Targeting the NF-κB and mTOR pathways with a quinoxaline urea analog that inhibits IKKβ for pancreas cancer therapy

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Running title: Quinoxaline urea analog inhibits pancreatic tumor growth

Keywords: Quinoxaline urea, IKKbeta, NF-kappaB, mTOR and Pancreatic Cancer

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Conflict of interest: None
Statement of 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 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, when administered chronically does not induce granulocytosis and protects mice from 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 pancreas cancer patients, these studies lay the foundation for a path to clinical trials with 13-197 or a close analog.

Abstract

Purpose: The presence of TNFα in ~ 50% of surgically resected tumors suggests that the canonical NF-κB and the mTOR pathways are activated. 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 kinome 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 TNFα mediated NF-κB transcription in cells with low-μM potency. 13-197 inhibited the phosphorylation of IκBα, S6K and eIF4EBP, induced G1 arrest and down regulated the expression of antiapoptotic proteins in pancreatic cancer cells. Prolonged administration of 13-197 did not induce granulocytosis and protected mice from 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.
Introduction

Since its discovery 27 years ago, NF-κB has been shown to regulate the expression of over 200 immune, growth and inflammation genes (1, 2). The clinically silent onset of pancreatic cancer has been attributed to the upregulation of pro-inflammatory 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 to non-malignant proliferating intestinal cells (5). These preclinical observations extend to pancreatic cancer patients: (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, down regulation 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).

IKKβ kinase (IKKβ) is a key kinase in the NF-κB pathway that is activated by proinflammatory cytokines such as tumor necrosis factor α (TNFα) (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). TNFα mediated activation of IKKβ is also known to result in phosphorylated tuberous sclerosis 1 (TSC1).
phosphorylation mediated suppression of TSC1 results in the activation of the mTOR pathway (15-17). Collectively these data suggest that IKKβ is a key node and upon activation by TNFα regulates transcription of genes through the p-IκBα / NF-κB axis and translation of the gene products through the mTOR / p-S6K / p-eIF4EBP axis.

The KRAS gene is mutated in > 90% of pancreatic carcinomas. Targeted inactivation of IKKβ in KrasG12D mice stalled PanIN lesions from developing into pancreatic ductal adenocarcinoma (PDAC) (18, 19). This establishes the mechanistic link between IKKβ and Kras mutations in the initiation of PDAC. This along with the observation that TNFα is found in ~50% of surgically resected tumor samples (16) makes a strong case for IKKβ as a target for pancreatic cancer therapeutic development.

Since its discovery the pharmaceutical industry has aggressively pursued the development of IKKβ inhibitors to treat diseases driven by chronic inflammation (20). Although several inhibitors passed the gamut of preclinical studies required to move compounds to the clinics only three of them were evaluated in the clinics and so far none have received FDA approval (21). One possible reason for this is the observed toxicity (ML-120B and TPCA1) in preclinical models (22, 23). Chronic administration of IKKβ results in granulocytosis and endotoxin-mediated mortality (23, 24). In the present study we characterize the mechanism of action and antitumor activity of an IKKβ inhibitor, 13-197, which has a quinoxaline urea chemotype. More importantly prolonged administration of 13-197 showed no detectable toxicity.
Materials and Methods

Chemicals and Reagents: The quinoxaline urea analog 13-197 was synthesized and purified (> 98%) as previously described (25, 26). Efavirenz (EFV) was obtained from Hetero Labs Ltd. (Hyderabad, India). HPLC-grade methanol, acetonitrile, ammonium acetate, ammonium formate, ammonium hydroxide, formic acid, and acetic acid were obtained from Fisher Scientific (Fair Lawn, NJ, USA).

Cell lines and culture conditions: Human pancreatic cancer cell lines Capan-2, MiaPaCa2, AsPC1, Hs766T and SUIT2 were cultured in Dulbecco’s modified eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 µg/mL streptomycin.

RBC Kinase assay: The appropriate substrate was freshly prepared in the kinase assay buffer (20 mM HEPES pH 7.5, 10 mM MgCl₂, 1 mM EGTA, 0.2 % Brij35, 0.02 mg/mL BSA, 0.1 mM Na₃VO₄, 2 mM DTT, 1% 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 h 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 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 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 HEPES, 10 mM KCl, 0.1 mM EDTA, 2 mM DTT) containing protease and phosphatase inhibitors. The cells were allowed to swell on ice for 30 min, 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 HEPES, 420 mM NaCl, 5 mM EDTA, 5 mM DTT, 10% glycerol) containing protease and phosphatase inhibitors was added. The samples incubated on ice with intermittent agitation over a period of 30 min 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 carried out 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% - 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-kB (p65) (Santa Cruz), rabbit polyclonal anti-p21 (Santa Cruz), rabbit polyclonal anti-E2F-1(Santa Cruz), mouse monoclonal anti-PCNA (Cell signaling), rabbit monoclonal anti-cyclin D1 (Epitomics), rabbit monoclonal anti-phospho S6K1(Cell signaling), rabbit monoclonal anti-S6K1 (Cell signaling), rabbit monoclonal anti-phospho 4EBP-1(Cell signaling), rabbit monoclonal anti-4EBP-1 (Cell signaling), rabbit anti-Ikbα (cell signaling), mouse anti-phospholKbα (cell signaling), rabbit anti-Bcl-xL (cell signaling), rabbit anti-survivin (Novus biological), rabbit anti-Mcl-1 (Santa cruz), mouse anti-XIAP (Santa cruz). Internal loading control for cytoplasmic fractions used mouse anti-β-actin.
(Sigma) and mouse anti-tubulin (Cell signaling) and, for nuclear fractions used mouse anti-PARP (Calbiochem) and goat polyclonal anti-H2B antibody (Santa Cruz). These primary antibodies were incubated with horse-radish peroxidase conjugated specific secondary antibodies. The signals were developed by using Supersignal west pico chemiluminescent substrate (Thermo scientific).

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

Proliferation assay: Cellular proliferation was determined by Alamar blue reduction assay method (Ab Serotec) according to manufacturer instructions. Briefly, MiaPaCa2 cells (5000 cells / well) were incubated with 13-197 in triplicate in a 96-well plate and then incubated for 24, 48, 72 and 96 h at 37°C. Alamar blue solution was added to each well (equal to 10% volume of the medium in the well) and incubated for 3 h at 37°C. The fluorescence of the solution was measured at 560nm excitation and 590nm emission using spectramax⁵⁶ fluorescence plate reader. Dose response curves were evaluated using the NCI algorithm:  

\[ T_z = \text{number of control cells at time } t_0, \ C = \text{number of control cells at time } t, \ T = \text{number of treated cells at time } t; \ 100*([T-T_z]/[C-T_z]). \]
experiment was repeated three times and statistical analysis was performed using unpaired Student’s t-test.

**Cell cycle analysis:** To determine the effect of 13-197 on the cell cycle progression, cell cycle analyses was carried out using cell Genescript kit according to manufacturer instruction. MiaPaCa2 cells (1x10^6) were plated in 100mm dish and serum starved for 24 h. Subsequently cells were treated with 13-197 (11 µM) in 0.1% DMSO and incubated for 24 h. Cells were trypsinized, washed in PBS and permeabilized with 70% ethanol for 1 h at 4ºC. The cell pellet was resuspended in PBS containing ribonuclease A at 37ºC for 30 min and stained with propidium iodide (50 µg/mL) for 30 min on ice. The DNA content was determined by flow cytometer (flow cytometry core facilities at UNMC). The experiment was repeated three times and the mean ± S. D. are represented and Student’s 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 protocol. Briefly, MiaPaCa2 cells were grown on cover slips and incubated with 13-197 (11 µM) in 0.1% DMSO for 24 h. 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 ± S. D. from three experiments and Student’s t-test was used to determine the significance.

**In vitro migration assay:** Migration assay was performed by using polyethylene teraphthalate (PET) transwell inserts as described previously (27). Briefly, MiaPaCa2 cells (1x10^6) 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 of 13-197 in 0.1%
DMSO. DMEM supplemented with 10% FBS was added to the bottom of each well. After 24 h 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 200X magnification). The results are from three 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 (1x10⁶) 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 13-197. DMEM supplemented with 10% FBS was added to the bottom of each well. After 24 h 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 x200). The results are from three independent experiments and represented as mean number of cells migrated per field.

**Pharmacokinetic studies:** Eight-week-old, healthy male C57/BL6 mice were purchased from Charles River Laboratories (Wilmington, MA). Sterilized 7012 Teklad diets (Harlan, Madison, WI) were used for mice, and water was provided ad libitum. In mice, 150 mg/kg oral dose was administered as a 75 µl of 50 mg/ml in cremaphor EL. At each of the following seven time points (0.5, 1, 2, 8, 24, 48 and 72hr) three 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 1500 × g for 10 min at 4°C within 1 hr of sample collection. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was
used for sample analysis. A hybrid ion trap-triple quadrupole (Q-Trap) MS was used to quantify all analytes by multiple reaction monitoring (MRM). For sample preparation, 1 ml of ice-cold ACN was added to 100 µl plasma samples pre-spiked with 4.0 µg/ml IS (EFV). Samples were then vortexed, and centrifuged at 16,000 × g for 10 min. The supernatant was aspirated, evaporated under vacuum, and reconstituted in a 100 µl 75% methanol. After centrifugation at 16,000 × g for 10 min, 10 µl 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). 100 µl aliquots of tissue homogenates were spiked with 10 µl of 4.0 µg/ml IS (EFV) and were extracted and analyzed similarly to plasma samples. The pharmacokinetic parameters were determined using the extravascular input non-compartmental analysis module of WinNonlin (version 1.5, Pharsight, Mountain View, CA). The absolute bioavailability (F) was calculated as the ratio between the AUC\(_{0-\infty}\) from oral and intravenous routes, after dose normalization using the following equation:

\[ \% F_{\text{oral}} = \frac{\text{AUC}_{\text{oral}} \times \text{Dose}_{\text{oral}}}{\text{AUC}_{\text{iv}} \times \text{Dose}_{\text{iv}}} \times 100 \]

**Biochemical toxicity assay:** To assess toxicity of 13-197 in animals, we analyzed leakage of alanine aminotransaminase (ALT) and aspartate aminotransaminase (AST) enzymes in the plasma. The plasma was collected from control and 13-197 treated animals using lithium heparin tubes (BD Microtainer). The enzyme assay was performed by using ALT and AST kit (Beckman coulter) according to manufacturer instructions (Regional Pathology Services at UNMC).
Leukocytes count and LPS challenge: BALB/c mice (National Cancer Institute, Frederic, MD, USA) were treated with 13-197 (150 mg / kg body weight, n=5) and vehicle control (Cremophor, n=5) p.o twice for four days. After 4th 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 performed by counting 100 leukocytes in randomly selected fields of view on blood smears. The mice were then challenged with LPS (30 mg / kg) i. p. and monitored for 72h.

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 (0.5x10^6 in 30µl) were injected into the pancreas using a 27 gauge needle. The abdomen was closed using two-layer suture with chromic catgut and ethilon suture. After 2 weeks of implantation, mice were randomized into two groups, 1) untreated control (cremophor, 100 µl p.o. daily) and 2) 13-197 treatment (150mg/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 for tumor weight and volume (V=π/6 (a × b × c), where a, b and c denotes length, width and height of the tumor mass). The tumor weight and volume were compared between the groups using unpaired Student’s t-test. Half portion of tumor tissue was formalin fixed and paraffin embedded for immunohistochemistry. The other half portion was snap frozen in liquid nitrogen and stored at -80ºC. Metastasis to other organs were examined, excised and stored at -80ºC.
**Proliferation index:** Formalin fixed, paraffin-embedded sections (5 µm) were stained with rabbit anti-Ki-67 antibody (neomarkers) as described previously (29). Results were expressed as percentage of Ki-67 positive cells ± SE per original magnification x400. A total of 5 fields were examined and counted from five tumors of control and treated groups. The values were subjected to one-way ANOVA and unpaired Student’s t-test.

**Microvessel density:** The pancreas tissue sections (5µm) from vehicle and 13-197 treated animals were stained with rabbit anti-CD31 antibody (Abcam) as described previously (29). The vessel density from area of interest were examined under microscope (original magnification x400) and counted. Any distinct area of positive staining for CD31 was counted as a single vessel. A total of 5 different fields from five different animals in each group were examined. The mean number of vessels ± SE per filed were obtained by using one-way ANOVA and unpaired Student’s 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 instructions (Leica Microsystems Inc., Buffalo Grove, IL, USA). Briefly, the pancreatic tumor tissue sections were incubated with the following primary antibodies Rabbit anti-Bcl-xL antibody (Cell Signaling Technology, Inc., Danvers, MA, USA), Rabbit anti-Mcl-1 antibody (Santa Cruz biotechnology, Inc., Santa Cruz, CA, USA), Rabbit anti-phospho-S6 Ribosomal protein (Ser 235/236) antibody (Cell Signaling Technology, Inc., Danvers, MA, USA). Three different pancreatic tumor tissues were used for each antigen. Pictures were captured by using iScan Coreo slide scanner (Ventana Medical Systems Inc., Tucson, AZ, USA). A semi-quantitative approach was
used to score the percentage of positive cells, +, <25% cells positive; ++, 25–50% cells positive; ++++, 50–75% cells positive; +++, 75-100% cells positive.

**Data processing and statistical analysis:** P-values were determined using one-way ANOVA in Sigma Plot and values < 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 quinoxaline analogs against a panel of cancer cell lines led to the identification of 13-197, which inhibited cell growth with low-μM 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. Since 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) from the mean of the screen. Results from the follow up 10-point dose response study are summarized in Figure 1A. The IC₅₀ values were in the low-μM range for four (TRKA, IKKβ, EPHA6 and Nek3) of the six 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 μM and 3.0 ± 0.1 μM respectively. Although TRKA is primarily localized to the brain, both TRKA and IKKβ are activated by cytokines (IL-1 and TNFα), which promote NF-κB mediated gene expression (33, 34).

TNFα stimulation regulates transcription via the p-IKKβ / p-IκBα / NF-κB axis and translation via the p-IKKβ / mTOR / p-S6K / p-elF4EBP axis (Figure 1B) (16, 35). TNFα 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 IκBα, S6K and elF4EBP1
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 IκBα levels (Figure 1C). 13-197 robustly inhibited phosphorylation of IκBα in the panel of pancreatic cancer cell lines and inhibited phosphorylation of IκBα in MiaPaCa2 cells in a dose- and time-dependent manner (Figure 1C). Inhibition of IκBα should result in reduced nuclear localization of p65, to test this we subjected MiaPaCa2 and Capan2 cells to 13-197 for 2h and probed the nuclear fraction of the lysate for p65 and PARP/H2B (Figure 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 2h followed by TNFα for an additional 4h. Under multiplexing conditions the cells were assayed for viability using Alamar Blue and the NF-κB transcription activity using the ONE-Glo luciferase system (Figure 1D). The results show that 13-197 inhibits TNFα stimulated NF-κB mediated luciferase expression with an IC$_{50}$ value of 8.4 ± 1.2 μM. 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 eIF4EBP1 treated with 13-197 for 4 and 24h (Figure 1C). The results showed decreased phosphorylation of S6K and eIF4EBP1. In summary the data suggests 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 μM potencies (Table S1). To evaluate the time dependent effects of 13-197, MiaPaCa2 cells were incubated with 13-197 (IC$_{50}$ = 11.4 ± 0.7 μM) and cell growth was monitored over time for 96 h (Figure 2A) (26). The results demonstrate 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 (Figure 2B). Consistent with the G1 arrest we observed reduced levels of the corresponding cell cycle markers E2F, PCNA and Cyclin D1 (Figure 2B) (36). Together the data demonstrate that 13-197 inhibits pancreatic cancer cell growth by inducing G1 arrest of cells.

13-197 down regulates 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) (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 to Bcl-xL, XIAP and Survivin at the 2h time point. However, longer incubation times at 20 μM shows a decrease in all proteins (Figure...
It is known the 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 is probably controlled by IKKβ directly while the others (Bcl-xL/XIAP/Survivin) are down regulated at the transcriptional level due to NF-κB inhibition. Consistent with the down regulation of antiapoptotic proteins, cells treated with 13-197 showed a 4-fold increase in the percentage of apoptotic cells when compared to untreated cells (Figure 2C). Together these data showed that 13-197 down regulates 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, ICAM-1 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% (Figure 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 (PK) difficulties account for more than 50% of drug development failures preventing new chemical entities (NCEs) 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 towards the biological target of interest, PK considerations have been moved to early stages of drug discovery, a significant paradigm shift in the pharmaceutical industry (42).
Lipinski’s rule of five 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 P (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 cremaphor EL, which is a commonly used excipient in drugs for PK 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 liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Figure 3A and 3B). The PK properties of 13-197 are described as an inset in Figure 3A and the tissue levels as an inset in Figure 3B. Although low (~ 5%) the results show that 13-197 is orally available, and demonstrates 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**

Since 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 h 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. Since the PK studies showed clearance through the liver the levels of
Alanine transaminase (ALT) and aspartate transaminase (AST), the plasma biochemical markers for hepatotoxicity were measured (44). Leakage of ALT and / or AST into the bloodstream 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 MTD for 13-197 is > 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 to control mice (Table S2). More importantly we observed a protective effect from LPS-induced mortality in mice pre-treated with 13-197 (Figure 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 TNFα stimulation. In the control experiment HEK293 cells were treated with DMSO followed by TNFα stimulation. This resulted in rapid phosphorylation of IκBα (5 and 10 min) and at 30 min post stimulation the absence of IκBα indicates phosphorylation mediated proteasomal
degradation. Re-expression of \( \text{IkB} \alpha \) at 60 and 120 min post stimulation suggests feedback inhibition of the pathway (\( \text{IkB} \alpha \) is a target gene of \( \text{NF-\kappaB} \)) (45). In the presence of 13-197 we observed modest inhibition of \( \text{IkB} \alpha \) phosphorylation and degradation at the early time points (5 and 10 min) at the same time points TPCA1 treatment resulted in complete inhibition. However, at the 60 and 120 min time points we observes a significant reduction in phospho-\( \text{IkB} \) levels (last two lanes of the gel) when compared to DMSO treated lanes (box, Figure 4B). This suggests that 13-197 does not inhibit the transient activation of IKK\( \beta \) but inhibits IKK\( \beta \) post activation which is probably found under sustained stimulation. The inhibition by 13-197 is unlike other known IKK\( \beta \) inhibitors such as TPCA1 (Figure 4C) that completely blocks TNF-\( \alpha \) induced phosphorylation of \( \text{IkB} \alpha \) (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 non-toxic and orally available, we evaluated its ability to reduce tumor growth and metastases in 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 tumors weights and
volumes were measured. We observed ~50% reduction in both the tumor weight and volume in the 13-197 treated animals compared to vehicle treated animals (Figure 5A). We also found fewer tumor nodules in other organs of 13-197 treated animals compared to vehicle treated animals (Table S3). The \textit{in vivo} effects are consistent with the \textit{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 Ki67 and CD31 in 13-197 treated tumors compared to vehicle controls (Figure 5B and 5C). 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 to vehicle treated tumors (Figure 6A and Table S4). This suggests that 13-197 perturbs both the NF-κB and mTOR signaling \textit{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 (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 (Figure 6B). Consistent with our PK 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 to vehicle treated mice, indicating the absence of hepatotoxicity (Figure 6C). We also conducted a macroscopic examination of the organs and found no obvious toxicity in the 13-197 treated animals.
Conclusion

The data shows 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 LPS-induced mortality. The later 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.

Acknowledgements

This work was supported in part by Eppley Cancer Center Pilot grant, UNeMed EI award, and NIH grants (CA009476, CA127239 and CA127297).
References


Figure legends

Figure 1. Kinome screen with 13-197 and mechanistic characterization in pancreas cancer cell lines (A) Chemical formula for quinoxaline urea analog, 13-197. A $^{32}$P-radiolabeled kinase assay profiling to identify the molecular target of 13-197. The bar graph shows the IC$_{50}$ values derived from a follow up 10-point dose response curve of six kinases. (B) A model for the TNF$\alpha$ induced regulation of NF-$\kappa$B and mTOR pathways by IKK$\beta$. (C) A panel of pancreatic cancer cell lines were treated with 20 $\mu$M 13-197 for 2h and the lysates were probed for p-I$\kappa$B$\alpha$. MiaPaCa2 cells were treated with 13-197 (0, 10 and 20 $\mu$M) for 2h (left) and at 20 $\mu$M for the indicated times (right) and probed for p-I$\kappa$B$\alpha$. Nuclear lysates of MiaPaCa2 cells treated with 12 $\mu$M of 13-197 for 2h probed for p65 and PARP levels. MiaPaCa2 cells were treated with 20 $\mu$M 13-197 for 4h and 24h. The cell lysates were probed for p-S6K and p-eIF4EBP1. (D) Inhibition of TNF$\alpha$ stimulated NF-$\kappa$B mediated transcription by 13-197.

Figure 2. Cellular characterization of 13-197 in pancreas cancer cell lines (A) MiaPaCa-2 cells were incubated with 11 $\mu$M of 13-197 and viability measured at indicated times. (B) MiaPaCa2 cells were treated with 11 $\mu$M 13-197 for 24h followed by cell cycle analyses (n = 3). Cell lysates were probed for cell cycle markers E2F, PCNA and Cyclin D1. *P < 0.05, **P < 0.005, ***P < 0.005. (C) MiaPaCa2 cells were treated with 13-197 at the indicated doses 2h and lysates probed for Mcl-1, Bcl-xL, XIAP and Survivin (left). MiaPaCa2 cells were treated with 20 $\mu$M of 13-197, lysates generated at the indicated time points and probed for Mcl-1, Bcl-xL and Survivin (right). MiaPaCa2 cells were treated with 11 $\mu$M of 13-197. After 24 h the cells were subjected to a live/dead assay (n=3). (D) Invasion of cells through matrigel coated microporous polycarbonate membrane was measured in the presence and absence of 13-197 (n=3). Transwell migration of cells in the presence and absence of 13-197 was measured after 24 h incubation (n = 3). **P<0.005 and ***P<0.0005

Figure 3. Pharmacokinetics in mice with 13-197 (A) Plasma levels of 13-197 in mice after oral administration. 13-197 concentrations in plasma are expressed as mean values (± SEM) at the 7 sampling times (0.5, 1, 2, 8, 24, 48, and 72 h; n=3). Non-compartmental pharmacokinetic parameters are summarized as an inset. (B) Tissue distribution of 13-197 in kidney, brain, lung, spleen, liver, and heart following oral administration of 150 mg/kg in mice (data is expressed as mean ± SEM, n=3). The area under the curve (AUC) for each tissue is described in the inset.

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**Figure 4. Toxicity studies in mice with 13-197** (A) Mice were either treated with vehicle (n = 5) or 13-197 (n = 3) at 150 mg/Kg twice daily for four days and challenged with 30 mg/Kg LPS. (B) The effect of 13-197 on the IκB phosphorylation and degradation kinetics in response to TNF-α stimulation in HEK293 cells. (C) Inhibition of TNF-α induced IκB phosphorylation by TPCA1 in HEK293 cells.

**Figure 5. Tumor growth and metastases with 13-197** (A) Nude mice with orthotopic MiaPaCa2 tumors were treated with 13-197 (150 mg/Kg) or vehicle orally daily for 30 days, the bar graph represents tumor weights and volumes at the end of the study. **P < 0.05. (B) Ki67 staining as a measure of proliferation index in tumor tissue. **P < 0.05. (C) CD31+ staining as a measure of microvessel density. **P < 0.05.

**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 18h after the last treatment. (C) Liver enzymes (AST and ALT) measured at the end of the study.
Figure 2

A) % MiaPaCa2 cell growth

B) % MiaPaCa2 Cells

C) 13-197 (μM) Mcl-1, Bcl-xL, XIAP, Survivin, Tubulin

D) No. of cells/field

E2F, PCNA, CyclinD1, H2B

G1, G2, S
Figure 3

A

- $T_{\text{max}}$ (h) = 1
- $C_{\text{max}}$ (ng/mL) = 386.3
- $K_{\text{a1}}$ (h$^{-1}$) = 0.14
- $\text{t1/2}$ (h) = 4.8
- $\text{Cl}$ (L/Kg/h) = 57.4
- $V_d$ (L/Kg) = 401.1
- AUCblood (ng/mL*h) = 2612.6

B

- AUC (ng/g*h)
  - Kidney = 71403
  - Brain = 556
  - Lung = 19750
  - Spleen = 14324
  - Liver = 99487
  - Heart = 26542
Figure 4
Figure 5
Figure 6
Targeting the NF-κB and mTOR pathways with a quinoxaline urea analog that inhibits IKK β for pancreas cancer therapy

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*Clin Cancer Res* Published OnlineFirst February 26, 2013.