Characterization and target identification of non-toxic IKKβ inhibitors for anticancer therapy

Elizabeth Blowers
University of Nebraska Medical Center

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CHARACTERIZATION AND TARGET IDENTIFICATION OF NON-TOXIC IKKβ INHIBITORS FOR ANTICANCER THERAPY

by

Elizabeth Coultas Blowers

A DISSERTATION

Presented to the Faculty of
the University of Nebraska Graduate College
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

Cancer Research Graduate Program

Under the Supervision of Professor Amarnath Natarajan

University of Nebraska Medical Center,
Omaha, NE

June, 2015

Supervisory Committee:
Jixin Dong, Ph.D.  Mayumi Naramura, M.D.  Rob Lewis, Ph.D.
Dedication

I would like to take this moment to express my gratitude to everyone who has supported me throughout my graduate career. Without your patience and assistance, this dissertation would not exist.

To begin, I would like to thank my graduate advisor Dr. Amar Natarajan for his constant guidance and mentorship throughout my graduate education. I have learned a great deal from him and our discussions continually influence how I think about and conduct research. Most importantly, his unwavering optimism has served as an ever-present reminder that tomorrow is another day to tackle the world’s problems.

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Additionally, I would like to thank the following researchers for their contributions to the following figures:

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CHARACTERIZATION AND TARGET IDENTIFICATION OF NON-TOXIC IKKβ INHIBITORS FOR ANTICANCER THERAPY

Elizabeth C. Blowers Ph.D.

University of Nebraska, 2015

Advisor: Amarnath Natarajan, Ph.D.

IKKβ is a key kinase in the canonical NF-κB pathway. Transient activation of IKKβ is required for normal immune response while sustained activation has been implicated in cancer. To date, no IKKβ inhibitor has been approved for clinical use as a chemotherapeutic, likely due to unexpected toxicities associated with blocking the transient activation of IKKβ. We hypothesized that an IKKβ inhibitor that allows transient activation of IKKβ, but not sustained activation of IKKβ, could inhibit cancer growth without inducing toxicity.

As such, we identified a small molecule inhibitor, 13-197, that allows the transient activation of IKKβ but not the sustained activation of IKKβ. 13-197 inhibits cancer growth without inducing toxicity. Structure activity relationship studies (SAR) were conducted with 13-197 analogs to determine substituents key for activity against IKKβ-mediated NF-κB activity and the anticancer effects of 13-197 were studied in pancreatic cancer cells. 13-197 inhibited NF-κB pathway proteins and decreased the expression of anti-apoptotic proteins.

To further explore options for the development of non-toxic IKKβ inhibitors, 25-4 was designed to feature the α-methylene-γ-butyrolactone targeting motif of the IKKβ and NF-κB inhibitor parthenolide. Parthenolide is active against IKKβ and NF-κB in cell-based studies but the drug has not been approved as an anticancer treatment despite its effects in cells and lack of toxicity in clinical trials. SAR studies with 25-4 identified structural features key to the small molecule’s activity and click chemistry with a 25-4 analog verified
the molecule’s activity towards IKKβ and NF-κB. Cell-based studies with 25-4 demonstrate the molecule’s anticancer activities, such as growth inhibition, inhibition of colony formation and induction of apoptosis. Furthermore, 25-4 synergized with the clinical chemotherapeutic cisplatin to inhibit the viability of cancer cells. Future studies will elucidate the mechanistic basis for non-toxic inhibition of IKKβ by 25-4 and will focus on improving the drug-like properties of the small molecule.

Overall, these studies demonstrate that IKKβ is a viable target for anticancer treatment and establish 13-197 and 25-4 as novel inhibitors of IKKβ with IC₅₀s comparable to known IKKβ inhibitors and synergistic activity with clinically-relevant chemotherapeutics.
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SAR Structure activity relationship
TNFα Tumor necrosis factor alpha
NF-κB Nuclear factor kappa B
IκBα inhibitor of nuclear factor κB
IKKβ IκB kinase β
DNA deoxyribonucleic acid
RNA ribonucleic acid
siRNA small interfering RNA
shRNA small hairpin RNA
mRNA messenger RNA
RNAi RNA interference
MyD88 Myeloid differentiation primary response gene 88
ICAM Intercellular adhesion molecule 1
LPS Lipopolysaccharide
TLR Toll-like receptor
PARP Poly (ADP-ribose) polymerase
FBS Fetal bovine serum
HEK Human embryonic kidney cells
DMEM Dulbecco’s modified Eagle’s medium
RPMI Roswell Park Memorial Institute-1640 medium
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
EDTA Ethylenediaminetetraacetic acid
DTT Dithiothreitol
SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis
HNTG HEPES, NaCl, Triton X-100, Glycerol

PBS Phosphate-buffered saline

PVDF Polyvinylidene difluoride

GAPDH Glycereraldehyde 3-phosphate dehydrogenase

TCEP Tris(2-carboxyethyl)-phosphine hydrochloride

TBTA Tris[(1-benzyl-1-H-1,2,3-triazol-4-yl)methyl]-amine

TEA Triethanolamine

RIPA Radioimmunoprecipitation assay

DMSO Dimethyl sulfoxide

XIAP X-linked inhibitor of apoptosis

IL-1 Interleukin 1

PMA Phorbol myristate acetate

CD Cluster of differentiation

RHD Rel homology domain

TA Transactivation

ANK Ankyrin

PEST Proline, glutamate, serine and threonine-enriched

KD Kinase domain

ULD Ubiquitin-like domain

SDD Scaffold and dimerization domain

NBD NEMO-binding domain

CC Coil coil domain

LZ Leucine zipper

Z Zinc-finger

E2, E3 enzyme 2 or enzyme 3

βTrCP beta-transducin repeat containing E3 ubiquitin protein ligase
SCF Skp1-Culin-Roc1/Rbx1/Hrt-1-F-box family
FADD Fas-associated protein with death domain
TRADD TNFR-associated protein with a death domain;
TRAF TNFR-associated factor
cIAP Cellular inhibitor of apoptosis
ubiq. Ubiquitin
TGFβ Transforming growth factor beta
TAK1 TGFβ-activated kinase 1
TAB TAK1-binding proteins
NIK NF-κB-inducing kinase
MOD Minimal oligomerization domain
SODD Silencer of death domain
TSC Tuberous sclerosis complex
NES Nuclear export sequence
NLS Nuclear localization sequence
BAFF B-cell activating factor
VEGF Vascular endothelial growth factor
ER Estrogen receptor
EMSA Electrophoretic mobility shift assay
IPMN Intraductal papillary mucinous neoplasm
PanIN Pancreatic intraepithelial neoplasia
PDAC Pancreatic ductal adenocarcinoma
HPV Human papilloma virus
hTERT Human telomerase reverse transcriptase
ATP Adenosine triphosphate
CIN Cervical intraepithelial neoplasia
mTOR Molecular target of rapamycin
RHEB Ras homolog enriched in brain
GTP Guanosine triphosphate
GDP Guanosine diphosphate
GTPase Guanosine triphosphatase
MEF Mouse embryonic fibroblast
PPM1A Protein Phosphatase, Mg2+/Mn2+ Dependent, 1A
PPM1B Protein Phosphatase, Mg2+/Mn2+ Dependent, 1A
PP2A Protein phosphatase 2 A
PP2C Protein phosphatase 2 C
SUMO Small ubiquitin-related modifier
A20 Tumor necrosis factor, alpha-induced protein 3
SOCS-1 suppressor of cytokine signaling 1
PDLIM2 PDZ and LIM Domain 2
Mdm-2 Mouse double minute 2 homolog
FOXO3a Forkhead box O3
E Embryonic
Akt Protein kinase B
EPHA6 ephrin type-A receptor 6
MAP4K4 mitogen-activated protein kinase kinase kinase kinase 4
Nek3 never in mitosis A-related kinase 3
STK25 serine/threonine protein kinase 25
TrkA tropomyosin receptor kinase A
IC Intracellular Concentration
Chapter 1: Background and Significance

Introduction to the NF-κB pathway

Nuclear factor kappa B (NF-κB) is a transcription factor that plays a key role in innate and adaptive immune responses, inflammation, cell growth and apoptosis. This pathway is activated by a myriad of stimuli such as tumor necrosis factor alpha (TNFα), interleukin 1 (IL-1), phorbol myristate acetate (PMA), ultraviolet radiation and stress to name a few. In unstimulated cells, dimers of NF-κB are sequestered in the cytoplasm by inhibitor of nuclear factor κB (IκB). In cells stimulated with the pro-inflammatory cytokine TNFα (Figure 1), IκBs are phosphorylated by the inhibitor of κB kinase (IKK) complex, polyubiquitinated, and degraded by the 26S proteasome, allowing NF-κB dimers to enter the nucleus and regulate transcription. The NF-κB pathway controls the expression of hundreds of genes, including genes encoding cytokines and chemokines, immunoreceptors, acute phase proteins, cell adhesion molecules, regulators of apoptosis and many transcription factors, among others. The chemokines and cytokines regulated by NF-κB include interferon gamma, many of the interleukins and TNFα to name a few. Immunoreceptors include CD80, which is necessary for co-stimulation of T cells, IgG and IgE heavy chains and the immunoglobulin light chain κ for which the transcription factor is named. The cell adhesion molecules regulated by NF-κB include fibronectin, ICAM-1 and P-selectin. Acute phase proteins

Figure 1: TNFα induces IKKβ-mediated NF-κB-driven transcription. Here, NF-κB pathway activation is driven by the pro-inflammatory cytokine TNFα and leads to the transcription of a κB DNA element-containing promoter.
include LPS binding protein\textsuperscript{16} and complement factor C4, which activates the extrinsic pathway of complement activation.\textsuperscript{19} Apoptotic genes controlled by NF-\kappa B include both pro-apoptotic and pro-survival proteins such as Bax,\textsuperscript{20} Bcl-xL,\textsuperscript{21} Bcl-2\textsuperscript{22}, Mcl-1,\textsuperscript{23} survivin,\textsuperscript{24} and X-linked inhibitor of apoptosis (XIAP).\textsuperscript{25} Important transcription factors regulated by NF-\kappa B include p53,\textsuperscript{26} c-myc,\textsuperscript{27} A20,\textsuperscript{28} c-fos,\textsuperscript{29} relb,\textsuperscript{30} c-rel,\textsuperscript{31} I\kappa B\alpha,\textsuperscript{32} nfkB1\textsuperscript{33} and 2.\textsuperscript{34} Many of these transcription factors actually regulate the activity of NF-\kappa B itself and serve to negatively regulate the pathway’s activity. Though very abridged, the list of NF-\kappa B-regulated genes given above demonstrates the importance of the tight regulation of this transcription factor in physiological and diseased states. As so many key processes are controlled by this pathway, improper or chronic activation of the pathway results in many diseases, including inflammatory diseases, immunodeficiency, autoimmunity and cancer.\textsuperscript{35}

The NF-\kappa B family of proteins

The NF-\kappa B family of proteins is comprised of five members including p65 (RelA), RelB, c-Rel and NF-\kappa B1 (p105) and NF-\kappa B2 (p100), which are proteolytically processed to p50 and p52, respectively. Each member of the family forms homo- or heterodimers and share a Rel homology domain (RHD) which facilitates dimerization and binding to \kappa B DNA elements. In resting cells, p65, RelB and c-Rel are bound to the inhibitory I\kappa Bs, which associate with the DNA-binding domains of the NF-\kappa B family members and yield them transcriptionally inactive. The pro-forms of p50 and p52, on the other hand, contain ankyrin repeats similar to those in the I\kappa Bs that serve as inhibitors of nuclear translocation. Unlike the other NF-\kappa B family members, p50 and p52 lack transactivation domains and thus serve as repressors of transcription unless bound to p65 or RelB.\textsuperscript{36} The domain architecture of the prototypical NF-\kappa B subunit p65 can be seen in Figure 2. Once free, NF-\kappa B dimers translocate to the nucleus where they may bind to promoter or enhancer regions containing the \kappa B consensus sequence 5’ GGGRNWYCC 3’ (where N represents any
base, R represents any purine, W represents an adenine or thymine and Y represents a pyrimidine). The κB site sequence, however, is quite degenerate and different NF-κB dimers show binding preferences for different sites leading to transcription of particular genes by specific dimers. The crystal structures of NF-κB dimers reveal that an Ig-like domain located in the N-terminus of NF-κB is responsible for the selectivity towards particular κB sites. Though 15 possible dimers have been shown to exist, the p50/p65 dimer is the most common dimer and is present in nearly all cell types.

The IκB family of proteins

The IκB family of proteins contains six members including IκBα, IκBβ, IκBε, IκBγ, Bcl-3 (B-cell lymphoma 3) and IκBζ. IκBα, IκBβ and IκBε are considered typical IκB proteins (along with the precursor proteins p105 and p100, which can be grouped as NF-κB or IκB family members) and serve to retain NF-κB dimers in the cytoplasm. Degradation of the typical IκBs occurs once the protein has been phosphorylated on two serine residues within the consensus sequence (DS*GXXS*) by the kinase IKKβ. For IκBα, which is the IκB that will be discussed in this dissertation, the phosphorylation occurs on residues 32 and 36. This phosphorylation event leads to recognition by βTrCP proteins.
and K48-linked polyubiquitination by the Skp1-Cul1-Roc1/Rbx1/Hrt-1-F-box family of E3 ligases (SCFβTrCP-E3 ligase complexes) and the E2 UbcH5 and subsequent degradation in the 26S proteasome, which consequently allows NF-κB dimers to translocate to the nucleus. Dimers composed of p65 and p50 are primarily associated with IκBα. Structural analysis of the complex composed of IκBα, p65 and p50 revealed that IκBα hides the nuclear localization sequence (NLS) of p65 but does nothing to mask the NLS of p50. As such, the balance between the NLS of p50 and the nuclear export sequence (NES) of IκBα results in shuttling of the complex between the nucleus and cytosol, though localization of the complex appears to be cytosolic. IκBα phosphorylation, ubiquitination and degradation, however, shifts the equilibrium of the complex towards the nucleus by eliminating the NES in IκBα and exposing the NLS of p65. IκBα is a better substrate for IKKβ than IκBβ or IκBε resulting in its rapid degradation.

IκBβ and IκBε are degraded more slowly than IκBα or not at all. Both IκBs are degraded and resynthesized upon stimulation of the NF-κB pathway but their kinetics differ from that of IκB and their roles in NF-κB signaling are still poorly understood. For example, deletion of IκBβ has no effect on the kinetics of NF-κB signaling but knocking IκBβ in to replace IκBα also does not affect the kinetics of the pathway, suggesting that resynthesis of the two proteins plays a major role in the cellular effects.

The atypical members of the IκB family include Bcl-3 and IκBζ and their roles are still not well understood. Bcl-3 features a transactivation domain and has been found in the nucleus in complex with p50 and p52 homo-dimers. In the case of p50, Bcl-3 may relieve transcriptional repression by removing p50 homodimers from κB elements. On the other hand, Bcl-3 may also stabilize p50 homodimers and prevent access of other TAD-containing NF-κB dimers to κB elements. With p52, Bcl-3 may potentiate transcriptional activation, particularly of Cyclin D1. IκBζ is not constitutively expressed but is up-regulated
in response to IL-1 and TLR4 ligands, but not TNFα and thus will not be discussed at length here.

As more data regarding the function of the IκB family members becomes available, it is increasingly clear that these proteins can no longer be considered only inhibitors of NF-κB signaling. Nevertheless, the main IκB discussed in this dissertation will be IκBα and its only important role for this work will be its binding to and prevention of translocation of p65/p50-containing heterodimers.

The IKK complex and canonical (IKKβ-dependent) NF-κB signaling

The IKK complex is a 550-900 kDa complex composed of IKKα (85 kDa), IKKβ (87 kDa), and IKKγ, which is also referred to as NEMO (48 kDa). The complex was originally identified by immunoprecipitation and anion exchange chromatography of fractionated whole cell extracts from HeLa cells that had been stimulated with TNFα. IKKα and IKKβ were subsequently established as catalytic subunits while NEMO was determined to be a regulatory subunit. IKKα and IKKβ share 51% sequence identities and are highly homologous in their kinase domains. A main structural difference between the two is the presence of an ubiquitin like domain (ULD) on IKKβ that is not predicted in IKKα. Furthermore, IKKα contains a nuclear localization sequence that is not present in IKKβ. NEMO, on the other hand, is structurally unrelated to IKKα and IKKβ and is composed of two coil-coil domains, a leucine zipper, a zinc finger domain and a minimal oligomerization domain (MOD) that may allow NEMO to oligomerize.

Though it has not been conclusively proven and the discrepancy between the molecular weights of the core components of the IKK complex and the observed molecular weight of the purified complex has caused much debate over the years, it is currently believed that the active IKK complex is composed of a tetramer of NEMO subunits that brings an IKKα/β heterodimer together. However, IKKβ activation of the NF-κB pathway
in cells lacking IKKα is clear evidence that homodimers also interact with NEMO.\(^1\) IKKα and IKKβ bind NEMO via a C-terminal NEMO binding domain (NBD). The regulation of the kinase activity associated with the IKK complex is not fully understood. NEMO-induced oligomerization is believed to induce a conformational change in IKKα/β heterodimers. IKKα/β heterodimers are then activated by upstream kinases such as TAK1 or trans-autophosphorylation of T-loop serine residues (177 and 181 of IKKβ) for canonical pathway activation.\(^45\) Dimerization of IKKα/β monomers is known to play a role in the activation or activity of the kinase. For example, dimerization mutations L654D/W655D and W655D/L658D exhibit complete or partial loss of activation in a manner that correlates with their degree of impairment in dimerization and failure to interact appropriately with NEMO. Additionally overexpression of IKKβ has been shown to result in activation. More recent studies suggest that IKKβ may exist as a dimer of dimers in cells and that neighboring dimers may be able to autophosphorylate one another to drive activation\(^46\) or an active kinase from a protomer in a neighboring dimer may activate an inactivate protomer in a separate dimer without forming a true tetramer.\(^47\)

In any event, it is widely accepted that the canonical NF-κB pathway is activated by inflammatory cytokines, such as TNFα, pathogen-associated molecules or antigen receptors. For this pathway to function properly, IKKβ activation is both necessary and sufficient and leads to the phosphorylation of IκBα in a NEMO-dependent manner. The noncanonical pathway is dependent on IKKα and is activated by BAFF, lymphotoxin-B or CD40 ligand, the upstream kinase NIK (NF-κB-inducing kinase) and phosphorylation of p100.\(^38\) As this dissertation focuses on the activation of the NF-κB pathway by IKKβ in response to the pro-inflammatory cytokine TNFα, the non-canonical pathway will not be discussed further.
Once activated by phosphorylation of its T-loop serine residues, IKKβ exhibits high activity and phosphorylates its substrate, IκBα, and autophosphorylates several C-terminal serine residues. The autophosphorylation results in decreased IKKβ kinase activity probably through another conformational change in the catalytic domain.\textsuperscript{48} The phosphorylated C-terminal serine residues provide binding sites for phosphatases such as PPM1A, PPM1B\textsuperscript{49} and PP2A\textsuperscript{50} that may dephosphorylate the T-loop serine residues. Another mode of regulation is through the phosphorylation of serine 740 in the NBD, which leads to the dissociation of the regulatory subunit NEMO. This also allows phosphatases to dephosphorylate the T-loop serine residues.\textsuperscript{2}

How else NEMO may regulate the activity of the IKK complex is under investigation. Mutation of the MOD of NEMO may hamper activation of NF-κB. Additionally, binding of NEMO to chains of polyubiquitin may also be key to activation of the IKK complex and many groups have demonstrated NEMO binding to upstream kinases with polyubiquitin chains, suggesting that NEMO may play a role in IKKβ activation by directing the IKK complex to upstream kinases. NEMO may also activate IKKβ in response to nuclear stimuli such as DNA damage. NEMO, free in the nucleus, may become SUMOylated and may then be exported to the cytoplasm where it can activate IKKβ and the NF-κB pathway. Furthermore, NEMO may also negatively regulate IKKβ activity through interaction with phosphatases (PP2A and PP2C) and deubiquitinases (CLYD and A20).\textsuperscript{1}

**Signaling upstream of the IKK complex**

Signaling upstream of the IKK complex involves many adaptor proteins and likely an upstream kinase (Figure 3). Following binding of TNFα, the cytoplasmic tail of TNFR1 (TNFα receptor 1) recruits a series of proximal adapter proteins through its death domain. TRADD (TNFR-associated protein with a death domain) and RIP1 (receptor-interacting
protein 1) bind to the death domain of TNFR1 through their own death domains though the mechanism is poorly understood. TRADD also recruits FADD (Fas-associated protein with death domain) again via their death domains and TRAF2/5 via a TRAF-binding domain in TRADD. TRAF2/5 in turn recruits cIAP1 and 2 (cellular inhibitor of apoptosis 1 and 2), which facilitates the recruitment of the ubiquitin chains required for the activation of the IKK complex. TRAF2, along with the cIAPs, mediates linear or K63-linked ubiquitination of RIP1. RIP1 and TRAF2 thus cooperate to recruit tumor growth factor β activated kinase (TAK1), the upstream kinase likely responsible for phosphorylation and activation of IKKβ and the IKK complex. TAK1 is recruited to the adapter proteins associated with TNFR1 via the TAK binding proteins TAB2 and TAB3, which bind to the K63 polyubiquitin chains on TRAF2, RIP1 or NEMO. Furthermore, NEMO has been shown to bind K63 polyubiquitin chains on RIP1 suggesting the ubiquitinated protein may bring the IKK complex and the upstream kinase TAK1 together to promote IKKβ activation.

**TNFα**

TNFα is synthesized as a 26 kDa, 233 amino acid propeptide that is cleaved by TNFα-converting enzyme into its 17 kDa, 157 amino acid form. TNFα binds to TNFR1, which is expressed on nearly all mammalian cells, as a homotrimer, resulting in the
dissociation of the silencer of death domain (SODD) protein that represses TNFR1 signaling in the absence of TNFα to activate the NF-κB pathway as previously described. Activated macrophages are the primary source of TNFα, though the propeptide can be synthesized by other cells, including fibroblasts and tumor cells.52

Inactivation of NF-κB pathway signaling

Unfortunately, the mechanisms that regulate the termination of NF-κB signaling are not yet well understood. It is known that IκBα along with p105 and p100 are target genes of NF-κB and serve as regulatory molecules that influence NF-κB activity. Newly synthesized IκBα, for example, can enter the nucleus and bind to NF-κB, causing it to shuttle back to the cytoplasm. Post-translational modification of NF-κB is also implicated in inactivation of pathway signaling. These post-translational modifications alter cofactor binding or can result in the displacement or degradation of NF-κB to terminate signaling.51 Acetylation of p65, for example, results in decreased DNA-binding affinity.53 The ubiquitin ligases SOCS-1 and PDLIM2 have been shown to drive proteasomal degradation of p65 bound to promoters.54-56

Phosphorylation of tumor suppressor proteins: NF-κB-independent IKK functions

Sequence analysis of IKKβ substrates using IκBα peptides revealed a consensus sequence in the C-terminus of p53. Phosphorylation of p53 typically leads to protein stabilization by blocking Mdm2-mediated ubiquitination and degradation of the protein; however, other phosphorylation events have been shown to lead to p53 degradation. Studies with IKKβ and p53 showed that phosphorylation of p53 at S362 and S366 by IKKβ resulted in β-TrCP-mediated ubiquitination and degradation of the protein. Furthermore, activation of the NF-κB pathway by doxorubicin, which induces DNA damage, resulted in IKKβ-induced p53 phosphorylation and degradation.57
Tuberous sclerosis complex 1 and 2 are tumor suppressor proteins that form a complex that controls the activity of molecular target of rapamycin (mTOR). TSC1 binds to TSC2 and protects it from ubiquitination and degradation. TSC2 is a GTPase-activating protein that converts RHEB-GTP to RHEB-GDP and its activity prevents the activation of mTOR by RHEB-GTP. IKKβ has been shown to phosphorylate TSC1 at S487 and S511 following activation by TNFα. This phosphorylation event results in the destruction of the TSC complex and activation of mTOR, which provides a link between inflammation via the NF-κB pathway and tumorigenesis via activation of the mTOR pathway.58

Phosphorylation of FOXO3a by IKKβ has also been found to lead to the protein’s ubiquitination and degradation in the absence of Akt. Activated Akt has been shown to phosphorylate FOXO3a, leading to the protein’s release from DNA and translocation to the cytoplasm where the protein binds 14-3-3 and becomes sequestered. In the absence of growth factor or survival stimuli, Akt is inactive and FOXO3a remains in the nucleus where it controls the transcription of genes involved in control of cell cycle progression and mitosis. However, in cells with inactive Akt, phosphorylation of FOXO3a by the IKK complex and subsequent degradation of the protein has been shown. Importantly, this regulation of FOXO3a by the IKK complex may contribute to tumor cell growth and tumorigenesis.59

IKK knock-out models

IKKα knock-out mice survive until a month past birth but have abnormal skin, lack whiskers, and have limbs that do not form properly. These mice show reduced activation of the NF-κB pathway in response to inflammatory signals but the remaining IKK complex (composed of IKKβ homodimers and NEMO) is still capable of IκBα phosphorylation and pathway activation in response to TNFα.60
In mice, IKKβ deletion is embryonically lethal at day E13 due to massive apoptosis in the liver and IKKβ knock-out mouse embryonic fibroblasts (MEFs) exhibit increased apoptosis following treatment with TNFα. The knock-out mice can be rescued by crossing IKKβ-/- mice with TNFR-/- mice but the resulting pups still only survive until one month past birth. IKKβ knock-out MEFs still respond to certain stimuli associated with the canonical IKKβ-IκBα-NF-κB pathway but deletion of NEMO results in a complete loss of this activity.

Mouse embryonic fibroblasts lacking NEMO are unable to respond to TNFα. Like IKKβ-/- mice, mice lacking NEMO die during embryogenesis due to liver apoptosis and fail to respond to pro-inflammatory, immune and pro-survival stimuli.

IKKα/β double knock-out mice die at E12 due to apoptosis and defective neuralation. TNFα, IL-1 and LPS are all unable to activate the NF-κB pathway in double knock-out MEFs. Though crossing the double knock-out mice with TNFα-/- mice does extend survival and yields mice more closely resembling IKKα-/- mice, these mice still die during embryogenesis (day E16.5) due to neural tube defects.
Chapter 2: The NF-κB pathway and cancer

**TNFα and the tumor microenvironment**

In 1863, Rudolf Virchow posed a link between inflammation and cancer and over a century and a half later that link has been generally accepted. Furthermore, it is now postulated that up to 20% of cancers are due to underlying inflammatory responses such as infection, autoimmune disease or inflammation due to unknown causes. Many correlations between inflammation and cancer exist, including: 1) the fact that patients with inflammatory diseases are at increased risk for a diverse array of cancers including cervical and ovarian cancers, 2) the risk of developing cancers such as breast or colon cancer is decreased by the use of non-steroidal anti-inflammatory drugs, 3) inflammatory pathways often operate downstream of oncogenic mutations, 4) inhibition of inflammatory pathways, such as the NF-κB pathway, or inflammatory cells reduces the incidence of cancer and 5) activation of inflammatory pathways or transfer of inflammatory cells promotes tumor development. Two pathways of inflammation exist in cancer including the extrinsic pathway, which is a pathway activated by inflammatory conditions that increase the risk of cancer, and the intrinsic pathway, which is activated by genetic alternations that activate inflammation. At least one of these pathways is active in most, if not all, tumors. This inflammation is caused by infiltrating immune cells, such as tumor-associated macrophages, that secrete pro-inflammatory cytokines, including TNFα into the tumor microenvironment.

The importance of TNFα and the NF-κB pathway has been clearly established in cancer. In resected tumor specimens, IKKβ is phosphorylated at S181 and thus activated in approximately 50% of tumor specimens, including breast, oral, stomach, colon, liver, and pancreatic tumor specimens. This phosphorylation is only seen in approximately 12% of normal tissues, suggesting that IKKβ and the NF-κB pathway are inappropriately...
activated in cancer. Furthermore, in breast cancer tissues, there is a near perfect correlation between the phosphorylation of IKKβ S181 and the presence of TNFα and the phosphorylation of IKKβ and the phosphorylation of IκBα, suggesting that TNFα in the tumor microenvironment drives the chronic activation of the NF-κB pathway (Figure 1).58 Though REL deletions, mutations and amplifications have been seen in human cancers, this continuous activation of IKKβ and the NF-κB pathway by TNFα in the tumor microenvironment likely plays a huge role in solid tumor formation.35

Activation of the Ras-Raf signaling pathway results in the production of chemokines and cytokines that promote tumorigenesis via the intrinsic pathway. Importantly, K-Ras is mutated in many cancers, including those of the pancreas, colon, small intestine and lung. However, the extrinsic pathway likely also plays a role in cancers such as pancreatic cancer where both inflammation prior to a genetic lesion and subsequent mutations in K-Ras drive cancer progression. The intimate relationship between Ras activation and activation of IKK and the NF-κB pathway will be discussed in detail in the next section.

The NF-κB pathway and solid tumors

Activation of the NF-κB pathway is implicated in a number of solid tumors, including colon,58, 68-70 pancreatic,71-73 lung,74, 75 breast58, 76-79 and ovarian80-82 cancers and has been
shown to play a role in inflammation, transformation, proliferation, angiogenesis, invasion, metastasis, chemoresistance and radioresistance. In this section, I will focus primarily on cancers that were used as model systems in this dissertation.

**Colon cancer**

Though no colon cancer cell lines were used in the body of work presented in this dissertation, the link between NF-κB activation and colon cancer is worth mentioning here. Deletion of IKKβ in enterocytes is associated with a decreased incidence of colitis-associated tumor formation. In this model, mice were treated with dextran sulfate sodium salt to mimic inflammatory bowel disease after an initial treatment with the procarcinogen azoxymethan, which results in tumor formation in the distal colon. A 75% decrease in tumor incidence was noted in the IKKβ knock-out mice compared to wild-type controls. This decrease was due to increased apoptosis in knock-out mice. Furthermore, deletion of IKKβ in myeloid cells decreased tumor incidence in the same model by 50% but this was not related to apoptosis and was likely due to blocking the transcription of one or more NF-κB-dependent genes. Additionally, phosphorylated IKKβ was detected in over 65% of all colon cancer specimens analyzed by Mein Chi Hung’s group, suggesting there is a connection between pro-inflammatory TNFα in the tumor microenvironment and colon cancer. Other groups have shown that NF-κB activation correlates with tumor progression and inhibition of NF-κB with a superrepressor IκBα (a nonphosphorylatable IκBα with serine residues 32 and 36 converted to alanine residues) sensitizes colorectal cancer cells to treatment with the chemotherapeutic CPT-11.

**Pancreatic cancer**

Pancreatic cancer is the fourth leading cause of cancer-related deaths in the US, with a 5-year survival rate hovering around 5%. Three types of precancerous lesions may form prior to the onset of pancreatic ductal adenocarcinoma. These lesions include
intraductal papillary mucinous neoplasms (IPMNs), which form in the main pancreatic duct or its branches, pancreatic intraepithelial neoplasias (PanINs), which form within intralobular ducts and mucinous cystic neoplasms (MCN), which are epithelial neoplasms that produce mucin. Each of these precancerous lesions are believed to represent the progression from normal pancreatic cells to pancreatic ductal adenocarcinoma.\textsuperscript{85, 86} The connection between mutant K-Ras and IKK activation has been well established in the development of pancreatic cancer.

K-Ras is mutated in approximately 90\% of pancreatic ductal adenocarcinomas and this mutation can be seen in as many as 40\% of PanIN lesions, suggesting the mutation is a very early event.\textsuperscript{87, 88} Furthermore, nearly half of ductal lesions displaying no atypia harbor the mutation and increased prevalence of this mutation is seen with increasing atypia.\textsuperscript{85} In mice, K-Ras mutation appears to be a primary event that results in the formation of premalignant ductal lesions that resemble human PanINs, but a second mutation is required for malignant progression.\textsuperscript{89} NF-\kappa B is constitutively activated in approximately 67\% of pancreatic adenocarcinomas\textsuperscript{90} and targeted inactivation of IKK\beta in KrasG12D mice has been shown to stall pre-malignant PanIN lesions from developing into pancreatic ductal adenocarcinoma (PDAC).\textsuperscript{72, 73} The necessity of this constitutive activation of NF-\kappa B for development of PDAC was made clear by work showing that pancreas-targeted IKK\beta inactivation inhibited NF-\kappa B activation and PDAC development in KrasG12D and KrasG12D;Ink4a/Arf\textasciitilde F/F mice.\textsuperscript{91} These mice develop PanIN lesions but do not progress to PDAC when IKK\beta is inactivated, supporting the importance of IKK\beta activation in pancreatic cancer and suggesting that inhibition of the kinase may serve to prevent the development of the disease.\textsuperscript{72}

The importance of IKK activation in K-RasG12V–driven pancreatic cancer has also been established. Knocking K-RasG12V into the pancreatic acinar cells of mice results in the development of PanINs and PDAC that perfectly resembles human disease.\textsuperscript{92}
However, if K-RasG12V is turned on in adult mice, the mice fail to develop PanINs and PDAC in the absence of chronic pancreatitis, the onset of which induces PanINs and PDAC. This work clearly demonstrates the necessity of inflammation for the formation of K-Ras-driven PDAC.93

Lung cancer

Lung cancer is another devastating disease with a 5-year survival rate of approximately 15%. To demonstrate the importance of IKKβ activation downstream of oncogenic mutation in a mouse model of non-small cell lung cancer, the researchers in Inder Verma’s group crossed IKKβfl/fl mice with KrasG12D mice to activate KrasG12D to initiate tumor formation but inactivate IKKβ upon transduction by Cre lentiviral vectors. The resulting KrasG12D/IKKβfl/fl mice survived longer (162 days median survival time compared to 114 days) than KrasG12D mice when each were infected with an appropriate lentiviral vector also encoding shRNA against p53 to speed tumor formation. Subsequent studies determined that these results were due to a pro-proliferation contribution of the NF-κB pathway in that particular model.94 Overexpression of IκBα in lung cancer models has been associated with increased sensitivity to paclitaxel, possibly through reduced VEGF expression.75

Breast cancer

There is a near perfect correlation between the presence of TNFα, activation of IKKβ and phosphorylation of IκBα in over one-third of breast cancer tissues. This activation of IKKβ has been associated with phosphorylation of TSC1, which leads to dissociation of the protein from TSC2 and the subsequent degradation of TSC2 and activation of mTOR to drive VEGF production. Furthermore, this phosphorylation of TSC1 correlates with decreased survival of patients from whom the tissues were collected.58
Estrogen receptor is expressed in approximately 70% of breast cancers and mediates the activation of estrogen, which is a key player in the initiation and progression of the disease. As the disease progresses, cancer cells lose their dependency for estrogen and become estrogen receptor negative (ER-) and this loss of ER has been shown to correlate with activation of the NF-κB pathway. Furthermore, ER is commonly used as a predictor of response to endocrine therapy, such as tamoxifen, for the treatment of breast cancer. DNA binding of NF-κB as measured by EMSA was much higher in ER- cell lines such as MDA-MB-231 cells than in ER+ cells lines and when an ER+ cell line was converted to an ER- cell line, NF-κB DNA binding activity doubled. Furthermore, the non-steroidal anti-inflammatory agent sulindac has been shown to drive apoptosis in breast cancer cells through inhibition of IKKβ. Importantly, MDA-MB-231 cells, which show high activation of the kinase, were more sensitive to the drug than breast cancer cells with low IKKβ activation. Additionally, transient transfection of an endocrine-resistant MCF-7 cell line that has an approximately 50% higher level of NF-κB activity due to constitutive activation of Akt with the nonphosphorylatable IκBα (S32A/S36A) restores NF-κB activity to levels similar in control cells. Furthermore, tamoxifen treatment of cells transfected with the nonphosphorylatable IκBα resulted in increased growth inhibition of the endocrine-resistance cells compared to tamoxifen treatment alone. Additionally, treatment with the IKKβ inhibitor parthenolide also enhanced tamoxifen-induced growth inhibition of the resistant cells.

Though NF-κB activation is more commonly associated with ER- breast cancers, NF-κB activity has also been shown in a subset of ER+ breast cancers and is associated with lower disease free survival. In these ER+ tumors, activation of NF-κB is shown to be higher in tumors with lower ER (21-87 fmol/mg ER vs. ≥100 fmol/mg ER).
The upregulation of NF-κB-related genes has also been implicated in inflammatory breast cancer, which is a rare and aggressive form of breast cancer. These genes include NF-κB genes such as NFKB1, RELA, NFKB2 and NEMO, tumor-promoting genes and genes that regulate angiogenesis, proliferation, apoptosis and immune responses.96

**Ovarian cancer**

IKKβ has been identified as a major promoter of a TLR4-MyD88-NF-κB pathway in epithelial ovarian cancer cells isolated from malignant ovarian cancer ascites and solid tumors. MyD88 is an adapter protein for TLR4 and expression of the protein allows signaling from TLR4 to NF-κB in MyD88+ epithelial ovarian cancer cells in response to LPS. Pathway activation by LPS induced increased growth and cytokine production in MyD88+ cells. Importantly, MyD88+ cells showed decreased sensitivity to the chemotherapeutic paclitaxel compared to MyD88- cells. In patients treated with paclitaxel and carboplatin following surgery, expression of MyD88 was correlated with decreased time until recurrence (23 months compared to 42 months for patients not expressing MyD88). Overall, these studies suggest that activation of the TLR4-MyD88-NF-κB pathway allows the epithelial ovarian cancer cells to constitutively secrete pro-inflammatory cytokines to promote tumor progression and chemoresistance.80

Subsequent work by the same group showed that treatment with LPS or paclitaxel results in increased NF-κB activity in MyD88+ cells but not MyD88- cells and that ectopic expression of IKKβ in MyD88- cells could induce production of the same cytokines produced by MyD88+ cells and restored TLR4 signaling. Furthermore, MyD88+ cells were shown to be resistant to apoptosis following treatment with high levels of TNFα while MyD88- cells were sensitive to the treatment. Analysis of miRNAs in MyD88+ and MyD88- cells identified hsa-miR-199a as being up-regulated in MyD88- cells compared to MyD88+ cells and a regulator of IKKβ in MyD88- cells, establishing a role for the IKKβ and the NF-κB pathway in ovarian cancer.81 A particularly important implication for this work is the role
it identifies for IKKβ and NF-κB in the development of resistance to paclitaxel. Though nearly 90% of ovarian cancer patients respond to treatments such as carboplatin and paclitaxel, most (again nearly 90%) will face recurrent disease. Pharmacological inhibition of the NF-κB pathway has been shown to increase ovarian cancer cell sensitivity to paclitaxel. Furthermore, analysis of 42 primary tumors derived from patients with follow-up data showed that patients with tumors expressing IKKβ in at least 25% of cells had reduced overall survival (21 months compared to 29 months) compared to patients with IKKβ expression in 25% or fewer of their tumor cells. Furthermore, pharmacological inhibition or RNA interference of IKKβ resulted in decreased ovarian cancer cell viability.

Cervical cancer

The role of NF-κB activation in cervical cancer is less clear than its role in previously discussed cancers. Infection with human papillomavirus (HPV) from the high risk subset of HPV is a major risk factor for the development of cervical cancer. The E6 and E7 genes carried by HPV-16, a prototypical high-risk HPV strain, are required to immortalize human epithelial cells. E6 expression results in p53 degradation and immortalization through activation of hTERT while the E7 protein functions to inactivate retinoblastoma and thereby promotes progression of the cell cycle. These proteins also regulate NF-κB and NF-κB activation has been shown to decrease following immortalization of the epithelial cells more frequently giving rise to cervical cancer. The mechanism by which HPV inactivates NF-κB signaling appears to be through inhibition of the IKK complex. In the context of HPV infection and HPV-immortalization of cells, it appears that NF-κB actually plays a role in controlling the proliferation of virally infected cells and inhibition of NF-κB using the IκBα superrepressor resulted in increased proliferation and colony formation of immortalized cells. Subsequent studies revealed that p65 functions to decrease expression of a short sequence of DNA upstream of HPV-16 E6 and thereby inhibits proliferation.
However, as cervical cancer progresses, the role of NF-κB seems to shift from inhibitory to activating. For example, NF-κB overexpression has been shown to increase HeLa and C33A cell viability while knockdown of NF-κB suppresses cell viability. Furthermore, overexpression of NF-κB results in increased colony formation by both cell lines, indicating that NF-κB promotes cervical cancer cell viability and growth. In these studies it was found that NF-κB overexpression also increased miR-130a levels while knockdown of the protein decreased miR-130a levels. Expression of this microRNA is key for regulating TNFα mRNA levels and the NF-κB/miR-130a/TNFα feedback loop may be responsible for maintaining a level of TNFα that promotes tumor growth and prevents apoptosis of cancer cells.107

This seemingly two-faced role of NF-κB during the progression of cervical cancer is supported by immunohistochemical analysis of human tissues. In a series of 106 cervical intraepithelial neoplasia (CIN lesions) and cervical cancer lesions analyzed, the NF-κB pathway was activated during the course of disease progression. In the normal cervix and early lesions (CIN1 lesions), p65 was localized exclusively to the cytoplasm but increased nuclear localization of p65 was seen during the progression of disease through CIN2 and CIN3 stages and the development of squamous-cell carcinoma of the cervix. As NF-κB serves to repress HPV transcription,108 the downregulation of NF-κB is likely required for initial infection and immortalization of cells but becomes less important during progression of the disease. Furthermore, NF-κB’s role in a myriad of tumor-promoting activities may select for its increased expression in precancerous lesions as the disease progresses, though the nuclear expression of NF-κB did not correlate with survival of patients, aggressiveness of disease or clearance or persistence of HPV infection.109 Nevertheless, inhibition of the NF-κB pathway with curcumin and aspirin has been shown
to sensitize cervical cancer cells to chemotherapeutic agents such as cisplatin,\textsuperscript{110} which is consistent with the pathway’s role in the development of chemoresistance.

Overall, the studies presented in this chapter establish TNF\textsubscript{α}, IKKβ and NF-κB as key mediators of tumorigenesis and demonstrate the pathway’s importance in the progression of many solid tumors types. The next chapter will focus on the considerable efforts that have been made to develop IKKβ inhibitors and the challenges associated with such a task.
Chapter 3: Targeting IKKβ

Rationale for targeting IKKβ

Nearly one-third of all cancers display an oncogenic mutation in Ras and IKKβ has been shown to be activated in 50% of tumors and its activation may serve as a second hit that results in the development of cancer. The case for pancreatic cancer, for example, is quite compelling. As mentioned previously, as many as 95% of human pancreatic carcinomas have been shown to harbor a mutation in codon 12 of the KRAS gene and NF-κB is constitutively activated in approximately 67% of pancreatic adenocarcinomas and correlates with lower overall survival of patients. Constitutive activation of NF-κB has great implications for cancer research as it may lead to the transcription of anti-apoptotic genes, among others, and chemoresistance. Importantly, knock-out of IKKβ stalls Kras-driven pancreatic cancer at PanIN lesions, demonstrating a mechanistic link between mutated Kras and IKKβ in the initiation of PDAC, suggesting IKKβ is an attractive target for anticancer therapy. Furthermore, research suggests that inhibition of IKKβ may increase the efficacy of many chemotherapeutics such as gemcitabine, paclitaxel and cisplatin.

Current IKKβ inhibitors

By the mid-2000’s research uncovering the undeniable role of the NF-κB pathway in inflammation and cancer had launched the development of many novel IKKβ inhibitors and the identification of natural products that blocked the kinase’s actions. The best-studied of these inhibitors include BAY 11-7085, ML120B, TPCA-1, BMS345541 and Bayer ‘compound A’ and the natural products curcumin and parthenolide. Despite promising results in pre-clinical models, none of these drugs have been clinically approved for the treatment of cancer, due to unexpected toxicities arising from targeting IKKβ or poor bioavailability. For example, pre-treatment of HeLa cells with ML120B for 1 hour followed by stimulation with TNFα (30 ng/mL) for 5 minutes to drive activation of IKKβ
and phosphorylation of IκBα, results in marked reduction in IκBα phosphorylation. Unfortunately, when mice were dosed with ML120B for 7 days, a decrease in peripheral blood T cell and B cell numbers and an increase in granulocyte neutrophils was observed and mice had to be removed from the treatment due to toxicity issues. These toxicities likely arise from inhibiting signaling through IKKβ required for proper immune functioning. The natural product parthenolide, on the other hand, did not display notable toxicity in a Phase I trial but instead suffered from the fact that a botanical product containing the natural product was not able to produce detectable levels of parthenolide in plasma. Taken together, these data suggest IKKβ represents a potential anticancer target, but its global inhibition is detrimental and careful design will be required to develop an IKKβ inhibitor that is viable for human use.

**Recent crystal structures of IKKβ**

Recent crystal structures of IKKβ have provided structural insight that is quite valuable for the rational design of IKKβ inhibitors. One, published in 2011, was of *Xenopus laevis* IKKβ and yielded a proper understanding of the domain architecture of IKKβ. In this structure, the kinase domain of IKKβ is at the N-terminus of the protein and an ubiquitin-like domain follows. A scaffold/dimerization domain that contains previously predicted leucine zipper (LZ) and helix-loop-helix (HLH) domains is then followed by the C-terminal NEMO binding domain (Figure 1A). Previous models for the activation and subsequent deactivation of IKKβ depended on interactions facilitated by the predicted, but non-existent, LZ and HLH domains. Furthermore, hydrophobic residues of the predicted LZ domain actually point inward in the crystal structure and, therefore, are unavailable for mediating dimerization as the previous models proposed. Additionally, the previously discussed MEK consensus sequence motif of SxxxS (S represents S177 and S181 for IKKβ) was found in the activation loop of IKKβ. The crystal structure of *Xenopus laevis* IKKβ (which is commonly referred to as xIKKβ) was obtained using an IKKβ of residues
4–675 with S177 and S181 mutated to glutamic acid in complex with inhibitor. The crystal

Figure 1: Crystal structure of Xenopus laevis IKKβ. A) Domain architecture of IKKβ showing the KD, ULD, SDD and NBD of IKKβ B) Crystal structure of Xenopus laevis IKKβ with the KD, ULD, SDD and N- and C-termini of the kinase labeled C) Tetramer (dimer of dimers) formed by xIKKβ D) Close-up view of the activation loops of neighboring protomers. This research was originally published by Nature Publishing Group. Xu, G. et al. Crystal structure of inhibitor of kappaB kinase beta. 2011. Nature. 2011: 472: 325-330.
structure captures IKKβ in a dimer resembling a pair of scissors with each kinase domain and ubiquitin-like domain representing handles and the scaffold/dimerization domain representing the blades of the shears, though the domains still intimately associate with one another (Figure 1B). The kinase domain exhibits a bilobal kinase fold, which is typical of all kinases, and the ubiquitin-like domain indeed has an ubiquitin fold and the hydrophobic surface patch of ubiquitin that is recognized by ubiquitin-binding proteins. The inhibitor binds to the hinge loop that connects the N- and C-lobes of the kinase domain of IKKβ, which is the same site where the adenine of ATP would bind and is the site targeted by most IKKβ inhibitors. Not surprisingly, the kinase domain is in a conformation that is not compatible with that of an active kinase. For example, the C-terminal anchor of the activation segment is in a conformation that results in loss of essential interactions with the catalytic center. Pulldowns with the C-terminal region of IκBα and full-length IKKβ or the ULD-SDD region of IKKβ showed that the ankyrin repeats and PEST region of IκBα interact with ULD-SDD IKKβ. Further studies demonstrated that the interaction directs the specificity of IKKβ to S32 and S36 of IκBa and away from its PEST sequence. Additionally, the interaction between the ubiquitin-like domain and the KD was found to allosterically position the KD in a conformation that is favorable for catalysis. Lastly, the SDD domain was found to mediate dimerization between the two IKKβ monomers in the dimer. The interactions key for dimerization were mainly hydrophobic interactions between residues localized towards the end of the bundle. The tetrameric nature of the IKKβ crystal structure derived in these studies suggests that IKKβ may form a dimer of dimers that allows one protomer to activate the neighboring dimer through autophosphorylation (Figure 1C and D).
A more recent crystal structure of human IKKβ residues 1-664 in complex with the staurosporine analog K252a captured an asymmetric dimer of IKKβ in which one IKKβ was phosphorylated at S177 and S181 while the other IKKβ protomer was not, yielding insight into the structural changes IKKβ undergoes when activated. The most significant differences between phosphorylated and nonphosphorylated IKKβ occurred in the N-terminal lobe and at the activation loop (Figure 2A). Furthermore, the SDD of the unphosphorylated protomer (which is in gray) is rotated 6° away from the KD compared to the orientation of the SDD of the phosphorylated protomer and its KD (Figure 2B).

**Figure 2: Crystal structure of an asymmetric dimer of human IKKβ.** A) Ribbon diagram of the asymmetric human IKKβ dimer and B) overlay of the unphosphorylated and phosphorylated human IKKβ protomers. C) Overlay of the KDs of unphosphorylated and phosphorylated human IKKβ bound to inhibitor. D) Comparison of the pSer interactions of pS181 of human IKKβ and activated PKA. The PKA activation loop is shown in gray while that of IKKβ in shown in red. A bound peptide substrate is shown in blue. This research was originally published in J. Biol. Chem. Liu, S. et al. Crystal structure of a human IkappaB kinase beta asymmetric dimer. J. Biol. Chem. 2013; 288: 22758-67.
The activation loop of the phosphorylated IKKβ (residues 166–194) assumes a conformation characteristic of an active kinase, as expected. The corresponding region of the unphosphorylated IKKβ is not well defined and is in a conformation that is not compatible with protein substrate binding (Figure 2C). As K252a binding is identical in both the phosphorylated and nonphosphorylated IKKβ protomers and packing of the two IKKβ protomers was equal, the differences in the flexibility and conformation of the two protomers is likely due to the differential phosphorylation. The three phosphate oxygens of pS181 interacted directly through ionic interactions with the cationic cluster formed by R144 from the catalytic loop and K171 from the activation loop. Furthermore, one of the phosphate oxygens formed hydrogen bonds to the main chain amide nitrogens of pS181 and T180 (Figure 2C). These interactions likely provide a link to the catalytic center and stabilize the correct orientation of the catalytic base D145 and allow for the formation of the polypeptide substrate pocket. This is similar to what is seen with other kinases, such as Protein Kinase A (PKA) (Figure 2D).47

These distinct conformations of the active and inactive IKKβ protomers reveals a well-formed peptide-binding pocket in active, but not inactive IKKβ (Figure 3A and B). Differences in the conformations of these crystal structures may allow targeting of active but not inactive IKKβ.47

Unlike xIKKβ, the human IKKβ dimer adopted an open conformation with its KDs well separated, which is in contrast to the closed conformation adopted by xIKKβ (Figure 4A and B). Furthermore, human IKKβ did not form a dimer of dimers but rather formed contacts with a crystal symmetry-related dimer at the KD and the N-terminal ends of the SDD to form a face-to-face KD-KD dimer. The activation loop (shown in red) in the nonphosphorylated human IKKβ molecule is close to the active site of the activated KD (Figure 5B), which is in contrast to what is seen with the dimer of dimers of xIKKβ (Figure
This orientation may facilitate the trans-phosphorylation of an unactivated KD by an active kinase domain.\(^{47}\)

**Figure 3: Comparison of the substrate binding sites in unphosphorylated and phosphorylated human IKKβ.** The substrate binding site is not formed in the unphosphorylated protomer (A) but is well-formed in the phosphorylated protomer (B), allowing the binding of a peptide substrate. K252a is shown as a stick model and the substrate peptide is shown in blue. This research was originally published in J. Biol. Chem. Liu, S. et al. Crystal structure of a human IkappaB kinase beta asymmetric dimer. J. Biol. Chem. 2013; 288: 22758-67.\(^{47}\)

**Historic IKKβ Inhibitors**

Historically, IKKβ inhibitors developed by pharmaceutical companies target the ATP binding site and thus the resting state of IKKβ. This mode of inhibition prevents the activation of IKKβ and likely results in the previously described toxicities. The crystal structure of the asymmetric IKKβ dimer sheds light on the possibility that the active, post-translationally modified form of IKKβ represents a unique form of the kinase that can be targeted. This is particularly valuable information when coupled with the finding that IKKβ is chronically activated by TNFα in the tumor microenvironment,\(^{58}\) which should result in a pool of phosphorylated IKKβ in cancerous cells (Figure 6). *We hypothesized that small molecules targeting the phosphorylated form of IKKβ that accumulates in cancer but not the resting, unphosphorylated form of IKKβ that is found in normal tissues could serve as non-toxic inhibitors of IKKβ and the NF-κB pathway.* As such, my dissertation work has focused on characterizing two small molecule inhibitors of IKKβ that we believe may target the activated form of the kinase to inhibit tumor growth without inducing toxicities.
Identification of novel IKKβ inhibitors 13-197 and 25-4

IKKβ activated by phosphorylation of its two T loop serine residues is found in nearly 50% of surgical tumor specimens but in only approximately 10% of normal tissues. Furthermore, there is a perfect correlation between the presence of the pro-inflammatory cytokine TNFα and phosphorylation of IKKβ, in addition to a perfect correlation between phosphorylation of IKKβ and phosphorylation of its substrate, IκBα, in these tissues. This suggests IKKβ is constitutively activated in tumor specimens in response to TNFα in the tumor microenvironment, which consequently leads to the constitutive activation of NF-κB.
As such, we sought to develop inhibitors of the NF-κB pathway. We took two routes to accomplish this task. First, we screened a library of novel small molecules for activity against TNFα-induced IKKβ-mediated NF-κB-driven transcription and identified the quinoxaline urea analog 13-197 as an inhibitor of the NF-κB pathway (Figure 7A). The quinoxaline core of 13-197 is considered a privileged scaffold as it is found in a number of natural products and commercially available drugs such as the smoking cessation drug Varenicline. A follow-up kinase screen and mechanism-based studies revealed that 13-197 inhibits NF-κB-driven transcription by targeting the kinase IKKβ. As information regarding the prevalence of TNFα in the tumor microenvironment and consequent activation of the NF-κB pathway has implicated over-activation of IKKβ, and not just mutation of IκBα or amplification or rearrangement of NF-κB subunits, in the constitutive activation of the NF-κB pathway, we tested this small molecule for anticancer activity in

Figure 5: Comparison of the Xenopus laevis and human IKKβ dimers. A) The activation loop of xIKKβ (which is shown in red) is far from the activation loop of the neighboring protomer. B) The KD of the nonphosphorylated human IKKβ molecule is close to the active site of the activated KD. This orientation may facilitate the trans-phosphorylation of an unactivated KD by an active kinase domain. This research was originally published in J. Biol. Chem. Liu, S. et al. Crystal structure of a human IkappaB kinase beta asymmetric dimer. J. Biol. Chem. 2013; 288: 22758-67.
cell lines and an orthotopic mouse model of pancreatic cancer and saw the small molecule was active without inducing toxicities associated with known IKKβ inhibitors.

The small molecule is a non-ATP competitive inhibitor of IKKβ and initial studies suggest 13-197 probably best occupies a pocket that is formed following the activation of resting IKKβ. Upon phosphorylation of S177 and S181 of IKKβ and activation of the kinase, the kinase undergoes a conformational change that creates potential binding sites that can be targeted by small molecules. Small molecules targeting these sites are likely to target the activated but not inactive form of the kinase. We believe 13-197 exhibits anticancer activity without inducing toxicity by inhibiting an activated form of IKKβ while allowing the transient activation of the protein. We hypothesize that constitutive activation of the NF-κB pathway by TNFα results in the sustained activation of the IKK complex in many solid tumors (See Figure 6 for model). We believe this sustained stimulation of IKKβ results in the accumulation of phosphorylated IKKβ that can be targeted by pharmacological inhibitors to allow successful targeting of the kinase for anticancer therapy. In our model, TNFα in the tumor microenvironment drives the chronic activation of IKKβ by phosphorylation of the kinase’s T loop serine residues (S177 and 181). Once phosphorylated on the T loop serine residues, IKKβ exhibits high activity and autophosphorylates a stretch of serine residues on its C-terminus, which provides docking sites for phosphatases that dephosphorylate the T loop serine residues to inactivate the kinase. Phosphorylation of the T loop serine residues of IKKβ is detectable minutes following stimulation with TNFα, making the transient activation of IKKβ very rapid. Furthermore, as phosphorylation of the C-terminal serine residues is an intramolecular event, this process should also occur quickly. However, the dephosphorylation of the kinase by phosphatases is a bimolecular event and is thus the rate-limiting step in the deactivation of IKKβ. Therefore, we propose that a hyperphosphorylated form of IKKβ in
which the T loop serine residues and C-terminal serine residues are phosphorylated accumulates in cancer. We hypothesize that this form of IKKβ is an ideal target for anticancer therapy and targeting this form will allow the transient activation of IKKβ that is required for normal immune functioning but will block the chronic activation of IKKβ that drives tumorigenesis. As such, we tested small molecule 13-197 for activity against activated IKKβ and preliminary experiments indicate the small molecule indeed targets activated IKKβ.

In our second approach to designing an inhibitor of the NF-κB pathway, we hoped to target IKKβ by designing a small molecule inhibitor that featured the α-methylene-γ-butyrolactone of the known IKKβ and NF-κB subunit p65 inhibitor parthenolide. After
screening a small library of \( \alpha \)-methylene-\( \gamma \)-butyrolactones against TNF\( \alpha \)-induced IKK\( \beta \)-mediated NF-\( \kappa \)B-driven transcription, we identified the small molecule 25-4 (Figure 7B) as an inhibitor of NF-\( \kappa \)B-driven transcription.\textsuperscript{131} This small molecule likely targets surface-exposed C179 of IKK\( \beta \) and a surface-exposed cysteine residue in NF-\( \kappa \)B, likely C38. C179, found in the activation loop of IKK\( \beta \), is primed for targeting as it sits between serine residues 177 and 181, which are phosphorylated to drive the activation of IKK\( \beta \).\textsuperscript{125, 132} Cysteine residue 38 in NF-\( \kappa \)B plays an important role in the protein’s activation of gene expression.\textsuperscript{133}

As briefly mentioned, parthenolide, a sesquiterpene lactone natural product, also targets IKK\( \beta \) and NF-\( \kappa \)B subunit p65 through covalent interaction with the sulfhydryl group of C179 of IKK\( \beta \) and C38 of NF-\( \kappa \)B.\textsuperscript{122, 130, 134, 135} The sulfhydryl groups on C179 of IKK\( \beta \) and C38 of NF-\( \kappa \)B covalently react with the Michael acceptor in the \( \alpha \)-methylene-\( \gamma \)-butyrolactone moiety of parthenolide, which results in the inhibition of the NF-\( \kappa \)B pathway.\textsuperscript{122} Parthenolide has displayed anticancer effects in cervical, breast, lung and pancreatic cancers and potentiates the effects of clinically approved chemotherapeutics such as gemcitabine, paclitaxel and cisplatin.\textsuperscript{136-138} Despite these remarkable activities, Parthenolide suffers from poor water solubility, is unstable under acidic and basic conditions and affords few opportunities for optimization due to structural complexity.\textsuperscript{139} Parthenolide’s core structure features a highly functionalized, fused cyclodecane lactone that is decorated by an epoxide, an \( E \)-alkene and four stereocenters and is trans fused to an \( \alpha \)-methylene-\( \gamma \)-butyrolactone.\textsuperscript{134} Recently, the first total synthesis of Parthenolide was reported.\textsuperscript{141} This complicated structure limits our ability to easily generate the analogs that would allow us to perform the extensive structure activity relationship (SAR) studies required to identify compounds with improved biological activity and drug-like properties.
Our main goal in our project with 25-4, which inhibits the NF-κB pathway by covalently binding to IKKβ and NF-κB, was to identify a highly modifiable core that, like parthenolide, contains the α-methylene-γ-butyrolactone moiety and is expected to target proteins through covalent interactions via its Michael acceptor. In our preliminary studies, 25-4 has been shown to inhibit phosphorylation of the IKKβ substrate IκBα, reduce cancer cell viability and induce caspase 3/7 activation and PARP cleavage in cancer cell lines. Furthermore, it has reduced tumor growth in an orthotopic model of ovarian cancer. Very importantly, the small molecule did not exhibit overt toxicity at therapeutic doses in mice and its structure affords us an opportunity to make structural changes that will allow us to conduct SAR studies that will potentially identify more potent and more specific inhibitors of the NF-κB pathway. As such, this project has identified a core that may be suitable for use as an IKKβ and/or p65 inhibitor and future studies will be aimed at improving the drug-like properties and specificity of the small molecule and identifying the form of IKKβ targeted by this non-toxic IKKβ inhibitor.

Treatment of cancer cells with small molecules 13-197 and 25-4 resulted in impaired IκBα phosphorylation, decreased cancer cell viability and increased apoptosis in cancer cell lines and decreased tumor growth and metastasis in orthotopic mouse models of cancer without inducing the toxicities associated with commercially available IKKβ inhibitors. We hypothesize their lack of toxicity is due to selective inhibition of phosphorylated IKKβ. Their selective inhibition of phosphorylated IKKβ allows the transient activation of IKKβ prior to inhibition. Allowing this transient activation of IKKβ appears to diminish toxicity in normal tissues while inhibition of sustained IKKβ activation appears sufficient for induction of cancer cell death, making phosphorylated IKKβ an attractive target for cancer drug discovery. The studies presented in this dissertation have been aimed at establishing 13-197 and 25-4 as small molecule inhibitors of IKKβ and have
identified functional groups key for their activity. Future studies will be aimed at determining if and how the small molecules selectively target phosphorylated forms of IKKβ and exploring the off-target effects of these small molecules.

**Figure 7: Small molecules 13-197 and 25-4.** A) The quinoxaline urea 13-197 and B) the spirocyclic α-methylene-γ-butyrolactone 25-4. Small molecule 13-197 was developed by Drs. Qianyi Chen, Rajkumar Rajule, Sandeep Rana and Yogesh Sonawayne. 25-4 was developed by Dr. Sandeep Rana.
Chapter 4: Materials and General Methods

Chemicals and reagents

The quinoxaline urea analog 13-197 was synthesized and purified (>98%) as previously described.\textsuperscript{126, 127}

The α-methylene-γ-butyrolactone 25-4 and its analogs were synthesized and purified as previously described.\textsuperscript{131} HPLC-grade methanol was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Triethanolamine (≥99.5%) was obtained from Sigma Life Science (St. Louis, MO, USA). Tris(2-carboxethyl)phosphine hydrochloride (98%) was obtained from Alfa Aesar (Ward Hill, MA, USA). 5-TAMRA azide was obtained from Lumiprobe (Hallandale Beach, Florida, USA). TAMRA-Azide-biotin was obtained from Click Chemistry Tools (Scottsdale, AZ, USA). CuSO4·5H2O (≥98%) VWR International (West Chester, PA, USA). Tris[(1-benzyl-1-H-1,2,3-triazol-4-yl)methyl]amine (97%) was obtained from Aldrich (St. Louis, MO, USA).

Cell lines and culture conditions

Human pancreatic cancer cell lines MIAPaCa-2, PANC-1, Capan-2 and SUIT-2, cervical cancer cell line HeLa and breast cancer cell line MDA-MB-231 were cultured in Dulbecco’s modified Eagle’s medium (DMEM) high glucose supplemented with 10% fetal bovine serum (FBS). Human embryonic kidney cells (HEK cells) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) low glucose supplemented with 10% fetal FBS. Human ovarian cancer cell line A2780 and pancreatic cancer cell lines Capan-2 and Hs766T were cultured in Roswell Park Memorial Institute-1640 medium (RPMI) supplemented with 10% FBS. Human lung cancer cell line luciferase A549 (generous gift from Prof. Brasier, University of Texas Medical Branch, Galveston, TX) was cultured in DMEM high supplemented with 10% FBS and 100 µg/mL hygromycin B. IKKα knock-out (−/−) and IKKβ knock-out (−/−) mouse embryonic fibroblast (MEF) cells (generous gift from Prof. I. Verma,
Salk Institute) were grown in DMEM high medium with 10% FBS and 1% Pencillin-Streptomycin as were wild-type MEFs.

Cell lysis

Cytoplasmic fractions were generated by harvesting cells on ice in Buffer A (10 mmol/L HEPES, 10 mmol/L KCl, 0.1 mmol/L EDTA, and 2 mmol/L DTT) containing protease and phosphatase inhibitors. The cells were allowed to swell on ice for 30 min and 1.25% 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 obtain nuclear fractions, Buffer C (20 mM HEPES, 420 mM NaCl, 5 mM EDTA, 5 mM DTT, 10% glycerol) containing protease and phosphatase inhibitors was added the remaining pellet. The samples were 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 -80°C until needed.

Whole cell lysates were generated by harvesting cells on ice in HNTG lysis buffer (20 mM HEPES, pH 7.5, 25 mM NaCl, 0.1% Triton X-100, 10% glycerol) containing protease and phosphatase inhibitors. The cells were allowed to swell on ice for 30 min and the samples were centrifuged in the cold. The resulting supernatant was transferred to a new tube and designated as a whole cell lysate. All samples were stored at -80°C until needed.

For click chemistry, cells were harvested via scraping in 1X PBS with protease and phosphatase inhibitors and sonicated for 15 min. Lysates were spun and the soluble fraction (supernatant) was collected and transferred to a new tube. All samples were stored at -80°C until needed.

Western blotting

Cytoplasmic fractions or whole cell lysates were prepared from control and treated cells as described above. The cell lysates (25 µg) were resolved in 4% to 20% denaturing polyacrylamide gel (Bio-Rad) and transferred to polyvinylidene difluoride (PVDF)
membranes (Millipore). The membranes were incubated with the following antibodies: rabbit anti-p-IKKα/β (Cell Signaling 2697), rabbit anti-IKKβ (abcam EPR6043), rabbit anti-IκBα (Cell Signaling 4812), mouse antiphospho-IκBα (Cell Signaling 9246) or mouse anti-PARP (Cell Signaling 9542). Internal loading control for cytoplasmic fractions and whole cell lysates used mouse anti-tubulin (Cell Signaling 3873) or GAPDH (Santa Cruz Biotechnology, Inc. sc-25778). These primary antibodies were incubated with horseradish peroxidase–conjugated specific secondary antibodies. The signals were developed by using Amersham ECL Prime Western Blotting Detection Reagent (General Electric Healthcare Life Sciences).

**Data processing and statistical analysis**

P-values were determined using one way ANOVA in SigmaPlot and values < 0.05 were considered significant.

IC₅₀ values were derived from plotting cell viability against drug concentration and curve-fitting the data using a four-parameter logistic curve in SigmaPlot.

**κB-luciferase assay**

A549 luciferase cells were seeded in white 96-well plates at a density of 50,000 cells per well and incubated overnight. Cells were then treated with the indicated concentrations of drugs for 2 hours. Cells were then stimulated with 20 ng/mL TNFα (Panomics) for 3 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 cells were incubated at room temperature for an additional 10 min before luminescence was measured at 1000 ms integration on a SpectraMax M5 plate reader.
Click chemistry

For click chemistry with purified proteins, purified IKKβ or p65 (250 ng in a total volume of 200 µL) was incubated with 10 µM 31-036 for 1 hour. 5-TAMRA azide (Lumiprobe) (0.1 mM from a 1 mM DMSO stock) and click chemistry reagents (1 mM tris(2-carboxyethyl)-phosphine hydrochloride (TCEP) from 50 mM stock in water, 1 mM CuSO4·5H2O from a freshly prepared 50 mM stock in water, and 0.1 mM tris[(1-benzyl-1-H-1,2,3-triazol-4-yl)methyl]-amine (TBTA) from 10 mM stock in DMSO) at a total volume of 48 µL were added for 1 hour and then the reaction was quenched with ice cold methanol and stored at -20°C to precipitate. The resulting precipitate was spun and the methanol was removed. The pellet was resuspended in 4% SDS buffer (4% SDS, 150 mM NaCl, 50 mM TEA pH 7.4) and 2X sample buffer (20% glycerol, 0.2% bromophenol blue, 1.4% β mercaptoethanol) to 1X. Samples were resolved on 4% to 20% denaturing polyacrylamide gel (Bio-Rad) and visualized using the Typhoon 9410 Variable Mode Imager.

For click chemistry in cells, cells were treated with the indicated concentration of 31-036 for 1 h and then cells were harvested as described above. 200 µg of total protein in 152 µL total volume was incubated with 48 µL of the same click chemistry cocktail described above to give 200 µL total volume. Click chemistry was continued as described above.

Cellular viability assay

Cellular viability was determined by alamarBlue Cell Viability Assay (Life Technologies) or PrestoBlue Cell Viability Assay (Life Technologies) according to manufacturer’s instructions. MIAPaCa-2, A2780, MDA-MB-231, Hs766T, AsPC-1, luciferase A549, SUIT-2 and PANC-1 cells were incubated with compound in at least duplicate in a 96-well plate and then incubated for 24, 48 or 72 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 or PrestoBlue solution was added to each well (equal to 10% volume of the medium in the well) and incubated for 30 minutes at 37°C. The fluorescence of the solution was
measured at 560 nm excitation and 590 nm emission using SpectraMax M5 \textsuperscript{e} fluorescence plate reader. Dose–response curves were evaluated using the National Cancer Institute (NCI) algorithm: \( T_z \) = number of control cells at time \( t_0 \), \( C \) = number of control cells at time \( t \), and \( T \) = number of treated cells at time \( t \); 100 \( \times \) \( [(T - T_z)/(C - T_z)] \).

**PARP cleavage**

Cells were treated as indicated and harvested by collecting media, trypsinizing cells, and centrifuging to obtain a combined cell pellet from all steps. Cells were lysed in radio immuno precipitation assay (RIPA) buffer (150 mM NaCl, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris, pH 8.0) and protein content was resolved on 4% to 20% denaturing polyacrylamide gel (Bio-Rad). PARP cleavage was determined via Western blotting using anti-PARP antibody.

**Caspase 3/7 assay**

3,000 SKOV3 cells/well were plated in a white, clear bottom, sterile 96 well dish in 95 \( \mu \text{L} \) and incubated overnight. The next day, cells were treated with 5 \( \mu \text{L} \) compounds to yield the final drug concentration indicated. Cells were incubated with compound for 24 h. AlamarBlue was added to each well and cells were incubated for another 3 hours. Fluorescence (560\textsubscript{ex}/590\textsubscript{em}) was measured on the SpectraMax M5 \textsuperscript{e} plate reader. 110 \( \mu \text{L} \) Caspase Glo-3/7 reagent was added to each well and mixed by the plate reader at 300-500 rpm for 30 seconds. The plate was incubated at room temperature for 1 h. Luminescence was measured on the SpectraMax M5 \textsuperscript{e} plate reader.

**Colony formation assay**

HeLa cells were plated in 6-well plates at a density of 500-1000 cells per well and cultured for 24 h, followed by drug treatment for 48 h at 37\textdegree C. DMSO was used as a control. Then, the supernatant fluid was replaced with fresh medium and the cells were cultured for 7 d. Cells were washed twice with PBS and then fixed with methanol for 15 min, followed by 0.1% crystal violet staining for 30 min at room temperature. Then, crystal violet was
carefully removed by immersing the plates in tap water. The plates were then air-dried at room temperature for colony counting, where colonies with more than 50 cells were counted.

**Immunohistochemistry analysis**

Hematoxylin and eosin staining of ovarian tumor tissues was performed by UNMC's Tissue Science Core Facility to identify tissue areas containing tumor. One core from each tumor was arranged into a tumor microarray and slides were generated from the microarray. The resulting slides were stained with antibody directed Mcl-1 using Leica bond polymer refine detection kit according to manufacturer's instructions (Leica Microsystems Inc.). Briefly, the ovarian tumor tissue sections were incubated with rabbit anti-Mcl-1 primary antibody (Santa Cruz Biotechnology, Inc. sc-819). Pictures were captured by using Coreo Au slide scanner (Ventana Medical Systems Inc.). From each image, a representative screen shot was captured at 40X magnification. Quantification of staining was done using these images.
Chapter 5: Evaluation of small molecule 13-197

Introduction to 13-197

13-197 was originally identified as a small molecule capable of inhibiting growth in a growth inhibition screen conducted with a panel of cell lines including A549 lung cancer cells, AsPC-1 pancreatic cancer cells, HT29 colon cancer cells, MDA-MB-231 breast cancer cells, PC3 prostate cancer cells, SKOV3 ovarian cancer cells and U-2 OS bone sarcoma cells. 13-197 was shown to induce caspase 3/7 activation in MDA-MD-231, PC3 and HeLa cell lines and PARP cleavage in HeLa cells in a Mcl-dependent manner.126, 127

To further study the apoptotic effects of 13-197, MIAPaCa-2 pancreatic cancer cells were treated with 13-197 and apoptosis was measured using the live/dead assay (Figure 1A). Treatment with 13-197 resulted in 4X more apoptosis in treated cells than in

Figure 1: 13-197 blocks cell cycle progression and induces cell death in MIAPaCa-2 cells. A) Treatment with 13-197 led to increased apoptosis in MIAPaCa-2 cells as measured by the live/dead cellular assay. B) Treatment with 13-197 led to an increase in the number of cells in the G1 phase of the cell cycle and reduced levels of E2F, PCNA and Cyclin D1. Reproduced from ref 128
control cells. Subsequent studies demonstrated induction of cell cycle arrest upon treatment with 13-197 (Figure 1B, left). These studies also showed reduced levels of the cell cycle markers E2F, PCNA and Cyclin D1 (Figure 1B, right), demonstrating that 13-197 inhibits MIAPaCa-2 cell growth through induction of G1 arrest.128

As the NF-κB pathway is known to control the transcription of genes that regulate growth, apoptosis and the cell cycle,142,143 processes which are all affected by 13-197, the small molecule was screened for activity in this pathway. For the screen, a lung cancer cell line (A549) that was specifically designed to express firefly luciferase under control of an NF-κB-driven promoter was used to monitor the activity of NF-κB in response to TNFα (Panomics). Cells were treated with the compounds for 2 h and then TNFα was added to cells to stimulate the NF-κB pathway for an additional 6 h. Under multiplexing conditions the cells were assayed for viability using AlamarBlue (Ab Serotec) and TNFα-induced IKKβ-mediated NF-κB-driven transcription using the ONE-Glo luciferase system (Promega). Treatment with 13-197 resulted in a 75% decrease in NF-κB-driven transcription compared to DMSO-treated cells.

As NF-κB is known to regulate invasion and metastasis,144 the ability of 13-197 to block invasion and migration of MIAPaCa-2 cells was also measured (Figure 2). 13-197 inhibited both the invasion and migration of MIAPaCa-2 cells by about 50%, demonstrating that 13-197 may inhibit both the growth of primary tumors and their metastasis.

**Figure 2: 13-197 inhibits invasion and migration of cancer cells.** Treatment with 13-197 led to decreased invasion and migration of MIAPaCa-2 cells. Reproduced from ref 128
With this building evidence that 13-197 could serve as an inhibitor of viability, cell cycle progression and invasion and migration of cancer cells, we decided to further investigate 13-197’s mechanism of action and its effects on cancer cells via its inhibition of the NF-κB pathway.
Results

13-197 was identified as an inhibitor of TNFα-induced IKKβ-mediated NF-κB-driven transcription in luciferase A549 cells. In a focused, follow-up screen using the same assay, 13-197 and quinoxaline urea analogs were screened for activity against TNFα-induced IKKβ-mediated NF-κB-driven luciferase expression at 20 µM to generate a small structure activity relationship (Figure 3). The well-studied NF-κB pathway inhibitor parthenolide was used as a positive control. Small molecule 13-197 features furan rings at the R1 positions and a bromo- substituent at the R2 position. Replacement of the R1 furan rings with pyridine rings (20-185) resulted in negligible loss of activity. Replacement of the R1 furan rings with a methyl group (20-162) had a greater effect on activity.

*Figure 3: Subset of a focused library screened to identify inhibitors of TNFα-driven IKKβ-mediated NF-κB activation.* Quinoxaline analogs were screened for activity against the NF-κB pathway at 20 µM. The core structure of the analogs featured in this screen and the positive control parthenolide is given above the graph and the structures for the quinoxaline analogs are given to the right.
Furthermore, substitution of the bromo-group at the R2 position of analog 20-162 with hydrogen yielded analog 13-123, which displayed no activity in the assay.

A follow-up screen against 318 kinases identified IKKβ as a molecular target of 13-197. The kinase screen was outsourced to Reaction Biology Corp. 13-197 was screened in duplicate against 318 kinases. Six kinases were identified as hits based on >2 SD (>95% confidence) from the mean of the screen (Figure 4). These kinases included ephrin type-A receptor 6 (EPHA6), mitogen-activated protein kinase kinase kinase kinase 4 (MAP4K4), IKKβ, never in mitosis A-related kinase 3 (Nek3), serine/threonine protein kinase 25 (STK25) and tropomyosin receptor kinase A (TrkA). The IC50 values were in the low-μM range for four of the six kinases inhibited by 13-197. The kinases TRKA and IKKβ had the lowest IC50 values at 1.9 ± 0.2 μM and 3.0 ± 0.1 μM respectively.

As IKKβ phosphorylates IκBα upon stimulation with TNFα, we hypothesized that treatment with 13-197 should block IκBα phosphorylation. To test this, HEK293 cells were pretreated with DMSO or 13-197 for 2 h and then stimulated with 20 ng/mL TNFα for increasing times (0, 5, 10, 15, 20, 30, 45, 60, 75, 90 and 120 min) to drive IKKβ activation and IκBα phosphorylation and degradation. Cells were harvested and the cytoplasmic fraction was probed for phosphorylated and total IκBα to measure IKKβ activity (Figure 5). In DMSO-treated HEK293 cells, stimulation with TNFα resulted in phosphorylation of IκBα.
by the 5 min time point. Thirty minutes after stimulation, most of the IκBα in the cell had been phosphorylated and degraded via phosphorylation-mediated proteasomal degradation. As IκBα is a target gene of NF-κB, IκBα re-expression was seen 45 min post-stimulation. The newly synthesized IκBα present at the 45 min time point was again phosphorylated by IKKβ. In the presence of 13-197, little inhibition of IκBα phosphorylation and degradation was seen at the early time points. A similar pattern to DMSO-treated cells is seen at the 30 min time point and at 75 minutes post-stimulation re-expression of IκBα is seen. However, a significant reduction in phospho-IκBα levels in comparison to DMSO-treated lanes is seen at the latest time points. The results of this experiment suggest that 13-197 does not inhibit the resting form of IKKβ that would persist prior to TNFα treatment but rather inhibits the phosphorylated form of IKKβ that persists approximately 1 h following TNFα stimulation.

To further test the hypothesis that 13-197 inhibits phosphorylated IKKβ and not resting IKKβ, we compared the activity of 13-197 to that of the commercially available, ATP-competitive IKKβ inhibitor TPCA-1118 (Figure 6). As before, cells were pretreated with
compound (13-197 or TPCA-1) and then stimulated with TNFα to drive activation of the NF-κB pathway. In 13-197-treated cells there is rapid phosphorylation of IκBα. However, when cells are treated with TPCA-1 a different pattern is seen. In cells treated with TPCA-1, there is no early phosphorylation of IκBα, demonstrating that TPCA-1 inhibits resting, unphosphorylated IKKβ and thus inhibits the rapid phosphorylation of IκBα seen within minutes of TNFα stimulation in DMSO- or 13-197-treated cells. These results further support the hypothesis that 13-197 preferentially targets phosphorylated IKKβ and establishes a unique mechanism of action for the small molecule.

![Figure 6: Inhibition of resting IKKβ by TPCA-1 but not 13-197.](image)

IKKβ was activated by TNFα (20 ng/mL) in HEK293 cells pretreated with vehicle, TPCA-1 or 13-197 (20 μM). Cell lysates were generated at early time points and probed for phospho-IκBα, total IκBα and tubulin. Reproduced from ref128

Since the knockout of IKKβ has been shown to stall the progression of Kras-driven pancreatic cancer at PanIN lesions in mice,72 and previous data had shown activity in pancreatic cancer cell lines,145 we next decided to explore the role of IKKβ in pancreatic cancer cell lines. RIPA whole cell lysates of Hs766T, MIAPaCa-2, Capan-2, Suit-2 and AsPC-1 pancreatic cancer cells lines were probed for phosphorylated and activated IKKβ (Figure 7). Robust phosphorylation of the kinase was seen in all 5 pancreatic cancer cell lines, suggesting that activation of the kinase indeed may play a role in pancreatic cancer.
To test if 13-197 would inhibit the activated IKKβ that was seen in the pancreatic cancer cell lines, Dr. Vashti C. Bryant and I treated a panel of pancreatic cancer cells with 13-197 and probed lysates for levels of phospho- and total IκBα (Figure 8). Treatment with 20 µM 13-197 for two hours resulted in decreased phosphorylation of the IKKβ substrate. Next, we treated MIAPaCa-2 pancreatic cancer cells with 13-197 and monitored phosphorylation of IκBα upon treatment with increasing doses of 13-197 or increasing exposure to a single concentration of 13-197 (Figure 9A). Additionally, we assessed the viability of a panel of cell lines over time using the AlamarBlue assay (Figure 9B and C). 13-197 inhibited IκBα phosphorylation and cell viability at low µM-potency.

![Figure 7: IKKβ is phosphorylated in pancreatic cancer cell lines. Pancreatic cancer cell lines were probed for phosphorylated and total IKKβ. Under basal conditions, IKKβ is phosphorylated in pancreatic cancer cell lines. This experiment was performed with Jacob Contreras.](image)

![Figure 8: 13-197 reduces IκBα phosphorylation in pancreatic cancer cell lines. Pancreatic cancer cell lines were treated with 13-197 at 20 µM for 2 hours and lysates were probed for phosphorylated and total IκBα. AsPC1, Capan-2 and Hs766T immunoblotting performed by Dr. Vashti C. Bryant. Reproduced from ref[128](image)
Given NF-κB’s role in anti-apoptotic signaling, we next investigated 13-197’s ability to induce apoptosis in cancer cells through down-regulation of anti-apoptotic proteins. To test this, I treated MIAPaCa-2 cells with 13-197 at increasing concentrations or for increasing periods of time and probed lysates for Bcl-xL, Mcl-1, survivin and XIAP, which are all known target genes of NF-κB. Dose-dependent decreases in the levels of Bcl-xL, survivin, Mcl-1 and XIAP were seen in cells treated with 10 or 20 µM 13-197 for 2 h (Figure 10, left panel). Furthermore, decrease of Bcl-xL, survivin and Mcl-1 levels was time-dependent with Mcl-1 levels decreasing within 30 min of 13-197 treatment (Figure 10, right panel), suggesting IKKβ may regulate the stability of Mcl-1 via phosphorylation of the protein. Overall, this data demonstrates that 13-197 down regulates anti-apoptotic proteins in both dose- and time-dependent manners.

![Figure 9](image)

**Figure 9: 13-197 reduces IκBα phosphorylation and viability of pancreatic cancer cells.** A) Treatment with 13-197 led to decreased expression of P-IκBα in pancreatic cancer cells. Furthermore, this inhibition of IκBα phosphorylation was dose dependent. Reproduced from ref\(^{28}\) B) Treatment with 13-197 reduced cell viability in a panel of pancreatic cancer cells. C) Representative dose-response curve with MIAPaCa-2 cells. Bars represent standard error of the mean for three separate experiments conducted at least in duplicate.
Due to these promising cell-based results, which demonstrated decreased NF-κB-driven transcription, decreased phosphorylation of IkBα, inhibition of phosphorylated IKKβ activity, decreased cell viability and increased apoptosis in cancer cells, small molecule 13-197 was moved into a mouse model of pancreatic cancer by collaborators, namely Dr. Prakash Radhakrishnan.\textsuperscript{128} An orthotopic mouse model of pancreatic cancer was used to investigate 13-197’s anticancer properties \textit{in vivo}. For this study, MIAPaCa-2 cells were orthotopically placed in the pancreas of nude mice and mice were allowed to heal after surgery. Tumors were given 2 weeks to grow and were palpable and the end of this period. Tumor-bearing mice were randomized and half the animals were treated orally with 150 mg/kg 13-197 in cremaphor daily while the other half was treated with vehicle control. At the end of 30 days the mice were sacrificed and the tumors weights and volumes were measured.

Tumor weights and volumes were reduced by 50% in 13-197-treated animals compared to vehicle-treated mice (Figure 11A). Fewer tumor nodules were seen in other organs of 13-197-treated animals compared to vehicle-treated animals (Figure 11B), which is consistent with our cell-based data demonstrating inhibition of MIAPaCa-2 cell growth and invasion and migration. Proliferation index and microvessel density were

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure10.png}
\caption{13-197 decrease levels of anti-apoptotic proteins in cancer cells. Treatment with 13-197 led to decreased expression of anti-apoptotic proteins Mcl-1, Bcl-xL, XIAP and survivin. Reproduced from ref \textsuperscript{128}}
\end{figure}
measured to assess the number of cells dividing in tumors and as measure of angiogenesis, respectively (Figure 11C and D). A reduction of both, as measured by Ki-67 staining and CD31 staining, respectively was seen in 13-197-treated mice compared to vehicle controls. Tumor tissues were scored by a pathologist (Dr. Subodh Lele) and decreased inflammation and increased necrosis was seen in 13-197-treated mice (Figure 11E). The levels of 13-197 in the pancreas, liver and serum were determined at the end of the study with the levels being the highest in the liver. Due to the high levels of 13-197 in the liver, levels of ALT and AST were measured in 13-197- and vehicle-treated mice (Figure 11F and G). No difference in the levels of these enzymes were seen between 13-197- and vehicle-treated mice, indicating an absence of liver toxicity (Figure 11G). Macroscopic examination of the organs of 13-197-treated and vehicle-treated mice found no obvious toxicities in 13-197-treated animals. Overall, these studies show that 13-197 is an orally available small molecule that exhibits excellent distribution and reduces tumor burden and metastases in a mouse model of pancreatic cancer without inducing toxicity.
Figure 11: Treatment with 13-197 reduces tumor burden and metastases in a mouse model of pancreatic cancer. A) Tumor weights and volumes in 13-197- and vehicle-treated mice at the end of the study. B) Number of animals with tumors and number of tumor nodules found in various organs. C) Ki-67 staining. D) CD31+ staining. E) Inflammation and necrosis scored by a pathologist (Dr. Subodh Lele). F) 13-197 levels in the pancreas, liver and serum 18 h after the last treatment. G) AST and ALT levels measured in mice at the end of the study. (**P < 0.05). The work depicted in this figure was performed by Dr. Prakash Radhakrishnan and collaborators from Tony Hollingsworth’s lab and Dr. Subodh Lele. Reproduced from ref 28.
On-going studies with 13-197

As other NF-κB pathway inhibitors have shown an ability to chemo sensitize cells to treatment with the clinical therapeutics gemcitabine and paclitaxel, we are investigating the ability of 13-197 to synergize with these compounds in pancreatic cancer cells. To accomplish this goal, I have determined IC\textsubscript{50} values for gemcitabine and paclitaxel in a panel of pancreatic cancer cell lines (Capan-2, MIAPaCa-2 and SUIT2 cells) for which 13-197 sensitivity has already been established (Figure 9, this chapter). A representative dose-response graph for MIAPaCa-2 cells is shown (Figure 12). MIAPaCa-2, Capan-2 and SUIT-2 cells were sensitive to the clinical inhibitor paclitaxel with IC\textsubscript{50} in the low (Capan-2 and MIAPaCa-2) or mid (SUIT-2) nM range while all displayed IC\textsubscript{50} to gemcitabine in the mid µM range. Interestingly, the two cell lines that are not sensitive to

<table>
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<th>Cell Line</th>
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<th>Gemcitabine EC\textsubscript{50} (µM)</th>
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<tr>
<td>Capan-2</td>
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<td>0.65 ± 0.20</td>
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<tr>
<td>MIAPaCa-2</td>
<td>0.002 ± 0.001</td>
<td>0.54 ± 1.32</td>
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<tr>
<td>SUIT-2</td>
<td>0.04 ± 0.01</td>
<td>0.24 ± 0.042</td>
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<tr>
<td>AsPC-1</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Hs766T</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
</tr>
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</table>

Figure 12: IC\textsubscript{50} values for paclitaxel and gemcitabine in five pancreatic cancer cell lines that are sensitive to 13-197 treatment. A) IC\textsubscript{50} values for paclitaxel and gemcitabine for a panel of 5 pancreatic cancer cell lines. Representative dose response curves for paclitaxel and gemcitabine in MIAPaCa-2 pancreatic cancer cells.
treatment with paclitaxel and gemcitabine, AsPC-1 and Hs766t, above 100 µM are sensitive to 13-197 at approximately 17 and 14 µM respectively,\textsuperscript{128} suggesting 13-197 may serve as a viable therapeutic for the treatment of pancreatic cancer resistant to current clinical inhibitors.

Using these IC\textsubscript{50} values as starting points, we will treat the cells lines with 13-197 and gemcitabine, 13-197 and paclitaxel or 13-197, gemcitabine and paclitaxel in combination at 5X their IC\textsubscript{50}s, their IC\textsubscript{50}s and subIC\textsubscript{50} concentrations of the clinical inhibitors in matrix format to test for possible synergism. CI values will be derived from pancreatic cancer cell treatments at the above concentrations for 72 hours. Cell viability will be determined via AlamarBlue assay and the fraction of cells dead for each treatment will be calculated. These values will be used to calculate combination index (CI) values using CalcuSyn and the Chou-Talalay method for calculating CI values. Using this method, a combination index will be calculated for a combination treatment based on the fraction of cells affected (fraction dead) by a particular treatment. CI values below 1 indicate synergism. Additive effects are indicated by a CI value of 1. A value above 1 indicates antagonism.\textsuperscript{147} As other NF-κB pathway inhibitors have shown synergism with gemcitabine and paclitaxel,\textsuperscript{80,82,114,115} we expect to see the same with 13-197.

Conclusions

The work presented in this chapter provides evidence that IKKβ is phosphorylated and activated in pancreatic cancer cells which leads to the phosphorylation of IκBα and subsequent activation of the NF-κB pathway. Treatment of HEK293 cells with the quinoxaline urea analog 13-197 results in decreased phosphorylation of IκBα an hour following stimulation with TNFα, suggesting 13-197 targets a phosphorylated, activated form of IKKβ but not resting IKKβ. In pancreatic cancer cells, 13-197 targets IKKβ to regulate transcription through the IKKβ/IκBα/NF-κB pathway, resulting in decreased
expression of anti-apoptotic proteins Bcl-xL, Mcl-1, survivin and XIAP and decreased viability of cancer cells. Furthermore, 13-197 exhibited low-μM potency in two cell lines (AsPC-1 and Hs766T) that are resistant to treatment with paclitaxel and gemcitabine. Lastly, work with collaborators has shown that 13-197 reduces tumor burden in a mouse model of pancreatic cancer without inducing toxicities associated with known IKKβ inhibitors. Further studies with the small molecule will be aimed at further refining the small molecule’s mechanism of action by identifying the form of IKKβ inhibited by 13-197.
Chapter 6: Evaluation of small molecule 25-4

Results

As mentioned previously, the small molecule 25-4 was designed to feature the same \( \alpha \)-methylene-\( \gamma \)-butyrolactone motif found in the natural product parthenolide, which has been shown to have activity against the NF-\( \kappa B \) pathway despite its limited success in clinical trials.\(^{122, 124}\) The available SAR with parthenolide analogs clearly shows that the Michael acceptor in the \( \alpha \)-methylene-\( \gamma \)-butyrolactone is critical for activity against the NF-\( \kappa B \) pathway.\(^{117}\)

Here we generated a biased library that features the \( \alpha \)-methylene-\( \gamma \)-butyrolactone core and tested this library for activity against TNF\( \alpha \)-induced IKK\( \beta \)-mediated NF-\( \kappa B \)-driven transcription activation again using our cell-based luciferase assay (Figure 1). At 20 \( \mu \)M, small molecule 25-4 displayed high activity in the screen, inhibiting NF-\( \kappa B \)-driven transcription by nearly 75%. Reduction of the double bond of the \( \alpha,\beta \)-unsaturated lactone resulted in compound 25-18, which showed no activity in the screen. 28-090, which is an acyclic analog of 25-4, showed reduced activity compared to 25-4, suggesting the \( \alpha,\beta \)-unsaturated lactone and rigidification of the Michael acceptor are necessary for the high activity of 25-4. Substitution of the hydrogen at the R1 position with a methyl group resulted in small molecule 28-097C, which showed activity comparable to that of 25-4 in the assay. 28-097A, which is an acyclic version of 28-097C, showed reduced activity in the assay, again indicating that rigidification of the Michael acceptor is a favorable feature. Compounds 28-131, 28-138 and 28-171 feature the same hydrogen at the R1 position as 25-4 but feature substitutions at the R3, R2 and R4, and R5 positions, respectively. 28-131 which features the iodo- substitution at R3 showed a moderate decrease in activity while substitution with methyl groups at the R2 and R4 positions (28-138) or a phenyl substitution at the R5 position (28-171) resulted in activity comparable to that of 25-4.
Overall, these SAR studies showed that the presence and rigidification of the Michael acceptor in the α,β-unsaturated lactone were key for activity.

To confirm that rigidification indeed resulted in increased activity and to determine if the Michael acceptor is critical for activity, we further compared the activity of 25-4, the reduced compound that lacks the Michael acceptor (25-18) and the acyclic compound 28-090 to that of parthenolide and a reduced parthenolide analog that lacks the Michael acceptor (Figure 2A). Consistent with previous reports, parthenolide showed more than 95% inhibition while the reduced version was much less active and only exhibited approximately 20% inhibition, demonstrating that the Michael acceptor in parthenolide is critical for NF-κB inhibitory activity (Figure 2B). Similarly the reduced analog 25-18 that lacks the Michael acceptor was >10-fold less active (approximately 5% inhibition) than its

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**Figure 1**: Novel α-methylene-γ-butyrolactone analogs exhibit activity against the NF-κB pathway. Inhibition of TNFα-induced IKKβ-mediated NF-κB activity by the α-methylene-γ-butyrolactone analogs. The core structure of the compounds featured in the focused library of α-methylene-γ-butyrolactone analogs and parthenolide is above the graph while each analog's structure can be found to the right. Library synthesized by Dr. Sandeep Rana.
corresponding analog with the Michael acceptor (25-4). Importantly the acyclic analog with the Michael acceptor (28-090) was approximately 2-fold less active than the cyclized version, suggesting that rigidification indeed increases activity against the NF-κB pathway.

In a follow-up dose-response study, 25-4 had low μM (IC\textsubscript{50} ~ 4 μM) inhibitory activity in the TNF\textalpha-induced IKKβ-mediated NF-κB-driven transcription activity assay (Figure 2C). Overall, these studies identified compound 25-4 with the \( \alpha \)-methylene-\( \gamma \)-butyrolactone as a potent NF-κB inhibitor. Like parthenolide, the NF-κB inhibitory activity of 25-4 requires the Michael acceptor. Additionally, rigidification of the Michael acceptor of 25-4 increases its NF-κB inhibitory activity.

We next used click chemistry, which is emerging as a powerful technique for determining the targets of covalent small molecules,\textsuperscript{148} to determine if 25-4 does indeed bind irreversibly to the parthenolide targets IKKβ and NF-κB.\textsuperscript{122, 130} For these studies, the 25-4 analog 31-036 (Figure 3), which is 25-4 with an alkyne handle at the R1 position, was used. The alkyne handle on 31-036 reacts with the azide handle on TAMRA-azide to covalently link the two, thereby tagging 31-036 and its target proteins with a fluorescent

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**Figure 2: Focused SAR with parthenolide, reduced parthenolide and 3 analogs.** A) Structure of parthenolide, reduced parthenolide and 3 analogs B) Evaluation of the above inhibitors in the TNF\textalpha-induced IKKβ-mediated NF-κB-driven transcription assay demonstrates the importance of the Michael acceptor and rigidification C) Dose-dependent inhibition of NF-κB activity by 25-4. 25-4, 25-18 and 28-090 synthesized by Dr. Sandeep Rana.
rhodamine tag. Recombinant IKKβ and NF-κB were incubated with 31-036 for 1 h at room temperature. Rhodamine azide and click reagents were added to the reaction mixture and incubated for an additional hour. At the end of the second hour the mixture was subjected to SDS-PAGE and the gel was imaged using the Typhoon 9410 Variable Mode Imager, an imager that produces digital images of fluorescent samples. The data summarized in Figure 3A shows fluorescent bands at molecular weights that correspond to IKKβ and NF-κB (with a 14 amino acid C-terminal truncation), demonstrating that 31-036 is a covalent inhibitor of IKKβ and NF-κB.

To determine if covalent binding to IKKβ results in the inhibition of the kinase activity of IKKβ, MBA-MD-231 breast cancer cells were incubated with 25-4, 25-18, 28-090, 28-097A and 28-097C (Figure 3B). The cells were lysed and the proteins were separated by SDS-PAGE, transferred to a membrane and probed with total and phospho-IκBα antibody. Since IκBα is a substrate of IKKβ, inhibition of the kinase activity of IKKβ
should result in reduced IκBα phosphorylation. Indeed we observed complete inhibition of IκBα phosphorylation in cells treated with 25-4 but no inhibition of IκBα phosphorylation in cells treated with the reduced compound 25-18 that lacks the Michael acceptor. The acyclic analog 28-090 showed partial inhibitory activity, which confirms that rigidification of the Michael acceptor indeed results in increased inhibition of IKKβ. Furthermore, 28-097C showed activity while its acyclic version, 28-097A, did not, supporting the observation that rigidification of the Michael acceptor is required for activity and confirming the results of our luciferase assay.

Studies with 25-4 demonstrated that the small molecule inhibits phosphorylation of IκBα in a dose-dependent manner in MIAPaCa-2 cells (Figure 3C). Cells were incubated with increasing doses of 25-4 or 25-18 for 2 h. Cells were harvested and immunoblotting with anti-phospho- and total IκBα antibodies was performed. 25-4 treatment, but not 25-18 treatment, reduced levels of IκBα phosphorylation, providing further evidence that 25-4 targets IKKβ while 25-18 does not display activity.

To determine if inhibition of the IKKβ-NF-κB pathway translates to anticancer activity, MDA-MB-231 and MIAPaCa-2 cancer cell lines were subjected to increasing concentrations of the IKKβ inhibitors for 3 days and the viability of the cells was assessed using PrestoBlue dye. The IC₅₀ values for the IKKβ inhibitors were derived by curve fitting the data. 25-4 showed dose- and time-dependent inhibition of cancer cell growth while 25-18 did not (Figure 4A and 4B) again demonstrating that the Michael acceptor is critical for anticancer activity. Furthermore, we subjected a panel of cancer cell lines to treatment with 25-4, 25-18 and three previously reported IKKβ inhibitors (13-197, Bayer VIII and TPCA-1). Importantly, the growth inhibitory activity of 25-4 was comparable to known IKKβ inhibitors (Table 1).
Figure 4: 25-4 decreases cancer cell growth. Dose-response studies with 25-4 and 25-18 in A) MDA-MD-231 and B) MIAPaCa-2 cells demonstrate that 25-4 dose- and time-dependently reduces cell viability of cancer cells while the small molecule lacking the Michael acceptor (25-18) does not.

Table 1: Inhibition of cancer cell growth by 25-4 and known IKKβ inhibitors. 25-4 inhibits the growth of cancer cells with IC50 values comparable to those of commercially available IKKβ inhibitors. IC50 values for 13-197 in MDA-MB-231 cells and IC50 values for Bayer VIII and TPCA-1 were determined by Smitha Kizhake.
Tian Zhou next helped us determine the ability of 25-4 and 25-18 to block colony formation using a clonogenic assay. Cells were sparsely plated and allowed to grow in the presence or absence of 25-4 or 25-18 for 7 days. Colonies were then stained using crystal violet and quantified (Figure 5A and B). In plates treated with 25-4 we observed a dose-dependent decrease in the number of colonies while we saw no such effect with 25-18.

Figure 5: 25-4 inhibits colony formation and induces caspase 3/7 activation in cancer cells. (A and B) Dose-dependent effects on colony formation of cervical cancer cells (HeLa) by 25-4 and 25-18. (C) Caspase 3/7 activity induced by 25-4, cisplatin and the combination in SKOV3 ovarian cancer cells. Tian Zhou performed the clonogenic assay pictured in A and B.

As the NF-κB pathway controls the transcription of many genes that regulate apoptosis, we evaluated the ability of 25-4 and 25-18 to induce caspase 3/7 (Figure 5C) and PARP cleavage (Figure 6), two key events during apoptosis, in cancer cell lines. Furthermore, we studied the ability of 25-4 to sensitize cells to treatment with cisplatin as platinum-based chemotherapeutics are the first line of treatment for many cancers, including ovarian cancer. In cells treated with 25-4 we observed modest induction of caspase 3/7 cleavage by itself and increased induction of caspase 3/7 cleavage in the
presence of a cisplatin. This is consistent with reports that implicate activation of NF-κB in chemoresistance. We also observed decreased/cleaved PARP in cells treated with 25-4. Importantly we observed no such effects in 25-18-treated cells. These studies clearly demonstrate that the biological effects induced by 25-4 are through the Michael acceptor functionality.

Due to these promising cell-based results, we next collaborated with investigators at Henry Ford Hospital to investigate if these anticancer effects in cell lines translate to mouse models. To do this, two studies were conducted. One aimed to determine the optimal doses of 25-4 and cisplatin that are required to inhibit tumor growth in an orthotopic ovarian cancer model and the second tested 25-4’s ability to synergize with cisplatin. For each, A2780 ovarian cancer cells were injected into the peritoneal cavity of nude mice and the tumors were allowed to establish. On day 3 of the first study, mice were dosed with one of three doses of 25-4 (1, 2.5 or 5 mg/kg given 5 days a week for 4 weeks) or one of two doses of cisplatin (2 or 4 mg/kg cisplatin given on days 5, 9, 14, and 21). In a

![Figure 6: Induction of PARP cleavage by 25-4. Treatment with 25-4 led to PARP cleavage in MIAPaCa-2 (pancreatic), SKOV3 and A2780 (ovarian) and HeLa (cervical) cancer cell lines.](image)
preliminary metabolism study conducted in mice, the plasma concentration of parthenolide was 0.112 µM one hour following oral dosing of 4 mg/kg of the drug.\textsuperscript{124} As the molecular weights (248.317 g/mol and 215.21 g/mol, respectively) and potency against TNFα-induced IKKβ-mediated NF-κB activity of parthenolide and 25-4 are comparable and the small molecules are expected to exert their effects similar mechanisms of action, we choose to treat mice with comparable doses of 25-4 (1, 2.5, and 5 mg/kg). Though PK studies were not conducted with 25-4, the goal of this study was just to establish that 25-4 exhibits anticancer properties in mice and is non-toxic in mice at a therapeutic dose. At the end of the study, the mice were sacrificed and the tumor weights determined (Figure 7). As other IKKβ inhibitors have shown toxicities in mice, it is important to note that no overt toxicity was observed in the mice treated with 25-4 (aside from blocked bowels at the highest dose). Additionally, approximately 40% reduction in tumor weights in mice

\textbf{Figure 7:} 25-4 decreases A2780 tumor weight alone and in combination with cisplatin in a mouse model of ovarian cancer. Dose-dependent effects on A2780 ovarian cancer cell growth by 25-4, cisplatin and the combination in an orthotopic model of ovarian cancer. P values were calculated only for 2.5 mg/kg 25-4 compared to vehicle and the combination of 25-4 and cisplatin compared to each single treatment. Mouse studies performed by Calvin Tebbe, Adnan R. Munkarah and Ramandeep Rattan, Josephine Ford Cancer Center.
treated with 25-4 (5 mg/kg) and 25% reduction of tumor weights in mice treated with cisplatin (2 mg/kg) were observed when compared to controls. To determine if 25-4 could chemosensitize ovarian tumors to cisplatin, a follow up study in which mice were treated with 2.5 mg/kg of 25-4 (5 days a week for 4 weeks) and 2 mg/kg of cisplatin (on days 5, 9, 14 and 21) was performed. The combination resulted in 60% reduction in tumor weights compared to the controls. These studies clearly demonstrate that 25-4 by itself has anticancer activity and the ability to chemosensitize ovarian tumors to cisplatin.

Next, with the help of Dr. Prakash Radhakrishnan we performed immunohistochemical analysis of the excised tumor tissue (Figure 8). Immunohistochemistry revealed that treatment with 25-4, cisplatin and the combination reduced levels of Mcl-1 staining by over 66%.

**On-going projects with 25-4**

To further validate that 25-4 binds to IKKβ and p65 to drive cancer cell death, we are conducting a series of experiments using click chemistry to determine the targets of 25-4. As the small molecule is designed to target surface-exposed cysteine residues, we first incubated the small molecule at 20 µM with a panel of cancer cell lines including luciferase A549 cells, which is the cell line we used to measure TNFα-induced IKKβ-mediated NF-κB-driven transcription activity, MBA-MD-231 cells, HeLa cells and SKOV3 cells for 1 h. Cells were harvested and 200 µg of whole cell lysate was incubated with TAMRA-azide and additional click reagents for 1 h. Samples were resolved by SDS-PAGE and fluorescent bands were imaged using the Typhoon (Figure 9). At this concentration, time point and Typhoon setting, the luciferase
A549 cells have the most distinct banding pattern but similar bands are seen in the other cell lines.

Moving forward, we characterized the bands (“click signature”) seen in MDA-MB-231 cells as we had accumulated growth and IKKβ inhibition data in these cells. First we performed a dose-response study with 31-036 and MDA-MD-231 cells (Figure 10, left) and saw that 31-036 binding to each of its targets in MDA-MD-231 cells was indeed dose-dependent. Next, we wanted to see how quickly 31-036 would bind to its targets in cells. To test this, we treated MDA-MD-231 cells with 31-036 for 5 min, harvested cells and performed click chemistry (Figure 10, right). In MDA-MD-231 cells, 31-036 binding to targets occurs within 5 min. Overall, these studies demonstrate that binding of 31-036 to target proteins in cells is dose-dependent and rapid.

To ensure that 25-4 and 31-036, which again is 25-4 with an alkyne handle at the R1 position, indeed bind the same targets in cells, a competition assay with the two small molecules was performed in MDA-MD-231 cells. MDA-MB-231 cells were incubated with
31-036 or 25-4 for 1 h or with 25-4 for 1 h followed by 31-036 for 1 h. Cells were harvested and click chemistry was performed (Figure 11). Pre-incubation with 25-4 decreased the fluorescent signal produced by 31-036 following click chemistry with 5-TAMRA azide, indicating the small molecules indeed bind the same targets within MDA-MB-231 cells.

To further study and identify all the proteins targeted by 31-036, click chemistry will be performed with 31-036 and a trifunctional TAMRA-biotin-azide. Cells will be treated with 31-036 and harvested, cell lysates will be incubated with TAMRA-biotin-azide and click chemistry will be performed. Pulldown of biotin with streptavidin beads will be performed to capture TAMRA-biotin-azide in complex with 31-036-targeted proteins. These proteins will then be sent out for identification via mass spectrometry (Figure 12 outlines these studies). Towards this goal, we have performed click chemistry with both 31-036 and TAMRA-azide, which has been used in all our previous studies, alongside click chemistry with 31-036 and the trifunctional TAMRA-biotin-azide (Figure 13). These studies show that click

![Figure 10: 25-4 binds to targets in MDA-MB-231 cells dose-dependently and rapidly. MDA-MB-231 cells were treated with the indicated concentrations of 31-036 for the indicated times, harvested and click chemistry with 5-TAMRA azide was performed on 200 µg protein. (Left) 31-036 binds dose-dependently to targets within 231 cells and (Right) binds within 5 min of treatment.](image)
chemistry with the TAMRA-biotin-azide has been optimized and the click signature with the trifunctional TAMRA-biotin-azide replicates the signature seen with TAMRA-azide (Figure 13).

Thus far, our studies with 25-4, 31-036 and click chemistry have demonstrated that 31-036 binding to targets within cells is dose-dependent and rapid and that the small molecule indeed binds the same targets as 25-4. Moving forward, we will use a combination of click chemistry and biotin pulldowns to determine the targets of 31-036, including which form(s) of IKKβ is targeted by the small molecule. Furthermore, we will conduct SAR studies to determine which structural changes to the scaffold can be made to increase specificity towards one target or another to characterize the phenotypic consequence of inhibiting each target of 31-036 and to explore which effects of the small molecule are through inhibition of IKKβ and which effects can be attributed to off-target interactions.

Figure 11: 25-4 and 31-036 bind similar targets in MDA-MB-231 cells. 231 cells were treated with 31-036 or 25-4 for 1 h or with 25-4 for 1 h followed by incubation with 31-036 for 1 h. Click chemistry was performed on 200 µg protein of resulting lysate. Pre-incubation with 25-4 reduces the intensity of the click signature of small molecule 31-036.
Figure 12: Schematic of on-going click chemistry studies. Cancer cells will be incubated with 31-036 to allow the small molecule to bind its targets in cell lysates (or intact cells). Next, lysate will be incubated with the trifunctional TAMRA-biotin-azide to label 31-036 with fluorescent rhodamine and biotin. The biotin probe now covalently linked to target proteins will be pulled down using streptavidin beads. Following pull down, samples will be run on SDS-PAGE and fluorescent bands will be imaged using the Typhoon to confirm pull down of trifunctional azide. Bands will be excised or tryptic digestion will be performed on protein attached to beads and protein targets will be sent for identification via mass spectrometry.

Figure 13: Click chemistry with TAMRA-azide and TAMRA-biotin-azide in cells and with pure p65 protein. The click signature for TAMRA-azide and TAMRA-biotin-azide is similar in cells and with pure protein. Again, this p65 has a 14 amino acid C-terminal truncation, resulting its reduced molecular weight.
Conclusions

A mechanism-based screen with a focused library of $\alpha$-methylene-$\gamma$-butyrolactone containing-analogs led to the identification of 25-4 as a potent inhibitor of TNF$\alpha$-induced IKK$\beta$-mediated NF-$\kappa$B-driven transcription activation. SAR studies revealed that rigidification of the $\alpha$-methylene-$\gamma$-butyrolactone increased the activity of the small molecule. Additionally, reduction of the Michael acceptor in 25-4 yielded 25-18, which was inactive in all assays it has been tested in. Our studies also showed that 25-4 binds covalently to both IKK$\beta$ and NF-$\kappa$B. Furthermore, 25-4 had anticancer activities that are comparable to known IKK$\beta$ inhibitors and sensitizes SKOV3 ovarian cancer cells to treatment with the clinical therapeutic cisplatin. 25-4 inhibited tumor growth by itself and in combination with cisplatin in an orthotopic ovarian cancer model to reduce tumor burden. Furthermore, treatment with the small molecule resulted in reduced levels of Mcl-1, which is in line with data seen with other IKK$\beta$ and NF-$\kappa$B inhibitors. Future studies with 25-4 will be aimed at determining the forms of IKK$\beta$ targeted by the small molecule, identifying the other targets of 25-4 and building specificity onto the small molecule’s core. Importantly, 25-4 is easy to modify and is a suitable starting compound for the extensive SAR studies required to identify compounds with improved biological activity, specificity and drug-like properties.
Chapter 7: Drug discovery and target validation, discussion of results and future directions and closing perspectives

Drug discovery and target validation

Due to the NF-κB pathway’s implication in cancer, our lab initiated two drug discovery projects centered on identifying inhibitors of this pathway. Many lines of evidence caused us to pursue the NF-κB pathway as a target, including the mounting proof that the pathway plays a key role in K-ras-driven cancers and is constitutively activated in cancers either by signaling from the tumor microenvironment or genetic aberrations. Furthermore, tissue specific deletion of pathway members has shown much promise in animal-models of disease and research suggests inhibition of this pathway could sensitize cancer cells to clinical chemotherapeutics such as paclitaxel, cisplatin and gemcitabine.

Small molecule screening typically involves the use of one of two types of screening strategies: either target/pathway-based screening (such as a kinase assay) or phenotype-based screening, which allows evaluation of a small molecule within cells (such as a screen for decreased angiogenesis). To begin searching for small molecule inhibitors of the NF-κB pathway, we chose a target-based screening strategy in which two libraries of small molecules were tested separately in a luciferase assay that monitored TNFα-induced IKKβ-mediated NF-κB-driven transcription in cells. Though this screen allowed us to identify inhibitors that targeted a member of the TNFα/IKKβ/NF-κB pathway, small molecules identified by assessing a particular target are very likely to interact with other proteins in cells and to affect other signaling pathways that may have phenotypic consequences in cells.

To further test a small molecule for specificity towards a particular target, multiple experiments can be performed. These include genetic modification of a target protein in cells to assess if deletion of the protein reproduces the same phenotype as inhibition of
the protein by the small molecule, overexpression or mutation of the protein to drive resistance to the small molecule or underexpression of the protein to drive sensitivity to the small molecule.\textsuperscript{152} Additionally, rescue experiments that can outcompete the effects of a small molecule can also be performed to assess off-target effects of a small molecule.\textsuperscript{153}

Furthermore, affinity methods can be used to assess the binding of small molecules to targets in cells. With this approach, it is assumed that the protein that binds a given small molecule with the highest affinity is the most likely mediator of the biological effect of the small molecule in cells. Furthermore, this method usually requires that 1) the target protein of a small molecule is abundant in cells or tissues and 2) the small molecule is still capable of interacting with its target when attached to resin or biotin, for example, in the case of affinity chromatography,\textsuperscript{154} or a reactive handle, in our case of click chemistry.

Lastly, mechanistic-based evaluation of a small molecule's activity can be performed. For example, inhibition of substrate phosphorylation by a kinase can be used to validate inhibition of kinase activity.

With these principles in mind, a ‘screening cascade’ can be developed to outline the biological assays that are required to validate the target of a small molecule. Figures 1 and 2 give an overview of the assays that were performed to validate IKK\(\beta\) inhibition by 13-197 (Figure 1) and 25-4 (Figure 2). In the following discussion, I will touch on each experiment conducted with the small molecules and outline how each experiment provides evidence that IKK\(\beta\) is targeted by these small molecules. Furthermore, I will suggest future experiments that will be performed to further validate the target of these small molecules and investigate possible off-target effects of the compounds. It is important to note that this ‘screening cascade’ like most is specific to a target of interest (IKK\(\beta\)), which limits the information given regarding off-target effects of compounds 13-197 and 25-4.
Validating the targets of small molecule inhibitors and RNAi

Many of the suggested experiments for determining off-target effects of the novel small molecules 13-197 and 25-4 include the use of RNAi or the genetic knockout or
modification of IKKβ. It is important to remember that although pharmacological inhibition, RNA interference and genetic knockout are all complementary methods for assessing the phenotype caused by the loss of a protein’s activity, these methods each have their own benefits and limitations. For example, small molecules can be costly and time-consuming to develop but their rapid induction of activity can prevent compensation by redundant pathways in cells. Additionally, they allow the probing of a specific function of a protein (kinase activity or a protein-protein or protein-DNA interaction) without abrogating all functions of the protein (a scaffolding function in the instance of kinase inhibition). RNAi interference and genetic knockout, on the other hand, can be more selective but long-term loss of a protein may allow for compensation and will result in the loss of all functions of a protein. Furthermore, it is key to remember that each method requires its own validation. Here I will briefly touch on validation techniques for small molecule inhibitors and RNAi approaches.

In the case of small molecules, SAR studies are required to demonstrate that modifying substituents of a core structure leads to a range of potencies against a target of interest. Furthermore, an analog with a very similar scaffold but substituent change that renders it inactive may need to be developed as a negative control (reduced 25-4 analog 25-18, for example). Additionally, a small molecule with a very different structure but same target and potency for that target can be selected as a positive control. As unrelated core structures are expected to have different off-target effects, comparison of the phenotype induced by this independent positive control and a novel small molecule under validation may give insights into target-independent effects. Lastly, rescue experiments involving the expression of a kinase, for example, that is resistant to an inhibitor of interest can be performed to study off-target effects. Small molecules should also display dose-dependency as this demonstrates saturatable inhibition of a target.
RNAi experiments also require validation and careful consideration. siRNA, for example, should be designed after careful analysis of the mRNA sequence being targeted to assure targeting of a desired isoform and targeting of a sequence that has little similarity to other mRNAs. Again, causality can be confirmed with siRNA. Firstly, a combined pool of siRNAs can be used to target a protein of interest. This minimizes off target effects in two ways. This allows the use of small concentrations of each siRNA, minimizing the off-target effect of each but giving efficient reduction in protein production. Furthermore, as each siRNA’s off-target effects are sequence, and not necessarily target specific, the use of two independent siRNAs to demonstrate the same phenotype upon target repression will also establish causality.\(^{153}\)

Additionally, controls such as mock transfection should be performed to control for use of transfection reagent. Also, reduction of mRNA should be monitored using RT-PCR and loss of protein should be verified by Western blot. Lastly, rescue experiments can also be performed with siRNA. Here, the expression of an RNAi-resistant transgene can be used to rescue the phenotype.\(^{153}\)

Additionally it is important to keep in mind that each method may result in a different phenotype, making it difficult to draw exact correlations between pharmacological inhibition and genetic inhibition.\(^{153}\) For example, if IKK\(\beta\)’s role as a member of the IKK complex (and not just as a catalytic subunit of the complex) is key for the protein’s activity in some biological process, inhibition of the protein using a pharmacological agent such as 13-197 or 25-4 may not affect its function in this particular pathway. However, loss of IKK\(\beta\) through RNAi or genetic knockout may induce a phenotype dependent on the loss of IKK\(\beta\) function as a scaffold. To test if IKK\(\beta\) plays a scaffolding role, catalytically inactive IKK\(\beta\) can be expressed in RNAi-targeted to cells to rescue the phenotype induced by loss
of IKKβ protein but not enzymatic activity. This phenotype should mirror that of pharmacological inhibition of the kinase.

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<th>1. Hit identification of 25-4</th>
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<td>Luciferase assay (TNFα-driven IKKβ-mediated NFκB-driven transcription assay)</td>
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<th>2. Structure Activity Relationship Studies</th>
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<td>Luciferase assay</td>
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<th>3.1 Cell-Free validation of target binding</th>
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<td>Click chemistry with pure IKKβ and p65</td>
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<th>4.1 Cell-based Studies: Mechanism-based studies</th>
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<td>• Detection of activated IKKβ in model cell lines</td>
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<td>- Detection of basal IκBα phosphorylation</td>
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<td>• Inhibition of IKKβ substrate phosphorylation</td>
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<th>4.2 Cell-based Studies: Phenotypic assays</th>
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<td>• Inhibition of cell proliferation</td>
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<td>• Induction of PARP cleavage</td>
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<td>• Inhibition of colony formation</td>
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<th>5. Animal-based Studies: Orthotopic tumor models</th>
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<td>• Decreased tumor weights</td>
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<td>• Inhibition of NFκB-driven transcription</td>
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<tr>
<td>- Reduced levels of Mcl-1</td>
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<td>(Mouse work performed by collaborators at Henry Ford Hospital, Mcl-1 staining performed by Dr. Prakash Radhakrishnan)</td>
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<th>6. Future Studies: Studies to assess off-target effects</th>
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<td>• Cell-free kinase assays with entire kinome</td>
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<td>- Will demonstrate effects similar to inhibition of IKKβ, p65</td>
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<td>• Overexpression of IKKβ</td>
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<td>- Will drive insensitivity to IKKβ inhibition</td>
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<td>• Expression of C179A IKKβ, C38S NFκB</td>
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<td>- C179A IKKβ will drive insensitivity to IKKβ inhibition</td>
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<td>- C38S NFκB will drive insensitivity to NF-κB inhibition</td>
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<td>• Mass spec identification of targets from biotin pulldowns following click chemistry with TAMRA-biotin-azide and 31-036 targeted proteins</td>
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<td>- Will identify additional cellular targets of 25-4 (31-036)</td>
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<td>• SAR studies to generate 25-4 analogs with specificity for smaller subsets of targets, single targets</td>
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<td>- Will give information regarding effects of inhibiting various targets</td>
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**Figure 2: Screening cascade for small molecule 25-4.** Like 13-197, 25-4 was identified as an inhibitor of TNFα-induced IKKβ-mediated NF-κB-driven transcription. Follow-up studies indicate that the small molecule targets IKKβ and p65. Future studies will be aimed at validating IKKβ and p65 as targets and will identify additional cellular targets of 25-4 using click chemistry.
Discussion of results presented in this dissertation

In the previous two chapters, I have introduced two novel small molecule inhibitors of IKKβ, 13-197 (Chapter 5) and 25-4 (Chapter 6), and have shown their promise as potential therapeutics for the treatment of cancer as single agents or in combination with clinical therapeutics (Figures 5.1, 5.2, 5.9, 5.10, 5.11, 6.4, 6.5, 6.6, 6.7 and 6.8 on pgs 42, 43, 50, 51, 53, 62, 63, 64, 65 and 66, respectively). In this final chapter, I will further expand upon the results presented for each small molecule and discuss how the effects of 13-197 and 25-4 correlate with the effects of genetic or pharmacological inhibition of the NF-κB pathway by others. Furthermore, I will touch upon the limitations of the work presented in this dissertation and discuss future experiments that could be performed to strengthen and further support our conclusions. I will discuss the implications of the work presented here and the newly published crystal structure of phosphorylated and non-phosphorylated human IKKβ protomers and conclude with my perspectives regarding the development of small molecule inhibitors of IKKβ.

Hit identification of and SAR studies with 13-197 and 25-4

Small molecules 13-197 and 25-4 contain a quinoxaline core and a lactone motif, respectively (Figure 3.7 pg 35). These two structures can be considered “privileged structures” as the quinoxaline core serves as a scaffold in a wide variety of molecules with biological activity and molecules containing α-methylene-γ-butyrolactones have been used for the treatment of a variety of indications. As such, chemists in the laboratory designed two small molecule libraries: one containing analogs featuring each structure.

My work with small molecule 13-197, which had been identified as an inhibitor of TNFα-induced IKKβ-mediated NF-κB-driven transcription by Dr. Vashti C. Bryant in a large primary screen (data not shown), began with studies focused on determining structure activity relationships for 13-197 and related quinoxaline analogs (Figure 5.3 pg
To perform this study, a focused subset of quinoxaline urea analogs was tested again for activity specifically against TNF$\alpha$-induced IKK$\beta$-mediated NF-$\kappa$B-driven transcription of firefly luciferase in our lung adenocarcinoma cell line genetically engineered to express firefly luciferase from a promoter containing a kB element. This study was performed to assess inhibition of NF-$\kappa$B activity for two reasons. The first of these reasons is that inhibition of TNF$\alpha$-induced IKK$\beta$-mediated NF-$\kappa$B-driven transcription of firefly luciferase would report on small molecule activity towards the NF-$\kappa$B pathway only. Assuming off-target effects of 13-197 and 25-4 did not induce alterations in transcription or translation of firefly luciferase or the small molecules did not target firefly luciferase itself, the effects of decreased luciferase activity could be attributed solely to modulation of the NF-$\kappa$B pathway. Secondly, inhibition of NF-$\kappa$B-driven firefly luciferase transcription at varying degree of potencies by multiple small molecules with a similar core but differing substituents establishes causality, further confirming that inhibition of the NF-$\kappa$B pathway by 13-197 (and 25-4, for that matter) was indeed due to inhibition of an NF-$\kappa$B pathway member. For studies with 25-4 (Figure 6.1 pg 58), the luciferase assay was used first to identify 25-4 as a small molecule inhibitor of an NF-$\kappa$B pathway member (as 13-197 had been identified by Dr. Vashti C. Bryant). Follow-up SAR studies using the luciferase assay gave insights into the key structural features of the small molecule and its analogs (Figure 6.2 pg 59).

The SAR studies identified substituents key for the activity of 13-197 and 25-4. For example, the presence of a five- or six-membered ring at the R1 positions of 13-197 was key for its activity (Figure 5.3 pg 45), suggesting this region of the small molecule makes key contacts with IKK$\beta$. Furthermore, a bromo-group at the R2 position of 13-197 was also key for the molecule’s activity again suggesting this group was key for proper binding to the target protein. These observations are being tested by Jacob Contreras who is
developing 13-197 analogs with modifications at the R1 positions of the small molecule. Testing of these analogs will yield further insight into the interactions made by these groups.

In the SAR studies conducted with 25-4 (Figures 6.1 and 6.2 pgs 58 and 59), reduction of the Michael acceptor resulted in loss of activity of the small molecule, indicating loss of activity against NF-κB pathway members. This observation is consistent with those for the reduction of the Michael acceptor of the natural product parthenolide. Upon reduction of its Michael acceptor, parthenolide loses its activity towards IKKβ. Furthermore, these studies demonstrated that rigidification of the Michael acceptor was key for 25-4 activity. This phenomenon suggests to us that rigidification of the small molecule likely reduces interactions with off-target proteins by decreasing the flexibility of the Michael acceptor and thus non-specific interactions with surface exposed cysteine residues. This study can be expanded through generation and testing of additional analogs that further explore the requirement for the Michael acceptor and its rigidification.

In luciferase screens with these small molecules, the known IKKβ inhibitor parthenolide was used as a positive control. In studies with 13-197, parthenolide was chosen as a control simply to demonstrate inhibition of NF-κB pathway activation by a known IKKβ inhibitor. Parthenolide’s use in the screen with 25-4 was two-fold. In this screen, it was again used a positive control but it was also chosen because it shares the α-methylene-γ-butyrolactone present in 25-4. Parthenolide’s use in this screen thus gave us information regarding the level of pathway inhibition that could possibly be expected upon treatment with 25-4 and its analogs. Furthermore, the use of reduced-parthenolide allowed us to demonstrate the requirement of the Michael acceptor for parthenolide’s activity in the screen and to establish a rationale for 25-18’s (reduced 25-4’s) lack of activity. 25-4 showed inhibition of NF-κB pathway activity that was comparable to inhibition by
parthenolide (75% vs 100%) (Figure 6.2 pg 59). Additionally, the IC$_{50}$ value for parthenolide in our TNF-α-induced IKKβ-mediated NF-κB-driven transcription assay is 4.7±1.5 µM, which was in line with the IC$_{50}$ value of 25-4 in this cell line.

Parthenolide showed approximately 55% inhibition of TNF-α-induced, IKKβ-mediated, NF-κB-driven transcription in the screens conducted with 13-197 (Figure 5.3 pg 45) while it showed nearly complete inhibition of NF-κB activity in screens with 25-4 and its analogs (Figure 6.2 pg 59). The parthenolide used in the studies with 25-4 was made fresh and specifically for testing alongside 25-4. Whenever I conducted the assays with 13-197, the parthenolide stock solution used was not made fresh but had instead been made by Dr. Vashti C. Bryant during her studies. Issues regarding the stability of this natural product in DMSO or over the course of many freeze-thaw cycles could have contributed to this discrepancy.

**Target Validation: 13-197**

The next experiments performed with 13-197 and 25-4 were directed at determining the molecular targets of the small molecules. For 13-197, the small molecule's activity towards a panel of kinases was determined in kinase assays carried out by Reaction Biology Corp. (Figure 5.4 pg 46). An initial screen was conducted in which 13-197 was tested at 1 µM against a panel of kinases. The resulting hits from these single concentration assays were then incubated with 13-197 at 10 concentrations starting at 100 µM compound with 9 1:3 serial dilutions to generate IC$_{50}$ values. Six kinases were identified as hits of 13-197 in the screen. These included TRKA, IKKβ, EPHA6, Nek3, MAP4K4 and STK25. The IC$_{50}$s for TRKA, IKKβ, EPHA6 and Nek3 were in the low µM range while those for MAP4K4 and STK25 were in the mid µM range. Due to their lower IC$_{50}$ values, we focused our attention on the kinases TRKA, IKKβ, EPHA6 and Nek3. MAP4K4, however, is known to be activated by TNF-α. TRKA plays a role in the
development and maturation of the central and peripheral nervous systems.\textsuperscript{156} EPHA6 is expressed mainly in the brain.\textsuperscript{157} Nek3 plays a role in the motility of breast cancer cells.\textsuperscript{158} Due to the physiological roles of these kinases, we chose to move forward by characterizing the effects of 13-197 on IKKβ as it is the main kinase in the NF-κB pathway. Nevertheless, we are mindful of the fact that inhibition of Nek3 and MAP4K4 may also play a role in the phenotypes induced by 13-197 treatment.

The human kinome contains 518 kinases\textsuperscript{159} while the panel used in the Reaction Biology Corp. screen only contained 318 kinases. Though the implications of this study are limited by the size of the panel used, the results establish 13-197 as an inhibitor of IKKβ and confirm that inhibition of NF-κB-driven transcription by 13-197 in the luciferase assay was indeed likely due to targeting of the NF-κB pathway. To further confirm inhibition of IKKβ by 13-197, Dr. Vashti C. Bryant, performed and \textit{in vitro} ELISA assay with IKKβ and 13-197. In this assay, 13-197 inhibited IKKβ with an IC\textsubscript{50} of approximately 14 µM, again confirming these results. These experiments are in line with experiments confirming inhibition of IKKβ by the commercially available IKKβ inhibitors TPCA-1\textsuperscript{118} and BMS-345541\textsuperscript{119} though our kinase screen was much more expansive (318 kinases vs 10 and 15 kinases, respectively). Though off-target effects through inhibition of TRKA, EPHA6 and Nek3 have not yet been assessed, we expect those through inhibition of MAP4K4 and STK25 to be minimized by the use of sub-IC\textsubscript{50} concentrations of 13-197 for these proteins. Through SAR studies we may be able to identify analogs of 13-197 that have increased specificity for IKKβ. The use of these small molecules, other known IKKβ inhibitors and RNA silencing of IKKβ alongside 13-197 in phenotypic assays (cell proliferation, death, angiogenesis) in which 13-197 is expected to display activity may give insight into off-target effects of 13-197 that could be mediated by inhibition of these proteins.
Target Validation: 25-4

As the α-methylene-γ-butyrolactone in 25-4 is also present in parthenolide, which has been shown to target C179 of IKKβ and C38 of NF-κB subunit p65\textsuperscript{122,130} we first sought to validate binding of 25-4 to these targets. To do this, we used click chemistry (Figure 6.3A pg 60), which is emerging as a powerful strategy for identifying the targets of covalent small molecules.\textsuperscript{148,160} For these experiments, an alkyne-tagged version of 25-4 (31-036) was synthesized by Dr. Sandeep Rana in the lab. Following incubation with live cells, cell lysates or pure protein the alkyne tag on this small molecule, which is now covalently bound to target, reacts with the azide tag of TAMRA-azide following incubation with TCEP, TBTA and CuSO\textsubscript{4}\times5H\textsubscript{2}O to generate a triazole product covalently linked to 31-036-targeted proteins. Tagged protein is then precipitated with methanol to remove excess, unbound TAMRA-azide and precipitated protein is resuspended in sample buffer, boiled and resolved on SDS-PAGE. Importantly, the intrinsic fluorescent properties of the fluorophore allows for the visualization of targets via in-gel fluorescence. Click chemistry with 25-4 analog 31-036 confirmed binding of the small molecule to both IKKβ and p65. As these results were in line with the activity of 25-4 in the luciferase assay, which reports specifically on the inhibition of NF-κB pathway members, we moved forward to further validate 25-4 as an inhibitor of IKKβ.

The pure p65 used for this experiment had a 14 amino acid truncation at the C-terminus, partially explaining its molecular weight of less than 65 kDa (Figure 6.3A pg 60). In our experiments, this commercially available p65 consistently runs at the shown molecular weight and reacts with anti-p65 antibody if the gel is transferred to a membrane and blotted for p65 following visualization of in-gel fluorescence (data not shown). For this experiment, we were unfortunately unable to generate a loading control. Following detection of in-gel fluorescence, the gel was stained with Bio-safe Coomassie. Unfortunately, the amount of protein loaded on the gel appears to be below the limit of
detection for the reagent. In subsequent experiments, equal loading may be confirmed by silver staining.

Through binding to IKKβ and NF-κB specifically was assessed in this experiment (Figure 6.3A pg 60), 25-4 and 31-036 are covalent small molecules that react with surface exposed cysteine residues in cells. Due to this mechanism of action, these small molecules likely bind targets besides IKKβ and p65. However, the point of this experiment was only to validate that 25-4 has targets within the NF-κB pathway that overlap with parthenolide’s targets as 25-4 contains the same Michael acceptor present in the NF-κB pathway inhibitor parthenolide and exhibited activity against NF-κB pathway activation in the luciferase screen.

Distinguishing IKKβ/NF-κB-dependent effects from IKKβ/NF-κB-independent effects: 13-197

Given that 13-197 has been shown to target kinases besides IKKβ (TRKA, EPHA6, Nek3, MAP4K4 and STK25), future studies will be aimed at determining the consequences of targeting these proteins alone and in combination with IKKβ. Experiments with siRNA and small molecule inhibitors will be conducted to determine how loss of these proteins’ activities and loss of IKKβ activity affect cellular function. For example, MiaPaCa-2 cells will be transfected with siRNA against each individual target to assess the consequence of inhibiting each individual target to provide insight into IKKβ-dependent and possible IKKβ-independent effects of 13-197. Additionally, siRNA against all 6 targets and siRNA against the 5 non-IKKβ targets will be pooled to compare the effects of silencing all targets of 13-197 and all non-IKKβ targets of 13-197. Furthermore, treatment with 13-197 will be compared to treatment with the pool of siRNA against all 6 of the small molecule's known targets and the 5 non-IKKβ targets of 13-197. If inhibition of IKKβ by 13-197 is indeed key for the small molecule's anticancer activity, transfection with the pooled siRNA of non-IKKβ targets will not yield the anticancer effects associated with 13-197 treatment.
(decreased cell viability, reduced levels of anti-apoptotic proteins). However transfection with siRNA against IKKβ alone or the pool of all 6 siRNAs including anti-IKKβ siRNA will recapitulate treatment with 13-197 if IKKβ inhibition is key for the molecule's activity. Furthermore, addition of an IKKβ inhibitor such as ML120B or TPCA-1 to cells transfected with the pool of non-IKKβ siRNAs should mimic all IKKβ-dependent effects of 13-197 treatment. Additionally, treatment with 13-197, ML120B and TPCA-1 will be compared to treatment with siRNA against IKKβ alone to validate that treatment with the small molecules replicates silencing of IKKβ. If the effects of 13-197 do not appear to be due solely to inhibition of IKKβ by 13-197 (for example, if treatment with 13-197 does not mimic treatment with IKKβ siRNA alone but more closely mimics treatment with the pool of siRNA against all 6 targets), siRNA against IKKβ will be tested in MiaPaCa-2 cells in combination with siRNA against the other targets one by one to test for sensitization to treatment with IKKβ siRNA. This may answer questions regarding the importance of targeting other pathway members upstream of IKKβ (MAP4K4) or those with known roles in cancer progression (Nek3).

Distinguishing IKKβ/NF-κB-dependent effects from IKKβ/NF-κB-independent effects: 25-4

Given that most of the targets of 25-4 are still unknown, assessing the IKKβ/NF-κB-dependent effects of 25-4 will be a challenging task. Experiments designed to identify the targets of 25-4 are currently underway. Briefly, cells will be treated with 25-4 analog 31-036, harvested and lysed. Next click chemistry will be performed as previously described with lysate and TAMRA-biotin-azide to covalently attach biotin to the 31-036 covalently linked to target proteins. Lysate will then be incubated with streptavidin beads to pull down the targets of 31-036. Mass spectrometry will be performed to identify these targets. A benefit of this methodology is that all proteins (enzymes, transcription factors, etc.) targeted by 31-036 can be identified using a single technique.
Once the targets of 25-4 analog 31-036 are known, studies assessing the IKKβ/NF-κB-independent effects of 25-4 can be conducted. Analysis of the known consequences of inhibiting 31-036 or 25-4 targets may provide insight into any IKKβ/NF-κB-independent effects on viability and apoptosis. Furthermore, click chemistry SAR studies with 31-036 analogs will be conducted to identify analogs with fewer targets. Treatment of cells with analogs that target IKKβ and p65 but distinct non-IKKβ and NF-κB targets of 25-4 analog 31-036 may give information regarding other targets that are essential for the anticancer properties of the parent compound 25-4. Once the targets of 31-036 are known and analogs targeting IKKβ, NF-κB and distinct subsets of 31-036's other targets have been identified, we may perform siRNA studies similar to those proposed for 13-197 with 31-036 analogs with only a few (~5 or fewer) targets. In addition to transfecting cells with each siRNA individually, we will also transfect cells with pooled siRNA against all the known targets of an analog. In these studies, we will measure decreased viability, induction of apoptosis, inhibition of IkBα phosphorylation and p65 nuclear translocation (to monitor IKKβ/NF-κB pathway inactivation) and the downstream consequences of inhibiting the non-IKKβ and NF-κB targets of the analogs. Comparison of the degree of decreased viability and induction of apoptosis in cells treated with different siRNAs against non-IKKβ and NF-κB targets may reveal that inhibition of other pathways contributes to the decreased cell viability and induction of apoptosis seen by 25-4.

Lastly, the described studies using C179A IKKβ and/or C38S p65 expressing cells will give further insight regarding the IKKβ and p65-independent effects of 25-4 (and 25-4 analogs). Cells expressing C179A IKKβ will be resistant to IKKβ inhibition by 25-4 while C38S expressing cells will be resistant to inhibition of p65 DNA binding by 25-4. Cells expressing both C179A IKKβ and C38S p65 will be resistant to inhibition of both IKKβ and p65 by 25-4. Overall, use of the C179A or C38S cell lines will elucidate the importance of targeting IKKβ or p65 while the use of cells expressing both C179A IKKβ and C38S p65
will give insight into the IKKβ and NF-κB-independent effects of 25-4. For example, if 25-4 treatment of C179A IKKβ/C38S p65 expressing cells still results in decreased cell viability or induction of apoptosis, we can conclude that 25-4’s anticancer effects may be partially due to inhibition of other targets. Identification of analogs that still target IKKβ and p65 but exhibit no anticancer activities in C179A IKKβ/C38S p65 expressing cells may identify analogs that are selective for the NF-κB pathway.

Though 25-4 has many targets and IKKβ and NF-κB-independent effects likely contribute to the anticancer effects of the small molecule, 25-4 is a small molecule that binds IKKβ and NF-κB, displays anticancer effects and is highly amenable to modification. As such, we intend to use the small molecule as a scaffold onto which we can, through functionalization, build specificity onto this molecule to design a more specific, yet still non-toxic, inhibitor of IKKβ and p65. The studies outlined in this dissertation provide preliminary evidence that IKKβ can be targeted with small molecules without inducing toxicity but future studies by our lab will be required to develop small molecule 25-4 as a specific inhibitor of the NF-κB pathway.

**Mechanism of action: 13-197**

As our luciferase assay reports on TNFα-induced IKKβ activation, we tested the ability of 13-197 to inhibit the phosphorylation of IκBα, the downstream target of IKKβ, following cellular stimulation with TNFα (Figure 5.5 pg 47). For this studies, cells were pretreated with 13-197 for 2 hours prior to stimulation with TNFα for increasing times. In the first of these studies, HEK293 cells were pretreated with DMSO or 13-197 for 2 hours and then stimulated with TNFα for 0, 5, 10, 15, 20, 30, 45, 60, 75, 90 and 120 minutes and levels of phosphorylated and total IκBα were monitored. In this experiment, 13-197 did not markedly decrease phosphorylation of IKKβ’s substrate at early time points but instead decreased phosphorylation of IκBα following the degradation and resynthesis of
IκBα in response to signaling through the NF-κB pathway (this phenomenon of IκBα degradation and resynthesis is not unique to our studies but rather appears to be a general feedback mechanism for regulating the activation of the pathway\textsuperscript{161}). To us, this experiment suggested that 13-197 was inhibiting a form of IKKβ that persists at later time points but is not present immediately following activation of the kinase.

It is well-accepted that IKKβ is phosphorylated upon its T loop serine residues to drive its activation. Once activated, the kinase autophosphorylates a stretch of its own C-terminal serine residues, which serves to dampen the kinase’s activity and provides docking sites for phosphates that can dephosphorylate the T loop serine residues to return the kinase to a resting state.\textsuperscript{2, 125} Using this model (Figure 3.6 pg 32) for the progressive phosphorylation of IKKβ, we hypothesized that 13-197 selectively targets IKKβ with its T loop phosphorylated but allows the activation of unphosphorylated IKKβ to yield brief signaling through the pathway.

As ML120B pre-treatment had already been shown to inhibit the early activation of IKKβ in response to stimulation with TNFα,\textsuperscript{117} we decided to compare inhibition of TNFα-induced IKKβ activation by 13-197 and the IKKβ inhibitor TPCA-1 (Figure 5.6 pg 48). HEK293 cells were pretreated with DMSO, TPCA-1 or 13-197 and then stimulated with TNFα for 5 or 10 minutes. Like ML120B, pre-treatment with TPCA-1 completely blocked phosphorylation of IκBα upon cellular stimulation with TNFα. 13-197, on the other hand, allowed marked phosphorylation of IκBα within 10 minutes of stimulation, again suggesting the small molecule allows the activation of IKKβ.

Both of these studies analyzed downstream effects of the activation of IKKβ in response to stimulation of the kinase with a known IKKβ activator and are nearly identical to experiments establishing ML120B as an inhibitor of IKKβ and similar to experiments establishing TPCA-1 as an IKKβ inhibitor.\textsuperscript{117, 118} However, these experiments have not
addressed the possibility that 13-197 targets a kinase upstream of IKKβ to block phosphorylation of IkBα or decreases the levels of IKKβ by inhibition of its transcription, for example. Immunoblotting for phosphorylated (and thus activated) IKKβ and total IKKβ alongside blotting for phosphorylated and total IkBα would establish that IKKβ was indeed activated (high IKKβ phosphorylation) and inhibited (low IkBα phosphorylation). However, these experiments were performed prior to our lab’s identification of suitable antibodies for detecting total and phosphorylated IKKβ. In future repeats of these experiments, lysates will also be probed for phosphorylated and total IKKβ.

Importantly, the mechanism of action established in these studies with 13-197 is in contrast to what is seen with ML120B and TPCA-1, which have both shown toxicities in animal models. As partial or transient inhibition of IKKβ has been hypothesized to be less toxic than complete inhibition of IKKβ, we continued our studies with this small molecule by testing it for efficacy against cancer cell lines (continued in next section discussing 13-197).

Mechanism of action: 25-4

As mentioned already, parthenolide covalently interacts with C179 of IKKβ and C38 of p65. We expect 25-4 to bind to these proteins through covalent interactions with the same residues through the α-methylene-γ-butyrolactone present in its structure. We have not tested this mechanism of action directly, though follow-up studies with 25-4 and its reduced analog 25-18 indicate that covalent interaction between the Michael acceptor and exposed cysteine residues of an NF-κB pathway member are indeed key for activity (Figures 6.1 and 6.2 pgs 58 and 59). Our first cell-based studies were used to validate the SAR seen with the butyrolactone analogs in the luciferase assay. Due to NF-κB’s known roles in disease progression, anti-apoptosis and chemoresistance in breast cancer we opted to study the pathway in breast cancer cells as well as pancreatic
cancer cells. Basal activation of the NF-κB pathway is known to be high in MDA-MB-231 cells\textsuperscript{78} and is confirmed by the high basal levels of phosphorylated IκB\textsubscript{α} in the MDA-MB-231 cells used in these studies (Figure 6.3B pg 60). The high expression of IKKβ in these cells has been linked to sensitivity to the IKKβ inhibitor sulindac, which drives apoptosis in MDA-MB-231 cells.\textsuperscript{78}

Reduced phosphorylation of IκB\textsubscript{α} was observed in MDA-MB-231 cells treated with 25-4 (Figure 6.3B pg 60), which is expected as 25-4 was active in the luciferase assay. Furthermore, treatment with the reduced version of 25-4 (25-18) resulted in no inhibition of IκB\textsubscript{α} activity, which again aligns with our results from the luciferase assay. As reduction of the α-methylene-γ-butyrolactone of parthenolide resulted in loss of binding to IKKβ\textsuperscript{122} and activity towards NF-κB-driven transcription in our luciferase assay, our observation that the Michael acceptor of 25-4 is key for activity is substantiated by work conducted with similar molecules.

Treatment with small molecule 28-090 (25-4 in which the Michael acceptor has been made less rigid), resulted in partial decrease in IκB\textsubscript{α} phosphorylation but the small molecule was less active than 25-4 (Figure 6.3B pg 60), suggesting rigidification of the Michael acceptor is key for activity towards IKKβ. This observation is further supported by the activity of cyclic 28-097C towards phosphorylation of IκB\textsubscript{α} but lack of activity by its acyclic analog 28-097A. Once more, the results of this experiment are in line with the results of the luciferase assay (Figure 6.1 pg 58). Additionally, the molecules tested for inhibition of IκB\textsubscript{α} phosphorylation were able to do so at concentrations similar to those used in the luciferase assay, demonstrating that the small molecules inhibit IKKβ activity in cells with the same rank potency they displayed in the previous assay designed to test their activity against the NF-κB pathway.
Again, we hypothesize that rigidification of the Michael acceptor decreases promiscuity of the cyclic small molecules by decreasing the number of interactions the Michael acceptor may make with surface-exposed cysteine residues. Due to their inherent flexibility, the acyclic 28-090 and 28-097A are able to interact with additional cysteine residues in cells, thereby decreasing their activity towards NF-κB pathway members and thus decreasing their activity towards IKKβ and NF-κB and reduction of IκBα phosphorylation and NF-κB-driven transcription. To further explore the importance of the Michael acceptor or rigidification of the Michael acceptor, additional analogs with modifications similar to those seen between 25-4 and 25-18 (reduction of Michael acceptor), 25-4 and 28-090 (loss of rigidity of Michael acceptor), and 28-097C and 28-097A (loss of rigidity of Michael acceptor) could be synthesized to further confirm this phenomenon.

To further test that 25-4 binds to C179 of IKKβ, cells expressing mutant C179A IKKβ (which cannot covalently interact with the α-methylene-γ-butyrolactone\textsuperscript{122}) could be treated with 25-4. Treatment of these cells with 25-4 should have no effect on IκBα phosphorylation if inhibition of IκBα phosphorylation in these cells is due to inhibition of IKKβ through covalent interaction with C179. However, if 25-4 is affecting a different residue, an upstream kinase or total levels of IKKβ, inhibition of IκBα phosphorylation will still be seen. Validation that C179A IKKβ still phosphorylates IκBα will be performed. Furthermore, experiments similar to those conducted with 13-197 (pre-treatment with DMSO or compound followed by stimulation with TNFα for increasing times) may be conducted to test if 25-4 binds resting IKKβ or selectively targets a phosphorylated form of IKKβ. Again, in these experiments, levels of phosphorylated and total IKKβ and phosphorylated and total IκBα will be assessed to monitor if the kinase is active at early time points and inhibited at late time points. However, as 25-4 also targets p65, it is
important to remember that resynthesis of IκBα may not occur in cells pretreated with 25-4 as the protein’s re-expression is driven by NF-κB translocation to the nucleus and induction of transcription. In such an event, the experiment will be modified such that treatment with 25-4, which binds its targets within 5 minutes (see 5 minute incubation with 31-036 followed by click chemistry, Figure 6.10 pg 68), will occur after stimulation with TNFα to drive phosphorylation and activation of IKKβ.

To test if inhibiting IKKβ gives the same effect as inhibiting both IKKβ and p65, cells expressing C38S p65 (which cannot covalently interact with a Michael acceptor but can still bind κB sites in DNA130) could be treated with 25-4 alongside wild-type cells. In this experiment, 25-4 will inhibit both IKKβ and p65 in wild-type cells but only IKKβ in C38S p65 cells. Prior to conducting this experiment alongside wild-type cells, p65 binding to DNA as well as levels of total and phosphorylated IKKβ and IκBα will be monitored in untreated, C38S p65 cells to ensure the pathway is functioning properly in the mutant cells. Upon treatment, levels of total and phosphorylated IKKβ and IκBα will be monitored and compared to levels in untreated cells to probe for inhibition of IKKβ as assessed by decreased IκBα phosphorylation. Additionally, p65 binding to DNA or translocation to the nucleus can be monitored to assess pathway inhibition. With C38S p65 cells, pretreatment with drug followed by TNFα stimulation could be performed, as p65 should still be functional in these cells and will still drive IκBα re-expression to give a readout (decreased IκBα phosphorylation) for IKKβ inhibition.

Overall, despite the limitations of this experiment, this mechanism-based study verified that 25-4 inhibited the phosphorylation of IKKβ’s substrate IκBα in MDA-MB-231 cells and validated observations from the SAR conducted in luciferase-expressing A549 cells.
Mechanism-based studies in cancer cells: 13-197

As previously mentioned, we next sought to test the ability of 13-197 to inhibit the phosphorylation of IκBα in cancer cells. As NF-κB pathway activation is implicated in pancreatic cancer\textsuperscript{72, 73, 90} and IKKβ activation is implicated in the development of K-ras-driven pancreatic cancers\textsuperscript{72, 93} we chose to study activation of IKKβ and the consequences of its inhibition in pancreatic cancer cells.

First, we tested a panel of cancer cell lines for phosphorylated IKKβ and found that IKKβ was indeed activated in these cells (Figure 5.7 pg 49). Next, we tested the ability of 13-197 to inhibit IκBα phosphorylation in pancreatic cancer cells (Figures 5.8 and 5.9A pgs 49 and 50). Treatment with 13-197 decreased IκBα phosphorylation in a panel of pancreatic cancer cells lines (AsPC-1, Capan-2, Hs766T, MIAPaCa-2 and SUIT-2) and reduced phosphorylation of IκBα in MIAPaCa-2 pancreatic cancer cells in a dose- and time-dependent manner. As NF-κB regulates a number of genes that play a role in the proliferation of cancer cells and genetic ablation or pharmacological inhibition of IKKβ has been shown to reduce cancer cell proliferation,\textsuperscript{66, 74} we next determined if inhibition of the pathway would result in decreased viability of cells (Figure 5.9B and C pg 50). Indeed, treatment with 13-197 decreased the viability of pancreatic cancer cells with low µM potency. Furthermore, the IC\textsubscript{50} of 13-197 in this panel of cancer cell lines is within the same range of potency of other small molecule inhibitors of IKKβ.

To further study the effects of 13-197 on cell proliferation, a collaborator, Dr. Prakash Radhakrishnan, analyzed the effects of 13-197 on the cell cycle and saw that treatment with the small molecule induces apoptosis, blocks cell cycle progression and reduces levels of PCNA.\textsuperscript{128} These effects are in line with those seen with other pharmacological inhibitors of IKKβ.\textsuperscript{74, 164, 165} As Bcl-xL, Mcl-1, XIAP and survivin are under the transcriptional control of NF-κB,\textsuperscript{21, 23-25} I treated MIAPaCa-2 cells with 13-197 and
probed lysates for expression of these anti-apoptotic proteins (Figure 5.10 pg 51). Treatment with the small molecule resulted in decreased levels of these proteins, providing a biological rationale for the results of Dr. Radhakrishnan’s studies. Furthermore, these results are in line with those seen in other models of pharmacological IKKβ inhibition or NF-κB pathway inhibition with the IκBα superrepressor. 166-168

Mechanism-based studies in cancer cells: 25-4

As the activation of the NF-κB pathway is implicated in the progression of not just pancreatic cancer but many others, including lung, 74, 75 breast, 58, 76-79 ovarian, 80-82 and cervical 107, 110 cancers and has been shown to play a role in inflammation, transformation, proliferation, angiogenesis, invasion, metastasis, chemoresistance and radioresistance, 83 we expanded the cells lines in our studies with 25-4 to encompass a diverse panel of cancer cell lines.

As mentioned previously, 25-4 showed activity against phosphorylation of IκBα in MDA-MD-231 breast cancer cells (Figure 6.3B pg 60). Additionally, 25-4 displayed dose- and time-dependent activity in MIAPaCa-2 pancreatic cancer cells (Figure 6.3C pg 60), which were established to have basal phosphorylation of IKKβ (Figure 5.7 pg 49) and basal phosphorylation of IκBα (Figures 5.9A and 6.3C pgs 50 and 60) suggesting that the NF-κB pathway is active in these cells. Furthermore, the IC50 for 25-4, as measured by cell viability assay, in these two cell lines was in the low µM range, which is again, in line with the IC50s for the known IKKβ inhibitors TPCA-1 and Bayer Compound VIII (Chapter 6, Table 1 pg 62). 25-18 was again inactive in these cell lines, supporting the argument that reduction of the Michael acceptor abolishes the small molecule’s activity. Furthermore, the reported IC50 values for parthenolide against acute myelogenous leukemia, lung, breast and pancreatic cancer cell lines is typically within the 0.5 to 17.5 µM range, 169-172 which is again in line with the potency of 25-4 in our studies.
As IKKβ and the NF-κB pathway are known to play a role in many tumorigenic properties of cancer cells, we next assessed the ability of 25-4 to inhibit colony formation (Figure 6.5A and B pg 63) and induce apoptosis in cancer cells (Figures 6.5C and 6.6 pgs 63 and 64). The colony formation assay was conducted as this assay monitors the reproductive viability of cancer cells after treatment with drug. Overexpression of NF-κB is associated with increased colony formation by cervical cancer cells while silencing of the protein results in decreased colony formation. To test if inhibition of IKKβ by 25-4 could prevent colony formation of cervical cancer cells, Tian Zhou performed colony formation assays in HeLa cells treated with DMSO, 25-4 and the reduced version of 25-4 (25-18). In the assay, cells were seeded at low density (500-1000 cells/well) in 6-well plates, given 24 hours to attach to the plate and treated with drug for 48 hours. The media above the cells was replaced and the cells were cultured for 7 additional days before fixation with methanol, staining and quantification of colonies. This method is similar to that used by others to assess clonogenecity of cells with NF-κB activation. Treatment with 25-4 dose-dependently inhibited colony formation while treatment with a dose of 25-18 equivalent to the highest dose of 25-4 had no activity.

For this study, additional IKKβ inhibitors such as parthenolide, ML120B, TPCA-1 or Bayer Compound VIII could have been tested to show similar effects by known IKKβ inhibitors. Furthermore, knockdown of IKKβ or p65 could also validate these results by showing effects similar to those of 25-4 treatment. Additionally, cells overexpressing IKKβ and p65 or cells expressing C179A IKKβ and C38S p65 could have been treated alongside wild-type cells to show resistance to treatment or rescue of the phenotype by cells that are expected to be resistant to 25-4 inhibition of IKKβ and p65.

NF-κB activation and IKKβ expression are associated with poor survival of ovarian cancer patients and chemoresistance of ovarian cancer cells. As inhibition of IKKβ has been shown to decrease viability of ovarian cancer cells and NF-κB pathway
inhibition has been associated with increased ovarian cancer cell sensitivity to the chemotherapeutic cisplatin,\textsuperscript{110} which is commonly used in the treatment of ovarian cancer,\textsuperscript{150} we next tested the ability of 25-4 to decrease cell viability in A2780 and A2780 cisplatin-resistant cells. Treatment with the small molecule indeed decreased cell viability of both cell lines (Chapter 6, Table 1 pg 62.). Again, the IC\textsubscript{50} values for the cells were in the low µM range. Furthermore, the IC\textsubscript{50} values for cisplatin in these lines were in the low µM range for A2780 cells while A2780 cisplatin-resistant cells were not sensitive to 20 µM cisplatin (data not shown). These studies illustrate the usefulness of 25-4 in the treatment of refractory disease. As most women who are treated for ovarian cancer will also face recurrent disease,\textsuperscript{97} on-going experiments are testing the ability of 25-4 to resensitize A2780 cisplatin-resistant cells to treatment with cisplatin to further assess the small molecule's usefulness in the treatment of refractory disease.

To explore the mechanisms behind the decreased viability seen in ovarian cancer cells treated with 25-4 and to determine if the small molecule would sensitize cells to treatment with cisplatin, SKOV3 ovarian cancer cells were treated with cisplatin, 25-4 or the combination and caspase 3/7 activity was monitored as activation of caspases is a hallmark of induction of apoptosis (Figure 6.5C pg 63).\textsuperscript{175} Treatment with 25-4 induced low levels of caspase 3/7 activation while induction of caspase activity was also low following treatment with cisplatin. When the two compounds were used in combination, marked induction of caspase 3/7 activity was observed, suggesting that 25-4 sensitizes SKOV3 cells to treatment with cisplatin. In this study, doses of 25-4 and cisplatin than drive very little induction of caspase 3/7 activation (5 µM) at the given time point (24 h) were chosen for two reasons. We reasoned that combination with 2 very lose doses of drug will result in fewer off-target effects and would induce the least toxicity \textit{in vivo}. Thus, selecting concentrations of drug that are not high enough to induce activation alone but give
induction together were chosen to reduce off-target effects and to reflect dosages we think would be most appropriate for in vivo use.

No combination index was calculated for this study with SKOV3 cells because a dose-response curve for caspase 3/7 activation by 25-4 alone and cisplatin alone was not calculated, which would be required to calculate a combination index.\(^\text{147}\) In this experiment, caspase 3/7 activation by 24 hour treatment with 5 µM drug (25-4 alone, cisplatin alone or 5 µM of each drug) only was assessed. Future studies assessing caspase 3/7 activation by the compounds can be conducted in a dose-response format so these values can be measured to calculate combination index values. Again, the same methods mentioned for rescuing the phenotype of 25-4-treated cells in previous phenotypic screens could be used to establish that inhibition of IKKβ is the primary mechanism driving decreased cell proliferation and induction of caspase 3/7 activity.

PARP is an enzyme that functions to repair DNA following cellular stress.\(^\text{176}\) The enzyme is the target of many proteases, including caspases, and its cleavage results in the production of PARP fragments that mediate diverse forms of cellular death.\(^\text{176}\) As inhibition of the NF-κB pathway induces apoptosis through inhibition of transcription of anti-apoptotic proteins\(^\text{21, 23-25}\) and PARP cleavage is a hallmark of induction of apoptosis,\(^\text{177}\) we treated various cancer cell lines with 25-4 to see if the proposed IKKβ inhibitor could induce PARP cleavage in cancer cells (Figure 6.6 pg 64).

MIAPaCa-2 and SKOV3 cells were treated with the indicated concentrations of 25-4 and 25-18 and etoposide for 24 h. In 24 h, 25-4 induced PARP cleavage in MIAPaCa-2 and SKOV3 cells and 25-18 was again inactive. Etoposide was used as a positive control in both of these experiments but does not show induction of PARP cleavage. MIAPaCa-2 and SKOV3 cell lines were both particularly sensitive to induction of PARP cleavage upon treatment with 25-4. Thus optimizing the treatment concentration and length of 25-4 exposure to induce PARP cleavage without inducing total cell death in these cell lines was
quite difficult. After performing initial dose-response experiments at the 48 hour time point (a time point when etoposide was expected to be active) and time-course experiments with multiple concentrations, the best concentration and drug exposure time for MIAPaCa-2 and SKOV3 cells appeared to be 24 hour treatment with 10 µM 25-4. As such, I opted to treat MIAPaCa-2 and SKOV3 cells with etoposide for only 24 hours instead of treating with etoposide for 48 hours starting 1 day after cell plating and treating with 25-4 for 24 hours starting 2 days after plating so an equivalent number of cells seeded on the same day would be treated with each drug. These experiments were carried out at the same time as those conducted with HeLa (48 hour treatment) and A2780 cells (96 hour treatment) and the same etoposide was used in these experiments and was indeed active 48 and 96 hours following treatment.

The range of treatment times required for induction of PARP cleavage in these cell lines is not surprising and has been noted with other small molecules. For example, staurosporine, a non-specific kinase inhibitor that inhibits IKKβ,\textsuperscript{178} has been shown to induce PARP cleavage in a matter of hours in some cells while induction of PARP cleavage takes at least a day in others.\textsuperscript{179} As such, the various time points required to induce PARP cleavage with 25-4 does not seem to be a phenomenon singular to these experiments. However, the lack of PARP cleavage by etoposide at the 24 hour time point in MIAPaCa-2 and SKOV3 cells is unlikely due to insensitivity to the drug. I suspect the lack of activity is due instead to the short time point for which cells were treated. As etoposide’s mechanism of action involves induction of DNA damage,\textsuperscript{180} at least one round of cell division would have needed to occur for etoposide to induce activation of PARP cleavage. Other small molecules that are known to inhibit signaling through the NF-κB pathway have to be shown to induce cleavage of caspases,\textsuperscript{181} which is required for their activation,\textsuperscript{182} within 4 hours of treatment. Such inhibitors or staurosporine may serve as
more appropriate controls in future experiments as they may induce their effects through the same pathway and at a relatively similar time point.

Interestingly, a decrease in total PARP but no cleaved PARP fragment was seen in MIAPaCa-2, SKOV3 and A2780 cells treated with 25-4. This is again likely due again to the difficulties associated with optimizing the concentration and length of 25-4 treatment. Time course experiments analyzing the activation of caspase 3 and caspase 7, which are known to cleave PARP, in these cells lines may yield a better idea of the time points and appropriate treatment concentrations so these experiments may be repeated to produce results more typical of a PARP cleavage assay (namely, presence of total PARP and production of a PARP cleavage fragment by 25-4 and a positive control that is known to induce DNA damage).

The difficulties associated with obtaining ideal results for this experiment are likely due to the nature of the experiment. The positive and negative controls chosen for this experiment (etoposide and 25-18, respectively) were indeed proper controls for showing induction of PARP cleavage and no effect on PARP cleavage (Figure 6.6 pg 64, A2780 and HeLa lines). However, due to the differential sensitivity of some cell lines to treatment with 25-4, some lines were not incubated with etoposide for a period of time sufficient to see induction of PARP cleavage by the small molecule (Figure 6.6 pg 64, MIAPaCa-2, SKOV3). Furthermore, treatment with 25-4 showed reduction of full-length PARP levels in all cell lines though a cleaved PARP fragment was not seen in all cell lines. This is likely due to the difficulty of selecting a treatment time at which the 89 kDa cleavage fragment is present in cells at a level detectable by immunoblotting. Future work may refine the timing issues associated with the studies by using a positive control that induces PARP cleavage as rapidly as 25-4. Furthermore, harvesting cells at different time points may yield 25-4-treated lysates with both full-length and the 89 kDa PARP fragment. Furthermore, other cleavage fragments of PARP are known to be generated by enzymes
other than caspases 3 and 7 including calpains and cathepsins, which can drive caspase-independent apoptosis. In future experiments, immunoblotting for these fragments can be performed to further study the cell death induced in cell lines by 25-4 treatment.

Animal-based studies: 13-197

To assess the ability of 13-197 to decrease tumor growth in a mouse model of pancreatic cancer, MIAPaCa-2 cells were orthotopically implanted into the flanks of nude mice, the mice were given two weeks to heal and were treated for 30 days with either vehicle control or 13-197. At the end of this study, tumor weights and volumes were analyzed as was metastasis (as measured by number of tumor nodules in other organs) and proliferation (Figure 5.11 pg 53). Treatment resulted in a decrease in all of these parameters. Though treatment with the IKKβ inhibitor TPCA-1 is associated with toxicity, mouse studies with the small molecule have shown reduced proliferation of lung cancer cells (as measured by Ki-67 staining), decreased PCNA staining and slower tumor growth, all of which mirror our observations in 13-197-treated mice and provide further proof that the effects of 13-197 are through inhibition of IKKβ. Furthermore, several groups have reported decreased tumor formation following inhibition of NF-κB activity by deletion of p65 or by overexpression of the IκBα superrepressor (nonphosphorylatable IκBα), suggesting again that the effects of 13-197 mirror inhibition of IKKβ.

The novel result of this experiment was the lack of toxicity seen with 30-day administration of 13-197, as ML120B and TPCA-1, for example, have proven toxic in previous studies. The lack of toxicity of the small molecule is likely due to the transient activation of IKKβ that was seen after stimulation of HEK293 cells with TNFα despite pre-treatment with 13-197. These studies offer initial validation of the theory that partially targeting IKKβ is a viable strategy for treating disease without inducing severe toxicity.
Future studies in our lab will be focused on purifying endogenous IKKβ from cells stimulated with TNFα for various times. Kinase assays with 13-197, purified IKKβ and IκBα will be performed to further validate that 13-197 selectively inhibits a phosphorylated form of IKKβ to yield the pattern of inhibition of IκBα phosphorylation seen upon pre-treatment with 13-197 and stimulation with TNFα. This will provide further proof that 13-197 escapes the toxicities associated with previously published IKKβ inhibitors by allowing early activation of IKKβ upon induction with TNFα.

Animal-based studies: 25-4

To continue investigating the anticancer properties of 25-4, our collaborators at Josephine Ford Cancer Center (Henry Ford Hospital) tested the small molecule in an orthotopic model of ovarian cancer. Two studies were conducted. In the first, a small dose escalation study with 25-4 was performed. Nude mice were injected with 2 million A2780 cells and after 3 days mice were divided into 25-4-treated, cisplatin-treated or untreated groups. The small molecule 25-4 was dosed at 1, 2.5 or 5 mg/kg 25-4 daily for 4 weeks while cisplatin was dosed at 2 or 4 mg/kg on days 5, 9, 14 and 21. This dosing regimen for 25-4 was chosen as it was expected to produce detectable levels of 25-4 in plasma and that for cisplatin was chosen to avoid toxicity in the mice. Mice were then sacrificed and tumor weights were analyzed (Figure 6.7 pg 65). Treatment with 1 mg/kg cisplatin resulted in ~15% reduction in tumor weights while treatment with 2.5 mg/kg resulted in ~20% reduction of tumor weights and 5 mg/kg treatment resulted in ~45% reduction in tumor weights. However, toxicity (blocked bowels) was noted at the highest dosage of the drug. Treatment with 2 mg/kg cisplatin resulted in ~25% reduction of tumor weights and treatment with 4 mg/kg resulted in ~75% reduction in tumor weights. Toxicity (blocked bowels) was also noted at the highest concentration of cisplatin. As NF-κB pathway inhibition has been shown to sensitize cancer cells to cisplatin and 25-4 sensitized
ovarian cancer cells to treatment with the drug in cell-based studies conducted in the lab, a follow-up study to assess the ability of 25-4 to sensitize A2780 cells in mice to cisplatin was performed. Following the same outline of the studies presented above, mice were treated with 2.5 mg/kg 25-4 and 2 mg/kg cisplatin in combination as these concentrations did not induce overt toxicity in mice but did reduce tumor weights. Treatment with the two in combination resulted in nearly ~70% reduction of tumor weight compared to approximately 20% (25-4) and 25% (cisplatin) reduction of tumor weights alone.

Immunohistochemistry studies were performed on ovarian tissues extracted from the mice following treatment (Figure 6.8 pg 66). Levels of Mcl-1 were analyzed in these tissues. Again, Mcl-1 is a known target gene of NF-κB and its upregulation likely plays a role in chemoresistance. In mice treated with 25-4, Mcl-1 staining was reduced by approximately 66%. Treatment with cisplatin again resulted in a decrease similar to that seen with treatment with 25-4. The combination had a similar effect.

Resistance to cisplatin has been associated with increased phosphorylation of IκBα and NF-κB activity in cisplatin-resistant A2780 cells and cisplatin treatment has been shown to be less effective at reducing IκBα phosphorylation and NF-κB activity in cisplatin-resistant cells. Furthermore, treatment of ovarian cancer cells, including A2780 cells, with cisplatin has even been shown to activate IκBα degradation in cells. This activation of the NF-κB pathway is hypothesized to be a mechanism by which cisplatin resistance develops and the up-regulation of anti-apoptotic proteins by NF-κB is likely key for the development of this resistance. As such, we expected treatment of A2780 tumor-bearing mice with cisplatin and 25-4 to lead to a synergistic decrease in tumor size as we hypothesized that treatment with 25-4 would counter any NF-κB pathway activation by cisplatin or rescue any lost NF-κB inhibitory activity of cisplatin in any A2780 cells that had developed resistance during the course of the study. As decreased levels of Mcl-1 (an
anti-apoptotic protein under transcriptional regulation of NF-κB) were seen in our cisplatin-treated mice and these levels were comparable to those seen in 25-4 treated mice, we concluded that our cisplatin-treated mice did not develop resistance to cisplatin and thus we did not observe the synergism in 25-4 and cisplatin-treated mice we hypothesized we would see. Given the downregulation of Mcl-1, and not upregulation of Mcl-1, by cisplatin, there was little potential for synergistic decrease of the protein’s levels in this study. That is to say, for these two small molecules that each reduced Mcl-1 staining by 66% to have shown synergistic decrease in Mcl-1 levels at these dosages, more than 100% reduction in the levels of Mcl-1 would have been required.

Future studies could focus on testing the combination of 25-4 and cisplatin in A2780 cisplatin-resistant cells, which in our hands have shown 25-4 sensitivity similar to A2780 cells (IC$_{50}$ = ~3 µM for A2780 cisplatin-resistant cells and ~2.3 µM for A2780 cells) but are insensitive to cisplatin at 5X the drug’s IC$_{50}$ for A2780 cells (A2780 cisplatin-resistant cells are insensitive at 20 µM while A2780 cells have an IC$_{50}$ of ~4 µM) (data not shown). However, the cisplatin-resistant cells used in our studies do not form tumors in mice and were thus unable to be tested in the mouse model outlined above. Furthermore, a study in which mice with A2780 tumors are treated with low doses of cisplatin to induce resistance and then treated with 25-4 in combination with cisplatin to test if 25-4 treatment can resensitize ovarian cancer cells to cisplatin could be designed. It would be interesting to see if cisplatin-resistant A2780 tumors display increased levels of phosphorylated IκBα, nuclear p65 and Mcl-1 during the development of resistance, suggesting that NF-κB activation does indeed drive chemoresistance.

The novelty of these studies was again the lack of toxicity noted with a therapeutic dose of 25-4 (2.5 mg/kg). Previous studies testing the inhibition of the NF-κB pathway in ovarian cancer have shown decreased tumor weights in mice.$^{189}$ Furthermore, NF-κB pathway inhibition by curcumin, an IKKβ inhibitor, has been shown to induce apoptosis in
ovarian cancer cells,\textsuperscript{190} which could be driven by reduced levels of the anti-apoptotic protein Mcl-1,\textsuperscript{185, 186} thus the activity of 25-4 in these models is aligned with the known consequences of inhibiting the NF-κB pathway in ovarian cancer cells. Again, treatment with cisplatin is known to induce NF-κB pathway activation in A2780 cells so the downregulation of Mcl-1 in cisplatin-treated cells and consequent lack of sensitization to cisplatin by 25-4 was unexpected.

To ensure that the effects of 25-4 in this study were due to the inhibition of the NF-κB pathway through inhibition of IKKβ and p65, two of its known targets, these studies could be expanded. More slides can be generated from our tumor microarray and analyzed for additional tumor properties that are known to be regulated by the NF-κB pathway such as proliferation (staining of Ki-67 or PCNA) and angiogenesis (CD31 staining).\textsuperscript{190} Furthermore, as the NF-κB pathway is known to regulate the expression of cyclins,\textsuperscript{191} staining for cyclins could be performed. Importantly, tissue staining of phosphorylated and total IKKβ, IκBα and p65 could be done to ensure the IKKβ was activated (high phospho-IKKβ staining) but inhibited (low phospho-IκBα, phospho-p65 staining) in tumor tissues.

Upon sacrifice, gastrointestinal complications (likely constipation or impaction) were noted in mice treated with the highest dosages of 25-4 and cisplatin. Constipation is the slow movement of feces through the large intestine, while impaction is the build up of hardened feces in the colon. Constipation is commonly caused by the intake of too little water while impaction may also be associated with both cardiac and respiratory symptoms.\textsuperscript{192} Unfortunately, the mice in the study were not being monitored for water intake or cardiac or respiratory symptoms and these symptoms were not obvious to researchers conducting the study, so it is difficult to determine the specific reason for the observed blockages. These gastrointestinal complications, however, are among the most
common side-effects of treatment with chemotherapeutics\textsuperscript{192} and can usually be alleviated if brought to the attention of a physician.

**Lack of toxicity: 13-197**

Our HEK293 cell-based studies that suggest 13-197 targets a later-acting, phosphorylated form of IKKβ while allowing early activation of IKKβ (Figure 5.5 pg 47) served as our first clue that the small molecule may not exhibit the toxicities associated with other inhibitors of the kinase. The most recently solved crystal structure of human IKKβ has given us further insight in the mechanism of action of 13-197, though future studies with the small molecule and protein will be required to validate these hypotheses.

Shenping Liu et al. obtained the crystal structure of a dimer of human IKKβ in complex with the ATP competitive inhibitor K252a. In this structure, one IKKβ protomer was phosphorylated while the other was not (Figure 3.2 pg 26). Analysis of the activation loop of phosphorylated and unphosphorylated IKKβ suggests that upon phosphorylation of the T loop serine residues, the activation loop of phosphorylated IKKβ moves by approximately 21Å and the conformational changes induced upon phosphorylation results in IKKβ protomers with distinct conformations. Though the ATP binding sites are nearly identical in either state, the conformational changes that occur upon phosphorylation could allow for the design of non-ATP competitive inhibitors of IKKβ that discriminate between active and inactive kinase. For example, phosphorylation of the IKKβ T loop serine residues creates a binding site for its substrate IκBα. Binding to this site in the active kinase is hypothesized to inhibit only the activated form of the kinase.\textsuperscript{47}

As our studies suggested that 13-197 preferentially targets phosphorylated IKKβ over nonphosphorylated IKKβ, which have nearly identical ATP binding sites, we outsourced kinase assays to Reaction Biology Corp. to test if 13-197 is a non-ATP competitive inhibitor. If the small molecule is preferentially targeting activated IKKβ, it
should be binding somewhere besides the ATP binding site. The results from these assays indicate that 13-197 is indeed a non-ATP competitive inhibitor (data not shown).

The substrate binding site is one of the main sites that undergoes a conformational change upon phosphorylation of the kinase. To test that 13-197 competes with substrate binding of phosphorylated IKKβ, a binding assay may be performed with active IKKβ, 13-197 and IκBα. Immunoprecipitation of activated IKKβ followed by immunoblotting for IκBα could be performed (and vice versa) after incubating the two proteins in the presence of increasing concentrations of 13-197. If 13-197 treatment decreases binding of IκBα to IKKβ, reduced levels of IκBα would be co-immunoprecipitated with IKKβ with increasing concentrations of 13-197. To perform these studies, phosphorylated and active IKKβ (or constitutively active S177/181E IKKβ) would be incubated with a nonhydrolyzable ATP as ATP binding is a prerequisite for substrate binding to enzyme\textsuperscript{193} followed by incubation with 13-197 and IκBα and immunoprecipitation. The use of activated IKKβ and nonhydrolyzable ATP would allow binding of both 13-197 and IκBα to the site of interest in active IKKβ. Crystalization of 13-197 in complex with active IKKβ would, however, best elucidate how the small molecule binds the kinase.

**Lack of toxicity: 25-4**

Studies with the natural product parthenolide, which features the same α-methylene-γ-butyrolactone that is present in IKKβ, binds to C179 of IKKβ and C38 of p65.\textsuperscript{122, 130} Studies with IKKβ and p65 mutants have shown that conversion of C179 of IKKβ to alanine and conversion of C38 to serine in p65 results in loss of inhibition by the small molecule as alanine and serine residues cannot react with the Michael acceptor of the natural product.\textsuperscript{122, 130} Using these same IKKβ and NF-κB mutants, we could confirm that binding to the proteins is through these same residues.
The newly solved crystal structure of human IKKβ with a phosphorylated and unphosphorylated protomer also gives insight into 25-4’s mechanism of action. In phosphorylated IKKβ, C179 (which is the target of parthenolide and the presumed target of 25-4) is solvent exposed. However, in unphosphorylated IKKβ, the T loop residues seem to be in a conformation that would not facilitate binding of 25-4 to C179. Unfortunately, critical residues of the activation loop were missing in the crystal structure of unphosphorylated IKKβ, so how accurately this structure reflects the true structure of unphosphorylated IKKβ is highly debatable. Moving forward, we will conduct work that establishes a mechanism for the lack of toxicity of 25-4. Experiments similar to those conducted with 13-197 and TNFα-stimulated cells will be performed. HEK293 cells will be pretreated with drug for 2 hours and then the cells will be stimulated with TNFα for increasing periods of time. If pre-treatment with 25-4 results in the inhibition of IKKβ present at later time points after TNFα stimulation, as was seen with 13-197, then 25-4 could indeed preferentially inhibit phosphorylated IKKβ and the theories generated by review of this crystal structure could indeed be valid. Again, this experiment may need to be modified such that cells are stimulated first and then treated with drug as 25-4 also inhibits NF-κB function and may thus block the resynthesis of IκBα and limit our abilities to assess IKKβ function as 25-4 treatment may affect our mechanistic readout (without IκBα resynthesis we cannot monitor IκBα phosphorylation at late time points) of its activity. Furthermore, 25-4 IC_{50} values for inhibition of IκBα phosphorylation by inactive and activated IKKβ could be determined using IKKβ purified from cells at different time points (prior to and after stimulation of TNFα) or S177/181A and S177/181E IKKβ (inactive, T loop nonphosphorylatable and constitutively active, T loop phospho-mimic IKKβ, respectively).
Though these pre-treatment followed by stimulation with TNFα studies can be readily conducted in our laboratory, identification of targets by mass spectrometry will give a much broader view into the off-target effects of the small molecule and a very focused answer to which form of IKKβ is targeted by 25-4. Briefly, streptavidin pulldowns of TAMRA-biotin-azide tagged 31-036-treated lysates will be performed to isolate proteins targeted by 31-036. These protein targets will be analyzed by mass spectrometry to determine binding partners of 31-036. Importantly, the structure of 25-4 is highly amenable to modification with functional groups that will allow us to perform subsequent SAR studies with 25-4 analogs. These studies will be aimed at identifying analogs that bind a single or a small subset of the targets of 25-4, so we may study the effects of targeting each protein that covalently interacts with the small molecule.

Interestingly, despite targeting both IKKβ and p65, 25-4, like parthenolide, displayed little toxicity in mice. Even if the small molecule preferentially targets activated IKKβ, it must also target an activated form of p65 to prevent total shutdown of the pathway. It is possible that prior to its degradation, IκBα’s interaction with p65 blocks 25-4 binding to p65, possibly through alteration of the protein’s structure. Though 25-4 is expected to bind to targets quickly (within 5 minutes), the time required for 25-4 to bind to p65 after the protein’s release from IκBα may be just long enough to allow a pool of p65 to translocate to the nucleus and drive transcription necessary for survival of normal cells, particularly T and B cell precursors as their death is associated with the inhibition of the NF-κB pathway. However, this level of NF-κB transcription may not be sufficient for the survival of cancer cells, providing a therapeutic window for the small molecule. Again, partial inhibition of the pathway with small molecules is predicted to be less toxic than complete and sustained inhibition of NF-κB pathway activity.
Click chemistry with 25-4 analog 31-036

We chose to pursue click chemistry followed by reaction with an azide- or biotin-azide-tagged TAMRA so we could perform visualization of targets via fluorescence and purification of targets via the high affinity interaction between biotin and streptavidin beads or resins, as these are emerging as very powerful techniques for the visualization and analysis of cellular targets of small molecules. Click chemistry reactions are particularly attractive for a number of reasons: 1) they are fast, high-yielding and relatively easy to optimize, 2) the azide and alkyne functional groups used in our reaction interact only with one another under the conditions of click chemistry and 3) click-based methods are compatible with the cellular environment allowing the reaction to occur in live cells. However, in designing a click probe, certain considerations must be taken. For example, the click probe must retain the binding properties of its parent small molecule (that is to say, 31-036 must display the same activity as 25-4). Furthermore, the click probe needs to retain cell permeability to be used in live cells. In the initial validation of 31-036 as a viable click probe, we incubated the probe with live cells at a concentration comparable to those used in studies with 25-4, washed away excess probe, harvested cells, performed the click reaction with TAMRA-azide and used in-gel fluorescence to ensure that the probe was indeed able to bind targets within live, intact cells (Figure 6.9 pg 67). We also performed dose- and time-response studies with the small molecule to investigate what concentration of the molecule was required to saturate our signal and how quickly the small molecule could bind targets (Figure 6.10 pg 68). These studies gave us an idea of the best concentration of the small molecule to use in follow-up studies and how short of an incubation we could perform with the small molecule.

Furthermore, we performed a very rudimentary competition assay with 31-036 and 25-4 (Figure 6.11 pg 69) based upon the methodologies of Kwok et al. to demonstrate that 25-4 and 31-036 bind similar targets in cells. In this experiment, cells were incubated
with 31-036 for 1 hour, 25-4 for 1 hour, or 25-4 for 1 hour followed by 31-036 for 1 hour. Cells were harvested, lysates were incubated with TAMRA-azide and resolved on SDS-PAGE and in-gel fluorescence was visualized. These experiments show that when cells are pre-treated with 25-4 prior to treatment with 31-036, the intensity of the 31-036 click signature is diminished. This experiment is limited by the fact that I was unable to obtain a loading control.

Additional experiments to outline binding of 31-036 to the same targets of 25-4 will be conducted in the future. In these experiments, the same treatment strategy as above will be performed (treatment with 31-036 only, treatment with 25-4 only, and treatment with 25-4 followed by treatment with 31-036). However, a wide range of 25-4 concentrations will be used. In these studies, concentrations of 25-4 that are equivalent to 1/10X, 1X, and 10X 31-036 with intervening concentrations will be used to show dose-responsiveness and complete loss of the 31-036 click signature.

The most recent click chemistry studies to have been conducted were reactions designed to ensure that TAMRA-azide and TAMRA-biotin-azide bind to the same targets in cells (Figure 6.13 pg 70). As the addition of a large group such as biotin may have affected the ability of the TAMRA-biotin-azide to access all the alkyne-tagged proteins (in their native state) available to TAMRA-azide,160 these initial studies were key for verifying that click reactions using TAMRA-biotin-azide would be similar to those with TAMRA-azide in native conditions. In these experiments, I verified that TAMRA-biotin-azide was indeed binding to the same targets in cells as assessed by a similar in-gel fluorescence click signature. Furthermore, I demonstrated that TAMRA-biotin-azide reacted with the alkyne tag of 31-036-bound p65. IKKβ was not used in this experiment due to difficulties associated with purchasing IKKβ in a buffer solution that does not contain compounds (glutathione and dithiothreitol) that will affect the reaction between 31-036 and the target
cysteine residue of IKKβ. Moving forward we will use this trifunctional TAMRA-biotin-azide to complete the mass spectrometry studies that have already been suggested.

**Determining the form of IKKβ targeted by 13-197 and 25-4**

We hypothesize that 13-197 and 25-4 inhibit IKKβ activity without inducing the toxicities associated with previously reported IKKβ inhibitors by specifically targeting activated IKKβ without binding resting, inactivated IKKβ. In this model (Figure 3.6 pg 32) IKKβ would be briefly activated by TNFα, allowing short activation of IKKβ prior to small molecule binding. We hypothesize that this brief activation provides enough signaling through the NF-κB pathway to allow proper immune functioning (data not shown). However, the inhibition of activated IKKβ by these small molecules would inhibit sustained activation of IKKβ. This selective inhibition of activated IKKβ appears to be sufficient to drive death or reduce viability of cancer cells (Figure 5.9, 5.10, 5.11, 6.4, 6.5, 6.6, 6.7 on pgs 50, 51, 53, 62, 63, 64, and 65, respectively and Table 1 pg 62).

To verify that 13-197 indeed inhibits a phosphorylated form of IKKβ, I have recently identified IKKβ antibodies that can be used to immunoblot and immunoprecipitate unphosphorylated and phosphorylated IKKβ. Using these antibodies, I will repeat the experiment shown in Figure 5.5 (pg 47) but will also immunoprecipitate IKKβ at various time points post-stimulation with TNFα (0, 5, 10, 15, 30, 45, 50, 90 and 120 min post-stimulation) and immunoblot lysate for both total and phosphorylated IKKβ and IκBα. If phosphorylated IKKβ is present at the time points where inhibition of IκBα phosphorylation by 13-197 is seen (90 and 120 min post-stimulation, for example) but little inhibition is seen at earlier time points, we may further assert that 13-197 indeed inhibits phosphorylated IKKβ. Furthermore, kinase and binding assays (see substrate binding experiments on pg 106) may be performed with immunoprecipitated phosphorylated IKKβ.
to further demonstrate that 13-197 blocks phosphorylation of IκBα by phosphorylated IKKβ.

Analysis of the forms of IKKβ inhibited by 25-4 will be more straightforward. The above experiment described for 13-197 can be performed with 25-4 though treatment with the small molecule will occur following stimulation with TNFα as 25-4 likely targets DNA binding by p65 and will block resynthesis of IκBα, leaving no substrate for IKKβ at late time points. Additionally, click chemistry with 25-4 analog 31-036 will be performed with lysates collected at the same time points post TNFα stimulation as described for 13-197. Pulldown with streptavidin beads will be performed following click chemistry with TAMRA-biotin-azide and pulled down protein will be resolved by SDS-PAGE and transferred to a membrane. The membrane will be immunoprobed for total and phosphorylated IKKβ. If 31-036 preferentially targets phosphorylated IKKβ, phosphorylated IKKβ will be pulled down in 31-036-treated lysates while nonphosphorylated IKKβ will not (will only see total IKKβ band in lanes with phosphorylated IKKβ). Again, endogenous IKKβ can be immunoprecipitated using anti-IKKβ antibody and kinase or IKKβ binding assays (visualized following click chemistry between 31-036 and TAMRA-azide) can be performed to demonstrate preferential inhibition of phosphorylated IKKβ by 25-4 and 31-036. Furthermore, phosphorylated IKKβ can be confirmed as a target of 31-036 in the mass spectrometry studies previously proposed for identifying the targets of 25-4 analog 31-036.

**Polypharmacology**

Drugs commonly interact with multiple targets in cells and although these off-target interactions are generally associated with harmful side-effects, there is growing evidence that drugs that interact with multiple targets may still, and in reality do, provide therapeutic benefit. This emerging paradigm is termed polypharmacology and describes a drug that
targets 1) multiple members within a single signaling pathway or 2) multiple proteins that each play a role in a different signaling pathway, though these pathways may be interconnected. Examples of polypharmacological agents include aspirin, which covalently modifies at least 120 targets in cells and is used to treat a variety of indications.\textsuperscript{194, 195} Furthermore, the anticancer medications sunitinib, pazopanib, sorafenib and dasatinib are known to inhibit multiple proteins. Not only do these drugs target multiple pathways and thus multiple processes known to drive cancer, but they also prevent drug resistance as they will still exert activity through their remaining targets even if one becomes resistant to treatment.\textsuperscript{194, 196}

With this in mind, though both 13-197 and 25-4 are known to target multiple proteins, we are confident future work to identify additional binding targets of 13-197 and 25-4 and subsequent optimization of these small molecules using structure-based drug design will reduce undesirable, off-target effects by these drugs. Furthermore, the ability of these small molecules to target two proteins upstream of NF-κB-driven transcription (IKKβ and MAP4K4 in the case of 13-197 and IKKβ and p65 in the case of 25-4) as well as other targets possibly relevant to cancer (Nek3 for 13-197 and unknown targets of 25-4) may be quite beneficial for overall activity in cells. Again, future studies with the compounds and their analogs will be required to test this.

**Rational design of IKKβ inhibitors**

In spite of the massive effort made by pharmaceutical companies to develop viable inhibitors of IKKβ, no such inhibitor has been approved for clinical use for the treatment of cancer. Nevertheless, cell- and animal-based studies continue to demonstrate the anticancer effects of IKKβ inhibition. The work outlined in this dissertation supports the hypothesis that IKKβ is indeed a viable target for anticancer research. By biasing our studies around a small molecule that allows the transient activation of IKKβ (13-197) and a targeting motif (α-methylene-γ-butyrolactone) of a natural product that inhibits IKKβ
without inducing severe toxicities, we were able to develop two small molecules that are active against IKKβ in cancer cell lines. Furthermore, these small molecules have shown promise in studies with clinically-approved therapeutics, again suggesting that IKKβ is a desirable target for anticancer research. Most importantly, these small molecules reduced tumor burden in mice without inducing toxicities, especially those associated with previously described IKKβ inhibitors. Future studies in our lab will outline the mechanistic basis for the non-toxic inhibition of IKKβ by these small molecules.

As new information regarding the structure of resting and phosphorylated IKKβ are made available, structure-guided drug discovery efforts focused on IKKβ will likely lead to the development of next-generation IKKβ inhibitors. These new small molecules, along with the previously discovered IKKβ inhibitors, will allow drug discovery groups to perturb and study the poorly-understood regulation of IKKβ’s activity by its complex members and positive and negative regulators. These studies will provide valuable insights that will allow researchers to develop targeted IKKβ inhibitors that inhibit diseased states of the kinase that persist in cancer and other diseases without affecting the transient activation of IKKβ that appears to be necessary for normal cellular functioning. Rational design of IKKβ inhibitors will be key for targeting the protein without inducing toxicity.
Chapter 8: References

References


