Targeting the NF-κB and mTOR Pathways with a Quinoxaline Urea Analog That Inhibits IKKβ for Pancreas Cancer Therapy

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Abstract

Purpose: The presence of TNF-α in approximately 50% of surgically resected tumors suggests that the canonical NF-κB and the mTOR pathways are activated. Inhibitor of IκB kinase β (IKKβ) acts as the signaling node that regulates transcription via the p-IκBα/NF-κB axis and regulates translation via the mTOR/p-S6K/p-eIF4EBP axis. A kinase screen identified a quinoxaline urea analog 13-197 as an IKKβ inhibitor. We hypothesized that targeting the NF-κB and mTOR pathways with 13-197 will be effective in malignancies driven by these pathways.

Experimental Design: Retrospective clinical and preclinical studies in pancreas cancers have implicated NF-κB. We examined the effects of 13-197 on the downstream targets of the NF-κB and mTOR pathways in pancreatic cancer cells, pharmacokinetics, toxicity and tumor growth, and metastases in vivo.

Results: 13-197 inhibited the kinase activity of IKKβ in vitro and TNF-α–mediated NF-κB transcription in cells with low-μmol/L potency. 13-197 inhibited the phosphorylation of IκBα, S6K, and eIF4EBP, induced G1 arrest, and downregulated the expression of antiapoptotic proteins in pancreatic cancer cells. Prolonged administration of 13-197 did not induce granulocytosis and protected mice from lipopolysaccharide (LPS)-induced death. Results also show that 13-197 is orally available with extensive distribution to peripheral tissues and inhibited tumor growth and metastasis in an orthotopic pancreatic cancer model without any detectable toxicity.

Conclusion: These results suggest that 13-197 targets IKKβ and thereby inhibits mTOR and NF-κB pathways. Oral availability along with in vivo efficacy without obvious toxicities makes this quinoxaline urea chemotype a viable cancer therapeutic. Clin Cancer Res; 19(8); 1–11. ©2013 AACR.

Introduction

Since its discovery 27 years ago, NF-κB has been shown to regulate the expression of more than 200 immune, growth, and inflammation genes (1, 2). The clinically silent onset of pancreatic cancer has been attributed to the upregulation of proinflammatory pathways such as NF-κB (3). NF-κB is constitutively active in pancreatic cancer cell lines and pancreatic adenocarcinoma but not in immortalized/non-tumorigenic pancreatic epithelial cells or normal pancreatic tissues (4). Pancreatic cancer cell lines show increased levels of NF-κB subunits compared with nonmalignant proliferating intestinal cells (5). These preclinical observations extend to patients with pancreatic cancer: (i) high expression of RelA (NF-κB subunit p65) was observed in 64% of histologically or cytologically verified advanced unresectable and/or metastatic disease, and (ii) this correlates with increased expression of NF-κB target genes and poor prognosis in this patient subgroup (6, 7). Conversely, downregulation of NF-κB (RelA) using siRNA sensitizes a subset of pancreatic cancer cells and pancreatic tumors in nude mice to gemcitabine (8, 9). Inhibiting constitutive NF-κB activity suppressed growth, angiogenesis, and metastasis of pancreatic cancer (10).

Inhibitor of IκB kinase β (IKKβ) is a key kinase in the NF-κB pathway that is activated by proinflammatory cytokines such as TNF-α (11). Upon activation, IKKβ phosphorylates IκBα, which leads to βTrCP-mediated ubiquitination and proteasomal degradation of IκBα (12, 13). Degradation of IκBα releases NF-κB, which translocates to the nucleus to activate gene expression (14). TNF-α–mediated activation of IKKβ is also known to result in phosphorylated tuberous
Translational Relevance

Biochemical and genetic studies implicate IKKβ in a number of diseases mediated by chronic inflammation, including cancer. This led the pharmaceutical industry to vigorously pursue development of small-molecule IKKβ inhibitors. Despite full characterization of these inhibitors in preclinical models, only three entered clinical trials and the U.S. Food and Drug Administration (FDA) has approved none. One of the reasons is toxicity; prolonged inhibition of IKKβ results in granulocytosis and endotoxin-induced mortality. Here, we have identified a small-molecule IKKβ inhibitor, 13-197, which when administered chronically does not induce granulocytosis and protects mice from lipopolysaccharide (LPS)-induced mortality. The latter observation has clinical implication in sepsis. The compound is orally available with extensive distribution to the peripheral tissues and inhibits pancreatic cancer growth in vitro and in an orthotopic model. Given the limited therapeutic options for patients with pancreas cancer, these studies lay the foundation for a path to clinical trials with 13-197 or a close analog.

Materials and Methods

Chemicals and reagents

The quinoxaline urea analog 13-197 was synthesized and purified (>98%) as previously described (25, 26). Efavirenz was obtained from Hetero Labs Ltd. High-performance liquid chromatography (HPLC)-grade methanol, acetone, ammonium acetate, ammonium formate, ammonium hydroxide, formic acid, and acetic acid were obtained from Fisher Scientific.

Cell lines and culture conditions

Human pancreatic cancer cell lines Capan-2, MiaPaCa2, AsPC1, HS766T, and SUIT2 were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin.

Reaction Biology Corporation kinase assay

The appropriate substrate was freshly prepared in the kinase assay buffer [20 mM/L HEPES pH 7.5, 10 mM/L MgCl2, 1 mM/L EGTA, 0.2% Brij35, 0.02 mg/mL bovine serum albumin (BSA), 0.1 mM/L Na3VO4, 2 mM/L dithiothreitol (DTT), 1% dimethyl sulfoxide (DMSO), and the required cofactors added individually for each kinase reaction]. The kinase was added to the solution and gently mixed and the compounds were then added in DMSO (final DMSO concentration of 1.4%). 33P-ATP (specific activity 0.01 µCi/µL final) was added to the mixture and incubated for 2 hours at room temperature. Kinase reaction was then spotted onto P81 ion exchange paper (Whatman # 3698-915), washed extensively in 0.75% phosphoric acid, and read on a scintillation counter.

For the follow-up screen, 10 mM/L DMSO solution of the compound was titrated 1:3 over 10 points into wells containing 100% DMSO. The diluted compounds were delivered into the reaction at 100 µM/L starting concentration with 1:3 serial dilution with the final DMSO concentration of 2.2%.

Western blot analysis

Cells were harvested on ice in buffer A (10 mM/L HEPES, 10 mM/L KCl, 0.1 mM/L EDTA, and 2 mM/L DTT) containing protease and phosphatase inhibitors. The cells were allowed to swell on ice for 30 minutes, 1% NP-40 was added, and the samples were centrifuged in the cold. The resulting supernatant was transferred to a new tube and designated as the cytoplasmic fraction. To the remaining pellet, buffer C (20 mM/L HEPES, 420 mM/L NaCl, 5 mM/L EDTA, 5 mM/L DTT, and 10% glycerol) containing protease and phosphatase inhibitors was added. The samples incubated on ice with intermittent agitation over a period of 30 minutes and were then centrifuged in the cold. The resulting supernatant was designated the nuclear fraction. All samples were stored at −20°C until needed. Western blot analyses were conducted as described previously with minor modifications (26, 27). Briefly, cytoplasmic and nuclear lysates were prepared from control and 13-197–treated cells by using nuclear extract kit (active...
motif). The cell lysates (50 μg) were resolved in 4% to 20% denaturing polyacrylamide gel (Bio-Rad) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). The blotted membranes were incubated with the following antibodies, mouse monoclonal anti-NF-κB (p65; Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-p21 (Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-E2F-1 (Santa Cruz Biotechnology, Inc.), mouse monoclonal anti-PCNA (Cell Signaling), rabbit monoclonal anticyclin D1 (Epitomics), rabbit monoclonal anti-phospho S6K1 (Cell Signaling), rabbit monoclonal anti-S6K1 (Cell Signaling), rabbit monoclonal anti-phospho 4EBP-1 (Cell Signaling), rabbit anti-4EBP-1 (Cell Signaling), rabbit anti-IkBα (Cell Signaling), mouse anti-phospho-IkBα (Cell Signaling), rabbit anti-Bcl-xL (Cell Signaling), rabbit anti-survivin (Novus Biologicals), rabbit anti-Mcl-1 (Santa Cruz Biotechnology, Inc.), mouse anti- XIAP (Santa Cruz Biotechnology, Inc.).

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kB-luciferase assay

A549 luciferase cells (generous gift from Prof. Brasier, University of Texas Medical Branch, Galveston, TX) were seeded in white 96-well plates at a density of 50,000 cells per well and incubated overnight. Cells were then treated with appropriate amounts of 13-197 (100 μg, 20 μg, 5 μg, 1.25 μg, 0.31 μg, and 0.063 μg/mL) in 0.2% DMSO for 2 hours. Cells were then stimulated with 20 ng/mL TNF-α for 4 hours. AlamarBlue (abSerotec) was added (which served as a control for seeding and viability) and the cells were incubated for 3 additional hours. ONE-Glo Luciferase reagent (Promega) was added to each well and luminescence was measured on a SpectraMax M5 plate reader (Molecular Diagnostics; ref. 28). IC₅₀ value was an average from 2 independent experiments run in triplicate.

Proliferation assay

Cellular proliferation was determined by AlamarBlue reduction assay method (Ab Serotec) according to manufacturer’s instructions. Briefly, MiaPaCa2 cells (5,000 cells/well) were incubated with 13-197 in triplicate in a 96-well plate and then incubated for 24, 48, 72, and 96 hours at 37°C. AlamarBlue solution was added to each well (equal to 10% volume of the medium in the well) and incubated for 3 hours at 37°C. The fluorescence of the solution was measured at 560-nm excitation and 590-nm emission using spectramax³⁵ fluorescence plate reader. Dose–response curves were evaluated using the National Cancer Institute (NCI) algorithm: $T = \frac{C}{t_0}$, $C =$ number of control cells at time $t$, $t_0 =$ number of control cells at time $t$, and $T =$ number of treated cells at time $t$. $100 \times \left(\frac{C - T}{T - C}\right)$. This experiment was repeated 3 times and statistical analysis was conducted using unpaired Student $t$ test.

Cell-cycle analysis

To determine the effect of 13-197 on the cell-cycle progression, cell-cycle analyses were carried out using cell GeneScript kit according to manufacturer instruction. MiaPaCa2 cells (1 × 10⁶) were plated in 100-mm dish and serum starved for 24 hours. Subsequently, cells were treated with 13-197 (11 μM/L) in 0.1% DMSO and incubated for 24 hours. Cells were trypsinized, washed in PBS, and permeabilized with 70% ethanol for 1 hour at 4°C. The cell pellet was resuspended in PBS-containing ribonuclease A at 37°C for 30 minutes and stained with propidium iodide (50 μg/mL) for 30 minutes on ice. The DNA content was determined by flow cytometry [flow cytometry core facilities at University of Nebraska Medical Center (UNMC; Omaha, NE). The experiment was repeated 3 times and the mean ± SD are represented and Student $t$ test was used to determine significance.

Live/dead cell assay

Apoptosis induced by 13-197 in MiaPaCa2 cells was assessed using Live/Dead Cell Assay Kit (Invitrogen) according to manufacturer’s protocol. Briefly, MiaPaCa2 cells were grown on cover slips and incubated with 13-197 (11 μM/L) in 0.1% DMSO for 24 hours. Cells were then stained with the assay reagents as per manufacturer instructions. Cell viability was determined under fluorescence microscopy by counting live (green) and dead (red) cells. Data are represented as mean ± SD from 3 experiments and Student $t$ test was used to determine the significance.

In vitro migration assay

Migration assay was conducted by using polyethylene teraphthalate (PET) Transwell inserts as described previously (27). Briefly, MiaPaCa2 cells (1 × 10⁶) were suspended in 1% FBS-containing DMEM and seeded on top of the PET membranes (8 μm, 24-well plates; BD Biosciences) with 11 μM/L of 13-197 in 0.1% DMSO. DMEM supplemented with 10% FBS was added to the bottom of each well. After 24 hours incubation, cells that migrated through the membrane were stained with Diff-Quick cell staining kit (Dade Behring, Inc.). The number of cells that migrated was counted (5 fields at ×200 magnification). The results are from 3 independent experiments and represented as mean number of cells migrated per field.

In vitro invasion assay

Pancreatic cancer cells invasion assay was evaluated by Matrigel-coated boydon chamber assay as described previously (27). Briefly, MiaPaCa2 cells (1 × 10⁶) were suspended in DMEM-containing 1% FBS and seeded on top of Matrigel-coated chambers (8 μm, 24-well plates; BD Biosciences) with 11 μM/L of 13-197 in 0.1% DMSO. DMEM supplemented with 10% FBS was added to the bottom of each well. After 24 hours of incubation, cells that invaded through the membrane were stained with Diff-Quick cell staining kit...
(Dade Behring, Inc.). The number of cells that invaded was quantified in 5 different random fields (original magnification $\times200$). The results are from 3 independent experiments and represented as mean number of cells invaded per field.

**Pharmacokinetic studies**

Eight-week-old healthy male C57/BL6 mice were purchased from Charles River Laboratories. Sterilized 7012 Teklad diets (Harlan) were used for mice, and water was provided ad libitum. In mice, 150 mg/kg oral dose was administered as a 75 mL of 50 mg/mL in Cremophor EL. At each of the following 7 time points (0.5, 1, 2, 8, 24, 48, and 72 hours), 3 mice were euthanized and their blood was collected by cardiac puncture in heparinized tubes and organs (liver, kidney, lung, heart, spleen, and brain) were snap-frozen. Plasma was separated by centrifugation of blood samples at 1,500 $\times$ g for 10 minutes at $4^\circ$C within 1 hour of sample collection. Liquid chromatography/tandem mass spectrometry (LC-MS/MS) was used for sample analysis. A hybrid ion trap-triple quadrupole (Q-Trap) mass spectrometry was used to quantify all analytes by multiple reaction monitoring (MRM). For sample preparation, 1 mL of ice-cold acetonitrile was added to 100 mL plasma samples prespiked with 4.0 $\mu$g/mL internal standard (efavirenz). Samples were then vortexed and centrifuged at 16,000 $\times$ g for 10 minutes. The supernatant was aspirated, evaporated under vacuum, and reconstituted in a 100 mL 75% methanol. After centrifugation at 16,000 $\times$ g for 10 minutes, 10 $\mu$L of each sample was used for LC-MS/MS analysis. Liver, kidney, lung, heart, spleen, and brain tissues were homogenized in deionized water (1:2 w/v). Hundred microliter aliquots of tissue homogenates were spiked with 10 $\mu$L of 4.0 $\mu$g/mL internal standard (efavirenz) and were extracted and analyzed similarly to plasma samples. The pharmacokinetic parameters were determined using the extravascular input noncompartmental analysis module of WinNonlin (version 1.5; Pharsight). The absolute bioavailability ($F$) was calculated as the ratio between the area under the curve (AUC)$_{0-\infty}$ from oral and intravenous routes after dose normalization using the following equation:

$$\%F_{\text{Absolute}} = \frac{\text{AUC}_{\text{Oral}} \times \text{Dose}_{\text{Oral}}}{\text{AUC}_{\text{IV}} \times \text{Dose}_{\text{IV}}} \times 100$$

**Biochemical toxicity assay**

To assess toxicity of 13-197 in animals, we analyzed leakage of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzymes in the plasma. The enzyme assay was conducted by using ALT and AST kit (Beckman couler) according to manufacturer’s instructions (Regional Pathology Services at UNMC).

**Leukocytes count and LPS challenge**

BALB/c mice (NCI, Frederick, MD) were treated with 13-197 (150 mg/kg body weight, $n = 5$) and vehicle control (Cremophor, $n = 5$) per os (p.o.) twice for 4 days. After fourth day treatment, thin blood smears were made by bleeding the tail vein. Blood smears were fixed and stained with Wright’s Giemsa. Differential counts were conducted by counting 100 leukocytes in randomly selected fields of view on blood smears. The mice were then challenged with lipopolysaccharide (LPS; 30 mg/kg) intraperitoneally (i.p.) and monitored for 72 hours.

**Orthotopic tumor model**

Pancreatic cancer cells were orthotopically placed into pancreas of nude mice as described previously (29). Briefly, MiaPaCa2 cells were harvested and resuspended in serum and dye free medium. Mice were anesthetized with ketamine–xylazine solution, a small left abdominal flank incision was made, and MiaPaCa2 cells (0.5 $\times$ 10$^6$ in 30 mL) were injected into the pancreas using a 27-gauge needle. The abdomen was closed using 2-layer suture with chromic catgut and ethilon suture. After 2 weeks of implantation, mice were randomized into two groups: (i) untreated control (Cremophor, 100 mL p.o. daily) and (ii) 13-197 treatment (150 mg/kg body weight, p.o. daily). Each group contains 15 animals. After 4 weeks of treatment the animals were sacrificed. The primary tumors in the pancreas were excised and measured in serum and dye free medium. Mice were anesthetized and orthotopic tumor model

**MicrovesSEL density**

The pancreas tissue sections (5 $\mu$m) from vehicle and 13-197–treated animals were stained with rabbit anti-Ki-67 antibody (neomarkers), as described previously (29). Results were expressed as percentage of Ki-67–positive cells $\pm$ SE per original magnification $\times400$. A total of 5 fields were examined and counted from 5 tumors of control and treated groups. The values were subjected to one-way ANOVA and unpaired Student t test.

**Proliferation index**

Formalin-fixed, paraffin-embedded sections (5 $\mu$m) were stained with rabbit anti-Ki-67 antibody (neomarkers), as described previously (29). Results were expressed as percentage of Ki-67–positive cells $\pm$ SE per original magnification $\times400$. A total of 5 fields were examined and counted from 5 tumors of control and treated groups. The values were subjected to one-way ANOVA and unpaired Student t test.
Immunohistochemistry analysis of Bcl-xL, Mcl-1, and phospho-S6 ribosomal protein in tumor samples

The tumor tissue were stained with Bcl-xL, Mcl-1, and phospho-S6 ribosomal protein using Leica bond polymer refine detection kit according to manufacturer’s instructions (Leica Microsystems Inc.). Briefly, the pancreatic tumor tissue sections were incubated with the following primary antibodies rabbit anti-Bcl-xL antibody (Cell Signaling Technology, Inc.), rabbit anti-Mcl-1 antibody (Santa Cruz Biotechnology, Inc.), rabbit anti-phospho-S6 ribosomal protein (Ser 235/236) antibody (Cell Signaling Technology, Inc.). Three different pancreatic tumor tissues were used for each antigen. Pictures were captured by using iScan Coreo slide scanner (Ventana Medical Systems Inc.). A semiquantitative approach was used to score the percentage of positive cells, +, <25% cells positive; ++, 25% to 50% cells positive; +++, 50% to 75% cells positive; +++++, 75% to 100% cells positive.

Data processing and statistical analysis

P values were determined using one-way ANOVA in Sigma Plot and values less than 0.05 was considered significant.

Results and Discussion

13-197 inhibits IKKβ and targets NF-κB and mTOR pathway proteins

Screening a focused library of quinoloxaline analogs against a panel of cancer cell lines led to the identification of 13-197, which inhibited cell growth with low-μmol/L IC₅₀ values and induced apoptosis in a Mcl-1–dependent manner (25, 26). Our next goal was to determine the molecular target of 13-197. Because multiple kinases are known to regulate Mcl-1 levels (30), 13-197 was screened in duplicate against a panel of 318 kinases. Six kinases were identified as hits based on >2 SD (>95% confidence interval) from the mean of the screen. Results from the follow-up 10-point dose response study are summarized in Fig. 1A. The IC₅₀ values were in the low-μmol/L range for 4 (TRKA, IKKβ, EPHA6, and Nek3) of the 6 kinases identified as hits. EPHA6 is highly expressed in the brain and is important for learning and memory (31). Nek3 is important in prolactin-mediated motility of cells and inhibiting Nek3 might be beneficial for a cancer therapeutic (32). We focused on the kinases TRKA and IKKβ as they had the lowest IC₅₀ values at 1.9 ± 0.2 and 3.0 ± 0.1 μmol/L, respectively. Although TRKA is primarily localized to the brain, both TRKA and IKKβ are activated by cytokines (IL-1 and TNF-α), which promote NF-κB–mediated gene expression (33, 34).

TNF-α stimulation regulates transcription via the p- IKKβ/p-1κBζ/1κBβ/IKKβ axis and translation via the p-IKKβ/mTOR/p-S6K/p-eF4EBP axis (Fig. 1B; refs. 16, 35). TNF-α is commonly found in the tumor microenvironment of pancreatic tumors and leads to activation of the canonical NF-κB pathway (18, 19). Therefore, if IKKβ is inhibited by 13-197 in pancreatic cancer, we expected to observe inhibition of 1κBζ, S6K, and eF4EBP1 phosphorylation. To test this, we subjected a panel of pancreatic cancer cell lines (AsPC1, Capan2, Hs766T, and SUIT2) to 13-197 and probed the lysates for phosphorylated 1κBζ levels (Fig. 1C). 13-197 robustly inhibited phosphorylation of 1κBζ in the panel of pancreatic cancer cell lines and inhibited phosphorylation of 1κBζ in MiaPaCa2 cells in a dose- and time-dependent manner (Fig. 1C). Inhibition of 1κBζ should result in reduced nuclear localization of p65, to test this we subjected MiaPaCa2 and Capan2 cells to 13-197 for 2 hours and probed the nuclear fraction of the lysate for p65 and PARP/12B (Fig. 1C). As expected, we observed decreased nuclear levels of p65. To determine if this leads to inhibition of NF-κB–mediated gene expression, we evaluated 13-197 in a NF-κB luciferase assay (28). A cancer cell line that is specifically designed to monitor the activity of NF-κB in response to TNF-α was treated with 13-197 for 2 hours followed by TNF-α for an additional 4 hours. Under multiplexing conditions, the cells were assayed for viability using AlamarBlue and the NF-κB transcription activity using the ONE-Glo luciferase system (Fig. 1D). The results show that 13-197 inhibits TNF-α–stimulated NF-κB–mediated luciferase expression with an IC₅₀ value of 8.4 ± 1.2 μmol/L. Together, these results show that 13-197 targets IKKβ, which is upstream of NF-κB nuclear translocation, and thereby inhibits NF-κB–mediated transcription. To determine inhibition of the mTOR pathway, we probed MiaPaCa2 lysates for phosphorylated S6K and eF4EBP1 treated with 13-197 for 4 and 24 hours (Fig. 1C). The results showed decreased phosphorylation of S6K and eF4EBP1. In summary, the data suggest that 13-197 targets IKKβ in pancreatic cancer cell lines and inhibits NF-κB and mTOR pathways.

13-197 inhibits MiaPaCa2 cell growth by arresting cells in the G1-phase

We next explored if IKKβ inhibition by 13-197 results in the inhibition of pancreatic cancer cell growth. A panel of pancreatic cancer cell lines subjected to 13-197 showed inhibition of cell growth with μmol/L potencies (Supplementary Table S1). To evaluate the time-dependent effects of 13-197, MiaPaCa2 cells were incubated with 13-197 (IC₅₀ = 11.4 ± 0.7 μmol/L) and cell growth was monitored over time for 96 hours (Fig. 2A; ref. 26). The results show that 13-197 inhibits pancreatic cancer cell growth both in a dose- and in a time-dependent manner. To determine if the growth inhibition induced by 13-197 is a result of cell-cycle arrest, MiaPaCa2 cells were treated with 13-197 and then subjected to cell-cycle analysis. Cells treated with 13-197 arrested in the G1-phase (Fig. 2B). Consistent with the G1 arrest, we observed reduced levels of the corresponding cell-cycle markers E2F, PCNA, and cyclin D1 (Fig. 2B; ref. 36). Together, the data show that 13-197 inhibits pancreatic cancer cell growth by inducing G1 arrest of cells.

13-197 downregulates expression of antiapoptotic proteins and induced apoptosis in MiaPaCa2 cells

Next, we probed the effect of 13-197 on apoptotic pathway proteins. Mcl-1 and Bcl-xL are antiapoptotic proteins that sequester the proapoptotic proteins (Bax/Bak; ref. 37).
XIAP and survivin belong to the IAP family and are inhibitors of caspase activation (38). In MiaPaCa2 cells treated with 13-197, we observed a significant decrease in the Mcl-1 levels when compared with Bcl-xL, XIAP, and survivin at the 2 hour time point. However, longer incubation times at 20 μmol/L shows a decrease in all proteins (Fig. 2C). It is known that Mcl-1, Bcl-xL, XIAP, and survivin are NF-κB target genes, therefore inhibition of NF-κB nuclear translocation by 13-197 would result in reduced protein levels. The different rates (Mcl-1 vs. Bcl-xL/XIAP/survivin) of degradation suggest that Mcl-1 levels are probably controlled by IKKβ directly, whereas the others (Bcl-xL/XIAP/survivin) are downregulated at the transcriptional level due to NF-κB inhibition. Consistent with the downregulation of antiapoptotic proteins, cells treated with 13-197 showed a 4-fold increase in the percentage of apoptotic cells when compared with untreated cells (Fig. 2C). Together, these data showed that 13-197 downregulates antiapoptotic proteins both in a dose- and time-dependent manner and thereby induced apoptosis in pancreas cancer cell lines.

NF-κB regulates the expression of several genes such as IL-8, VEGF, ICAM1, and MMP-9 that are implicated in angiogenesis, invasion, and metastasis (39, 40). We observed that 13-197 inhibited both the invasion and migration of MiaPaCa2 cells by about 50% (Fig. 2D). This suggests that 13-197 has the potential to not only inhibit growth of tumors but also inhibit metastasis.

13-197 is orally available with extensive distribution to peripheral tissues. Pharmacokinetic difficulties account for more than 50% of drug development failures preventing new chemical entities (NCE) from reaching the market, whereas toxicity issues and lack of efficacy account for only 30% of...
development failures (41). As a result, in addition to paying attention to the traditional concern of attaining potency and selectivity toward the biologic target of interest, pharmacokinetic considerations have been moved to early stages of drug discovery, a significant paradigm shift in the pharmaceutical industry (42).

Lipinski’s rule of 5 serves as a guide to determine if compounds will be orally bioavailable (43). Lipinski’s rule states a compound with the following properties, molecular weight (M. Wt.) < 500 Da, < 5 hydrogen bond donors, <10 hydrogen bond acceptors, and a log (an octanol–water partition coefficient) < 5 is likely to be orally available. Analysis of 13-197 (M. Wt. = 474 Da, hydrogen bond donors = 2, hydrogen bond acceptors = 7, and log P = 3.94), suggested that it would be orally available. 13-197 was formulated in Cremophor EL, which is a commonly used excipient in drugs for pharmacokinetic and tissue distribution studies.

A mass spectrometry method was established to determine 13-197 levels in plasma and tissue. Mice were dosed orally with 150 mg/kg of 13-197 and sacrificed at indicated time points. 13-197 levels in blood and various tissue samples were determined by LC-MS/MS (Fig. 3A and B). The pharmacokinetic properties of 13-197 are described as an inset in Fig. 3A and the tissue levels as an inset in Fig. 3B. Although low (~5%), the results show that 13-197 is orally available and shows extensive distribution to peripheral tissues, especially liver and kidney, which are the primary organs of elimination.

Maximum-tolerated dose of 13-197 under acute administration

Because IKKβ inhibitors ML-120B and TPCA1 showed toxicity in mice (22, 23), we conducted a preliminary dose-escalation study to assess acute toxicity in Balb/c mice. Mice were gavaged with increasing 13-197 doses (vehicle, 150, 450, and 900 mg/kg) and monitored for 24 hours at which time they were sacrificed and plasma isolated. All the mice were alive and none of the mice, even at the highest dose, showed any visible signs of toxicity. Because the pharmacokinetic studies showed clearance through the liver, the levels of ALT and AST, the plasma biochemical markers for hepatotoxicity, were
measured (44). Leakage of ALT and/or AST into the blood stream indicates liver damage. Plasma ALT and AST were measured in vehicle and 13-197–treated Balb/c mice are within error range indicating a lack of hepatotoxicity (data not shown). Together, these show that the maximum-tolerated dose (MTD) for 13-197 is more than 900 mg/kg.

13-197 does not induce granulocytosis and protects mice from LPS-induced mortality

Prolonged treatment (twice daily for 4 days) with the Millennium IKKβ inhibitor ML-120B resulted in granulocytosis and LPS-induced mortality (23, 24). This is because chronic inhibition of IKKβ in neutrophils results in the processing of pro-IL-1β to IL-1β by the serine protease PR3. This was shown to induce a feed forward loop upon LPS treatment resulting in a sustained release of IL-1β from macrophages leading to death (23). We treated 13-197 twice daily for 4 days and measured neutrophil levels by blood smears. The mice were then challenged with LPS. Our results did not show an increase in the neutrophil levels in the treated mice when compared with control mice (Supplementary Table S2). More importantly, we observed a protective effect from LPS-induced mortality in mice pretreated with 13-197 (Fig. 4A).

To gain insight into this protective effect, we investigated the effect of IκBα phosphorylation in cells treated with and without 13-197 followed by TNF-α stimulation. In the control experiment, HEK293 cells were treated with DMSO followed by TNF-α stimulation. This resulted in rapid phosphorylation of IκBα (5 and 10 minutes) and at 30 minutes poststimulation the absence of IκBα indicates phosphorylation-mediated proteasomal degradation. Reexpression of IκBα at 60 and 120 minutes poststimulation suggests feedback inhibition of the pathway (IκBα is a target gene of NF-κB; ref. 45). In the presence of 13-197, we observed modest inhibition of IκBα phosphorylation and degradation at the early time points (5 and 10 minutes) at the same time points TPCA1 treatment resulted in complete inhibition. However, at the 60 and 120 minutes time points, we observed a significant reduction in phospho-IκB levels (last 2 lanes of the gel) when compared with DMSO-treated lanes (box, Fig. 4B). This suggests that 13-197 does not inhibit the transient...
activation of IKKβ but inhibits IKKβ postactivation, which is probably found under sustained stimulation. The inhibition by 13-197 is unlike other known IKKβ inhibitors such as TPCA1 (Fig. 4C) that completely blocks TNF-α–induced phosphorylation of IkBα (46, 47). This suggests that 13-197 does not block the transient activation of the pathway in neutrophils and thereby protects them from LPS-induced mortality.

13-197 reduced tumor growth and metastasis in an orthotopic pancreas cancer model

Once we established that 13-197 is nontoxic and orally available, we evaluated its ability to reduce tumor growth and metastases in an orthotopic pancreas cancer model. MiaPaCa2 cells were placed in the pancreas of nude mice and the mice were allowed to heal after surgery. The tumors were allowed to grow for 2 weeks at which time they were palpable. The tumor-bearing mice were randomized and half the animals were treated orally with 13-197 at 150 mg/kg in cremaphor daily. It is difficult to accurately measure tumor volumes over time, therefore the endpoint was time based as opposed to survival. At the end of 30 days, the mice were sacrificed and the tumor weights and volumes were measured. We observed approximately 50% reduction in both the tumor weight and volume in the 13-197–treated animals compared with vehicle–treated animals (Fig. 5A). We also found fewer tumor nodules in other organs of 13-197–treated animals compared with vehicle–treated animals (Supplementary Table S3). The in vivo effects are consistent with the in vitro data, which suggested inhibition of tumor growth and metastases by 13-197. Proliferation index and microvessel density are measures of the number of cells dividing in the tumor and angiogenesis respectively. We observed a reduction of both Ki-67 and CD31 in 13-197–treated tumors compared with vehicle controls (Fig. 5B and C). Immunohistochemistry studies showed that 13-197–treated tumors had reduced levels of Mcl-1 (NF-κB target gene) and phospho-S6 ribosomal protein (mTOR target) as compared with vehicle–treated tumors (Fig. 6A and Supplementary Table S4). This suggests that 13-197 perturbs both the NF-κB and mTOR signaling in vivo and is consistent with cell-based studies. The pathologist was blinded and the tumor tissues were scored for inflammation and necrosis. The results indicate 13-197 suppresses inflammation and increases necrosis in 13-197–treated mice (Supplementary Table S5). These results are consistent with the inhibition of NF-κB. We also determined the 13-197 levels in the pancreas, liver, and serum at the end of the study (Fig. 6B). Consistent with our pharmacokinetic data, the highest drug levels were found in the liver. Therefore, to probe for hepatotoxicity, we measured the levels of ALT and AST in 13-197– and vehicle–treated mice. We did not observe a difference in the ALT or AST levels in 13-197–treated mice when compared with vehicle–treated mice, indicating the absence of hepatotoxicity (Fig. 6C). We also conducted a macroscopic examination of the organs and found no obvious toxicity in the 13-197–treated animals.

Conclusion

The data show that 13-197 is a promising IKKβ inhibitor that targets NF-κB and mTOR pathway in pancreas cancer cell lines. 13-197 inhibits pancreas cancer cell growth by inducing G1 arrest and induces apoptosis by reducing levels of the antiapoptotic proteins. Prolonged treatment of 13-197 does not induce granulocytosis and protects mice from...
Figure 6. Studies with 13-197-treated in vivo samples. A, immunohistochemical analysis of Mcl-1 and phospho-S6 ribosomal protein in tumor samples. B, 13-197 levels measured in the pancreas, liver, and serum 18 hours after the last treatment. C, liver enzymes (AST and ALT) measured at the end of the study.

LPS-induced mortality. The latter observation will have therapeutic implications against sepsis. 13-197 is orally available and reduces tumor growth and metastases in vivo. Together, these results lay the foundation for clinical evaluation of 13-197 either alone or as part of a combination for the treatment of pancreatic cancer.

Disclosure of Potential Conflicts of Interest
A. Natarajan, P. Radhakrishnan, V.C. Bryant, and R.N. Rajule have ownership interest (including patents) in a pending patent. No potential conflicts of interest were disclosed by the other authors.

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