

BMS-754807, a Small-Molecule Inhibitor of Insulin-like Growth Factor-1 Receptor/Insulin Receptor, Enhances Gemcitabine Response in Pancreatic Cancer

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Abstract

Gemcitabine has limited clinical benefits in pancreatic ductal adenocarcinoma (PDAC). Insulin-like growth factor (IGF) signaling proteins are frequently overexpressed in PDAC. The therapeutic potential of BMS-754807, a small-molecule inhibitor of IGF-type 1 receptor (IGF-1R) and insulin receptor (IR), and gemcitabine was evaluated in experimental PDAC. Cell proliferation and protein expression were measured by WST-1 assay and immunoblotting. Tumor growth and survival studies were conducted in murine xenografts. PDAC cells expressed phospho-IGF-1R protein. BMS-754807 and gemcitabine inhibited cell proliferation of PDAC cells; the combination of BMS-754807 with gemcitabine had additive effects. Addition of BMS-754807 decreased gemcitabine IC₅₀ from 9.7 μmol/L to 75 nmol/L for AsPC-1, from 3 μmol/L to 70 nmol/L for Panc-1, from 72 to 16 nmol/L for MIA PaCa-2, and from 28 to 16 nmol/L for BxPC-3 cells. BMS-754807 caused a decrease in phospho-IGF-1R and phospho-AKT proteins in AsPC-1 and Panc-1 cells. BMS-754807 and gemcitabine caused an increase in PARP-1 and caspase-3 cleavage. Net tumor growth inhibition in BMS-754807, gemcitabine, and BMS-754807+gemcitabine groups was 59%, 35%, and 94% as compared with controls. Effects of therapy on intratumoral proliferation and apoptosis corresponded with tumor growth inhibition data. BMS-754807 also caused a decrease in phospho-IGF-1R and phospho-AKT in tumor tissue lysates. Median animal survival (controls: 21 days) with BMS-754807 was 27 days ($P = 0.03$), with gemcitabine 28 days ($P = 0.05$), and in the BMS-754807+gemcitabine combination group, 41 days ($P = 0.007$). The strong antitumor activity of BMS-754807 in experimental PDAC supports the potential of BMS-754807-induced mechanisms for clinical PDAC therapy. *Mol Cancer Ther*; 11(12); 2644–53. ©2012 AACR.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive human cancers with extremely poor prognosis and 5-year survival is less than 5% (1). Each year, more than 40,000 individuals are diagnosed with PDAC and this incidence nearly equals mortality mostly due to late clinical presentation, early and aggressive local and metastatic progression, and high resistance to conventional therapies. Gemcitabine (Gem), a nucleoside analog that exerts its cytotoxic effects mainly by being

incorporated into the DNA strand and inhibit DNA synthesis, is still the standard chemotherapeutic treatment of locally advanced and metastatic PDAC (2). However, the efficacy of gemcitabine as a single agent remains modest, with a median progression-free survival of approximately 6 months in randomized clinical trials and a 12-month survival of less than 20% (2–4). Currently, several clinical trials are underway to explore combination treatment benefits of gemcitabine with either cytotoxic agents or targeted agents toward development of more effective and less toxic therapeutic strategies for PDAC.

Although the causes of highly aggressive nature of PDAC are not well-understood, previous studies have shown the association of overexpression of several growth factors and their receptors during the progression of PDAC including EGF, fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), VEGF, and insulin-like growth factor (IGF) (5, 6). The IGF system consists of ligands IGF-1, IGF-2, insulin, and IGF type-1 receptor (IGF-1R), insulin receptor (IR), and hybrid receptors. Activation of the IGF system stimulates proliferation, differentiation, angiogenesis, metastasis, survival, and resistance to anticancer therapies in many cancers (7),

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supporting the IGF system as an attractive therapeutic target. In the IGF system, IGF-1R is the primary mitogenic receptor that is overexpressed and has increased tyrosine kinase activity in several cancer types including PDAC (6, 8, 9). The binding of 2 IGF-1R ligands, IGF-1 and IGF-2, leads to the activation of several downstream signal transduction pathways including the phosphatidylinositol 3'-kinase (PI3K)/AKT and mitogen-activated protein kinase (MAPK) signaling pathways (10, 11). IGF-1R shares significant structural homology with IR. Although the binding of insulin to IR is primarily directed to regulate glucose homeostasis, this binding has also been shown to stimulate mitogenic signaling in cancer cells (12). IR has 2 isoforms, the classic IR-B that binds insulin leading to predominantly metabolic effects (13), and IR-A that binds insulin and IGF-2 causing proliferative effects (14). IR also has the ability to form hybrid receptors with IGF-1R (15, 16), which increases the complexity in targeting the IGF signaling pathway and suggests that the inhibition of both IR and IGF-1R may be beneficial for cancer therapy.

The strategy to target IGF-1R with specific monoclonal antibodies is attractive, as IGF-1R is the primary mitogenic receptor responsible for transducing IGF-1 and IGF-2, and as glucose homeostasis remains intact because of lack of IR-B inhibition (17, 18). However, monoclonal antibodies binding specifically to IGF-1R and not to IR could potentially provide escape mechanism for insulin and IGF-2 signaling, leading to limited clinical response. Interestingly, hyperglycemia and evidence of insulin resistance was observed clinically even with the IGF-1R antibody therapy (19). Tyrosine kinase inhibitors can more indiscriminately regulate the kinase domain activity of all IGF system receptors as their primary sequence share 84% homology in the kinase domain with near complete conservation in the ATP binding pocket (20). BMS-754807 is a potent oral, reversible small molecule tyrosine kinase inhibitor of the IGF-1R/IR family kinases. BMS-754807 has shown antitumor activity in a broad range of tumor types *in vitro* and *in vivo* (21–23). This drug is currently under phase I clinical investigation for the treatment of a variety of human cancers. In the present study, we evaluated the *in vitro* and *in vivo* efficacy of BMS-754807 alone and in combination with gemcitabine in an attempt to identify a more effective PDAC therapeutic strategy.

Materials and Methods

Cell culture and reagents

The human PDAC cell lines AsPC-1, BxPC-3, MIA PaCa-2, and Panc-1 were obtained from the American Type Culture Collection (ATCC) and cultured in RPMI-1640 medium (Sigma Chemical Co.) supplemented with 10% FBS at 37°C in a humidified 5% CO₂ atmosphere. Each cell line was tested and authenticated by ATCC. Cells were initially grown and multiple aliquots were cryopreserved. All the cell lines were used within 6 months after resuscitation. BMS-754807 was purchased from Active Biochemicals Co. Limited. Gemcitabine was purchased from

Eli Lilly Corporation. The cell proliferation reagent WST-1 was purchased from Roche Diagnostic Corporation.

Cell viability assay

Cell viability of PDAC lines was evaluated by the colorimetric WST-1 assay. The measurement is based on the ability of viable cells to cleave the sulfonated tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) by mitochondrial dehydrogenases. PDAC cells (4,000 cells/well) were plated in a 96-well plate in regular growth medium, and after 16 hours the medium was replaced with 2% FBS containing medium. After 5 hours of incubation, the cells were treated with BMS-754807 and gemcitabine. The range of concentrations used (100 nmol/L to 10 μmol/L) for BMS-754807 and gemcitabine was comparable with their clinically achievable concentrations. After 72 hours, 10 μL WST-1 reagent was added in each well followed by additional incubation for 2 hours. The absorbance at 450 nm was measured using a microplate reader.

Western blot analysis

Subconfluent monolayers of PDAC cells were treated with BMS-754807 (10 μmol/L) and gemcitabine (10 μmol/L). The cells were lysed after 24 hours as previously described (24). To obtain tumor lysates, tumor tissues obtained from the *in vivo* tumor growth experiment were immediately snap-frozen in liquid nitrogen and stored at –80°C. These samples were crushed in liquid nitrogen using a sterilized mortar, resuspended in lysis buffer, and extracts were sonicated on ice for 10 minutes. Supernatants were recovered by centrifugation at 13,000 rpm, protein concentrations were measured and equal amounts of total protein were separated by SDS-PAGE. Proteins were transferred to PVDF membranes (Bio-Rad) followed by blocking with 5% milk in TBS-T for 1 hour. The membranes were incubated overnight at 4°C with the following antibodies: total IGF-1R, phospho-IGF-1R (Tyr1135/1136)/IR (Tyr1150/1151; #3024), total AKT, phospho-AKT (Ser473), cleaved poly (ADP-ribose) polymerase-1 (PARP-1), cleaved caspase-3 (all from Cell Signaling Technology), α-tubulin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; both from Sigma). The membranes were then incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (Pierce Biotechnologies) for 1 to 2 hours. Specific bands were detected using the enhanced chemiluminescence reagent (ECL, PerkinElmer Life Sciences) on autoradiographic film and quantitated by densitometry.

Subcutaneous tumor growth study

Animal studies were conducted in accordance with the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center. Athymic female nude mice (4–6 weeks old) were used to establish the subcutaneous xenograft model for tumor growth as previously described (25). AsPC-1 cells (0.75×10^6) were subcutaneously injected in each mouse. The mice

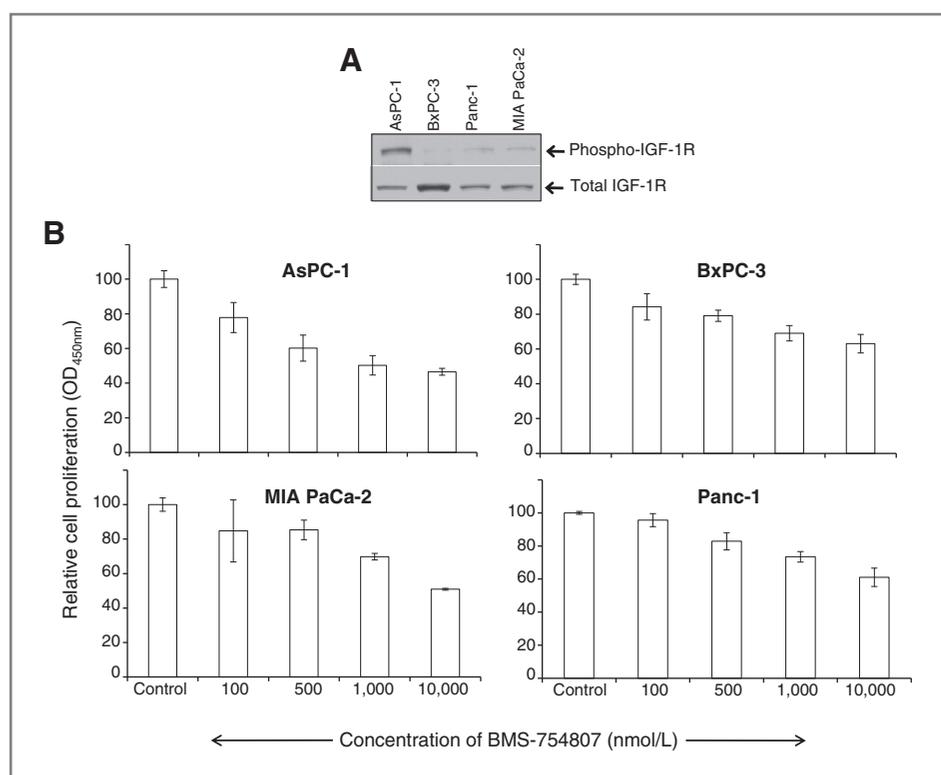


Figure 1. BMS-754807 inhibits the *in vitro* cell proliferation of PDAC cells AsPC-1, BxPC-3, MIA PaCa-2, and Panc-1. **A**, total cell extracts of PDAC cells were analyzed by immunoblotting for phospho-IGF-1R and total IGF-1R. **B**, PDAC cells were plated on 96-well plates and treated with 100, 500, 1,000, and 10,000 nmol/L concentrations of BMS-754807. After 72 hours, 10 μ L WST-1 reagent was added in each well and incubated for 2 additional hours. The absorbance at 450 nm was measured using a microplate reader. The resulting number of viable cells was calculated by measuring absorbance of color produced in each well. Data are the mean \pm SD of triplicate determinations.

were weighed twice a week. Fourteen days after tumor cell injection, all mice had measurable tumor. Mice were then randomly grouped ($n = 6-8$ per group) and treated intraperitoneally with PBS (control), BMS-754807 (25 mg/kg in 100 μ L PBS, 5 times a week), and gemcitabine (100 mg/kg in 100 μ L PBS, twice weekly) for 14 days. The tumor size was measured twice weekly, and tumor volume (V) was calculated using the formula $V = \frac{1}{2}(L \times W)^2$, whereby $L =$ length and $W =$ width. Net growth in tumor size for each mouse was calculated by subtracting tumor volume on the first treatment day from that on the last day. After completion of treatment, all mice were euthanized, tumors were dissected, weighed, and processed for histological, immunohistochemical and Western blot analysis.

Immunohistochemical analysis

Tumor tissue specimens were fixed in 4% paraformaldehyde and embedded in paraffin. Intratumoral proliferation was measured by Ki67 nuclear antigen staining as per manufacturer's protocol (Abcam). Briefly, paraffin-embedded tissue sections were cut (5 μ m), deparaffinized, and rehydrated. Antigen retrieval was carried out by heating the tissue sections in citrate buffer. The tissue sections were incubated with CAS blocking buffer followed by 1-hour incubation with Ki67 antibody (1:200 dilution) and 40 minutes of incubation with Cy3 (1:200 dilution) secondary antibody. Slides were mounted using mounting solution containing 4',6-diamidino-2-phenylindole (DAPI; Invitrogen). Intratumoral proliferative index was evaluated by calculating the Ki67-positive cells from 5

different high-power fields in a blinded manner. Intratumoral apoptotic activity was evaluated by staining tissue sections with the Apoptag Apoptosis Detection Kit according to the manufacturer's instructions. Fluorescence microscopy was used to detect fluorescent signals using the IX81 Olympus microscope equipped with a Hamamatsu Orca digital camera (Hamamatsu Corporation) and a DSU spinning confocal unit using Slidebook software (Intelligent Imaging Innovations).

Animal survival analysis

Animal survival studies were conducted using 6- to 8-week-old female severe combined immunodeficient (SCID) mice (26). The mice were intraperitoneally injected with AsPC-1 (0.75×10^6) cells and body weight was measured twice a week. Two weeks after tumor cell injection, mice were randomly grouped ($n = 6-8$ per group) and treated intraperitoneally with PBS (control), BMS-754807 (25 mg/kg in 100 μ L PBS, 5 times a week), and gemcitabine (100 mg/kg, twice weekly) for 2 weeks. Animals were euthanized when turning moribund according to predefined criteria (27, 28). Animal survival was evaluated from the first day of treatment until death.

Statistical analysis

Statistical significance was analyzed by the 2-tailed Student t test using GraphPad Prism 4 Software (GraphPad Software). *In vitro* cell proliferation data are expressed as mean \pm SD. Statistical analysis for normally distributed *in vivo* study data was conducted by ANOVA for multiple group comparison and Student t test for the individual

group comparison. Survival study statistics were evaluated with StatView for Macintosh (SAS) by nonparametric survival statistics and log-rank testing. Values of $P < 0.05$ were considered to represent statistically significant group differences.

Results

BMS-754807 inhibits PDAC cell proliferation

Analysis of human PDAC cells AsPC-1, BxPC-3, MIA PaCa-2, and Panc-1, revealed that all 4 lines expressed IGF-1R and phospho-IGF-1R. Expression of phospho-IGF-1R was in the following order: AsPC-1>Panc-1>MIA PaCa-2>BxPC-3 (Fig. 1). BMS-754807 dose-dependently inhibited PDAC cell proliferation, with an inhibition in cell proliferation at 10 $\mu\text{mol/L}$ of 54%, 37%, 49%, and 39% in AsPC-1, BxPC-3, MIA PaCa-2, and Panc-1 cells, respectively. Interestingly, inhibition in cell proliferation of

PDAC cells by BMS-754807 seems to correlate to their expression of phospho-IGF-1R (Fig. 1).

BMS-754807 addition decreases gemcitabine IC₅₀ in PDAC lines

Gemcitabine, a standard anti-PDAC chemotherapy agent, inhibited cell proliferation of PDAC lines in a dose-dependent manner. At 500 nmol/L, the inhibition in cell proliferation was 27%, 65%, 55%, and 29% in AsPC-1, BxPC-3, MIA PaCa-2, and Panc-1 cells, respectively (Fig. 2). The combination of BMS-754807 with gemcitabine had enhancing inhibitory effects. Including BMS-754807 at its IC₂₅ decreased the gemcitabine IC₅₀ from 9.7 $\mu\text{mol/L}$ to 75 nmol/L for AsPC-1, from 28 to 16 nmol/L for BxPC-3 cells, from 72 to 16 nmol/L for MIA PaCa-2, and from 3 $\mu\text{mol/L}$ to 70 nmol/L for Panc-1 cells, respectively (Fig. 2).

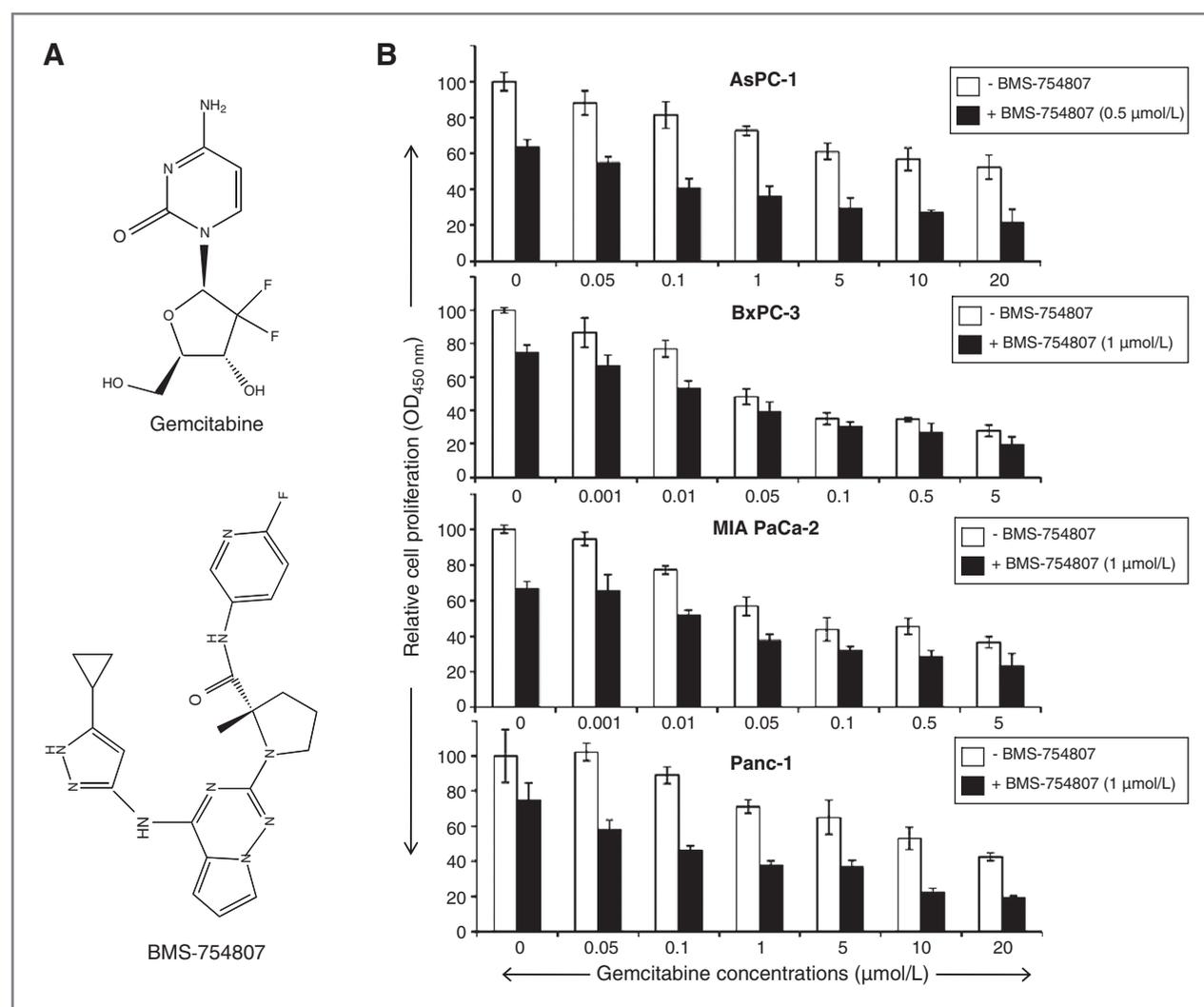


Figure 2. Additive effects of BMS-754807 and gemcitabine on PDAC cell proliferation inhibition. A, structures of gemcitabine and BMS-754807. B, AsPC-1, BxPC-3, MIA PaCa-2, and Panc-1 cells were plated on 96-well plates and treated at concentrations between 0.001 and 20 $\mu\text{mol/L}$ gemcitabine, either alone or in combination with an IC₂₅ dose of BMS-754807. After 72 hours, 10 μL WST-1 reagent was added in each well and incubated for 2 additional hours. The absorbance at 450 nm was measured using a microplate reader. The resulting number of viable cells was calculated by measuring absorbance of color produced in each well. Data are the mean \pm SD of triplicate determinations.

BMS-754807 blocks IGF signaling proteins and induces apoptosis

The effect of BMS-754807 on the IGF/receptor axis was investigated using AsPC-1 and Panc-1 PDAC cell lines that were most responsive to this drug in terms of causing a shift in the gemcitabine IC₅₀. Immunoblot analysis revealed that BMS-754807 blocked the expression of phosphorylated IGF-1R and downstream signaling protein phospho-AKT in both cell lines. The effect of BMS-754807 on chemotherapy-induced apoptosis was also evaluated by analyzing cleavage of caspase-3 and PARP-1 proteins as markers of apoptosis. Gemcitabine and BMS-754807 as single agent induced expression of cleaved caspase-3 and PARP-1, whereas the combination of BMS-754807 with gemcitabine caused additive effects on induction in cleavage of these apoptosis-related proteins (Fig. 3).

BMS-754807 inhibits the growth of PDAC xenografts and enhances gemcitabine antitumor response

BMS-754807 significantly inhibited the growth of AsPC-1 xenografts. Treatment of AsPC-1 tumor-bearing mice with BMS-754807 (25 mg/kg, 5 times a week for 2 weeks) resulted in statistically significant net tumor growth inhibition of 59% ($P = 0.02$) as compared with the PBS-treated control group (Fig. 4A and B). The evaluation of gemcitabine alone treatment in this model resulted in net tumor growth inhibition of 35% ($P = 0.05$). The combination treatment of AsPC-1 tumor-bearing mice with BMS-754807 and gemcitabine resulted in a 94% inhibition in net tumor growth ($P = 0.0007$) as compared with

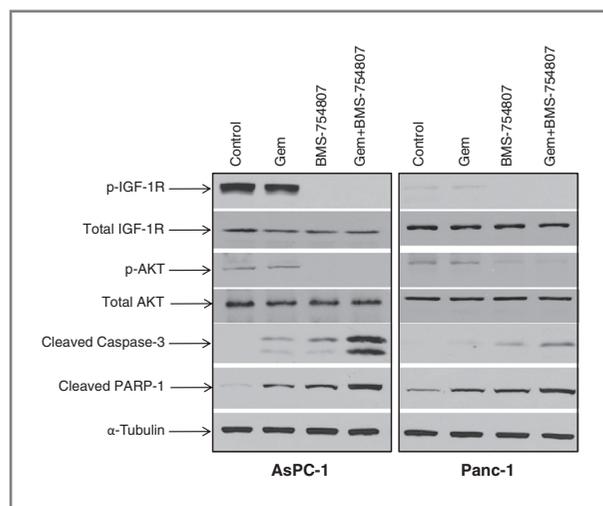


Figure 3. BMS-754807 and gemcitabine effects on the IGF-signaling pathway and apoptosis-related proteins. A subconfluent monolayer of human PDAC cells AsPC-1 and Panc-1 was treated with BMS-754807 (10 $\mu\text{mol/L}$) and gemcitabine (10 $\mu\text{mol/L}$), either alone or in combination for 16 hours. Total cell extracts were analyzed by immunoblotting for p-IGF-1R, total IGF-1R, p-AKT, total-AKT, cleaved PARP-1, cleaved caspase-3, and α -tubulin (loading control) proteins. Data are representative of 2 independent experiments with similar results.

controls (Fig. 4A and B). The difference in net tumor growth inhibition in the combination group was significantly different as compared with gemcitabine monotherapy ($P = 0.003$) or BMS-754807 monotherapy ($P = 0.04$).

Mechanisms of antitumor activity of BMS-754807, either alone or in combination with gemcitabine, were examined by Western blot analysis of protein lysates from AsPC-1 xenografts. A significant decrease in the expression of phospho-IGF-1R was observed in the BMS-754807-treated groups. BMS-754807 treatment also decreased the expression of the downstream signaling protein phospho-AKT (Fig. 4C). Evaluation of intratumoral apoptosis by analyzing expression of cleaved caspase-3 and cleaved PARP-1 proteins revealed that BMS-754807 and gemcitabine both induced cleavage of caspase-3 and PARP-1, and that the combination of these 2 agents had additive effects on cleavage of these apoptosis-related proteins (Fig. 4C).

No significant change in mice body weight was observed in the BMS-754807, gemcitabine, or combination therapy groups (Supplementary Fig. S1).

BMS-754807 inhibits intratumoral proliferation, induces apoptosis and enhances gemcitabine response

Further investigation of mechanisms of the antitumor activity of BMS-754807 by immunohistochemical analyses of tumor tissues revealed that the tumors of BMS-754807-treated mice presented a decreased tumor cell proliferation rate. Intratumoral proliferative index decreased by 29% ($P = 0.038$) in the BMS-754807-treated group as compared with controls. Gemcitabine monotherapy caused a 37% decrease in intratumoral proliferation as compared with controls ($P = 0.0005$). The combination of BMS-754807 and gemcitabine resulted in 39% decrease in proliferative activity as compared with control group ($P = 0.001$). However, the decrease in the intratumoral proliferative index in the combination treatment group was not significantly different than either therapy alone (Fig. 5).

Examination of intratumoral apoptosis in tumor tissues revealed a 3.9-fold increase in apoptotic index in the BMS-754807 monotherapy group ($P = 0.004$) and a 4.4-fold increase in the gemcitabine-treated group ($P = 0.003$) as compared with the control group. The combination of BMS-754807 and gemcitabine led to a 6.4-fold induction in intratumoral apoptosis ($P = 0.0005$). Induction in intratumoral apoptosis in the combination treatment group was significantly higher than in the BMS-754807 monotherapy ($P = 0.037$) or gemcitabine monotherapy ($P = 0.003$) groups (Fig. 5).

BMS-754807 increases animal survival and further enhances gemcitabine survival benefits

In AsPC-1 PDAC murine xenograft study, the median survival of nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice was 21 days in the control

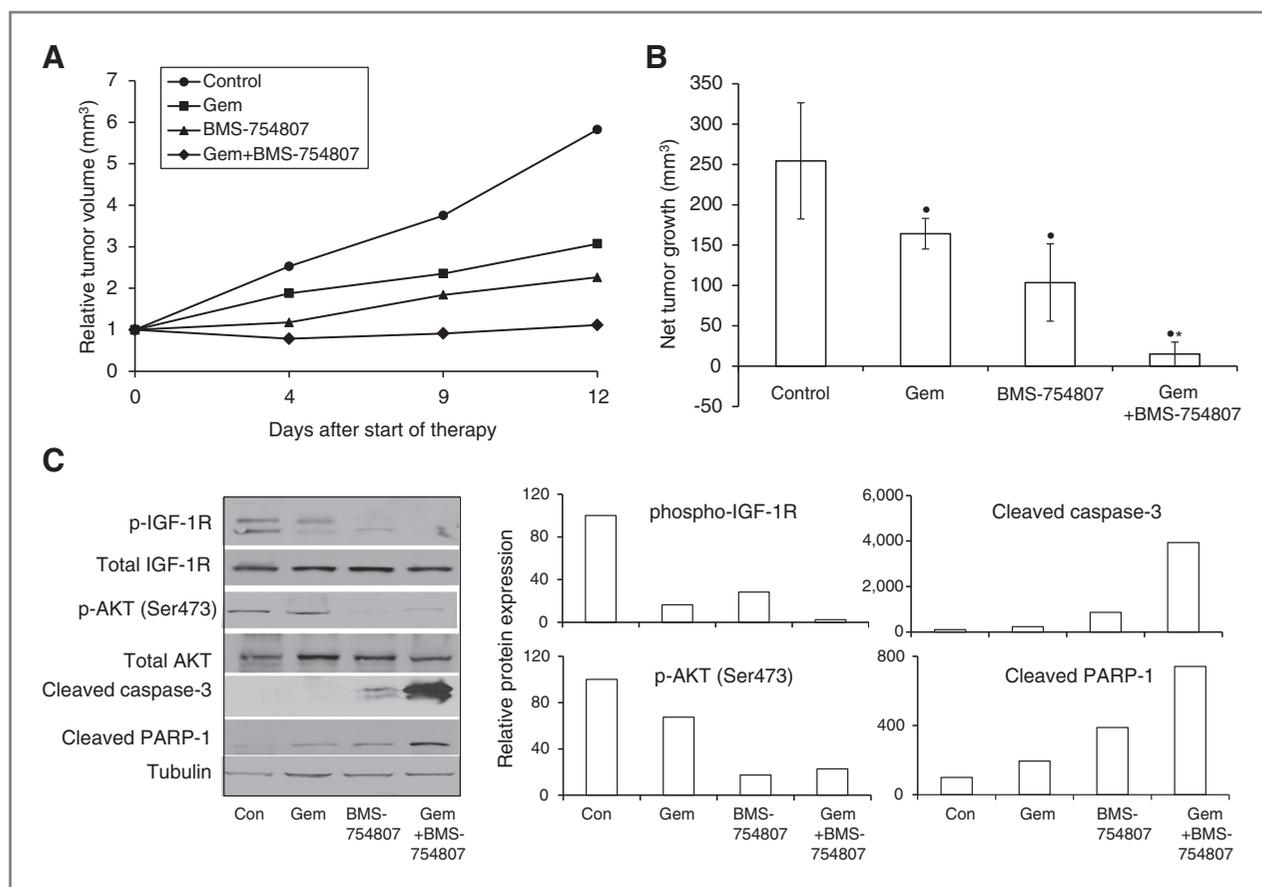


Figure 4. BMS-754807 and gemcitabine inhibit growth of established local PDAC tumor. Nude mice were subcutaneously injected with AsPC-1 cells and treated with BMS-754807 and gemcitabine for 2 weeks. Tumor growth was measured twice a week using calipers. **A**, relative tumor volume is calculated by dividing the tumor volume at any time by the tumor volume at the start of treatment. **B**, net tumor growth was calculated by subtracting tumor volume on the first treatment day from that on the final day. Data are representative of mean values \pm SD from 6 to 8 mice per group. \bullet , significant difference ($P < 0.05$) versus control; *, significant differences ($P < 0.05$) compared with monotherapy groups. **C**, BMS-754807 blocks IGF signaling proteins and induces apoptosis-related proteins. Tumor lysates were prepared from tumor tissue samples obtained from AsPC-1 tumor-bearing mice and were analyzed by immunoblotting. The intensity of bands was quantitated by densitometry and is represented in the bar graph after normalizing values with α -tubulin expression. Data are representative of 2 independent experiments with similar results.

group. Median survival of mice was increased by BMS-754807 treatment to 27 days ($P = 0.03$ vs. controls). Gemcitabine monotherapy resulted in a median survival of 28 days ($P = 0.05$ vs. controls). The combination of BMS-754807 and gemcitabine had additive effects on mouse survival, with the median survival extended to 41 days. The median survival in the BMS-754807+gemcitabine group was significantly higher as compared with controls ($P = 0.007$) or the 2 monotherapy groups ($P = 0.01$; Fig. 6).

Discussion

Most PDAC cases are unresectable at the time of presentation, due to unspecific and indistinct early symptoms. Also, local recurrence rate is very high in completely resected PDAC patients and they eventually require palliative treatment. Intrinsic and acquired chemoresistance to conventional chemotherapy continues to impact devastating prognosis in PDAC. Gemcitabine, the first-line

adjuvant treatment of advanced and metastatic PDAC, provided some clinical benefits but a modest survival advantage (2). The majority of the gemcitabine based-combination chemotherapy regimens for PDAC have failed to show any survival benefit (29, 30), except the EGF receptor inhibitor erlotinib that was approved in combination with gemcitabine based on a small but statistically significant improvement in overall survival (31). Recent advances in understanding of the molecular mechanisms of PDAC progression, however, have highlighted potential therapeutic targets for which combination therapy strategies seem promising. IGF-1R activation seems to be such a mechanism important for progression of several malignancies, and disruption of IGF-1R activation has been shown to inhibit growth and motility of a wide range of cancer cells, both *in vitro* and in animal models (32, 33). IGF-1R overexpression has also been correlated with tumor aggressiveness (34, 35), whereas IGF-1-deficient mice have reduced capacity to

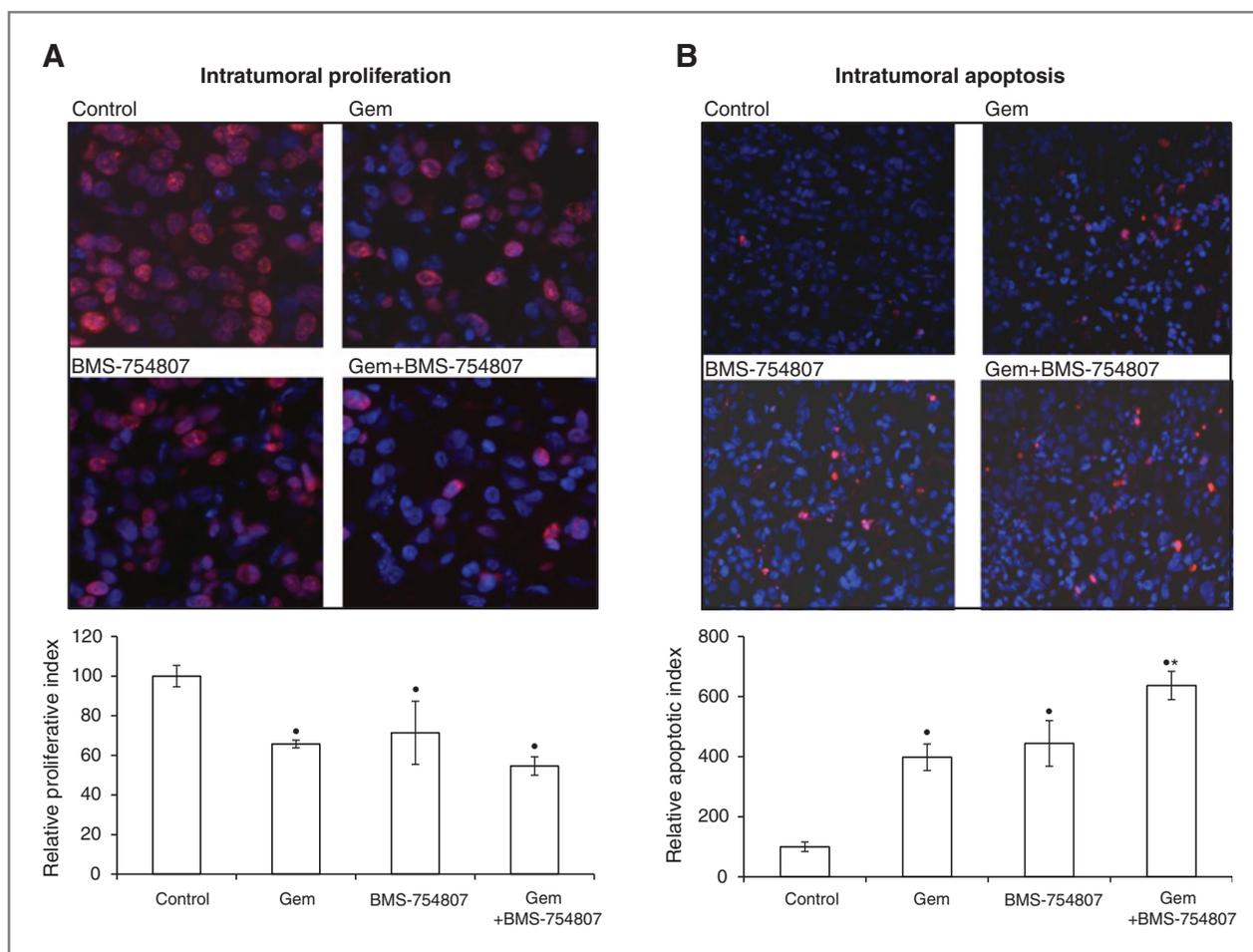


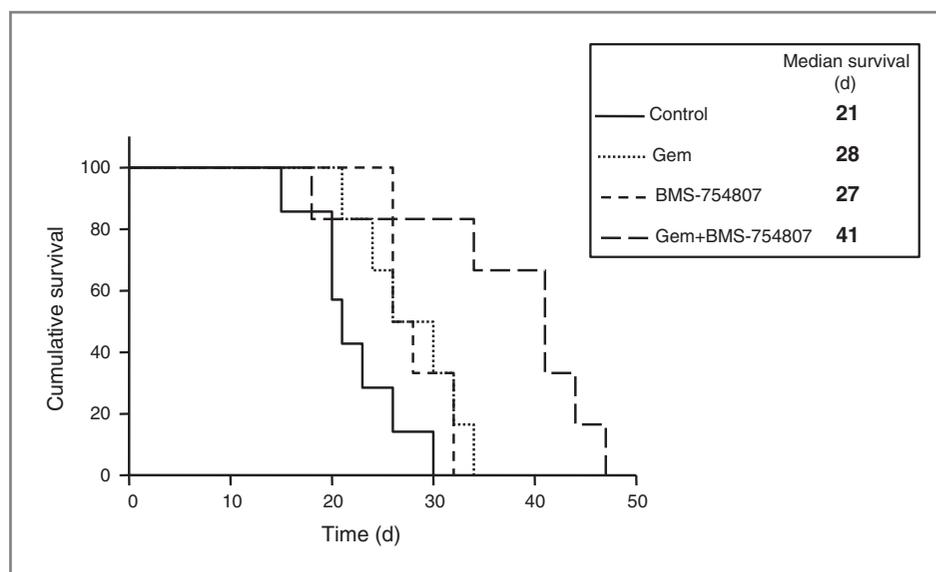
Figure 5. Effects of BMS-754807 and gemcitabine therapy on intratumoral proliferative and apoptotic activity. Nude mice were subcutaneously injected with AsPC-1 cells (0.75×10^6) and treated with BMS-754807 and gemcitabine, either alone or in combination, for 2 weeks. **A**, intratumoral proliferation was measured by immunostaining tissue sections with Ki67 nuclear antigen, and slides were photographed under a fluorescent microscope. Ki67-positive cells were counted in 5 different HPFs. **B**, intratumoral apoptosis was measured by staining tumor tissue section with terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) procedure and photographed under a fluorescent microscope. TUNEL-positive apoptotic cells were counted in 5 different HPFs. For both immunostaining experiments, the data are expressed as the mean \pm SD. ●, significant difference ($P < 0.05$) versus control; *, significant differences ($P < 0.05$) compared with BMS-754807 and gemcitabine monotherapy groups.

support tumor growth and metastasis (36). Several approaches have been used to block IGF-1R-related tumorigenicity including antisense strategies, RNA interference, blocking antibodies, and tyrosine kinase inhibitors (37, 38). The blockage of IGF-1R signaling has been shown to enhance chemotherapy response in several cancer types in laboratory studies (39–41). Because IGF-1R and IR are highly homologous, antagonist development was initially focused toward monoclonal antibodies that selectively target IGF-1R and not affect IR signaling, which could lead to dysregulation of glucose homeostasis (42). Initial clinical studies with IGF-1R antibodies showed some responses in monotherapy (43) and in combination with cytotoxic chemotherapy (44). However, patients receiving antibody therapy have hyperglycemia indicating the potential of undesirable metabolic side effects of such therapy (45). Several reports showed the

involvement of stimulation of IR by insulin or IGF-2 in cancer cell progression (14, 46) suggesting that IGF-1R and IR both are therapeutic targets and inhibition of both receptors may be required for maximum tumor growth inhibition. Our present study thus explored the effects of BMS-754807, a small-molecule inhibitor of both the receptors IGF-1R/IR, in combination with gemcitabine in PDAC.

Previous studies have shown the expression of IGF-1R in pancreatic cancer cells (6). We observed a differential expression of phospho-IGF-1R and IGF-1R in AsPC-1, BxPC-3, MIA PaCa-2, and Panc-1 cells, indicating a possible involvement of aberrant IGF signaling in PDAC progression. Interestingly, BMS-754807 inhibited the proliferation of all PDAC cells tested; importantly, in all lines there were additive inhibitory effects in combination with gemcitabine. The sensitivity of PDAC cells against BMS-

Figure 6. Effects of BMS-754807 and gemcitabine therapy on the overall survival of mice. AsPC-1 cells (0.75×10^6) were injected intraperitoneally in SCID mice and treatment started after 2 weeks with BMS-754807 (25 mg/kg, 5 times a week) and gemcitabine (100 mg/kg, 2 times a week) for 2 weeks. The curve represents the animal survival time from the beginning of therapy. The *P* values for survival differences compared with controls were 0.05 (Gem), 0.03 (BMS-754807), and 0.007 (Gem+BMS-754807); *P* values for comparisons to single agent groups were less than 0.01 (Gem+BMS-754807).



754807 seemed to be directly correlated with the expression of phospho-IGF-1R. We found interesting that the 2 cell lines with greater resistance to gemcitabine had greater IGF-1R expression and greater inhibition after exposure to BMS-754807; a rationale to combine these 2 agents may therefore be specifically sensible to enhance clinical PDAC treatment effects. Additional validation of involvement of IGF signaling in PDAC comes from our finding that a PI3K/mTOR inhibitor (NVP-BEZ235) enhances gemcitabine response as PI3K/mTOR may serve as downstream component of IGF signaling pathway (27). Therefore, further evaluation of combination of NVP-BEZ235 and BMS-754807 seems promising for PDAC clinical application. As previously reported in a human rhabdomyosarcoma cell line (21), we observed that in PDAC cells BMS-754807 treatment caused a decrease in phosphorylated IGF-1R and its downstream signaling protein phospho-AKT, and increased cleavage of apoptosis-related caspase-3 and PARP-1 proteins. These molecular signaling changes are likely operational in the BMS-754807-induced inhibition in cell proliferation and induction in apoptosis, and seem to represent good markers of *in vivo* activity testing and for clinical validation.

A recent study reported that BMS-754807 has some antitumor activity in multiple tumor models including epithelial, mesenchymal, and hematopoietic cancer cells (21). We observed that in a PDAC murine xenograft model, BMS-754807 inhibited local tumor growth as single agent and more importantly, caused additive effects in combination with gemcitabine. Analysis of intratumoral proliferation and apoptosis seem to be correlated with tumor growth inhibition data. Evaluation of target proteins in tumor tissue lysates showed that BMS-754807 treatment decreased levels of the activated form of IGF-1R and AKT, and induced levels of apoptosis-related proteins, indicating that the BMS-754807 therapy is

indeed managing to affect these targets within the local tumor model.

Active PDAC progression involves multiple distinct mechanisms including induction in cancer cell proliferation, migration, differentiation, angiogenesis, and inhibition of cancer or stromal cell apoptosis. Previous studies in our laboratory have shown the benefits of targeting multiple pathways in different cellular compartments including epithelial, vascular, and stromal elements for PDAC treatment (27, 47–49). Gemcitabine itself has antiproliferative and proapoptotic effects on tumor cells, endothelial cells, and fibroblasts (27, 28). The molecular mechanisms for the enhancement in antitumor activity of gemcitabine by BMS-754807 addition is not completely clear, but it is likely caused by augmentation of its antiproliferative and proapoptotic activities and possibly not just restricted to the tumor cells of epithelial origin. Because of the multifactorial nature of the disease and limited effectiveness of gemcitabine, combination therapies targeting multiple pathways provide promising strategies for clinical evaluation. There is some evidence that combination therapy is superior to monotherapy for the management of pancreatic cancer (31). Recently, combination of gemcitabine with nab-paclitaxel has been shown to have substantial antitumor activity in patients with advanced pancreatic cancer in a phase I/II trial (50). On the basis of our findings, combination of gemcitabine and BMS-754807 is a reasonable clinical trial option for phase II evaluation after establishing toxicity of this combination.

Dysregulation of glucose homeostasis is an important concern of using BMS-754807 and therefore the advantage of inhibition of both IGF-1R and IR signaling might have to be balanced with the potential for metabolic side effects. We have not obtained any evidence for *in vivo* toxicity during a 2-week treatment course, but have no data on whether long-term inhibition of the IGF-1R and IR functions remains tolerable. A previous study reported only a

short-term increase in serum glucose or insulin levels at doses up to 12.5 mg/kg, with an average weight change of -1.5 g at 25 mg/kg, but no mortality (21). In our study, no significant change in mouse body weight was observed by BMS-754807 therapy, but blood glucose measurements were not obtained. In conclusion, our study indicates that BMS-754807 can mediate mechanism-specific antitumor activity in experimental PDAC, and it significantly improves gemcitabine response. These findings corroborate the rationale of blocking multiple pathways of IGF and insulin signaling, and support the potential of BMS-754807 as targeting agent for clinical PDAC therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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