Pancreatic adenocarcinoma is one of the most lethal and poorly understood human malignancies. Because of the lack of effective systemic therapies, the 5-year survival rate for patients with pancreatic adenocarcinoma has remained at 1%–3%, without change over the past 25 years (1). Hence, development of novel chemotherapeutic approaches that reduce the intrinsic drug resistance of this disease poses one of the greatest challenges in pancreatic cancer research.

Nuclear factor κB (NF-κB) and activator protein-1 (AP-1) are key transcriptional factors that orchestrate expression of many genes involved in inflammation, oncogenesis, and apoptosis (2,3). NF-κB is constitutively activated in numerous hematologic malignancies and solid tumors (4–7), including pancreatic cancer (8), and its activation can suppress proapoptotic signaling pathways through the expression of several antiapoptotic genes (5). The exact function of AP-1 in cellular responses to genotoxic stress has not been thoroughly studied.

**Modulation of Pancreatic Cancer Chemoresistance by Inhibition of TAK1**

Davide Melisi, Qianghua Xia, Genni Paradiso, Jianhua Ling, Tania Moccia, Carmine Carbone, Alfredo Budillon, James L. Abbruzzese, Paul J. Chiao

Manuscript received September 28, 2010; revised May 24, 2011; accepted May 31, 2011.

**Correspondence to:** Davide Melisi, MD, PhD, Experimental Pharmacology Unit, Istituto Nazionale per lo studio e la cura dei Tumori—Fondazione “G. Pascale,” via M. Semmola, 80131 Naples, Italy (e-mail: d.melisi@istitutotumori.na.it; dave.melisi@gmail.com) or Paul J. Chiao, PhD, Department of Molecular and Cellular Oncology, Unit 107, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd, Houston, TX 77030 (e-mail: pjchiao@mdanderson.org).

**Background**

TGF-β-activated kinase-1 (TAK1), a mitogen-activated protein kinase kinase kinase, functions in the activation of nuclear factor κB (NF-κB) and activator protein-1, which can suppress proapoptotic signaling pathways and thus promote resistance to chemotherapeutic drugs. However, it is not known if inhibition of TAK1 is effective in reducing chemoresistance to therapeutic drugs against pancreatic cancer.

**Methods**

NF-κB activity was measured by luciferase reporter assay in human pancreatic cancer cell lines AsPc-1, PANC-1, and MDAPanc-28, in which TAK1 expression was silenced by small hairpin RNA. TAK1 kinase activity was targeted in AsPc-1, PANC-1, MDAPanc-28, and Colo357FG cells with exposure to increasing doses of a selective small-molecule inhibitor, LYTAK1, for 24 hours. To test the effect of LYTAK1 in combination with chemotherapeutic agents, AsPc-1, PANC-1, MDAPanc-28 cells, and control cells were treated with increasing doses of oxaliplatin, SN-38, or gemcitabine in combination with LYTAK1. In vivo activity of oral LYTAK1 was evaluated in an orthotopic nude mouse model (n = 40, 5 per group) with luciferase-expressing AsPc-1 pancreatic cancer cells. The results of in vitro proliferation were analyzed for statistical significance of differences by nonlinear regression analysis; differences in mouse survival were determined using a log-rank test. All statistical tests were two-sided.

**Results**

AsPc-1 and MDAPanc-28 TAK1 knockdown cells had a statistically significantly lower NF-κB activity than did their respective control cell lines (relative luciferase activity: AsPc-1, mean = 0.18, 95% confidence interval [CI] = 0.10 to 0.27; control, mean = 3.06, 95% CI = 2.31 to 3.80; MDAPanc-28, mean = 0.30, 95% CI = 0.13 to 0.46; control, mean = 4.53, 95% CI = 3.43 to 5.63; both P < .001). TAK1 inhibitor LYTAK1 had potent in vitro cytotoxic activity in AsPc-1, PANC-1, MDAPanc-28, and Colo357FG cells, with IC50 between 5 and 40 nM. LYTAK1 also potentiated the cytotoxicity of chemotherapeutic agents oxaliplatin, SN-38, and gemcitabine in AsPc-1, PANC-1, and MDAPanc-28 cells compared with control cells (P < .001). In nude mice, oral administration of LYTAK1 plus gemcitabine statistically significantly reduced tumor burden (gemcitabine vs gemcitabine plus LYTAK1, P = .03) and prolonged survival duration (median survival: gemcitabine, 82 days vs gemcitabine plus LYTAK1, 122 days; hazard ratio = 0.334, 95% CI = 0.027 to 0.826, P = .029).

**Conclusions**

The results of this study suggest that genetic silencing or inhibition of TAK1 kinase activity in vivo is a potential therapeutic approach to reversal of the intrinsic chemoresistance of pancreatic cancer.

J Natl Cancer Inst 2011;103:1190–1204
completely elucidated (9), but it could be associated with the concomitant activation of other pathways known to mediate survival, including NF-κB. In particular, Lamb et al. (10) demonstrated that the AP-1 transcription factor JunD cooperates with NF-κB to increase the expression of prosurvival genes that contain both NF-κB- and AP-1-binding sites in their promoters. Because much of the cytotoxicity of chemotherapeutic agents occurs through apoptosis, the coactivation of NF-κB and AP-1, which can synergistically and effectively suppress the apoptotic potential of chemotherapeutic agents, could be a crucial obstacle to effective treatment of cancer.

We recently demonstrated that an autocrine stimulation of interleukin 1 alpha (IL-1α), primarily mediated through induction of AP-1 activity, accounted for the constitutive activation of NF-κB (11) and thus for the metastatic behavior of pancreatic cancer (12). During immune and inflammatory responses, detailed investigation of IL-1-induced tumor necrosis factor (TNF) receptor associated factor (TRAF)-6 signaling demonstrated activation of NF-κB through two parallel signaling pathways (13,14), depending on differential activation of two mitogen-activated protein kinase kinase kinases (MAP3Ks), MEKK3 (MAP3K3), or the TGF-β-activated kinase-1 (TAK1; MAP3K7) (15).

TAK1 was originally identified as a MAP3K, which can be rapidly activated in response to TGF-β signal transduction (16). In vitro studies have demonstrated that overexpression of a dominant negative version of TAK1 inhibits both the activation of NF-κB and the mediator of AP-1 induction, c-Jun N-terminal kinase (JNK) (17), thus increasing the sensitivity of cells to apoptosis induced by TNF-α (18). Mice carrying an epidermal-specific deletion of the TAK1 gene developed severe skin inflammation caused by impaired activation of NF-κB and JNK in response to TNF, which resulted in a massive apoptosis of keratinocytes much greater than those observed in IκB kinase beta (IKKβ) and IKKγ deletion models (19). A mouse model with TAK1 conditionally deleted in T cells was used to demonstrate that TAK1 is essential for in vivo thymocyte development and activation. The loss of TAK1 in the thymocytes prevented the activation of IKK, NF-κB, and JNK and sensitized the mutant cells to activation-induced apoptosis (20). Using a B cell-conditional TAK1-deficient mouse model, Sato et al. (21) demonstrated that TAK1 is essential for toll-like receptor, IL-1 receptor, TNF receptor, and B cell receptor cellular responses and signaling pathways leading to the activation of JNK and/or NF-κB. Suppression of TAK1 signaling by dominant negative TAK1 reduced NF-κB activation in human head and neck squamous cell carcinoma (22) and breast cancer cell lines (23).

Cellular inhibitor of apoptosis 2 (cIAP-2) is a member of the inhibitor of apoptosis (IAP) family of proteins (24) that regulate programmed cell death by directly inhibiting caspases (25) and by targeting proapoptotic components of the TNF-α signaling pathways for ubiquitin degradation (26). The overexpression of cIAP-2 is a common and early event in the progression of pancreatic cancer. Although the expression of cIAP-1 is constantly high in both normal and neoplastic pancreatic tissues, cIAP-2 mRNA levels are statistically significantly higher in pancreatic cancer than in normal pancreatic tissue (27). A sequence analysis of the cIAP-2 promoter revealed two critical NF-κB-binding sites and two potential AP-1-binding sites (28).

**Material and Methods**

**Cell Lines and Reagents**

Human papillomavirus type 16 early gene 6- and 7-immortalized and nontumorigenic human pancreatic ductal epithelial (HPDE) cells, used as control cells in this study, have been described previously (29). Human pancreatic cancer cell lines AsPc-1, PANC-1, Hs766t, MiaPaCa-2, and CFPAC-1 were purchased from the American Type Culture Collection (Manassas, VA). MDA-Panc-28 and Panc-48 cell lines were obtained from the laboratory of Dr Marsha L. Fraizer and Dr Douglas B. Evans. The Colo357FG cell line was obtained from the laboratory of Dr Isaiah J. Fidler. All cell lines used in this study were authenticated using DNA
fingerprinting at the genomic core facility at Wayne State University (2009) and maintained as monolayer cultures in Dulbecco’s modified Eagle medium (Life Technologies, Gaithersburg, MD) that contained 4.5 g/L glucose, glutamine, and nonessential amino acids and was supplemented with 10% heat-inactivated fetal bovine serum and penicillin (100 IU/mL)–streptomycin (100 µg/mL) in an atmosphere of 5% carbon dioxide and 95% air at 37°C (30).

LYTAK1 (Cancer Cell Biology; Lilly Research Laboratories, Indianapolis, IN) is an orally active TAK1 kinase–selective inhibitor (TAK1 Kᵢ = 13 nM; p38 Kᵢ > 20 µM; IKKβ Kᵢ > 20 µM). For in vitro assays, LYTAK1 was dissolved in 100% dimethyl sulfoxide (DMSO) at a stock concentration of 1 mM. The concentration of DMSO did not exceed 0.1% in any assay. For in vivo studies, LYTAK1 was dissolved in the SX-1292 oral vehicle (1% sodium carboxymethyl cellulose, 0.5% sodium laurel sulfate, and 0.05% antifoam; Eli Lilly, Indianapolis, IN) and administered by mouth. Gemcitabine (Gemzar; Eli Lilly) and oxaliplatin (Eloxatin; Sanofi-Aventis, Paris, France) were used as lyophilized products, which were then dissolved in sterile saline or 5% dextrose solution, respectively.

**Nude Mouse Orthotopic Xenograft Model**

Female athymic nude mice (NCI-nu), which were 6- to 8-weeks old, were purchased from the Animal Production Area of the National Cancer Institute—Frederick Cancer Research Facility (Frederick, MD). All mice were housed and treated in accordance with the guidelines of The University of Texas M. D. Anderson Cancer Center’s Animal Care and Use Committee, and the mice were maintained in specific pathogen-free conditions. The facilities were approved by the Association for Assessment and Accreditation of Laboratory Animal Care and met all current regulations and standards of the US Departments of Agriculture and Health and Human Services and the National Institutes of Health.

To produce pancreatic tumors, pancreatic cancer cells were harvested from subconfluent cultures by brief exposure to 0.05% trypsin and 0.02% EDTA. Trypsin activity was stopped with medium containing 10% fetal bovine serum, and the cells were washed once in serum-free medium and resuspended in serum-free Hanks balanced salt solution. Only suspensions consisting of single cells with more than 90% viability were used for the injections.

The orthotopic injection of pancreatic cancer cells was performed as described previously (30). Briefly, the mice were anesthetized with a 1.5% isoflurane–air mixture. A small incision in the left abdominal flank was made, and the spleen was exteriorized. Tumor cells (1.0 × 10⁶ cells in 50 µL of Hanks balanced salt solution) were injected subcapsularly in a region of the pancreas just beneath the spleen. A 30-gauge needle, 1 mL disposable syringe, and calibrated push button–controlled dispensing device (Hamilton Company; Reno, NV) were used to inject the tumor cell suspension. A successful subcapsular intrapancreatic injection of tumor cells was identified by the appearance of a fluid bleb without intraperitoneal leakage. To prevent such leakage, a cotton swab was held over the injection site for 1 minute. One layer of the abdominal wound was closed with wound clips (Auto-clip; Clay Adams, Parsippany, NJ). The mice tolerated the surgical procedure well, and no anesthesia-related deaths occurred.

**Generation of TAK1 Knockdown Cell Lines**

To silence the expression of TAK1, two human TAK1 small hairpin RNA (shRNA) target sequences were chosen: 5′GAGGAAAGCCTTTATTTGTATT3′ and 5′CCCAATGGCTTATCCTACATT3′. These sequences were cloned into an FG12 lentivirus vector (gift from Dr Qin Xiaofeng, The University of Texas M. D. Anderson Cancer Center, Houston, TX) with Bam HI and Hind III. The shRNA vector and packaging vectors were cotransfected into 293T cells. The virus-containing supernatants were collected after 72 hours of transfection and filtered through a 0.45 µm filter (Corning, Inc., Germany). The AsPc-1, PANc-1, and MDAPanc-28 cells were transduced by the lentivirus in the presence of the polycation Polybrene, and the stably transduced cells were then sorted by green fluorescent protein activity.

TAK1 shRNA rescue was performed by stably coexpressing wild-type murine TAK1 and human TAK1 shRNA by lentiviral infection in AsPc-1 and MDAPanc-28 cells. For the NF-κB reporter gene assay, 1 µg each of the wild-type κB, reporter plasmids containing the firefly luciferase reporter gene, and the pRL-TK plasmid containing the Renilla luciferase gene under the control of the herpes simplex virus thymidine kinase promoter as an internal control were cotransfected into cells in triplicate by the lipotransfection method (FuGENE 6; Roche, Indianapolis, IN), according to the manufacturer’s recommendations. The activities of both firefly and Renilla luciferases were determined 48 hours after transfection with the dual luciferase reporter assay system (Promega, Madison, WI). The luciferase activities were normalized to the Renilla luciferase activity of the internal control.

**Assessment of TAK1 Expression and Activation**

HPDE and human pancreatic cancer cell lines were washed twice with cold phosphate-buffered saline and lysed at 4°C into radioimmunoprecipitation assay buffer (50 mM Tris–HCl [pH 8], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate). Each lysate (20 µg of protein) was separated by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and probed (1 : 1000) with a rabbit polyclonal antibody against TAK1 and a monoclonal mouse antibody against caspase-3, TCDD-inducible poly(ADP-ribose) polymerase (TIPARP/PARP-1), phospho-p38, p38, and β-actin (Santa Cruz Biotechnology, Inc, Santa Cruz, CA), or cIAP-2 (BD Biosciences, San Jose, CA). For immunoprecipitation experiments, whole-cell lysates were prepared with lysis buffer (50 mM Tris–HCl [pH 7.9], 0.5 mM EDTA, 150 mM NaCl, 5% glycerol, 0.5% NP-40, 1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride, 1 mmol/L phenylmethylsulfonyl fluoride, 1 µmol/L DTT, 10 µg/mL aprotinin, and 10 µg/mL leupeptin). Cell lysates (2 mg) were immunoprecipitated with 10 µL rabbit anti-TAK1 antibody (A301-915A, Bethyl Laboratories, Montgomery, TX, US) at 4°C under rotary agitation for 4 hours, and then the immunocomplexes were captured by 40 µL protein A/G agarose/sepharose bead slurry (Santa Cruz Biotechnology, Inc) at 4°C for 2 hours. The agarose/sepharose beads were collected by centrifugation and washed three times with cold lysis buffer. The agarose/sepharose beads were boiled in 3X sample buffer for 5 minutes. Each lysate (20 µL of protein) was
separated by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and probed (1:1000) with a rabbit polyclonal antibody against phospho-TAK1 (No. 4531, Cell Signaling Technology, Inc, Boston, MA). Immunoreactive proteins were visualized with Lumi-Light western blotting substrate (Roche), according to the manufacturer’s instructions.

**Electrophoretic Mobility Shift Assay**

Nuclear extracts of AsPc-1, Panc-1, and MDAPanc-28 pancreatic cancer cells were prepared according to the method of Andrews and Faller (31). DNA-binding assays for NF-kB and AP-1 proteins were performed with 10 μg of nuclear extracts as described by Chiao et al. (32). The wild-type double-stranded oligonucleotides containing the NF-κB and AP-1 site were obtained from Santa Cruz Biotechnology, Inc, and labeled with 32P to be used as probes. The reactions were analyzed on 4% polyacrylamide gels containing 0.25 x TBE (Tris/Borate/EDTA) buffer.

**Induction of Pancreatic Tumors in Mice to Assess Effect of TAK1 Silencing**

Fifteen athymic mice bearing orthotopic AsPc-1, AsPc-1shTAK1, or AsPc-1TAK1shRNA pancreatic tumors (n = 5 per group) were euthanized by carbon dioxide inhalation at the median survival duration of the control group (63 days). To assess tumor growth, a digital image of resected tumors was acquired with a Leica MZ16 stereoscopic dissecting microscope (Leica Microsystems Inc., Wetzlar, Germany) equipped with a Hamamatsu Orca ER cooled CCD digital camera (Hamamatsu Photonics K.K., Japan) coupled to a data acquisition computer running the image acquisition software Image-Pro version 6.0 (Media Cybernetics, Inc., Bethesda, MD, USA). Tumor volume was measured using the formula π/6 × larger diameter × (smaller diameter)², as reported previously (33).

**Treatment of AsPc-1, Panc-1, and MDAPanc-28 TAK1 Knockdown Cells to Determine Sensitivity to Gemcitabine, SN-38, and Oxaliplatin**

On day 0, AsPc-1, Panc-1, and MDAPanc-28 pancreatic cancer cells transduced with lentiviruses expressing TAK1-specific shRNA or a scramble sequence as control were seeded at a density of 1.0 × 10⁶ cells/well. On the following day, the cells were treated with increasing doses of gemcitabine, SN-38, or oxaliplatin. On day 2, the medium containing drugs was removed, the cells were washed twice with phosphate-buffered saline, and fresh medium was added. After 5 days of incubation, the MTT (Sigma-Aldrich) assay was used to obtain relative estimates of viable cell number. Three independent experiments with four replicates each were performed.

**Determination of Apoptosis In Vitro**

The extent of apoptosis was determined by fragmented DNA detection as previously described by Matassov et al. (34). Briefly, low–molecular weight DNA was extracted from lysed pancreatic cancer cells by adding an equal volume of phenol : chloroform : isomyl alcohol (25 : 24 : 1) to the samples. DNA was precipitated by adding 5 M NaCl to a final concentration of 300 mM and 2.5 volumes of 100% ethanol. Dried DNA samples were resuspended in TE buffer and separated by agarose gel electrophoresis. Photographs were taken on an UV transilluminator.

**Exposure of Human Pancreatic Cell Lines to TAK1–Selective Inhibitor LYTAK1**

To measure the activity of the TAK1–kinase selective inhibitor LYTAK1 in vitro, pancreatic cancer cell lines AsPc-1, Panc-1, MDAPanc-28, and Colo357FG were exposed for 24 hours to increasing doses of LYTAK1. On day 0, AsPc-1, Panc-1, MDAPanc-28, and COLO357FG pancreatic cancer cells were seeded at a density of 1.0 × 10⁶ cells per well. After 4 days of incubation, the cells were treated with increasing doses of LYTAK1 or DMSO as control. On day 2, the medium containing drugs was removed, the cells were washed twice with phosphate-buffered saline, and fresh medium was added. After 5 days of incubation, the MTT (Sigma-Aldrich) assay was used to obtain relative estimates of viable cell number. Three independent experiments with four replicates each were performed.

**Treatment of Pancreatic Cancer Cell Lines with TAK1–Selective Inhibitor LYTAK1 and Gemcitabine, SN-38, and Oxaliplatin to Test Effect of TAK1 Inhibition in Combination With Chemotherapeutic Agents**

On day 0, AsPc-1, Panc-1, and MDAPanc-28 pancreatic cancer, and human papillomavirus type 16 early gene 6- and 7-immortalized/ nonimmortalized HPDE control cells were seeded at a density of 1.0 × 10⁶ cells per well. On the following day, the cells were treated with increasing doses of gemcitabine, SN-38, or oxaliplatin alone or in combination with LYTAK1 at doses corresponding with the IC₅₀ measured for each cell lines or DMSO as control. On day 2, the medium containing drugs was removed, the cells were washed twice with phosphate-buffered saline, and fresh medium was added. After 5 days of incubation, the MTT (Sigma-Aldrich) assay was used to obtain relative estimates of viable cell number. Three independent experiments with four replicates were performed.

**Pharmacokinetic and Pharmacodynamic Studies**

Activation of p38 kinase in peripheral blood mononuclear cells (PBMC) was determined as previously described by Zhao et al. (35). Briefly, female BALB/c mice were randomly assigned (n = 3 per group) to receive escalating doses of LYTAK1 (0, 1, 3, 10, 30, or 100 mg/kg) for 2 hours, or 6.9 mg/kg of LYTAK1 at different time points (2, 4, 6, 8, 12, 16, 20, 24 hours). Whole blood from anesthetized mice was collected via cardiac puncture in heparin tubes at room temperature. Additional whole blood was collected to generate plasma with EDTA for compound exposure analysis. The whole blood was stimulated ex vivo with recombinant murine TNF-α (10 ng/ml; Roche) for 5 minutes at 37°C. PBMC surface staining was conducted simultaneously with the incubation of fluorescein isothiocyanate (FITC)–conjugated rat anti-mouse Ly-6G antibody (1:250) (BD Biosciences) and allophycocyanin (APC)-conjugated rat anti-mouse Ly-6G antibody (1:250) (BD Biosciences). To lyse red blood cells and fix white blood cells, Lyse/Fix buffer (BD Biosciences) was then added to cells and vortexed. For intracellular staining, stimulated cells were washed and resuspended in permeabilization medium B with phycoerythrin-conjugated rat anti-phospho-p38 (1:250) (pT180/pY182; BD Biosciences) containing 1% mouse BD Fc Block
Figure 1. Determination of in vitro and in vivo proapoptotic phenotype by silencing TGF-β-activated kinase-1 (TAK1) expression. A) Western blot analysis for the expression of TAK1 in an immortalized and non-tumorigenic human pancreatic ductal epithelial (HPDE) and eight different pancreatic cancer cell lines as indicated. B) Western blot analysis for the expression of TAK1 in AsPc-1, PANC-1, and MDAPanc-28 pancreatic cancer cells transduced with lentiviruses expressing TAK1-specific small hairpin RNA (shRNA) or a scramble sequence as control. C) TAK1 shRNA rescue experiment. Nuclear factor κB (NF-κB) reporter gene assay in AsPc-1 and MDAPanc-28 cells transduced with lentiviruses expressing TAK1-specific small hairpin RNA (shRNA) or a scramble sequence as control. The luciferase activities were normalized to the Renilla luciferase activity of the internal control. Error bars indicate 95% confidence intervals of two independent experiments performed in triplicate. Asterisks indicate statistical significance (P<.001) compared with scramble, as determined by two-sided one-way analysis of variance and Dunnett test. D) Western blot analysis for the autophosphorylation of TAK1 and E) the phosphorylation of p38 in AsPc-1, PANC-1, and MDAPanc-28 pancreatic cancer cells transduced with lentiviruses expressing TAK1-specific shRNA or a scramble sequence as control. F) Light-microscopic phenotype in AsPc-1, PANC-1, and MDAPanc-28 pancreatic cancer cells transduced with lentiviruses expressing TAK1-specific shRNA or a scramble sequence as control. Scale bars = 100 μm. G) Electrophoretic Mobility Shift Assay analysis for the NF-κB p65/p50 and p50/p50 complexes and H) activator protein-1 (AP-1) (continued)
Antitumor Activity of Oral LYTK1 Plus Chemotherapeutic Agents In Vivo in AsPc-1 Pancreatic Tumor Orthotopic Xenografts

AsPC-1 orthotopic tumor-bearing mice were randomly assigned (n = 5 per group) to receive one of the following on a weekly schedule: 1) 6.9 mg/kg of oral LYTK1 (days 1–5), 2) 25 mg/kg of intraperitoneal gemcitabine (day 3), 3) 10 mg/kg of intraperitoneal oxaliplatin (day 3), 4) 25 mg/kg of intraperitoneal irinotecan (day 3); and their combinations: 5) gemcitabine (25 mg/kg, intraperitoneal, day 3) plus LYTK1 (6.9 mg/kg, orally, days 1–5), 6) oxaliplatin (10 mg/kg, intraperitoneal, day 3) plus LYTK1 (6.9 mg/kg, orally, days 1–5), 7) irinotecan (25 mg/kg, intraperitoneal, day 3) plus LYTK1 (6.9 mg/kg, orally, days 1–5), or 8) vehicles only (50 µL of oral vehicle, days 1–5 plus 50 µL sterile saline, intraperitoneal, day 3) as control. Treatments were continued for 4 weeks.

All mice were weighed weekly and observed for tumor growth. Bulky disease was considered present when the tumor burden was prominent in the mouse abdomen (tumor volume ≥ 2000 mm³). When at least three of the five mice in a treatment group presented with bulky disease, the median survival duration for that group was considered to have been reached. At the median survival duration of the control group, the tumor growth in mice in all groups was evaluated using the bioluminescence emitted by the tumor cells. Bioluminescence imaging was conducted using a cryogenically cooled IVIS 100 imaging system coupled to a data acquisition computer running Living Image software (Xenogen, Hopkinton, MA). The mice were euthanized by carbon dioxide inhalation when evidence of advanced bulky disease was present, which was considered the day of death for survival evaluation.

Immunohistochemistry to Determine Expression of NF-κB- and AP-1-Induced Protein cIAP-2 in Tumors From Mice Treated With LYTK1, Gemcitabine Singly and in Combination

To determine the expression of the NF-κB- and AP-1-induced protein cIAP-2, the p65 subunit of NF-κB, and the NF-κB-induced protein IL-6 in treated mice, another group of 24 mice bearing orthotopic AsPC-1 pancreatic tumors was randomly allocated (n = 6 per group) to receive the following on a weekly schedule: 1) 6.9 mg/kg of oral LYTK1 (days 1–5), 2) 25 mg/kg of intraperitoneal gemcitabine (day 3), 3) gemcitabine (25 mg/kg, intraperitoneal, day 3) plus LYTK1 (6.9 mg/kg, orally, days 1–5), or 4) vehicles only (50 µL of oral vehicle, days 1–5 plus 50 µL sterile saline, intraperitoneal, day 3) as control. Treatments were continued for 4 weeks. Tumors were excised 1 day after the end of treatments. Formalin-fixed paraffin-embedded tissue sections were subjected to immunostaining using the streptavidin–peroxidase technique, with diaminobenzidine as a chromogen. For antigen retrieval, the sections were subjected to microwave heating in 0.01 mol/L citrate buffer (pH 6.0). Sections were incubated at 4°C overnight with primary rabbit anti-human monoclonal antibodies anti-NF-κB p65 (Cell Signaling Technology) (1 : 20 dilution), anti-IL-6 (Santa Cruz Biotechnology, Inc) (1 : 100 dilution) or mouse anti-human monoclonal antibody anti-cIAP-2 (BD Biosciences) (1 : 25 dilution). Slides were washed in Tris-buffered saline buffer and then incubated for 30 minutes with the appropriate horseradish peroxidase–conjugated secondary antibody. The slides were counterstained with Meyer’s hematoxylin (Peroxidase Detection System; Leica Microsystems Inc., Wetzlar, Germany). To ensure antibody specificity, consecutive sections were incubated with isotype-matched control immunoglobulins and in the absence of the primary antibody. In these cases, no specific immunostaining was detected. The expression of cIAP-2, p65, and IL-6 was detected as nuclear and cytoplasmic brown staining of varying intensity in neoplastic cells. The slides were evaluated independently using light microscopy by two pathologists who were blinded to the treatments.

Statistical Analysis

The statistical significance of differences in NF-κB activity was determined by one-way analysis of variance and Dunnett test. The results of in vitro proliferation were analyzed for statistical significance of differences by nonlinear regression analysis and are expressed as means and 95% confidence intervals [CIs] for at least three independent experiments performed in quadruplicate. The statistical significance of differences in p38 phosphorylation in mouse PBMC was determined by one-way analysis of variance and Dunnett test. The statistical significance of differences in tumor growth was determined by the Mann–Whitney test; differences in survival duration were determined using a log-rank test. All statistical tests were two-sided, and a P value less than .05 indicated statistical significance. All statistical analyses were performed using GraphPad Prism software version 4.0c for Macintosh (GraphPad Software, San Diego, CA).

Results

Effects of TAK1 Silencing on NF-κB and AP-1 DNA-Binding Activities and Apoptotic Cascade

To study the role of TAK1 in pancreatic cancer chemoresistance, we initially evaluated the expression of TAK1 protein in eight pancreatic cancer cell lines and in the immortalized and nontumorigenic athymic mice bearing orthotopic AsPC-1, AsPC-1 scramble, or AsPC-1 TAK1shRNA pancreatic tumors (n = 5 per group) were euthanized by carbon dioxide inhalation at the median survival duration of the control group (63 days). A digital image of resected tumors was acquired to assess tumor growth.

Figure 1 (continued).

DNA-binding activity in AsPC-1, PANC-1, and MDAPanc-28 pancreatic cancer cells transduced with lentiviruses expressing TAK1-specific shRNA or a scramble sequence as control. I) Western blot analysis for the expression of cellular inhibitor of apoptosis 2 (cIAP-2), caspase-3, PARP-1, TAK1, and β-actin in the same cells as indicated. J) Fifteen
HPDE cell line, demonstrating the overexpression of TAK1 in all of the malignant cell lines compared with the non-tumorigenic control (Figure 1, A).

To test our hypothesis that TAK1 might be responsible for the resistance of pancreatic cancer to the proapoptotic effect of chemotherapeutic agents by increasing the NF-κB and AP-1-mediated transcription of cIAP-2, we used two pooled shRNA sequences to knock down the expression of TAK1 in three different pancreatic cancer cell lines, AsPc-1, PANC-1, and MDAPanc-28. These cell lines were transduced with lentiviruses expressing TAK1-specific shRNAs or a scramble sequence. With this approach, we were able to markedly reduce the expression of TAK1 in all three cell lines (Figure 1, B). TAK1 shRNA rescue experiments with wild-type murine TAK1 were performed as controls. Whereas AsPc-1 and MDAPanc-28 TAK1 knockdown cells had a statistically significant lower NF-κB activity than did their respective control cell lines (relative luciferase activity: AsPc-1 scramble control cells, mean = 3.06, 95% CI = 2.31 to 3.80 vs AsPc-1 TAK1 shRNA cells, mean = 0.18, 95% CI = 0.10 to 0.27; P < .001; MDAPanc-28 scramble control cells, mean = 4.53, 95% CI = 3.43 to 5.63 vs MDAPanc-28 TAK1 shRNA cells, mean = 0.30, 95% CI = 0.13 to 0.46; P < .001), the same cells stably expressing wild-type murine TAK1 demonstrated an NF-κB activity similar to the control cell lines (relative luciferase activity: AsPc-1 TAK1 shRNA plus murine TAK1, mean = 2.40, 95% CI = 1.86 to 2.94; MDAPanc-28 TAK1 shRNA plus murine TAK1, mean = 4.47, 95% CI = 2.80 to 6.14) (Figure 1, C). Knocking down the expression of TAK1 suppressed polyUb-induced TAK1 activity or autophosphorylation because phosphorylated TAK1 proteins were undetectable (Figure 1, D) and reduced the phosphorylation of p38, a downstream target of the TAK1-MKK6 intracellular pathway (36) (Figure 1, E).

TAK1 knockdown cells had a markedly different light-microscopic appearance compared with the control cells, including a more rounded cell morphology and a dramatic increase in intercellular adhesion that induced these cells to grow more as conglomerates or clusters when compared with the monolayer growth pattern observed in the control cells (Figure 1, F).

To determine the effect of silencing TAK1 on the constitutive activation of NF-κB and AP-1 in pancreatic cancer, we evaluated the DNA-binding activity of these two transcriptional factors in knockdown and control cell lines. We demonstrated that knocking down the expression of TAK1 dramatically suppresses the DNA-binding activity of both NF-κB (Figure 1, G) and AP-1 (Figure 1, H) in the pancreatic cancer cell lines compared with the respective controls.

Inhibition of the transcriptional activities of NF-κB and AP-1 completely suppressed the expression of the antiapoptotic protein cIAP-2 (Figure 1, I). Suppression of cIAP-2 released a higher basal level of activation of the apoptotic cascade as demonstrated by an increased level of caspase-3 and PARP-1 cleavage products (Figure 1, I) and by the fragmentation of genomic DNA as shown below.

In vivo, these effects translated into a slower orthotopic growth of AsPC-1 TAK1 knockdown pancreatic tumors compared with the AsPC-1 scramble or AsPC-1 parental tumors as controls (tumor volume, mm³: AsPC-1 tumors, mean = 1129, 95% CI = 452.5 to 1805; AsPC-1 scramble control tumors, mean = 1177, 95% CI = 731 to 1623; AsPC-1 TAK1 shRNA tumors, mean = 124.4, 95% CI = 60.65 to 188.2, P = .0012) (Figure 1, J).

Taken together, these data demonstrate that TAK1 is an essential mediator of the constitutive activation of NF-κB and AP-1 in pancreatic cancer cells.

**Effects of TAK1 Silencing on Chemoresistance of Pancreatic Cancer Cells**

To test our hypothesis that TAK1 would be responsible for the chemoresistance of pancreatic cancer, we determined the sensitivity of the TAK1 knockdown and control cell lines to three chemotherapeutic agents with different mechanisms of action that are either currently used or have been recently studied in combination with gemcitabine for pancreatic cancer treatment: The DNA-intercalating agent oxaliplatin, the topoisomerase I inhibitor SN-38, and the nucleoside analog gemcitabine. AsPc-1, PANC-1, and MDAPanc-28 TAK1 knockdown cells demonstrated a statistically significantly higher sensitivity to these chemotherapeutic agents than did their respective control cell lines (Figure 2 and Table 1). With the exception of the already gemcitabine-sensitive PANC-1 cells, after knocking down TAK1, we observed a statistically significant increase in the sensitivity to gemcitabine for the moderately resistant AsPc-1 and the extremely gemcitabine-resistant MDAPanc-28 cells (Figure 2 and Table 1). All three knockdown cell lines were statistically significantly more sensitive than control cells to the same extent to the irinotecan active metabolite SN-38 (Figure 2 and Table 1). A dramatic chemosensitization to oxaliplatin was observed in all three knock down cell lines (AsPc-1 TAK1 knockdown cells, IC₅₀ = 3.48E-007 M vs control cells, IC₅₀ = 7.33E-006 M; PANC-1 TAK1 knockdown cells, IC₅₀ = 1.86E-007 M vs control cells, IC₅₀ = 5.38E-006 M; MDAPanc-28 TAK1 knockdown cells, IC₅₀ = 2.48E-007 M vs control cells, IC₅₀ = 4.52E-006 M; all P < .001, Table 1). In particular, whereas the IC₅₀ values for oxaliplatin in control pancreatic cancer cells were higher than those achievable in patients (37), we found that silencing TAK1 decreased the IC₅₀ for oxaliplatin into the range of plasma concentrations achievable in patients.

Because several studies have shown that the proapoptotic stimuli elicited by the cytotoxic agents activate the antiapoptotic function of NF-κB (2), we determined the activation of NF-κB after treatment with IC₅₀ doses of the cytotoxic agents. Whereas the control cells already demonstrated a substantial NF-κB DNA-binding activity that could not be further augmented by exposure to the cytotoxic agents, the TAK1 knockdown cells were unable to increase their low DNA-binding activity, even if treated with IC₅₀ doses of the cytotoxic agents (Figure 3, A). In this regard, whereas the control pancreatic cancer cells were protected from apoptosis, the TAK1 knockdown cells demonstrated a high induction of apoptosis as demonstrated by PARP-1 cleavage and DNA fragmentation (Figure 3, B). These data suggest that TAK1 is an important mediator of chemoresistance in pancreatic cancer.

**Inhibition of TAK1 Kinase Activity and Chemosensitization of Pancreatic Cancer Cells In Vitro**

To establish TAK1 as a pharmacologically relevant target for the chemosensitization of pancreatic cancer, we first tested the activity of the TAK1–kinase selective inhibitor LY-TAK1 in vitro. Four
pancreatic cancer cell lines, AsPc-1, PANC-1, MDAPanc-28, and Colo357FG, were exposed for 24 hours to increasing doses of LYTA1. LYTA1 had potent in vitro cytotoxic activity, demonstrating an IC{50} between 5 and 40 nM (AsPc-1: IC{50} = 5.1E-009 M, 95% CI = 3.9E-009 to 6.6E-009 M; Panc-1: IC{50} = 1.4E-008 M, 95% CI = 1.18E-008 to 1.67E-008 M; MDAPanc-28: IC{50} = 3.93E-008 M, 95% CI = 2.0E-008 to 7.72E-008 M; Colo357FG: IC{50} = 1.2E-008 M, 95% CI = 1.11E-008 to 1.3E-008 M) (Figure 4, A). At this dose range, the kinase activity of TAK1 was markedly inhibited as indicated by the suppression of the autophosphorylation of TAK1 and the phosphorylation of p38 (Figure 4, B).

To demonstrate that the inhibition of the kinase activity of TAK1 would suppress the activation of NF-{kappa}B, we studied the effect of LYTA1 on the DNA-binding activity of this

Table 1. Differences in IC{50} for gemcitabine, SN-38, or oxaliplatin in pancreatic cancer cells transduced with lentiviruses expressing TAK1-specific shRNA, or a scramble sequence as control

<table>
<thead>
<tr>
<th>Chemotherapy agent</th>
<th>AsPC-1</th>
<th>PANC-1</th>
<th>MDAPanc-28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gemcitabine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scramble</td>
<td>1.27E-07</td>
<td>2.97E-08</td>
<td>1.25E-08</td>
</tr>
<tr>
<td>shTAK1</td>
<td>3.25E-08</td>
<td>2.38E-08</td>
<td>3.25E-08</td>
</tr>
<tr>
<td>Fold change</td>
<td>4.3</td>
<td>1.4</td>
<td>52.8</td>
</tr>
<tr>
<td>P</td>
<td>&lt;.001</td>
<td>0.087</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>IC{50}, M (95% CI)</td>
<td>9.02E-008 to 2.3E-008</td>
<td>3.6E-008 to 1.91E-008</td>
<td>6.1E-006 to 3.75E-006</td>
</tr>
<tr>
<td>SN-38 IC{50}</td>
<td>3.18E-08</td>
<td>4.66E-09</td>
<td>9.95E-09</td>
</tr>
<tr>
<td>M (95% CI)</td>
<td>1.94E-008 to 3.71E-009</td>
<td>7.6E-009 to 1.77E-009</td>
<td>1.3E-009 to 2.8E-009</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>7.33E-006</td>
<td>3.48E-007</td>
<td>5.38E-006</td>
</tr>
<tr>
<td>IC{50}, M (95% CI)</td>
<td>5.98E-006 to 2.34E-007</td>
<td>4.5E-006 to 1.05E-007</td>
<td>6.4E-006 to 3.31E-007</td>
</tr>
</tbody>
</table>

* Human pancreatic cell lines: AsPC-1, PANC-1, and MDAPanc-28; P values determined by nonlinear regression analysis; All statistical tests were two-sided. CI = confidence interval; IC{50} = half-maximal inhibitory concentration; TAK1 = TGF-{beta}-activated kinase-1; shTAK1 = TAK1 gene silenced with short hairpin RNA.
transcriptional factor. We observed that in the concentration range indicated, LYTAK1 was able to completely suppress the constitutive activation of NF-κB in the pancreatic cancer cell lines (Figure 4, C).

To test our hypothesis that the pharmacological inhibition of TAK1 would modulate the chemoresistance of pancreatic cancer, we determined whether the addition of LYTAK1 resulted in increased in vitro antitumor activity when combined with classic chemotherapeutic agents. AsPc-1, PANC-1, and MDAPanc-28 pancreatic cancer cells, and HPDE cells as control were treated for 24 hours with doses of gemcitabine, SN-38, or oxaliplatin corresponding with the IC_{50} for the control cells or saline as control. A) Electrophoretic Mobility Shift Assay analysis was performed to determine the DNA-binding activity of NF-κB p65/p50 and p50/p50 complexes. B) Apoptosis was measured by western blot analysis for PARP-1 cleavage and DNA fragmentation detected by agarose gel electrophoresis. Similar results were obtained with three independent experiments.

![Figure 3](image-url)
LYTAK1 Pharmacokinetic and Pharmacodynamic Studies in Mice

To confirm the effects of LYTAK1 on in vivo TAK1 inhibition and to assess the potential therapeutic dose of the compound in mice, we next investigated whether oral treatment with LYTAK1 affected the p38 phosphorylation level in the mouse PBMCs. Consistent with the cell-based study (Figure 4, B), 2 hours of in vivo treatment with LYTAK1 statistically significantly suppressed p38 phosphorylation in mouse PBMC ($P < .001$), with a total effective dose (TED)$_{50}$ of 1.8 mg/kg and TED$_{90}$ of 6.9 mg/kg (Figure 6, A). When mice were treated with a single dose of 6.9 mg/kg of LYTAK1, p38 phosphorylation in PBMC was statistically significantly suppressed compared with LYTAK1-untreated control, and the effect lasted at least 8 hours (mean fluorescence intensities, 2 hours: 97% of control; mean fluorescence intensities, 8 hours: 75% of control, $P < .001$, Figure 6, B). LYTAK1 activity was positively associated with drug concentration in plasma.

Effects of LYTAK1 in AsPc-1 Mouse Orthotopic Xenograft Tumors

To demonstrate that TAK1 kinase is a viable target for the chemosensitization of pancreatic cancer in vivo, we determined whether LYTAK1 could cooperate with chemotherapeutic agents at clinically relevant doses to inhibit growth of human pancreatic cancer cells in an orthotopic xenograft nude mouse model.

Forty mice were orthotopically injected with AsPc-1 human pancreatic cancer cells and randomly assigned to eight groups (n = 5 per group) to be treated as indicated. At the median survival duration of mice in the control group (day 66), LYTAK1 and
gemcitabine as single agents were completely inactive. However, the mice treated with the combination of both LYTAK1 and gemcitabine experienced a statistically significant reduction in tumor burden (photons per second: gemcitabine, mean = 1.86E+007, 95% CI = 2.5.7E+006 to 4.3E+007, gemcitabine plus LYTAK1, mean = 2.2E+006, 95% CI = -2.3E+006 to 6.7E+006, P = .032) (Figure 7, A and B). Accordingly, only the mice treated with the LYTAK1 plus gemcitabine combination demonstrated a statistically significantly prolonged median survival duration (control vs gemcitabine, median survival = 66 vs 82 days, P = .066; control vs gemcitabine, SN-38, and oxaliplatin alone or in combination with LYTAK1 at doses corresponding with the IC50 measured for each cell line or dimethyl sulfoxide (DMSO) as control. DMSO-treated cells were assigned a value of 100% and designated as control. Means and 95% confidence intervals of three independent experiments performed in quadruplicate are shown. Curves were fitted by nonlinear regression analysis.

Figure 5. Inhibition of TGF-β-activated kinase-1 kinase activity and chemosensitization of human pancreatic cancer cells in vitro. Percent survival of AsPC-1, PANC-1, and MDAPanc-28 pancreatic cancer cell lines, and human papillomavirus type 16 early gene 6- and 7-immortalized/nontumorigenic human pancreatic ductal epithelial (HPDE) control cells after treatment with increasing molar concentrations (log scale) of gemcitabine, SN-38, and oxaliplatin alone or in combination with LYTAK1.

Figure 6. LYTAK1 pharmacokinetic and pharmacodynamic models. BALB/c mice were treated (n = 3 per group) either with A) increasing doses of LYTAK1 for 2 hours or with or B) 6.9 mg/kg of LYTAK1 for different time points as indicated. Mouse blood was stimulated with tumor necrosis factor α (TNF-α), and TAK1 activity in peripheral blood mononuclear cells (Ly-6G-/CD11b+) was determined by flow cytometry with intracellular staining of p38 phosphorylation. Blue bars indicate mean fluorescence intensities (MFI) for phosphorylated p38 (p-p38). Error bars are SEs. Asterisks indicate P < .001 significance level compared with TNF-α-stimulated LYTAK1-untreated control, as determined by two-sided one-way analysis of variance and Dunnett test. Percentages indicate p-p38 MFI reduction compared with TNF-α-stimulated LYTAK1-untreated control. Red curves indicated drug exposure concentrations. Error bars are SEs.
LYTAK1, median survival = 66 vs 87 days, \( P = .066 \); control vs gemcitabine plus LYTAK1, median survival = 66 vs 122 days, hazard ratio = 0.2959, 95% CI = 0.01869 to 0.6421, \( P = .014 \); gemcitabine vs gemcitabine plus LYTAK1, median survival = 82 vs 122 days, hazard ratio = 0.334, 95% CI = 0.027 to 0.826, \( P = .029 \) (Figure 7, C). Similar but not statistically significant trends were observed in mice treated with the combination of LYTAK1 plus oxaliplatin or irinotecan (Supplementary Figure 1, A and B, available online). With the exception of two mice that died of complications following injections in groups 4 and 7, all regimens were well tolerated. No weight loss or other signs of acute or delayed toxicity were observed.

Immunohistochemical analyses were carried out to determine the expression of the NF-\( \kappa \)B- and AP-1-induced protein cIAP-2, the p65 subunit of NF-\( \kappa \)B, and the NF-\( \kappa \)B-induced protein IL-6. Vehicle-treated AsPC-1 tumors showed a constitutive activation of NF-\( \kappa \)B and AP-1 as demonstrated by a strong expression of c-IAP-2, IL-6, and p65 (Figure 7, D). The expression of these proteins was induced in tumors treated with gemcitabine. Tumors from mice treated with LYTAK1 as single agent demonstrated no expression of cIAP-2 and only a very weak cytoplasmic expression of p65 and IL-6. Tumors from mice treated with the gemcitabine plus LYTAK1 combination demonstrated no expression of the NF-\( \kappa \)B- and AP-1-induced protein cIAP-2 and only a weak cytoplasmic expression of p65 and, in turn, of IL-6 when compared with controls (Figure 7, D).

**Discussion**

Pancreatic cancer is one of the most lethal human cancers and will continue to be a major unsolved health problem in the 21st century. The major challenge is to develop effective therapeutic strategies that target the unique molecular alterations of pancreatic cancer and to integrate these targeted agents into established combination chemotherapy regimens to improve patient survival. To our knowledge, this study is the first to demonstrate that the genetic silencing or the inhibition of the kinase activity of TAK1 is a valid approach to revert the intrinsic chemoresistance of pancreatic cancer.

![Figure 7](image-url)

**Figure 7.** Antitumor activity of oral LYTAK1 plus chemotherapeutic agents in vivo in AsPc-1 pancreatic tumor orthotopic xenografts (\( n = 40 \), 5 mice per group). A) Tumor volume was quantified as the sum of all detected photons within the region of the tumor per second. **Error bars** are 95% CI. *\( P = .0317 \), gemcitabine vs LYTAK1 plus gemcitabine by two-sided Mann–Whitney test. B) A digital grayscale image of each mouse was acquired, which was followed by the acquisition and overlay of a pseudocolor image representing the spatial distribution of detected photons emerging from active luciferase within the mouse. C) Mice were killed by carbon dioxide inhalation when evidence of advanced bulky disease was present. Survival was estimated from the day of pancreatic cancer cells orthotopic injection until the day of death. Differences among survival duration of mice in each group were determined by log-rank test. D) Immunohistochemical analysis. Serial paraffin sections from AsPc-1 tumors treated as indicated were stained with antibodies to the nuclear factor \( \kappa \)B (NF-\( \kappa \)B)– and activator protein-1–induced protein cellular inhibitor of apoptosis 2 (cIAP-2), the p65 subunit of NF-\( \kappa \)B, and the NF-\( \kappa \)B-induced protein interleukin 6 (IL-6).
TAK1 has recently emerged as a central regulator of diverse physiological processes including development, metabolism, and immune and stress responses leading to the activation of the transcription factors NF-κB and AP-1 (38). We demonstrated that silencing the expression of TAK1 leads to a proapoptotic phenotype in pancreatic cancer cells in vitro and in turn to their statistically significantly higher sensitivity to the antitumor activity of chemotherapeutic drugs with different mechanisms of action.

To confirm the results we obtained through genetic silencing of TAK1 expression and to validate TAK1 kinase as a potential therapeutic target for treatment of pancreatic cancer patients, we investigated the antitumor activity of the TAK1–kinase selective inhibitor LY-TAK1 in vitro and in vivo as a single agent and in combination with gemcitabine, oxaliplatin, or irinotecan. In a different study, screening for compounds that can inhibit TAK1 kinase activity resulted in the isolation of one natural compound, 5Z-7-oxozeaenol, a resorcylic lactone of fungal origin with a selective TAK1-inhibiting activity (39). Together with anti-inflammatory activity, 5Z-7-oxozeaenol has been recently shown to potentiate in vitro TRAIL-induced apoptosis in lung cancer cells (40). To our knowledge, this study is the first to demonstrate that targeting the kinase activity of TAK1 with the selective inhibitor LY-TAK1 potentiates in vitro the antitumor activity of gemcitabine, oxaliplatin, or irinotecan against a panel of pancreatic cancer cells. More importantly, we evaluated the activity of LY-TAK1 in an orthotopic model of pancreatic cancer. Our results demonstrated that the TAK1-selective inhibitor LY-TAK1 administered orally at a dose of 6.9 mg/kg for 5 days a week for 4 weeks significantly enhanced the antitumor activity of gemcitabine.

The potent in vitro antitumor activity of the pharmacological inhibition of TAK1 kinase activity in contrast to the milder proapoptotic phenotype demonstrated by the TAK1 knockdown pancreatic cancer cell lines could be attributable to different reasons. LY-TAK1 is a potent and selective kinase inhibitor resulting in the almost complete inhibition of TAK1 activity, which in part may explain its cytotoxicity in vitro as single agent. Alternatively, shRNA did not completely silence the expression of TAK1, which may explain why TAK1 knockdown cells survived. Furthermore, our unpublished data from somatic cell (HCT116) knockout experiments indicated that complete ablation of TAK1 is lethal, consistent with the embryonic lethal phenotype noted in TAK1 knockout mice (21).

A second possible explanation for the potent in vitro antitumor activity of LY-TAK1 in contrast to the milder proapoptotic phenotype obtained by silencing TAK1 expression could rely on the lower in vitro secretion of the potent activator of apoptosis TNF-α by the TAK1 knockdown cells in comparison to their respective control cell lines (Supplementary Figure 2, available online). These differences in the secretion of TNF-α (AsPC-1 > PANC-1 > MDAPANC-28) are also consistent with the different in vitro sensitivity of the pancreatic cancer cell lines to LY-TAK1 (LYTAK1 IC50: AsPC-1 = 5.13 nM; PANC-1 = 14 nM; MDAPanc-28 = 39.3 nM).

This study, however, had some limitations. There is a wide consensus that appropriate preclinical tumor models should be used for the validation of sensitivity to targeted agents (41). The orthotopic xenograft tumor models used in this study are limited by the artificial microenvironment in an immune compromised host and do not faithfully recapitulate the histopathologic features of the human disease. These characteristics could account for their high sensitivity in vivo to oxaliplatin and irinotecan, explaining the measurable but not statistically significant enhancement of the antitumor activity of these drugs when combined with the TAK1-selective inhibitor. Moreover, the characteristics of the preclinical models used in this study could impair the generalizability of the results observed to human patients.

In line with other murine models carrying a tissue-specific deletion of the TAK1 gene (19–21), during the preparation of this article, two studies reported that conditional ablation of TAK1 in liver parenchymal cells causes an early (6 weeks) spontaneous apoptosis of hepatocytes and cholangiocytes (42,43). This led to chronic hepatitis, cholestasis, liver fibrosis, and to a subsequent compensatory liver cell proliferation that at later time (16–33 weeks) drove carcinogenesis in the liver. The additional deletion of the receptor for TNF-α was able to statistically significantly suppress liver damage and inflammation (42). Whereas the spontaneous apoptosis of TAK1 knockout liver parenchymal cells is consistent with our observations in pancreatic cancer cells, these studies could raise concerns about the potential hepatic toxicity of the pharmacological inhibition of TAK1. As indicated before, in our work, all regimens were well tolerated, and no weight loss or other signs of acute or delayed toxicity were observed. The low levels of TNF-α in athymic nude mice kept under axenic conditions could explain the safety of LY-TAK1 in comparison to the spontaneous apoptosis and inflammation observed in mice not kept under axenic conditions (42,43), and, in particular, the lack of activity in vivo of LY-TAK1 as single-agent treatment. In the absence of other activators of apoptosis, the pharmacological inhibition of TAK1 induced apoptosis of pancreatic cancer cells only when combined with proapoptotic chemotherapeutic agents.

Given its complex role in pancreatic cancer biology, targeting the TGF-β signaling pathways represents a challenging but important opportunity for the development of novel treatments for pancreatic cancer patients (44,45). In a different study (30), we investigated whether targeting the TGF-β signaling through inhibition of TGF-β receptor I/II kinase activity was a valid therapeutic approach to suppress growth and metastatic spread of pancreatic cancer. Because we observed that targeting the kinase activity of the TGF-β receptor suppressed the Smad-dependent pathway and, in turn, statistically significantly inhibited pancreatic cancer metastasis in mice, we found that the combination of TGF-β receptor inhibition and gemcitabine resulted in a relatively minor effect on pancreatic tumor growth and mouse survival. More recently, it has been demonstrated that the activation of TAK1 by TGF-β occurs in a TGF-β receptor kinase–independent manner (46). These results suggest that targeting the kinase activity of TGF-β receptors I/II would not be likely to suppress the activation of TAK1, thereby explaining the weak potentiation of gemcitabine observed (30).

Since the 1990s, the research for pancreatic cancer therapy has been focused on several molecules that are early players in the signal transduction cascade, with particular interest in membrane...
receptors such as the epidermal growth factor receptor (EGFR) (47). From the results of the clinical trials with inhibitors of this receptor in pancreatic (48) and colorectal cancer (49), we have learned that the single mutation of K-Ras, the most common genetic alteration in pancreatic cancer, is probably able to circumvent the antitumor activity of anti-EGFR approaches. Alternatively, strategies for the direct inhibition of the final players of the signal transduction cascade, such as the transcriptional factor NF-κB, still face enormous challenges (2). As a member of the MAP3K family, TAK1 is unique (38). More than a single enzyme, it may be considered rather as the active component of a larger protein signaling complex made up of a variety of proteins such as TAK1-binding proteins (TABs) and TRAF6, explaining its role as a key regulator of several signal transduction cascades. We believe that targeting nonredundant cytosolic mediators of the signal transduction cascade such as TAK1 could represent a better approach to inhibiting key processes in tumor cells.

In conclusion, this study demonstrates that the genetic silencing or the inhibition of the kinase activity of TAK1 is a valid approach to reverting the intrinsic chemoresistance of pancreatic cancer. LYTAK1 is the first TAK1-selective inhibitor shown to be active in vivo in mice and thus warrants further development for the treatment of pancreatic cancer in humans.

References


**Funding**

This work was supported in part by the Associazione Italiana per la Ricerca sul Cancro (Start-Up Grant No. 10129), the American-Italian Cancer Foundation, and the Sassi Foundation for Medical Research grants (to D.M.). This work was also supported in part by the Core grant (CA16672 to P.J.C.) and the National Cancer Institute and the National Institutes of Health (R01CA109405 and R01CA097159 to P.J.C.).

**Notes**

We acknowledge the many helpful suggestions and support of the members of our laboratories. We thank Dr Rosa Calemma and Dr Stefania Scala (Clinical Immunology Unit, Fondazione “G. Pascale,” Naples, Italy) for performing secreted TNF alpha enzyme-linked immunosorbent assay measurements. We thank Michele Simbolo and Annamaria Di Filippo for technical assistance, and Adele De Caro for administrative assistance (Experimental Pharmacology Unit, Fondazione “G. Pascale,” Naples, Italy). We thank Kristi M. Speights (Department of Scientific Publications, The University of Texas M. D. Anderson Cancer Center, Houston, TX) for editorial assistance. D. Melisi and Q. Xia contributed equally to this work. The authors declare no direct or indirect financial interest in or arrangement with the companies whose products were used in this study.

**Affiliations of authors:** Experimental Pharmacology Unit, Istituto Nazionale per lo Studio e la Cura dei Tumori—Fondazione “G. Pascale,” Naples, Italy (DM, TM, GP, CC, AB); Department of Gastrointestinal Medical Oncology (DM, JLA), Department of Surgical Oncology (OX, JL, PJC), and Department of Molecular and Cellular Oncology (PJC), The University of Texas M. D. Anderson Cancer Center, Houston, TX; Program of Cancer Biology, The University of Texas Graduate School of Biomedical Sciences at Houston, Houston, TX (PJC).