nu/nu mice were purchased from NCI, C57Bl/6 mice were purchased from The Jackson Laboratory, and severe combined immunodeficient mice were obtained from an on-campus supplier. At sacrifice, the pancreas and tumor were excised and weighed en bloc to determine primary tumor burden. Metastases were identified through visual inspection of the surface of the liver, diaphragm, peritoneal surfaces, and lymph nodes. Samples were fixed in 10% formalin (Sigma) or snap frozen in liquid nitrogen for further studies.

Early intervention model. Six- to 8-wk-old athymic nu/nu female mice were injected with MIA PaCa-2 cells (1 × 106) as described (13). Animals were randomized following tumor cell injection into treatment groups and therapy was initiated 1 wk following tumor cell injection. Therapy with TRAIL (20 mg/kg) alone or in combination with JP1201 (0.2, 0.6, 2.0, and 6.0 mg/kg) was given thrice weekly (Monday, Wednesday, Friday) through the lateral tail vein for a total of six injections. All drugs were diluted in saline. Animals were sacrificed 2.5 wk after the cessation of therapy.

Late intervention model. On day 28 after tumor cell injection, therapy was initiated and continued for six doses. The dosing was similar except that JP1201 was only given at 6.0 mg/kg and gemcitabine was dosed at 175 mg/kg on day 28 and at 100 mg/kg on days 30, 37, and 40. A replicate experiment with gemcitabine at 25 mg/kg given six times over 2 wk was also performed. The animals were sacrificed on day 42, 2 d following the last treatment.

Syngenic model. Panc02 cells were injected orthotopically into C57Bl/6 mice, and therapy was initiated on day 21 after tumor cell injection. Therapy consisted of saline, gemcitabine alone (25 mg/kg), JP1201 (6.0 mg/kg), and the combination of gemcitabine and JP1201. The schedule of therapy was the same as above.

Survival study. MIA PaCa-2 tumors were established in nude mice in an identical manner to the late intervention study. Therapy was given on the same schedule with gemcitabine at 25 mg/kg. Following therapy, animals were weighed thrice weekly and assessed for weight loss or ascites. Assessment of animals was done by an experienced observer blinded to the treatment group. Animals were sacrificed for humane purposes if weight loss was >15% of body weight or if they had ascites and weight gain of >10%. These animals were counted as a death on the day of sacrifice.

Transgenic model of pancreatic ductal adenocarcinoma. p48-Cre KrasG12D: Ink4a/Arflox/lox mice (14, 15) were genotyped shortly after birth, and therapy with saline, gemcitabine (25 mg/kg), or JP1201 (6.0 mg/kg) combined with gemcitabine was delivered by i.p. injection thrice weekly. End point and survival studies were performed as described above.

Pharmacokinetics. MIA PaCa-2 tumor cells were implanted into the pancreas of athymic nu/nu mice. Forty-one days later, the animals were placed in groups of three and given gemcitabine alone (100 mg/kg to 2 mg total, i.p.), JP1201 alone (6 mg/kg to 0.12 mg total, i.v.), or a combination of gemcitabine and JP1201. Animals were sacrificed at varying times after dosing (5 and 30 min, and 2, 8, 12, and 24 h), and plasma and tumors were sampled. The processing and analytic methods used for the detection of gemcitabine (dFdC), JP1201, and the gemcitabine inactive metabolite dFdU are described in detail in the online Supplementary Material.

Histology

Formalin-fixed tissues were embedded in paraffin and sectioned (10 μm) by the Molecular Pathology core at University of Texas Southwestern Medical School where routine H&E staining was also performed. Antibodies were used at 5 μg/mL and included goat anti-proliferating cell nuclear antigen (PCNA; SC-9857, Santa Cruz Biotechnology), rabbit anti-human TNFα (ab6671, Abcam), and rat anti-mouse TNFα (506302, Biologend). Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining was performed per the manufacturer’s instructions (Promega).

Statistics

Data were analyzed using the GraphPad software (GraphPad Software). Results are expressed as mean ± SEM. Data
were analyzed by t test or ANOVA and results are considered significant at \( P < 0.05 \).

**Results**

**JP1201 + TRAIL slows the growth of orthotopic pancreatic tumors**

Smac mimetics induce apoptosis *in vitro* when combined with TRAIL or TNFα (9). JP1201 did not induce apoptosis in MIA PaCa-2 as a single agent *in vitro*; however, addition of JP1201 decreased the IC_{50} of TRAIL from 4.5 to 1.2 nmol/L. We assessed how JP1201 in combination with TRAIL affected pancreatic cancer growth *in vivo*. In this early intervention model, mice bearing MIA PaCa-2 tumors were treated with saline, TRAIL, or TRAIL + JP1201 at 0.2, 0.6, 2, or 6 mg/kg starting 1 week following tumor cell injection and sacrificed 2.5 weeks after the last dose of therapy (Fig. 1A). Therapy with TRAIL + JP1201 at doses of 2 or 6 mg/kg reduced the final size of pancreatic tumors by \( \sim 50\% \) (control, 0.46 g versus 0.23 and 0.24 g, respectively; Fig. 1B). TRAIL alone had a modest effect on the size of xenografts compared with saline. Tumors were removed en bloc. At the time of sacrifice, we observed significantly less tumor burden and more residual normal pancreas in mice treated with TRAIL + JP1201. Histologic analysis showed extensive tumor burden in saline-treated animals. In contrast, mice treated with combination therapy using the highest doses of JP1201 showed scattered foci of tumor growth with a large amount of residual normal pancreas (Fig. 1C).

**Figure 1.** JP1201 in combination with TRAIL is effective in controlling pancreatic tumor xenografts. A, early intervention treatment algorithm. MIA PaCa-2 cells (1 × 10^6) were injected into mice on day 0. Therapy was initiated on day 7 after tumor cell injection (TCI). Therapy was given by i.v. or i.p. injection every other day (Monday, Wednesday, and Friday) for 2 wk on the days indicated. Mice received saline, TRAIL (20 mg/kg), or TRAIL + JP1201 (JP, at 0.2, 0.6, 2.0, or 6.0 mg/kg). Animals were sacrificed 2.5 wk after the last dose of therapy (D36). B, a scatter plot of tumor weights at the time of sacrifice showing the mean ± SEM as well as the weight of each tumor from individual mice. *, \( P < 0.05 \) by one-way ANOVA with Dunn’s posttest. C, representative H&E staining of formalin-fixed, paraffin-embedded sections of tumor tissue from mice treated with saline or JP1201/TRAIL. Top, images at \( \times 100 \) total magnification (scale bar, 100 μm); bottom, \( \times 400 \) total magnification (scale bar, 50 μm). White box, area magnified. D, the mean ± SEM number of metastases in each treatment group as well as the number of metastatic events in each animal is shown in the scatter plot. Metastases were evaluated by careful visual inspection at the time of necropsy. Note that the animals treated with JP1201/TRAIL at the highest dose did not have any visible metastases. The data represent a single animal experiment with seven to nine animals in each treatment group.
In addition to controlling primary tumor burden, metastases were reduced by JP1201 + TRAIL. There were no metastatic events in the TRAIL + JP1201 (6 mg/kg) group, compared with 15 total metastatic events in control animals (Fig. 1D). These results show that JP1201 sensitizes pancreatic tumors to therapy with the death receptor ligand TRAIL. Animals treated with combination therapy did not display any weight loss or other signs of systemic toxicity.

### Inhibition of XIAP improves sensitivity to gemcitabine

Despite being the standard of care for pancreatic cancer, gemcitabine is only minimally effective. Blocks in apoptosis, including overexpression of IAPs, might mediate chemoresistance in pancreatic cancer (16). To determine if JP1201 affected the sensitivity in vitro to gemcitabine, we screened a panel of cell lines against gemcitabine alone, JP1201 alone, or the combination (Table 1). We found that in general, a low dose (100 nmol/L) of JP1201 enhanced the sensitivity of pancreatic cancer cells to gemcitabine by ∼20-fold. However, the range of fold reduction was wide, with two cell lines showing no change in the IC₅₀ of gemcitabine (Capan-2 and Hs 766T), whereas PANC-1 cells showed a 170-fold reduction.

Because JP1201 binds to and inhibits IAPs, we hypothesized that siRNA-mediated knockdown of IAPs would increase sensitivity of pancreatic cancer cells to gemcitabine similar to treatment with JP1201. We found that siRNA-mediated knockdown of XIAP but not cIAP1 or cIAP2 sensitized MIA PaCa-2 and PANC-1 (Fig. 2) cells to treatment with gemcitabine. The increase in sensitivity was similar to treatment with JP1201 (Fig. 2). These data expand the work in other pancreatic cancer cell lines (8, 17), which show that resistance to chemotherapy is mediated in part by XIAP.

### JP1201 + gemcitabine is efficacious for established pancreatic tumors

A challenge in treating pancreatic cancer is the fact that patients typically present at an advanced stage. To more closely parallel human disease presentation, we performed a late intervention study. MIA PaCa-2 tumors were grown for 28 days (tumor burden, ~500 mg) before the initiation of therapy. Therapy was given as six doses over 2 weeks. A cohort of animals from each group was sacrificed 24 hours after the first dose of therapy (day 29) to acquire tissue for histologic analysis (see below). The remaining animals were sacrificed on day 42, 2 days following the end of treatment. As shown in Fig. 3A, treatment with JP1201 + TRAIL or gemcitabine reduced tumor weight significantly (***, P < 0.001 versus saline). JP1201 alone also had a significant effect (*, P < 0.05 versus saline) on pancreatic tumor weight, despite having little effect as a single agent in vitro.

Comparison of mean tumor weights on day 42 to the mean weight of tumors harvested on at day 29 (mean of 15 tumors harvested) shows that animals treated with JP1201 in combination with gemcitabine or TRAIL had reductions in mean tumor size of ~50%, whereas mice treated with saline or gemcitabine had increased mean tumor weight by 150% and 50%, respectively (Fig. 3B). Treatment with JP1201 alone caused a cessation of tumor growth. These data strongly suggest that mice treated with combination therapy underwent tumor regression rather than growth inhibition. To confirm this robust effect, we performed histologic analysis, which...

### Table 1. JP1201 sensitizes pancreatic tumor cells to gemcitabine

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Gemcitabine (nmol/L)</th>
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<th>Fold reduction JP1201 (μmol/L)</th>
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<td>2</td>
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<tr>
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<tr>
<td>Pan02</td>
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NOTE: Cell growth assays were performed in 96-well format for 5 days (n = 8/condition/assay). On day 0, cells were plated; on day 1, drugs were added in 4-fold dilutions. The highest dose of gemcitabine was 2,000 nmol/L. The highest concentration of JP1201 alone was 100 μmol/L. On day 5, the relative cell number was estimated using MTS (Promega; final concentration, 333 μg/mL); plates were incubated for 1 to 3 h at 37°C and read at 490 nm. Drug sensitivity curves and IC₅₀ values were calculated using in-house software. The number of independent assays performed and the median gemcitabine IC₅₀ ± SD (nmol/L) for gemcitabine alone and gemcitabine + JP1201, the fold reduction in gemcitabine IC₅₀ in the presence of JP1201 (100 nmol/L), and sensitivity to JP1201 alone are displayed. Shaded rows indicate cell lines that were tested in vivo.
revealed large poorly differentiated tumor tissue in the saline- and gemcitabine-treated animals with little evidence of remaining normal pancreas tissue. However, treatment with JP1201 alone or in combination with gemcitabine or TRAIL revealed reduced primary tumor burden and an increase in the amount of residual normal pancreas (dark purple), as seen by H&E analysis (Supplementary Fig. S1). A replicate experiment revealed similar results (Fig. 3C). In the replicate experiment, the dose of gemcitabine was decreased to 25 mg/kg. We also evaluated the response of orthotopic BxPC-3 tumors using a similar dosing strategy and found that treatment with JP1201 in combination with gemcitabine was effective at controlling tumor size (saline, 0.94 g; combination therapy, 0.35 g).

Impaired drug delivery is proposed as a complicating factor in the poor response of pancreatic tumors to standard therapy. With this in mind, we evaluated the general pharmacokinetic parameters of gemcitabine, its inactive metabolite dFdU, and JP1201 in mice bearing large orthotopic MIA PaCa-2 tumors (Supplementary Table S1). Fifty-four mice with a mean ± SD pancreas/tumor of 0.47 ± 0.2 g were used for the study. We found that the plasma half-life and area under the curve of gemcitabine were elevated by combination with JP1201; however, the contribution to the dramatic increase in response in mice treated with combination therapy is unclear. We also identified a surprisingly long plasma and tumor half-life of JP1201, 300 to 400 minutes and 900 to 1,000 minutes, respectively.

**JP1201 maintains effectiveness as a chemosensitizer in a syngenic model**

To determine if JP1201 + gemcitabine remained effective in a syngenic model of pancreatic cancer, we performed a therapy experiment in C57Bl/6 mice using the murine pancreatic cancer cell line Pan02. Therapy was started 3 weeks after tumor cell injection, and animals were sacrificed on day 35. The combination of JP1201 + gemcitabine was effective at reducing tumor burden in this fully immunocompetent model (Fig. 3D). Of note, 5 of 10 mice in the combination therapy group had no identifiable tumor at the time of necropsy, whereas 100% of the saline-treated mice and mice treated with single-agent therapy had significant pancreatic tumor masses. We found in this model that gemcitabine (25 mg/kg) induced splenomegaly, which was then exacerbated by the combination with JP1201 (spleen weight: saline, 0.11 g; gemcitabine, 0.19 g; JP1201, 0.11 g; JP1201/gemcitabine, 0.25 g).

**JP1201 reduces metastatic disease**

We assessed metastatic burden in the xenograft late interventions studies and found that overall metastatic incidence was 55% in the saline-treated animals; this was reduced to 35%,
43%, 31%, and 14% by treatment with gemcitabine, JP1201, JP1201 + gemcitabine, and JP1201 + TRAIL, respectively.

**JP1201 induces apoptosis and TNFα expression in orthotopic tumors**

To assess induction of apoptosis after therapy, MIA PaCa-2 tumors from animals sacrificed on days 29 and 42 (Fig. 3A) were analyzed. TUNEL immunofluorescence revealed that there was an acute induction of apoptosis in the tumor following administration of JP1201 combined with gemcitabine. However, after the full course of therapy, there was no difference in the TUNEL signal between any of the treatment groups (Fig. 4A). We also evaluated cell proliferation by immunohistochemistry for PCNA. PCNA reactivity was elevated in the combination (JP1201/gemcitabine) at the early time point (day 29); however, single-agent and combination therapy significantly reduced PCNA reactivity at the late time point (Fig. 4B). Furthermore, cell proliferation in tumors treated with JP1201 and the combination (JP1201/gemcitabine) were reduced significantly compared with treatment with gemcitabine alone (Fig. 4B).

TNFα has been implicated in the response to Smac mimetics (18). Therefore, we evaluated the level of TNFα protein in tumors from each group at early (day 29) and late (day 42) time points. TNFα levels were increased modestly by combination therapy at the early time point, but were elevated dramatically by JP1201 alone or in combination with gemcitabine at the late time point (Fig. 4C and D). We believe that the TNFα detected is of human tumor cell origin. Pan02 tumor tissue from controls and animals treated with JP1201 alone or in combination with gemcitabine revealed sparse reactivity with the rabbit anti-TNFα antibody used in Fig. 4 (Supplementary Fig. S2) and a rat anti-mouse TNFα antibody (data not shown). These results are consistent with PCR-based analysis of Pan02 tumor tissue, which did not show induction of TNFα (data not shown). Taken together, our observations suggest that Pan02 cells do not produce TNFα upon treatment with JP1201 and that the majority of...
TNFα staining observed in MIA PaCa-2 xenografts is of tumor cell origin. Additionally, we evaluated if TNFα induction was detectable in vitro after treatment with JP1201 or siRNA-mediated XIAP knockdown (Supplementary Table S2). Cells (MIA PaCa-2, PANC-1, AsPC-1, and BxPC3) were treated with JP1201 (100 nmol/L) or subjected to XIAP knockdown. Conditioned medium was harvested 12, 48, and 96 hours posttreatment.

Figure 4. Induction of apoptosis using combination therapy. A to D, tumors from animals sacrificed 1 d after initiation of therapy [early (D29)] or after the cessation of therapy [late (D42)] were formalin fixed and paraffin embedded. A to C, data from a minimum of four tumors from each group were normalized to the saline group and are representative of at least two independent assays on the same tumor tissue. A, TUNEL analysis was performed and analyzed using immunofluorescence. *, *P < 0.05; **, *P < 0.01; ***, *P < 0.001 versus saline by one-way ANOVA. B, cell proliferation was measured by immunofluorescence for PCNA. **, *P < 0.01; ***, *P < 0.001 by one-way ANOVA. C and D, the expression level of TNFα was determined by immunofluorescence. *, *P < 0.05; ***, *P < 0.001 by one-way ANOVA. D, representative images of TNFα (green) immunofluorescence; scale bar, 50 μm. GEM, gemcitabine; JP, JP1201.
and analyzed for TNFα levels by ELISA. These results show that during this time frame, a single treatment with JP1201 or knockdown of XIAP does not consistently or robustly induce TNFα expression in these cell lines.

**JP1201 + gemcitabine prolongs survival in mice with orthotopic pancreatic tumors**

Animal models frequently show therapy-induced tumor growth inhibition, but these drugs often fail to transition to the clinical setting (19). Although we show tumor regression in a model of advanced cancer, we investigated if the addition of JP1201 to gemcitabine would improve survival. The use of objective end points, such as survival, may help to improve the clinical success of agents validated in animal models (19). Tumor establishment and dosing schedule were identical to that in Fig. 3A. Following therapy, animals were monitored and sacrificed when they showed objective signs of tumor burden or when they appeared moribund (as determined by a blinded observer). Rx, treatment; Ns, not significant. B, p48-Cre;KRasG12D;Ink4a/Arflox/lox PDAC animals were treated with saline, gemcitabine, or the combination of JP1201/gemcitabine starting at 4 wk of age. Therapy was delivered i.p. continuously thrice weekly until sacrifice. C, a second cohort of PDAC animals underwent 4 wk of therapy (saline, n = 5; gemcitabine, n = 4; JP1201/gemcitabine, n = 6) and were sacrificed at 8 wk of age. Tumors were excised and weighed, and a scatter plot showing mean ± SEM tumor weight is displayed. Compared with control (1.124 ± 0.1135 g), JP1201/gemcitabine significantly reduced tumor growth (0.3833 ± 0.1492 g). **, P < 0.01. Each panel represents a single animal experiment.

**Discussion**

The prognosis for patients with pancreatic cancer is poor (1). Chemotherapeutic options are limited and the standard of care, gemcitabine, improves survival only minimally (21). Resistance to apoptosis is a critical event in tumorigenesis as it allows for the development of mutations, survival in harsh conditions, and proliferation in an anchorage-independent fashion (22). Efforts to increase therapy-induced apoptosis are appropriate and attractive for cancer chemotherapy (5, 22). We show that JP1201, a novel Smac mimic, robustly improves the efficacy of standard chemotherapy in

Figure 5. Combination of JP1201 with gemcitabine enhances survival of mice with pancreatic cancer. A, mice bearing established MIA PaCa-2 tumors were treated using the late intervention protocol described in Fig. 3A. Mice were treated with saline (black line), gemcitabine (GEM, 25 mg/kg; green line), JP1201 (JP, 6.0 mg/kg; red line), or the combination of JP1201/gemcitabine (blue line) thrice weekly for 2 wk. Animals were monitored and sacrificed when they showed objective signs of tumor burden or when they appeared moribund (as determined by a blinded observer). Rx, treatment; Ns, not significant. B, p48-Cre;KRasG12D;Ink4a/Arflox/lox PDAC animals were treated with saline, gemcitabine, or the combination of JP1201/gemcitabine starting at 4 wk of age. Therapy was delivered i.p. continuously thrice weekly until sacrifice. C, a second cohort of PDAC animals underwent 4 wk of therapy (saline, n = 5; gemcitabine, n = 4; JP1201/gemcitabine, n = 6) and were sacrificed at 8 wk of age. Tumors were excised and weighed, and a scatter plot showing mean ± SEM tumor weight is displayed. Compared with control (1.124 ± 0.1135 g), JP1201/gemcitabine significantly reduced tumor growth (0.3833 ± 0.1492 g). **, P < 0.01. Each panel represents a single animal experiment.
pancreatic cancer xenografts, syngeneic tumors, and spontaneous transgenic tumors in mice. In vitro mechanistic studies showed that inhibition of XIAP is critical for the sensitization of Mia PaCa-2 and PANC-1 cells. Although in vivo treatment with JP1201 resulted in induction of TNFα, these data suggest that JP1201 promotes apoptosis through two potential mechanisms that result in enhanced activity of gemcitabine.

TRAIL is known to induce apoptosis in a variety of cell lines and is currently being pursued as a potential cancer therapy. We show here a moderate effect of TRAIL monotherapy in vivo and potent antitumor activity in animals that were treated with the combination of JP1201 and TRAIL. This mirrors the in vitro data with Mia PaCa-2 cells. Other studies in glioma (23) and pancreatic cancer (10) models show that TRAIL alone is ineffective, but TRAIL combined with inhibition of XIAP can induce apoptosis and inhibit tumor growth. These studies showed a reduction in tumor burden and/or an increase in survival, consistent with the results presented here. Thus, our data support that Smac mimetics in combination with TRAIL is a promising anticancer strategy.

Because gemcitabine is the standard of care for pancreatic cancer, we determined if inhibition of XIAP would sensitize tumors to gemcitabine therapy. We found that knockdown of XIAP, but not related IAPs, using siRNA increases sensitivity to gemcitabine in pancreatic cancer cell lines. These data expand the work on other pancreatic cancer cell lines, which shows that resistance to chemotherapy is mediated in part by XIAP (8, 17). We also show that the sensitization to gemcitabine with XIAP knockdown is strikingly similar to the sensitization seen with JP1201 in vitro, supporting the concept that JP1201 sensitizes pancreatic cancer to gemcitabine through inhibition of XIAP.

We also explored whether JP1201 would sensitize tumors to gemcitabine treatment. We showed a significant decrease in tumor weights in animals treated with the combination of JP1201 and gemcitabine compared with saline in a late intervention model in both immunocompromised and immunocompetent mice. JP1201 sensitization was also evident at lower doses of gemcitabine. In a separate experiment, this effect translated into increased survival in tumor-bearing animals. These data alone are very suggestive that Smac mimetics in combination with TRAIL is a promising anticancer strategy.

Because gemcitabine is the standard of care for pancreatic cancer, we determined if inhibition of XIAP would sensitize tumors to gemcitabine therapy. We found that knockdown of XIAP, but not related IAPs, using siRNA increases sensitivity to gemcitabine in pancreatic cancer cell lines. These data expand the work on other pancreatic cancer cell lines, which shows that resistance to chemotherapy is mediated in part by XIAP (8, 17). We also show that the sensitization to gemcitabine with XIAP knockdown is strikingly similar to the sensitization seen with JP1201 in vitro, supporting the concept that JP1201 sensitizes pancreatic cancer to gemcitabine through inhibition of XIAP.

We also explored whether JP1201 would sensitize tumors to gemcitabine treatment. We showed a significant decrease in tumor weights in animals treated with the combination of JP1201 and gemcitabine compared with saline in a late intervention model in both immunocompromised and immunocompetent mice. JP1201 sensitization was also evident at lower doses of gemcitabine. In a separate experiment, this effect translated into increased survival in tumor-bearing animals. These data alone are very suggestive that Smac mimetics combined with standard therapy could greatly improve pancreatic cancer patient response to therapy. This study adds to the growing body of literature that show the potency of Smac mimetics in combination with various standards of care, such as a study done in a glioblastoma model in which Smac mimetics were shown to enhance tumor cell death after radiation treatment (24). We further explored the effect of combination therapy in an aggressive transgenic model of pancreatic cancer and again saw increased survival.

We also found that treatment with JP1201 induced TNFα in vivo. Previous reports (12, 25, 26) have shown that Smac mimetic-sensitive cell lines produce TNFα, can be induced to produce more TNFα after Smac mimetic treatment, and are dependent on TNFα for Smac mimetic-induced cell death. However, there are currently no published data indicating that Smac mimetics induce TNFα in resistant cell lines, such as Mia PaCa-2. Possible explanations the potential of prolonged treatment with JP1201 to induce TNFα production. Alternatively, the in vivo tumor microenvironment may be required to facilitate TNFα production after treatment with JP1201 or other Smac mimetics. In that regard, it is worth noting that although the pancreatic cancer cell lines showed in vitro resistance to JP1201, the xenografts showed reduction of tumor burden with JP1201 as a monotherapy, which might be linked to the production of TNFα. These observations suggest that alternative pathways that promote cell death may be engaged. TNFα expression has been linked to noncanonical activation of NF-κB as a result of cIAP1/2 degradation (25, 27). NF-κB can in turn stimulate autocrine expression of TNFα (25, 26, 28, 29), which activates receptor-interacting protein kinase 1 through the TNF receptor and subsequently induces caspase activation. In support of this, preliminary studies suggest that NF-κB actively participates in JP1201-mediated sensitization of pancreatic tumor cells, as treatment of cells with gemcitabine, JP1201, and an IKK inhibitor (SC-514) increased the IC50 for gemcitabine 3- to 13-fold compared with JP1201 + gemcitabine (data not shown). Thus, JP1201 could promote apoptosis by inducing the degradation of cIAP1/2, resulting in NF-κB activation, and inhibiting the activity of XIAP; both could potentiate the efficacy of gemcitabine (30).

In summary, resistance to therapy-induced apoptosis is a major roadblock in the treatment of patients with pancreatic cancer. Another significant challenge is the lack of predictive power of preclinical models of pancreatic cancer. In an effort to address both of these challenges, we have shown potent antitumor activity of a unique Smac mimic in animal models of pancreatic cancer that are robust and clinically relevant. We believe that our data support the development of agents designed to sensitize pancreatic tumors to apoptosis and suggest that JP1201 deserves serious consideration for clinical investigation.

Disclosure of Potential Conflicts of Interest

H. Sun: employment, Joyant Pharmaceuticals. R.A. Brekken: commercial research grant, Joyant Pharmaceuticals. The other authors disclosed no potential conflicts of interest.

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