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Raf-1 kinase inhibitor protein modulation of the cellular response to chemotherapeutic drugs and PDE5 inhibitors

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Submitted in fulfilment of the requirements for the
Degree of Doctor of Philosophy

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University of Glasgow

Say first, of God above or Man below
What can we reason but from what we know?

…He who thro' vast immensity can pierce,
See worlds on worlds compose one universe,
Observe how system into system runs,
What other planets circle other suns,
What varied being peoples every star,
May tell why Heav'n has made us as we are:
But of this frame, the bearings and the ties,
The strong connexions, nice dependencies,
Gradations just, has thy pervading soul
Look'd thro'; or can a part contain the whole?

…But all subsists by elemental strife;
And passions are the elements of life.
The gen'ral order, since the whole began,
Is kept in Nature, and is kept in Man…

…Where, one step broken, the great scale's destroyed:
From Nature's chain whatever link you like,
Tenth, or ten thousandth, breaks the chain alike.
And if each system in gradation roll,
Alike essential to th'amazing Whole,
The least confusion but in one, not all
That system only, but the Whole must fall…

…All Nature is but Art unknown to thee;
All chance direction, which thou canst not see;
All discord, harmony not understood;
All partial evil, universal good:
And spite of Pride, in erring Reason's spite,
One truth is clear, *Whatever is, is right.*

From an Essay on Man, Epistle I: Of the Nature and State of Man, With Respect to the Universe by Alexander Pope

Declaration

I declare that the work described in this thesis has been carried out by me unless otherwise cited or acknowledged. It is entirely of my own composition and has not, in whole or in part, been submitted for any other degree.

Anne Marie Reid

September 2010

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On that note and in the words of George Thorogood, it’s time for me to “Get a haircut, and get a real job!”

Cheers

AM

Abstract

RKIP was initially discovered as an endogenous inhibitor of the ERK and NF-κB pathways, and was also shown to prolong the activation of GPCRs *via* inhibition of the GRK2 protein. Now increasing evidence has linked RKIP to a metastases suppressing and chemo-sensitising role in cancer cells.

The chemo-sensitising effect of RKIP was investigated in a colon carcinoma cell line using a variety of chemotherapeutic agents from conventional agents to newer targeted therapies.
Initial results suggested that role of RKIP in the modulation of chemotherapeutic drug response was at the level of apoptosis; there did not appear to be great observable effects in the cell proliferative response and the cell cycle distribution of the colon carcinoma cells after treatment with selected agents.
Apoptosis modulation by RKIP occurred after treatment with doxorubicin, FasL, paclitaxel and TRAIL. TRAIL-treated colon carcinoma cells displayed increased cell death as the levels of RKIP within the cell were increased. In contrast, doxorubicin, FasL and paclitaxel-treated cells displayed a scaffold-like response as the levels of RKIP were increased in the cell; with WT RKIP-expressing cells being more sensitive to doxorubicin, FasL and paclitaxel-induced apoptosis than low or high RKIP-expressing colon carcinoma cells. There was no modulation of 5-FU, cisplatin and etoposide-induced apoptosis by RKIP. Indeed, these three agents did not appear to induce cell death in this colon carcinoma cell line.
RKIP modulation of chemo-sensitivity has never been shown before in a colon carcinoma cell line and this is the first time that doxorubicin and FasL-induced apoptosis has been shown to be modulated by RKIP. Further, it is shown here, for the first time, that the modulation of chemotherapy-induced apoptosis by RKIP can change depending upon the cytotoxic drug employed as treatment.

TRAIL and FasL, both members of the TNF super-family, were selected for further analysis due to the distinctive cell death responses observed as a consequence of the levels of RKIP within the cell. WT RKIP cells were sensitive to FasL treatment, and high RKIP cells were most sensitive to TRAIL administration.

Increased sensitivity of high RKIP-expressing colon cells to TRAIL treatment appeared to involve up-regulation of the DR5 receptor; down-regulation of the anti-apoptotic molecule Bcl-xl; pIKK which activates the NF-κB pathway; and TRAF2 which has been shown to activate the NF-κB pathway. Whether RKIP directly interacts with these molecules is unknown however RKIP has been shown to bind upstream activators of the NF-κB pathway and another TRAF subtype TRAF6.
YY1 expression was evident in the TRAIL-treated cells but the expression was unchanged as the levels of RKIP within the cell were altered.

The FasL-treated cells also displayed decreased pIKK levels as the levels of RKIP were increased; it is possible that NF-κB was behaving as both pro- and anti-apoptotic within this cell line. Thus RKIP inhibition of the NF-κB pathway may have prevented FasL-induced apoptosis in the high RKIP-expressing colon carcinoma cells.
The expression of TRAF6, which has been shown to bind RKIP, displayed a scaffold-like response with WT RKIP-expressing cells having the highest TRAF6 expression. This was also the case for the transcriptional regulator YY1, thus it is possible that both YY1 and TRAF6 were behaving in a pro-apoptotic-like manner in the WT RKIP-expressing cells.
TRAF2 was also evident in the FasL-administered cells but the expression did not change regardless of the levels of RKIP within the cell.

Overall, it appears that differential expression of TRAF adaptor proteins is responsible for the contrasting responses of TRAIL and FasL-treated cells with low, WT and high RKIP expression. Utilisation of particular TRAF adaptors or TRAF combinations by the TRAIL and Fas receptors may also account for the pro- and anti-apoptotic roles of the NF-κB pathway, and the recruitment or down-regulation of other proteins dependent upon the cell stimulus. How RKIP affects these proteins requires further investigation, however these results are exciting and novel, and strengthen evidence surrounding the role of RKIP in chemo-sensitivity.

On another note, RKIP has been shown to bind the PDE5 inhibitor PF-3717842, therefore investigation of the effects of the PDE5 inhibitors sildenafil citrate and vardenafil citrate on RKIP inhibition of the ERK pathway in a colon carcinoma cell line were examined. The effects of the PDE5 inhibitors were compared to the cell migration inhibitor locostatin that has been shown to bind and inhibit RKIP, and prevent the RKIP-Raf-1 interaction. With TPA

and EGF stimulation, locostatin appeared to act in a manner consistent with its known function as an RKIP inhibitor. The PDE5 inhibitors sildenafil citrate and vardenafil citrate displayed a similar trend to that of locostatin, although their effects on the ERK pathway were not as potent. It is possible that after EGF stimulation, the strong activation of B-Raf was over-shadowing the subtle effects of the drug treatments. Under growth conditions, the RKIP inhibitor locostatin did not appear to behave as an inhibitor of RKIP nor did the PDE5 inhibitors sildenafil citrate and vardenafil citrate. It is possible that the strong activation of various growth and proliferative cascades was impinging upon the ERK pathway, were overshadowing the drug effects, or resulting in off-target (RKIP-unrelated) effects of the drugs.

In summary, the role of RKIP within the cell is becoming an increasingly exciting avenue of research and is consistently yielding new and interesting roles and interactions within the cell. Understanding and elucidating the roles of this intriguing protein within the cell will not only strengthen our knowledge of signal transduction regulation and modulation, but may also provide a new source of targeted therapy and means of manipulation in the treatment of cancer and chemotherapeutic drug resistance.

Table of Contents

List of Figures

CHAPTER 1: GENERAL INTRODUCTION

CHAPTER 2: MATERIALS AND METHODS

CHAPTER 3: RKIP MODULATION OF CHEMOTHERAPEUTIC DRUG RESPONSE

APPENDIX

Abbreviations

5-FU	5-fluorouracil
AC	adenylyl cyclase
AD	Alzheimer's disease
ADP	adenosine-5'-diphosphate
ANK	ankyrin-repeat motif
ANOVA	analysis of variance
Apaf-1	apoptotic protease-activating factor 1
ATM	ataxia telangiectasia mutated
ATP	adenosine-5'-triphosphate
ATR	ATM-Rad3-related
Bcl-2	B-cell lymphoma 2
BrdU	Bromo deoxyuridine
BSA	bovine serum albumin
cAMP	adenosine 3',5'-cyclic monophosphate
CARD	caspase activation and recruitment domain
CDK	cyclin dependent kinase
C/EBP	CCAAT/enhancer binding protein
cGMP	guanosine 3',5'-cyclic monophosphate
Chk	checkpoint kinase
CHOP	C/EBP homologous protein
CRD	cysteine rich domain
CREB	cAMP response element-binding protein
DD	death domain
DED	death effector domain
DISC	death inducing signalling complex
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
DR	death receptor
dTMP	deoxythymidine monophosphate
dUMP	deoxyuridine monophosphate
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial–mesenchymal transition
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FACS	fluorescence-activated cell sorting
FADH	flavin adenine dinucleotide (reduced form)
FADD	Fas-associated protein with DD
FasL	Fas ligand
FBS	foetal bovine serum
FdUMP	fluorodeoxyuridine monophosphate
FdUTP	fluorodeoxyuridine triphosphate
FT-ICR MS	Fourier-Transform Ion Cyclotron Resonance Mass Spectrometry
FUTP	fluorouridine triphosphate
G-protein	guanosine nucleotide binding protein

GADD153	growth arrest- and DNA damage-inducible gene 153
G1 phase	Gap1 phase
G2 phase	Gap2 phase
GC	guanylyl cyclase
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GPCR	G-protein coupled receptor
Grb2	growth factor receptor-bound protein 2
GRK2	G-protein coupled receptor kinase-2
GST	glutathione-S-transferase
GTP	Guanosine-5'-triphosphate
HCNP	hippocampal neurostimulating peptide
H Ras	Harvey sarcoma virus
IκB	inhibitor of κB protein
IKK	IκB kinase
IL-1	interleukin 1
JNK	c-Jun NH2-terminal kinases
Kd	dissociation constant
KO	knock out
K Ras	Kirsten sarcoma virus
M phase	Mitosis phase
MAPK	mitogen activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MDCK	Madin-Darby canine kidney
MEK	MAPK/ERK kinase
MEKK1	MEK kinase 1
MI	microsatellite instability
MMR	mismatch repair
MOMP	mitochondrial outer membrane permeability
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NEMO	NF-κB essential modifier
NER	nucleotide excision repair
NES	nuclear export sequence
NF-κB	nuclear factor-kappa B
NIK	NF-κB inducing kinase
NLS	nuclear localisation sequence
NO	nitric oxide
NOS	nitric oxide synthase
N Ras	neuroblastoma
OPG	osteoprotegerin
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PDE	3',5'-cyclic nucleotide phosphodiesterase
PEBP	phosphatidlyethanolamine-binding protein
PKA	cAMP dependent protein kinase
PKB	protein kinase B
PKC	protein kinase C
PKG	cGMP dependent protein kinase

pERK	phosphorylated ERK
pMEK	phosphorylated MEK
pRKIP	phosphorylated RKIP
PTB	phosphotyrosine binding
Raf	rapidly accelerated fibrosarcoma
Rb	retinoblastoma protein
Rel	reticuloendotheliosis
RBD	Ras binding domain
RHD	Rel homology domain
RIP	receptor-interacting protein
RKIP	Raf-1 kinase inhibitor protein
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
RTK	receptor tyrosine kinase
S phase	synthesis phase
sGC	soluble Guanylate Cyclase
SH2	Src-homology 2
Shc	Src homology 2 domain-containing-transforming protein C1
SoS	Son of Sevenless
TAD	transcriptional activation domain
TAB2/3	TAK1 binding protein 2/3
TAK1	transforming growth factor-β (TGFβ)-activated kinase-1
TBS	Tris buffered saline
TBST	TBS plus tween-20
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
TPA	12-O-Tetradecanoylphorbol-13-acetate
TRADD	Tumor necrosis factor receptor type 1-associated death domain protein
TRAF	tumour necrosis factor receptor associated factor
TRAIL	tumour necrosis factor-related apoptosis-inducing ligand
Tris	Tris (hydroxymethyl) aminomethane
TS	thymidylate synthase
Tween 20	polyoxyethylene sorbitan monolaurate
WT	wild type
XIAP	X-linked inhibitor of apoptosis
YY1	yin yang 1

CHAPTER 1:
GENERAL INTRODUCTION

1.1 Cancer

Cancer is arguably one of the most devious diseases afflicting society today. It is a silent, random killer and is responsible for hundreds of thousands of deaths per year in the UK alone [1]. Cancer is the collective term for over 100 diseases associated with abnormal and uncontrolled cell growth and proliferation, eventually leading to tissue invasion and metastases formation [2]. Many cell-types can produce malignancies, but in order to become neoplastic cells must acquire at least half a dozen essential traits [3]. These include growth signal autonomy as well as resilience to growth inhibitory stimuli; the ability to evade apoptosis and undergo limitless cell division; and finally cells must be able to sustain angiogenesis eventually leading to tissue invasion and metastases formation [3]. In short, cancer cells have discovered the sought-after Holy Grail and unlocked the key to Immortality.

The alterations in cancer arise due to multiple genetic and epigenetic changes that result in the dysregulation of molecules and signalling pathways involved in the control of cell growth, proliferation and apoptosis. There are two classes of genes that are the important players in this twisted game of Life; the proto-oncogenes and the tumour suppressor genes. One example is the tumour suppressor protein p53 that is thought to be mutated in over 50% of all cancers [4]. An example of the proto-oncogenes is the Ras GTPase super-family that is involved in the control of several important transduction cascades including the mitogen activated protein kinase (MAPK) pathway. This protein family is believed to be abnormally expressed and dysregulated in approximately 30% of human tumours [5]. These two examples are well documented in a wide range of cancers, however within each single cancer cell there are thought to be as many as 10,000 genetic errors [6].

Although cancer cells must achieve at least six basic milestones before they become neoplastic, the road to this cancerous phenotype is not always the same. Cancer cells achieve Immortality through their own individual evolutions. This is what makes cancer such a formidable enemy and so difficult to kill.

1.2 Apoptosis

In order to function as part of a living and healthy system, cells must recognise when to die. As dramatic and irrational as it may sound, programmed cell death or apoptosis is crucial for the maintenance of homeostasis, and is vitally important in the immune response and defence against viral invasion [7]. Aberrations in apoptosis or the apoptotic machinery is a common trick devised by cancer cells to remain alive indefinitely.

Apoptosis was first described in 1972 by Kerr *et al.* [8] and should not be confused with disordered or traumatic cell death that occurs in necrosis, or the large scale protein degradation that occurs in autophagy [8, 9]. Cells undergoing apoptosis display a number of morphological and biochemical features. These features occur several hours after the proverbial crossing of the Rubicon and include, but are not exclusive to the following; cell shrinkage, membrane blebbing and other membrane changes, chromatin condensation and nuclear DNA fragmentation [8, 9].

1.2.1 Caspases

Central to the apoptotic machinery, and the major culprits for the biochemical and morphological changes associated with apoptosis, are a family of cysteine proteases called caspases [9]. Caspases contain cysteine at their active site and only cleave substrates at the carboxyl terminus of aspartic acid bonds [10, 11], hence the C-asp in the name caspase. Unlike many proteases, caspases are extremely specific in their substrate choice and act as protein modifiers rather than degraders. The substrate specificity of caspases is determined by the four amino acid residues present to the amino terminus of the active site [10, 11]. As a result of their high specificity, activation of particular caspases can lead to physiologically distinct functions. For example, some substrates are cleaved later than others, and the cleavage of a substrate may also be cell-type specific [10]. The substrates cleaved by caspases are numerous; from nuclear and cytoplasmic proteins to protein kinases and other signal transduction-related poteins [10]. The cleavage of substrates and the subsequent cellular disassembly by caspases occurs with military precision in the cell.

Despite their isoform-specific function, modifying the activity of the caspases will ultimately affect programmed cell death. This is a strategy that is employed by cancer cells to evade apoptosis [12-15].

Caspases are synthesised within the cell as enzymatically inert zymogens and consist of an N-terminal prodomain and p10 and p20 domains that are found in the mature enzyme. The mature enzyme forms a heterotetramer that consists of two active sites and two p10/p20 heterodimers [10]. Activation of inert zymogens to the mature enzyme occurs *via* proteolytic cleavage of the zymogen between the p10 and p20 domains, and sometimes between the prodomain and p20 domain [11]. These sites are all aspartic acid bonds so it is possible that autocatalytic cleavage is occurring [16]. This cleavage leads to a caspase cascade resulting in the cleavage and activation of pro-apoptotic proteins and also the inhibition of anti-apoptotic molecules [10].

Activation of the initiator caspases is thought to occur *via* two very different mechanisms; the induced proximity/extrinsic pathway involving death receptors; or by association with a regulatory subunit, also called the intrinsic pathway, which utilises the mitochondria [16, 17]. Both the extrinsic/death receptor and intrinsic/mitochondrial cell death pathways converge at the activation of the executioner caspases 3, 6 and 7 [16, 17].

1.2.2 Death receptor activation of apoptosis (external cell death pathway)

The extrinsic pathway involves members of the Tumour Necrosis Factor Receptor (TNF-R) family, mainly Fas (also called CD95 or APO-1) and Tumour Necrosis Factor-related Apoptosis-Inducing Ligand (TRAIL)/APO-2 receptors (DR4 and DR5) [17, 18]. Their actions on caspases are mediated *via* adaptor proteins that form bridges between the death receptor and the relevant initiator caspase. These associations are possible due to domain-domain interactions between the molecules [19]. There are two major domains involved in these interactions; the death domain (DD) and the death effector domain (DED) [19]. Examples of where these domains can be found include the DD in the cytoplasmic region of Fas and adaptor proteins such as Fas-associated DD protein (FADD) and receptor-interacting protein (RIP). The DED can be found in FADD, and caspase 8 and 10 zymogens [19]. Upon ligand binding to the death receptor, the death receptors form

membrane bound signalling complexes by aggregating [17]. Following receptor oligomerisation, adaptor proteins such as FADD interact with the death domains on the cytoplasmic tail of the receptors resulting in the formation of the death inducing signalling complex – DISC [17, 19]. Procaspases 8/10 are then recruited to the DISC *via* these adaptor proteins due to the domain-domain interactions mentioned previously, leading to high local concentrations of procaspase 8/10 [17, 19]. This allows autocatalytic cleavage of the inert zymogens to occur, resulting in the production of mature and activate caspases, and so the initiation of apoptosis.

During recruitment of adaptor proteins and caspase activation, FADD can also interact with tumour necrosis factor receptor associated factors (TRAFs), tumor necrosis factor receptor type 1-associated death domain protein (TRADD) and RIP [20-22]. TRAFs, TRADD and RIP are employed mainly by other TNF family members to activate signalling cascades such as the nuclear factor-kappa B (NF-κB) pathway and the c-Jun NH2-terminal kinase (JNK) pathway. This allows TNF receptor modulation of these pathways which is essential for cell growth, proliferation and also for stress responses [23-26]. TNF family members often use these adaptors to recruit FADD in order to induce apoptosis.

The death receptors used by FasL or TRAIL directly recruit FADD to initiate apoptosis [27], and do not generally employ secondary signalling *via* TRAFs or other adaptors to signal to NF-κB or JNK pathways as potently as other TNF family members [28]. Reports have shown that FADD has a role in secondary signalling from Fas and TRAIL receptors, but it is mainly involved in the apoptotic response [22, 25, 29].

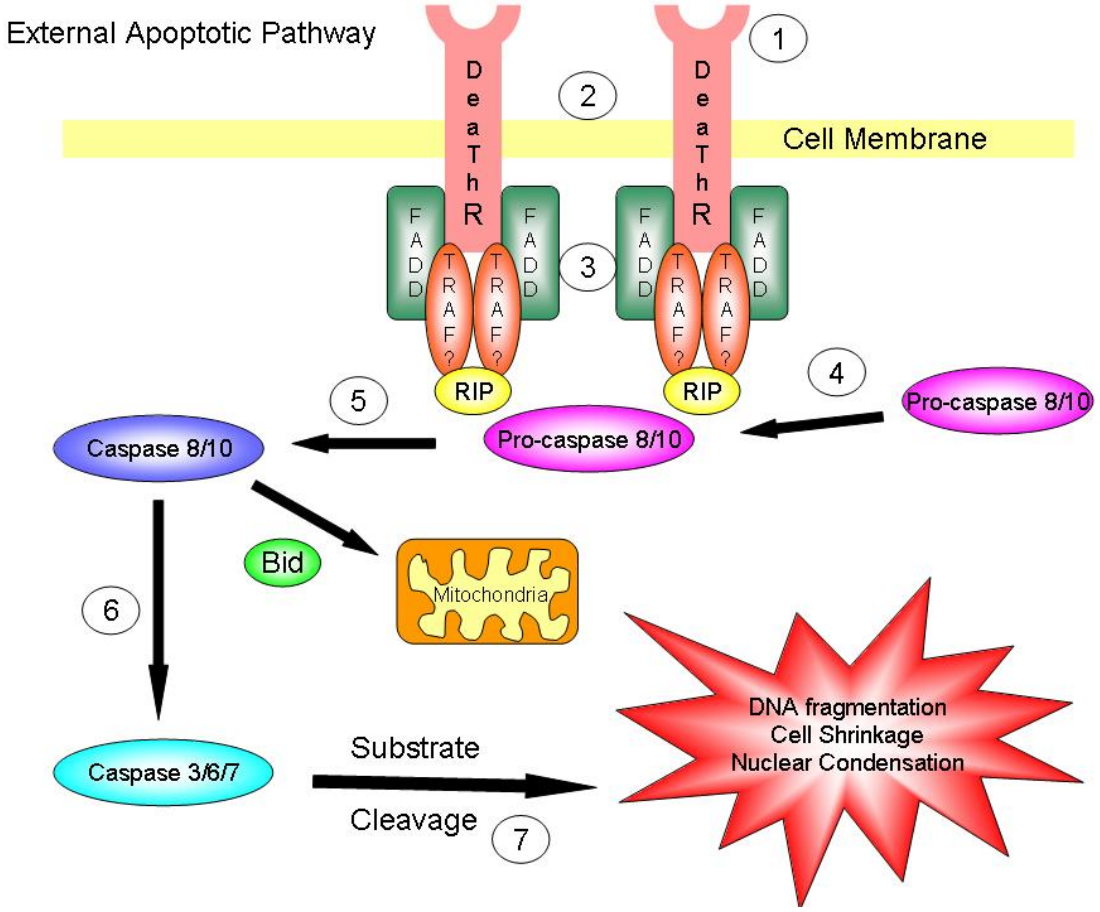


Figure 1.1: Schematic representation of the external apoptotic machinery. 1. Ligand binding to death receptor. 2. Death receptor oligomerisation. 3. Recruitment of adaptor protein such as FADD (and occasionally RIP, TRAFs for NF-κB or JNK involvement) leading to DISC formation. 4. Procaspase 8/10 recruitment to DISC. 5. Activation of inert caspase 8/10 zymogens. 6. Triggering of caspase cascade leading to the activation of executioner caspases. 7. Cleavage of apoptotic substrates resulting in the biochemical and morphological changes associated with apoptosis.

Once the initiator caspases 8 and 10 are activated by death receptors, the caspase cascade occurs leading to the activation of the executioner caspases 3, 6 and 7. Once these executioner caspases are activated, they can cleave substrates leading to the timely destruction of cellular components. Finally, caspase 8 can cleave the pro-apoptotic Bcl-2 family member Bid to link death receptor initiation of apoptosis to mitochondria-induced cell death [30, 31].

1.2.3 Mitochondrial induction of apoptosis (internal cell death pathway)

In contrast to the extrinsic pathway, the intrinsic pathway is not activated by death receptors or their associated extracellular ligands. Instead, this pathway is initiated by intracellular signals released as a result of cellular stress, for example by the withdrawal of growth factors or by treatment with cytotoxic agents [32]. These endogenous signals act as pro-apoptotic factors and subsequently lead to an increase in mitochondrial outer membrane permeability (MOMP), which causes the release of mitochondrial mediators of caspase-dependent cell death [33]. One of the major mediators released is the flavoprotein cytochrome c [34]. Release of this protein from the outer mitochondrial membrane to the cytosol results in oligomerisation of apoptotic protease-activating factor 1 (Apaf-1) molecules into a wheel-shaped signalling complex called the apoptosome [35]. Apaf-1 contains the caspase activation and recruitment domain (CARD) at the amino terminus, a central nucleotide-binding oligomerisation domain (NOD) and multiple C-terminal WD40 repeats thought to bind cytochrome c [36, 37]. Adenosine-5'-diphosphate (ADP) bound to Apaf-1 retains Apaf-1 in an inactive and auto-inhibited state. Upon release of cytochrome c and binding (possibly to the WD40 repeats), in the presence of adenosine-5'-triphosphate (ATP), Apaf-1 molecules oligomerise to form the active apoptosome [36, 37].

The role of the apoptosome is to activate the initiator caspase in this pathway, caspase-9 [35]. The apoptosome results in aggregation of procaspase-9 and allows dimerisation, and not autocatalytic cleavage as previously thought, of the zymogens. This in turn leads to the formation of the active enzymes [35]. The caspase cascade occurs as mentioned previously and programmed cell death follows [10]. The major domain involved in these interactions is the CARD domain, present in procaspase 9 and 2 as well as in the adaptor protein Apaf-1 [19].

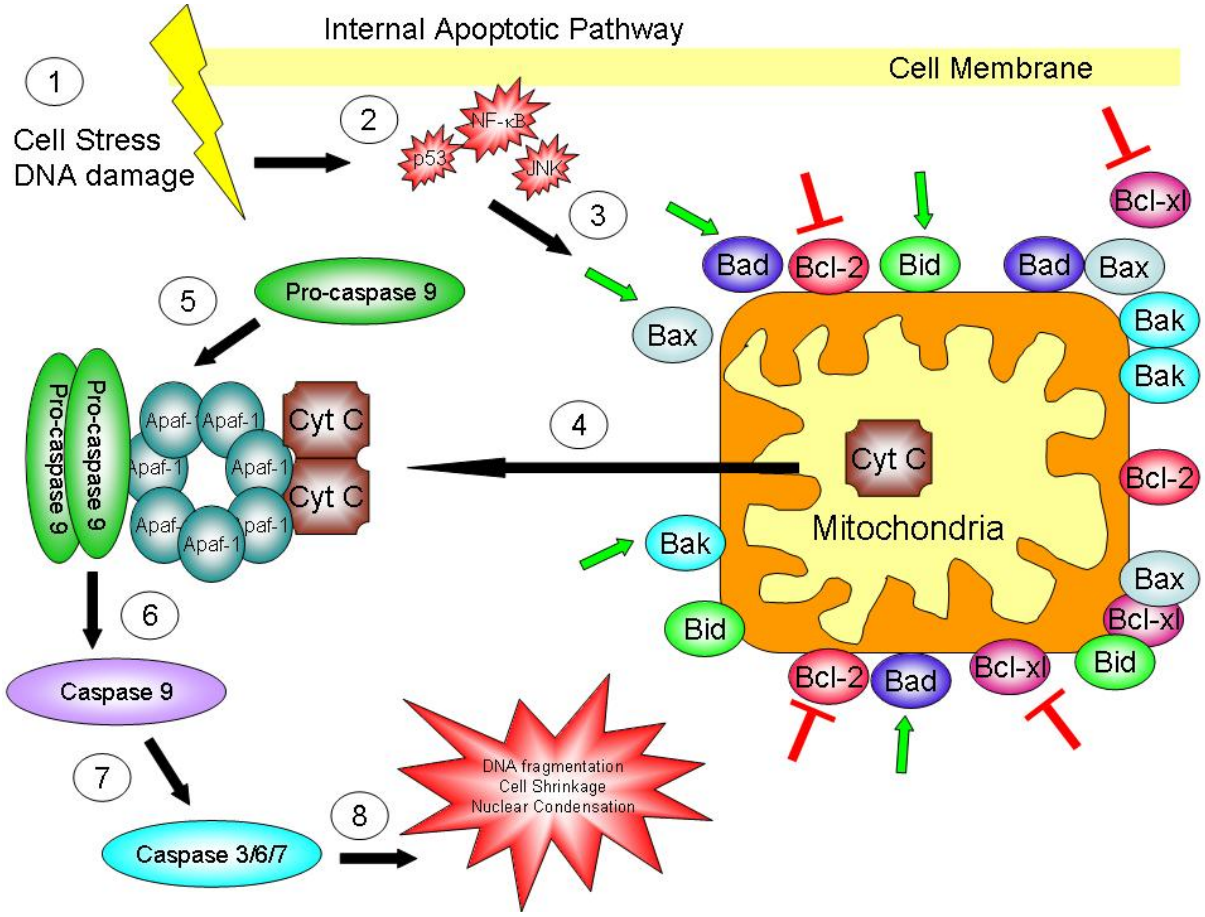


Figure 1.2: Schematic representation of the mitochondrial apoptotic pathway. 1. Cell stress or DNA damage. 2. Release of proteins and mediators involved in the cell stress response. 3. Modulation of pro- and anti-apoptotic members of the Bcl-2 family of proteins. 4. Release of cytochrome C into the cytosol due to increased MOMP. 5. Formation of the apoptosome containing the adaptor Apaf-1, cytochrome C and procaspase 9. 6. Activation of the inert zymogen procaspase 9. 7. Initiation of caspase cascade leading to activation of executioner caspases. 8. Substrate cleavage resulting in the morphological and biochemical changes associated with apoptosis.

Prior to formation of the apoptosome, members of the B-cell lymphoma 2 (Bcl-2) family are involved in the modulation of MOMP, leading to the regulated release of cell death mediators from the mitochondria. It is important to understand the regulation of the release of these mitochondrial factors by members of the Bcl-2 family since these factors are crucial for apoptosome formation [16].

There are three groups of Bcl-2 family members, 1, 2 and 3 [38]. Group 1 members are anti-apoptotic proteins whereas proteins in groups 2 and 3 are promoters of cell death [38, 39]. The key function of all three groups of Bcl-2 family members is to regulate the release of pro-apoptotic molecules from the outer mitochondrial membrane to the cytosol, in

particular the protein cytochrome c [34]. The release or inhibition of these mitochondrial factors is dependent upon the relative quantity of pro- and anti-apoptotic members of this family in the cell [34]. Indeed, Bcl-2 family members are mainly involved in the formation of heterodimers with each other, and so an increase in pro-apoptotic members or a decrease in anti-apoptotic members will lead to the initiation of cell death or *vice versa* [38, 40].
All Bcl-2 family members share a conserved carboxyl-end transmembrane region that localises these molecules to the outer mitochondrial membrane, to the endoplasmic reticulum (ER) or to the nuclear envelope [41]. Moreover, pro-death members of the Bcl-2 family all possess a BH3 (Bcl-2 homology) region that is absolutely essential for pro-apoptotic activity [38, 39, 42].
The modulation of cytochrome c release into the cytosol from the mitochondria by Bcl-2 family members is essential for the activation of the internal death pathway. Once released, cytochrome c is involved in the formation of the apoptosome [35]. Here, adaptor proteins are able to activate caspase 9 zymogens resulting in the initiation of the caspase cascade that leads to cell death [35].

Finally, during cell stress, as the cell attempts to repair itself or overcome a transcriptional or a translational block, numerous proteins and mediators are activated. These include p53, transcription factors such as NF-κB and C/EBP homologous protein (CHOP) and members of the MAPK family, in particular the JNK (JNK1, JNK2 and JNK3) and the four p38 kinases (α, β, γ and δ) [32, 43, 44]. All these stress response proteins can induce or repress members of the Bcl-2 protein family, and thus modulate the cellular apoptotic response.

1.3 Chemotherapy

Chemotherapy is one of the treatments of choice in the battle against cancer. It has travelled a long way since the discovery that nitrogen mustards could be used as a treatment for lymphomas - tumours of lymphoid cells in the immune system [45]. All chemotherapeutic agents activate apoptotic pathways within cancerous cells in order to induce cell death [46]. Conventional cytotoxic drugs such as doxorubicin and 5-fluorouracil (5-FU), and the prototype nitrogen mustards, generally initiate the intrinsic or mitochondrial apoptotic cascade by inflicting stress upon the cell [46, 47]. Other cytotoxic drugs bind to death receptors at the cell surface and activate the external apoptotic pathway resulting in cell death [46, 47].

1.3.1 Cell stress induction of apoptosis *via* chemotherapeutic agents

Anti-cancer compounds cause cell stress, which in turn activates a number of mechanisms that result in the induction of programmed cell death. Alkylating agents such as cisplatin, carboplatin and doxorubicin intercalate with the deoxyribose nucleic acid (DNA) helix leading to the blockade of replication and transcription [47-49]. Anti-metabolites, like pyrimidine analogues (5-FU and capecitabine) inhibit nucleotide and DNA synthesis [47, 49-51].

Chemotherapy-induced cellular stress does not occur by affecting DNA, and as a result protein synthesis, alone. The enzyme topoisomerase is responsible for the unwinding of the DNA helix during replication. Therefore topoisomerase inhibitors (etoposide and irenotecan) have been used to treat a wide range of cancers [52-54]. Furthermore, taxane-based compounds like paclitaxel (Taxol) are used as chemotherapeutic agents because they interfere with microtubule formation by stabilising and polymerising the essential cellular protein tubulin [55-57].

Regrettably, these types of cytotoxic treatment affect all of the cells in the body and not just the cancerous cells. In the body, normal cells are being replaced all the time, particularly skin cells and hair follicles, therefore normal cells are affected alongside cancer cells. This is why chemotherapy has major side effects including skin lesions and baldness. The rationale of conventional therapies is that because cancer cells are growing faster than normal cells, they are affected to a greater degree by the chemotherapeutic treatment.

1.3.2 The targeted therapy revolution

Unfortunately, cancer cells have developed tricks to evade chemotherapy. Some cancers over-express proteins that pump the drug out of the cells. Other cancers recruit proteins that allow the cell to overcome DNA damage that would normally kill a cell [58-60].
Each individual cancer cell within a tumour may have at least 10000 genetic errors/mutations compared to a normal cell [6]. Some of these errors may render the cell resistant to chemotherapy, possibly by increasing the cellular levels of anti-death proteins and decreasing the levels of pro-death proteins [13].
However, the wise words of Edgar Allan Poe may provide us with the 'magic bullet' that we have been searching for. Poe philosophised that; "The boundaries which divide Life from Death are at best shadowy and vague. Who shall say where one ends, and where the other begins?" Drinking from the Elixir of Life always has its drawbacks. By manipulating cell growth and resisting death, cancer cells may be exposing their Achilles' heel.
This hypothesis has led to cancer research moving towards "targeted therapy" approaches for overcoming the resistance mechanisms of cancer cells and killing them [60]. Targeted therapy involves the use of a drug that specifically interferes with a molecule that is involved in the formation of a cancerous phenotype [61, 62]. The hope is that the use of targeted therapy will be more clinically successful because it will be designed to overcome the resistant forms of cancer, and also reduce the side-effects that are associated with chemotherapy treatment.

Better understanding of the signalling components that are responsible for the formation of neoplasms has led to the development of molecular targeted therapy [63]. The epidermal growth factor receptor (EGFR) is up-regulated in certain cancer types so antibodies against

this receptor (*e.g.* cetuximab) have been commercially employed [62]. In addition, inhibitors of MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK) and apoptosis-inducing drugs are currently undergoing clinical trials as promising treatments for this disease [44, 64].

One of these apoptosis-inducing drugs is TRAIL, which is an agonist of the death receptors mentioned earlier. TRAIL acts synergistically with conventional agents to enhance cancer therapy [65]. Most importantly, TRAIL has promise as an anti-cancer agent as it is selective for cancer cells [66, 67]. This is because decoy TRAIL receptors are present on normal cells. These decoy receptors possess the same extracellular components but do not have the apoptosis-activating machinery [67]. Cancer cells do not express these decoy receptors thus they are highly susceptible to TRAIL treatment [67].

Another group of apoptosis-inducing drugs are the Bcl-2 inhibitors. Cancer cells over-express the anti-death members of the Bcl-2 family so drugs that target these anti-death proteins would be very effective. It is possible that this group of drugs may even recruit pro-death molecules [68]. Because cancer has been dependent on these anti-death proteins to ensure immortality, removal of these molecules restores cellular mortality and makes them sensitive to conventional chemotherapy regimes [68, 69]. Like TRAIL, these Bcl-2 inhibitors are being used in combination with conventional treatments to successfully battle cancer.

In summary, targeted therapy aims to exploit the weaknesses of cancer cells by turning the molecules they manipulate to stay alive, against them.

1.3.3 Chemotherapeutic agents and the induction of cell death

A number of cytotoxic agents are available. Drugs were selected for analysis in this study based on ease of acquisition; routine use in tumours; and mechanism of action. The drugs that were selected for analysis and how they activate the apoptotic machinery will be briefly reviewed.

1.3.3.1 5-fluorouracil (5-FU)

It has been over 50 years since the cytotoxic properties of 5-FU were identified [50]. 5-FU is a member of the fluoropyrimidines and an analogue of the DNA base uracil. The structure of 5-FU contains fluorine instead of hydrogen at the C-5 position. This allows the molecule to be taken up by the cell by facilitated transport, the same way in which uracil is internalised [50].

a) b)

Figure 1.3: Comparison of the structural differences in 5-FU compared to uracil. a) Structure of uracil. **b)** Structure of 5-FU, showing the addition of the fluoride atom on the uracil structure.

Once inside the cell, 5-FU is metabolised to form the active compounds fluorodeoxyuridine triphosphate (FdUTP), fluorodeoxyuridine monophosphate (FdUMP) and fluorouridine triphosphate (FUTP) [50, 70]. The FdUMP metabolite is responsible for inhibiting the enzyme thymidylate synthase (TS). TS is an important enzyme in the biosynthesis of pyrimidines; it is involved in the production of deoxythymidine monophosphate (dTMP) by reductive methylation of deoxyuridine monophosphate (dUMP) [70]. This process is essential for DNA repair and replication as it is the only source of new thymidylate in the cell [70].

Furthermore, the FdUTP and FUTP metabolites are then incorporated into the DNA and RNA resulting in toxicity of the RNAs – rRNA, tRNA and snRNA - which in turn leads to profound cellular damage [70]. For this reason, 5-FU is an extremely efficient inhibitor of DNA replication and synthesis, and so prevents the formation of new daughter cells.

1.3.3.2 Cisplatin

Cis-diamminedichloroplatinum II (CDDP or cisplatin) has had a significant impact on the treatment of cancer since its discovery more than 30 years ago [71]. The primary target of this neutral and inorganic compound is DNA. Cisplatin leads to the formation of DNA-protein and DNA-DNA intra- and inter-strand crosslinks by reacting with nucleophilic sites on the purine bases of the DNA molecules [48, 72]. In order to chelate with the DNA, cisplatin first undergoes spontaneous aquation reactions during which the chloride ligands on the cisplatin molecule are exchange for water molecules [48].

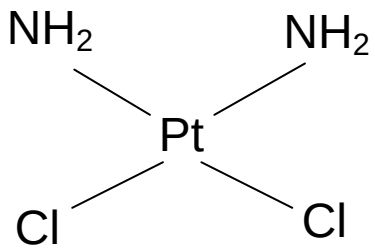


Figure 1.4: Structure of cisplatin. The structure of cis-diamminedichloroplatinum II, more commonly known as cisplatin; showing the two chloride ligands which undergo aquation reactions, allowing cisplatin to chelate with DNA molecules.

The creation of DNA-DNA and DNA-protein adducts results in damage and de-stabilisation of the DNA molecules which in turn leads to the activation of DNA damage proteins and problems with DNA replication [48, 72]. Activation of DNA damage proteins plays an important role in the initiation of apoptosis, the ultimate goal of anti-cancer therapy. The DNA damage proteins can lead to repair of the DNA or induce programmed cell death if repair is insufficient [43, 73, 74]. Indeed, nucleotide excision repair (NER) is one of the most important routes for the removal of platinum adducts. This is supported by studies that show that there is clear correlation between the cytotoxicity of cisplatin and the number and persistence of DNA-cisplatin adducts formed [72, 75]. Furthermore, a defect in the NER pathway is known to be associated with cisplatin hyper-sensitivity. Normal levels of response to cisplatin resume once the pathway is re-instated in the cells.

The mismatch repair (MMR) complex is also important in cisplatin-induced apoptosis. The MMR complex has evolved to maintain the integrity of the genome by rectifying errors

that occur normally in DNA synthesis, these errors are often in the form of microsatellites [75]. If the MMR complex cannot repair these errors and the associated cisplatin-induced genome instability, then apoptosis is initiated. Furthermore, down-regulation or mutation of components involved in this process has been implicated in cisplatin resistance [72, 75].

Cisplatin therefore activates substantial cell stress networks within the cell in addition to disrupting DNA replication; the culmination of these actions leads to the initiation of apoptosis.

1.3.3.3 Doxorubicin

Doxorubicin (or Adriamycin) is an anthracycline antibiotic derived from *Streptomyces* bacteria. It has various methods of inducing cell death within neoplastic tissues [76].
Doxorubicin can covalently cross-link with DNA molecules leading to the formation of DNA adducts. Like other DNA chelators, such as cisplatin, increased adduct formation is associated with increased activation of DNA damage responses [76, 77]. Furthermore, the quinine structure within the doxorubicin molecule allows for electron reduction leading to the generation of free radicals and reactive intermediates. Production of these compounds has adverse effects on the cell organelles and lipid membranes as well as on the DNA [76, 77].

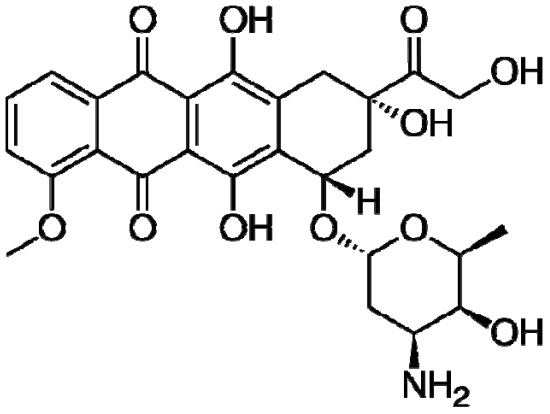


Figure 1.5: Structure of doxorubin. The structure of doxorubin (adriamycin) displaying the quinine structure which allows the generation of free radicals and reactive intermediates. Doxorubicin can also intercalate with DNA and lead to the formation of toxins which induce DNA strand breakages.

Interference with nuclear enzymes within the cell contributes significantly to the cytotoxicity of doxorubicin. The double and single strand breaks in the DNA, that prevent DNA biosynthesis, occur *via* the inhibition of the function of DNA helicase [76]. Finally, like etoposide, doxorubicin can result in the formation of molecules that interfere with topoisomerase II activity [76]. Topoisomerase II is required for the maintainance of DNA topology, and the inhibition of this enzyme results in the initiation of the DNA damage response and subsequently apoptosis [76].

Overall, doxorubicin-induced DNA damage and protein inhibition makes this a highly potent anti-cancer and cell stress-inducing compound, thus it has been successfully used to treat a wide range of malignancies.

1.3.3.4 Etoposide

Etoposide is a derivative of naturally occurring compounds called podophyllotoxins that are found in plants. They have been used throughout history as medication by many cultures [52]. In 1984, the primary target of etoposide was discovered to be the mammalian enzyme DNA topoisomerase II [53]. Topoisomerase II enzymes are ATP-dependent nuclear enzymes that introduce transient double strand breaks in the DNA molecule. This allows the topology to be manipulated which is essential for DNA synthesis, recombination and transcription [54]. There are two topoisomerase II isoforms present in the cell – alpha and beta isoforms - and they are produced at different stages in the cell cycle [54].

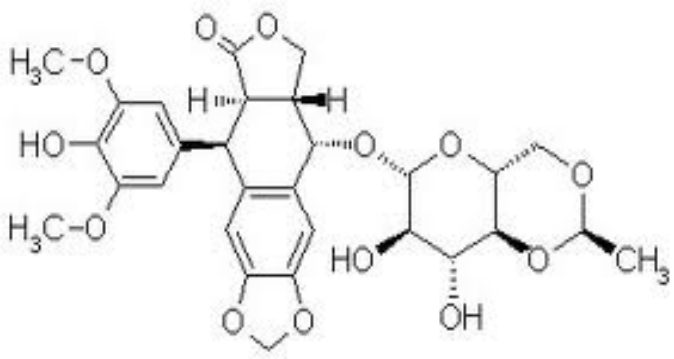


Figure 1.6: Structure of etoposide. The structure of etoposide; etoposide induces the formation of topoisomerase II toxins. These poisons lead to single and double-strand breakages within the genome of cells.

Etoposide, which acts on the alpha isoform, induces the formation of physiological topoisomerase II toxins that introduce "cleavage complexes" into the genome [53, 54]. When the cell attempts to overcome these blockades *via* MMR, the DNA fractures into permanent double strand breaks leading ultimately to the initiation of apoptosis [73, 78]. Etoposide-induced activation of the DNA damage response and protein inhibition makes this a highly efficacious anti-cancer agent.

1.3.3.5 Fas ligand (FasL)

Fas ligand (FasL or CD95L) is a member of the tumour necrosis factor (TNF) family of cytokines [28]. The TNF family is involved in major cellular activities such as immune surveillance, apoptosis, cell cycle and differentiation [28]. Like many members of this family, expression of FasL is under tight regulation [28]. FasL binds to the Fas receptor (Fas or CD95), a well-expressed transmembrane protein of 45 kDa [79]. Receptor-ligand binding leads to activation of the death receptor apoptotic pathway described earlier [79]. Fas signalling is essential for apoptosis, and any loss or mutation within this machinery can result in severe abnormalities and predispose individuals to certain infections and disease states [79, 80]. Furthermore, many chemotherapeutic drugs and radiotherapy depend upon Fas signalling to mediate their cytotoxic effects on neoplastic cells. The combination of these Fas-induced effects make FasL a potent agent in the battle against cancer [81-87].

1.3.3.6 Paclitaxel

Paclitaxel (or Taxol) is another naturally occurring compound that was purified in 1971 from the Pacific yew tree *Taxus brevifolia*, as part of a large-scale National Cancer Institute programme [56, 57]. Paclitaxel is a complex diterpene and belongs to the taxane drug class of molecules. The sidechains attached to the ester link of paclitaxel are essential for the cytotoxic activity of this compound [55, 57].

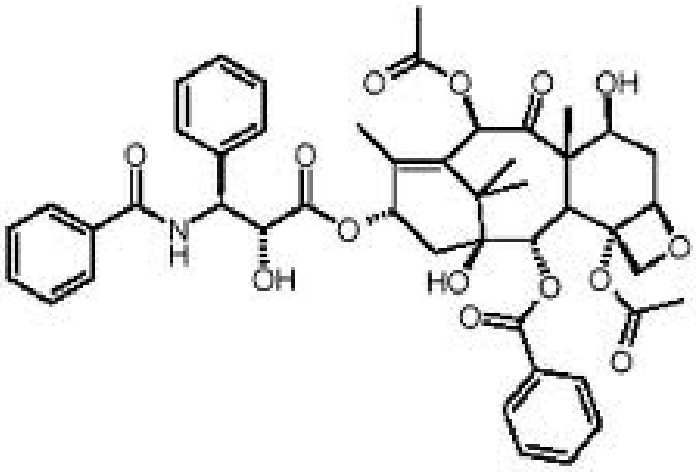


Figure 1.7 Structure of paclitaxel. The diterpene structure of paclitaxel displaying the ester sidechains; the ester sidechains are crucial for the toxicity of paclitaxel.

The most prominent mode of action for paclitaxel within the cell is to stabilise microtubules. Microtubules are critical components of the cytoskeleton and are essential for many cellular processes including cell shape, motility, division and intracellular transport [55, 88]. Unlike the vinca alkaloids and other anti-mitotic agents, paclitaxel binds to the polymeric form of α/β-tubulin rather than the soluble tubulin dimers [55, 88]. This shifts the equilibrium from soluble tubulin dimers to polymerisation; which in turn leads to microtubule stabilisation [55, 88]. This stabilisation of microtubules leads to mitotic arrest as the dynamic structural changes fail to occur. The induction of apoptosis is currently thought to occur from an arrested Gap1 (G1)-like state [55].

Like other microtubule-active drugs, paclitaxel also induces Bcl-2 hyper-phosphorylation. This leads to a loss in the Bcl-2 anti-apoptotic function, shifting the equilibrium in the cell in favour of cell death. The modulation of Bcl-2 proteins by anti-cancer drugs is nicely reviewed in the following references [44, 89]. In summary, by stabilising microtubules and hyper-phosphorylating Bcl-2, paclitaxel promotes the initiation of apoptosis within the cell, which makes it a highly effective chemotherapeutic agent.

1.3.3.7 Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)

TRAIL was first discovered in 1995 during a search for TNF family members [90]. The TNF super-family is substantially involved in a number of cellular processes ranging from growth and differentiation to apoptosis and the immune response [28].

TRAIL binds to five different receptors; DR4 and DR5 which mediate the apoptotic response, two decoy receptors DcR1 and DcR2 which do not possess the intracellular domains required for the initiation of apoptosis, and finally osteoprotegerin (OPG) which is involved in bone remodelling [91]. In terms of expression, TRAIL is ubiquitously expressed compared to other ligands of the TNF family. When TRAIL binds to DR4 and DR5, the external apoptotic cascade is activated [91]. Furthermore, TRAIL has been extensively researched as a potential anti-cancer compound due to its apparent selectivity for neoplastic cells [66, 67]. In contrast to normal cells, many cancer cells do not express decoy TRAIL receptors but only express DR4 and DR5 receptors [67]. Thus the selectivity of TRAIL makes it a potent apoptosis-inducer and anti-cancer agent.

1.3.3.8 Summary of chemotherapeutic agents

In summary, the chemotherapeutic agents chosen for this study act in a number of different ways to initiate cell death within neoplastic cells; some agents are conventional and radically affect normal tissues as well as cancerous tissues, while other agents are amongst the first of the targeted therapy-based compounds to be used in anti-cancer care. All the chemotherapeutic agents described herein are highly effective, but all of them also have weaknesses, primarily that they can be overcome by cancer cells leading to chemotherapeutic drug resistance.

1.4 Cancer and the involvement of signal transduction pathways

The main focus of this research is to elucidate the mechanisms by which impaired cell signalling affects the responses of cancer cells to chemotherapy. Previous studies have demonstrated a link between the failure of signalling and the development of a cancerous phenotype [2, 3]. Common signalling cascades implicated in the formation of cancerous phenotypes are those of the MAPK family and the NF-κB pathway [5, 92-100].

1.4.1 MAPK pathways

The MAPK family consists of three major evolutionarily conserved subtypes of protein kinases; the extracellular signal-regulated kinases (ERK 1 and ERK 2), the JNK (JNK1, JNK2 and JNK3) and the four p38 kinases (α, β, γ and δ) [101-104]. In general, the ERK subtype has major roles in transducing signals from the G-protein coupled receptors (GPCRs), cytokines and growth factors to modulate cell growth, differentiation and proliferation [102, 104]. In contrast, the JNK and p38 kinases are predominantly activated through stress or inflammatory stimuli, although not exclusively, and their downstream effects are seen in apoptotic, transcriptional and auto-immune responses [102, 104].

The general pattern of behaviour for these proline-directed, serine/threonine kinase family members is: upon stimulation by an external signal, a small "activator" protein (generally a guanosine nucleotide-binding protein (G-protein) or adaptor protein) is recruited. This leads to the activation of the first component of the cascade, the MAPK kinase kinase (MAPKKK) [101]. The MAPKKK then specifically phosphorylates a MAPK kinase (MAPKK), which in turn phosphorylates a specific MAP kinase (MAPK) with a specific threonine and tyrosine (T-X-Y) sequence [105, 106]. This phosphorylation occurs in a highly regulated manner [101].

MAPK Pathway	ERK	p38	JNK
Stimulus	Growth Factors	Growth Factors, Stress, Cytokines	Growth Factors, Stress, Cytokines
↓ Activator	RasGTP	TRAF6-TAB1/2	Rac1
↓ MAPKKK	A-Raf, B-Raf, C-Raf (Raf-1), Mos, Tpl2	MLK3, TAK1, MEKK4, ASK1	MEKK1/4, DLK, MLK1-4, LZK, ASK1, TAK1, ZAK
↓ MAPKK	MEK1/2	MKK3/6	MKK4/7
↓ MAPK	ERK 1/2	p38 α/β/γ/δ	JNK 1/2/3
↓ Transcription Factors	C-Myc, ELK-1, Ets, c-Fos,	CHOP, ATF2, MNK, MSK, MEF2	Jun, ATF2, RNPK, p53, NFAT 4, Shc

Figure 1.8: Diagrammatic representation of the MAPK framework. The three common MAPK pathways: the ERK, p38 and JNK pathways. Information presented in this figure was derived from the following references [101-104].

The individual motifs of the MAPKKKs allow them to recognise a range of stimuli. This means that the cell is able to activate the appropriate transduction pathway, based on the stimulus, and respond in a suitable manner [101]; thus introducing flexibility into this signal transduction system. Furthermore, the substrates of the MAPK component are proteins that are involved in the control of intracellular events from cell proliferation and differentiation to transcription and apoptosis to name a few [101-104, 107]. The description of the MAPK system has been simplified in this report. It is important to bear in mind that there is cross-talk between the components of the three families, as well as input from other, unrelated signal transduction cascades. All of these play a role in the maintenance of the cell/tissue and regulate the signal transfer with the environment. Moreover, the importance of these kinase families is strengthened by their roles in diseases such as cancer and auto-immune disorders. Members of all three subtypes have been

discovered to be dysregulated and/or atypically expressed in cancer and are often targeted in the treatment of this disease [108].

1.4.2 The ERK pathway

The ERK pathway is important in many cellular processes including cell growth and proliferation. In fact, this signalling nodule is used by many cytokines and growth factors for signal transduction to the intracellular environment [102, 107]. One example is ligand binding to receptor tyrosine kinase (RTK) molecules on the cell surface. RTKs are membrane spanning proteins that contain an external ligand binding domain at the N-terminus and an intracellular tyrosine kinase domain at the C-terminus [109]. RTKs generally exist as monomers (but can occasionally form inactive oligomers) that upon activation dimerise, leading to auto-phosphorylation of the tyrosine residues at the C-terminal and activation of the RTK receptor [110]. Phosphorylated tyrosine residues can bind proteins containing Src-homology 2 (SH2) or phospho-tyrosine binding (PTB) domains such as the adaptors growth factor receptor-bound protein 2 (Grb2) and Src homology 2 domain-containing-transforming protein C1 (Shc) [111-113]. Grb2 and Shc also contain domains that allow them to recruit and interact with various other proteins involved in the induction of the signal cascade. These include the guanine nucleotide exchange factor Son of Sevenless (Sos). Sos, *via* a guanosine diphosphate (GDP) to guanosine-5'-triphosphate (GTP) exchange, mediates the activation of the small GTPase Ras. This is the first and critical step required for the initiation of this pathway [114-117].

The next stage involves the recruitment and activation of Raf (rapidly accelerated fibrosarcoma) kinases by Ras proteins at the cell membrane. This leads to the classical three-tiered signaling module described earlier (Figure 1.8). There are three Raf kinase isoforms, A-Raf, B-Raf and C-Raf/Raf-1, all of which contain three conserved regions (CR1, CR2 and CR3). All three Raf isoforms are subject to independent regulation and have their own distinct functions [118]. Further, there are four subtypes of Ras proteins; H-ras (Harvey murine sarcoma virus), the alternatively spliced K-ras (Kirsten murine sarcoma virus) of which there are two isoforms A and B, and finally N-ras (human neuroblastoma). All the Ras isoforms display varying binding affinities for the different Raf protein isoforms [118].

In the Raf proteins, the first conserved region (CR1) includes a cysteine-rich domain (CRD) and a Ras-binding domain (RBD), both of which are crucial for the recruitment of this protein to the cell membrane [118]. The Raf proteins are thought to be retained in an inactive state in the cytosol by 14-3-3 dimers. This is supported by evidence that conserved 14-3-3 binding domains are found in the CR2 domain of Raf proteins [106, 114, 118, 119]. The third CR3 domain contains the kinase or catalytic region of the molecule as well as the activation segment [118].

The activation stage for all three Raf proteins is highly complex and involves numerous phosphorylation and de-phosphorylation events amongst other critical processes such as protein-protein interactions and protein-lipid interactions [114, 118, 120]. There is currently more information available for Raf-1 (C-Raf) thus in this report, we will focus on the activation sequences for this isoform.

In addition to binding 14-3-3 dimers, Raf-1 is phosphorylated at two sites, S259 and S621. Both of these modifications serve to keep Raf-1 in an inactive state within the cell [118, 120]. For this reason, one of the first stages in the activation of this molecule, after membrane recruitment, includes two de-phosphorylation events at S259 and S621. These de-phosphorylation events allows dissociation of Raf-1 from the 14-3-3 proteins [118, 120]. A further two phosphorylation sites, S43 and S233, are involved in Raf-1-Ras binding and Raf-1-14-3-3 association respectively. These must be modified to allow the liberation of the Raf-1 kinase [118, 120]. Activation of Raf-1 kinase activity is achieved through the phopsphorylation of S338 in the negative-charged regulatory region (N region). Other less established phosphorylation sites include Y341, T491 and S494 [118, 120].

A-Raf follows a similar activation process to Raf-1. In contrast, B-Raf contains an S445 residue, similar to the S338 present in Raf-1, which is constitutively active. For this reason, translocation of B-Raf to the membrane results in activation of this protein [118]. This ease of activation may explain why B-Raf displays a more potent kinase activity for MAPK/ERK kinase (MEK) compared to both A-Raf and Raf-1 [121].

Once Raf-1 is activated, it induces the phosphorylation of MEK kinases at S218 and S222. Interestingly, this appears to be the sole requirement for the activation of MEK kinase activity. This is a stark contrast to the complexities of the Raf kinases [122]. Furthermore, the subsequent activation of the ERK 1 and ERK 2 kinases by MEK kinases involves phosphorylation at residues T183 and Y185 [105, 123].

Upon activation, the ERK kinases are translocated to the nucleus and interact with a number of transcriptional targets.

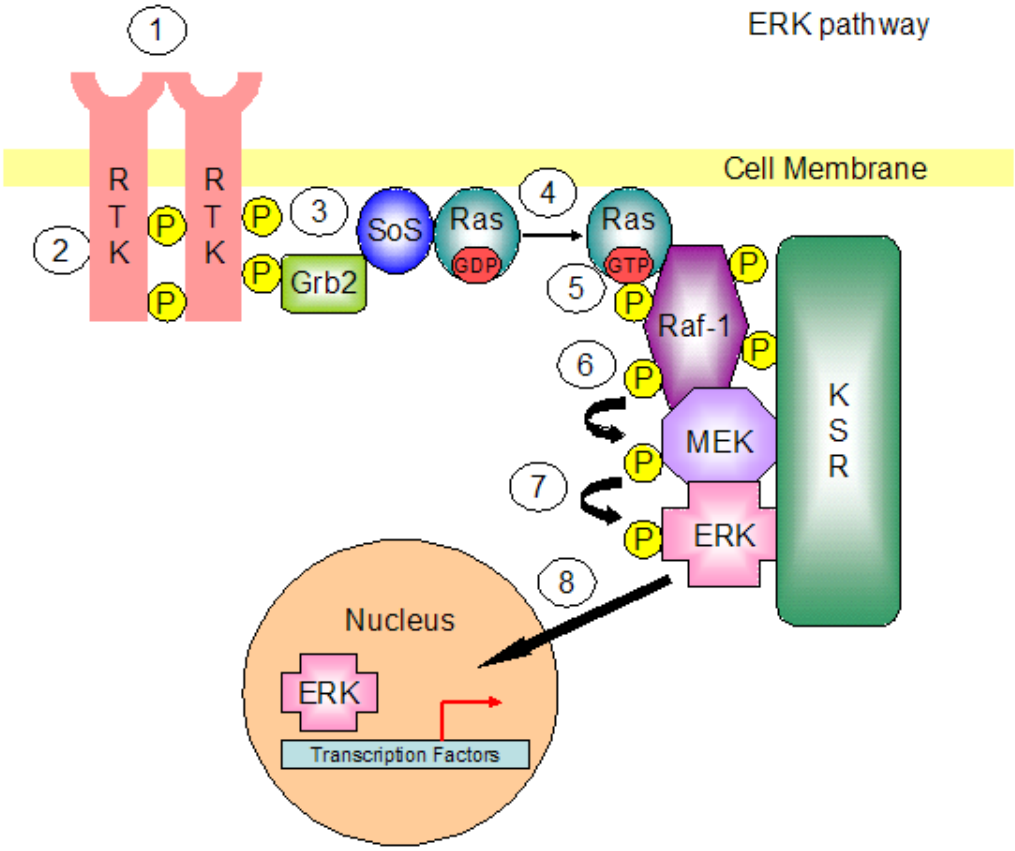


Figure 1.9: Schematic representation of the ERK pathway. 1. Ligand interaction with receptor tyrosine kinase. 2. Dimerisation of receptors and auto-phosphorylation of tyrosine residues. 3. Recruitment of adaptors such as Grb2 to the activated receptor. 4. Ras GDP-GTP exchange *via* the guanine nucleotide exchange factor SoS. 5. Recruitment and activation of the Raf-1 kinase by Ras. 6. Raf-1 phosphorylation and activation of MEK. 7. MEK phosphorylation and activation of ERK. 8. ERK translocation to the nucleus and subsequent interaction with transcriptional targets that affect growth, proliferation and differentiation.

Activation of ERK *via* the Ras-Raf-MEK framework is also thought to involve scaffold proteins such as Kinase Suppressor of Ras (KSR) amongst others. The functions of these scaffold proteins in MAPK signalling are extensively reviewed by Morrison *et al.* [124]

ERK has over 150 known substrates and therefore has an important role in the growth and proliferative processes within the cell, in maintaining cellular integrity and as a determinant of cellular fate [94, 100, 107]. Better understanding the modulation of the ERK pathway and dysregulation of this pathway in neoplasias is critical.

1.4.3 The Nuclear Factor-kappa B (NF-κB) pathway

NF-κB is a transcription factor that is involved in the regulation of genes that are associated with the immune response, apoptosis and cell proliferation. A plethora of stimuli induce the activation of this transcription factor [125]. NF-κB exists not as a single protein but as various hetero- and homodimers of the reticuloendotheliosis (Rel) protein family [97]. There are two classes of Rel proteins: the first class includes RELA (p65), RELB and c-Rel proteins; and the second class contains the large precursors NF-κB1 (p105) and NF-κB2 (p100) [97]. All Rel proteins contain an N-terminal Rel homology domain (RHD) that is required for nuclear localisation, transcriptional activity and the formation of dimers. The C-terminus is involved in the modulation of activity and contains unrelated transcriptional activation domains (TADs) [126, 127]. The precursors p105 and p100 have ankyrin repeat motifs (ANK) at the carboxyl terminus which are removed upon ubiquitin-dependent proteolysis, this results in the formation of the mature proteins p50 and p52 [126, 127].

The activity of these homo- and heterodimers are regulated by two main pathways, the canonical and non-canonical pathways.

1.4.3.1 Canonical pathway

Dimers containing RELA, c-Rel and p50 are regulated by the canonical pathway. Extracellular ligands that initiate the canonical signal cascade include pro-inflammatory cytokines (*e.g.* tumour necrosis factor alpha (TNF-α), interleukin 1 (IL-1)) and by-products of bacterial invasion [128].

The Rel subunits are retained in an inactive state in the cytoplasm by a family of inhibitory proteins known as the inhibitor of κB proteins (IκB proteins). There are three main subtypes of IκB, IκBα, IκBβ and IκBε (although there are 7 IκB family members) [129, 130]. The IκB proteins have an amino-terminal regulatory domain and ANK repeat motifs at their carboxyl terminus whose function is to conceal a conserved nuclear localisation sequence (NLS) that is present in the RHD of the NF-κB subunits [129, 130]. However, even in the absence of stimulation, NF-κB complexes may translocate to the nucleus. This is counteracted by nuclear export sequences (NES) on the IκB proteins which allow quick return of the NF-κB complexes back to the cytoplasm [130].

Relief of IκB inhibition on NF-κB subunits is achieved *via* phosphorylation of IκB at two conserved serines residues, S32 and S36. This results in the targeting of the IκB proteins for ubiquitin-proteasome degradation [129, 131]. Phosphorylation of IκB proteins is performed by the IκB kinase (IKK) complex; particularly the IKKβ subunit. This complex contains three core subunits, two catalytic subunits called IKKα and IKKβ, and a regulatory subunit called the NF-κB essential modifier (NEMO) [127, 129, 131-133]. Activation of the IKK complex is thought to involve recruitment of the IKK complex to the membrane by members of the TNF superfamily *via* adaptor proteins downstream of the receptor. Ligands that employ this cascade include TNF-α and IL-1. Upon ligand-receptor binding, a receptor complex is formed which includes various adaptor proteins from the tumour necrosis factor receptor associated factor (TRAF) family, tumor necrosis factor receptor type 1-associated death domain protein (TRADD) and receptor interacting protein (RIP) [129, 130, 133]. This receptor complex recruits the IKK complex leading to ubiquitination of K63 on the protein NEMO [134]. This attracts kinases like the transforming growth factor-β (TGFβ)-activated kinase-1 (TAK1), which phosphorylates the activation loop of the IKKβ subunit at S177 and S181 [134, 135]. TAK1 is recruited and activated through the ubiquitin binding proteins TAK1-binding proteins (TAB) 2 and 3 (TAB2 and TAB3) [136]. TAB2 and TAB3 contain C-terminal zinc finger domains that bind K63-linked polyubiquitin chains [136]. The IκB inhibition of NF-κB dimers is relieved by the phosphorylation of the IKK complex [129, 131] which then phosphorylates S32 and S36 on IκB bound to NF-κB dimers. The NK-κB dimers are then free to translocate to the nucleus where they can regulate the expression of a number of transcriptional targets.

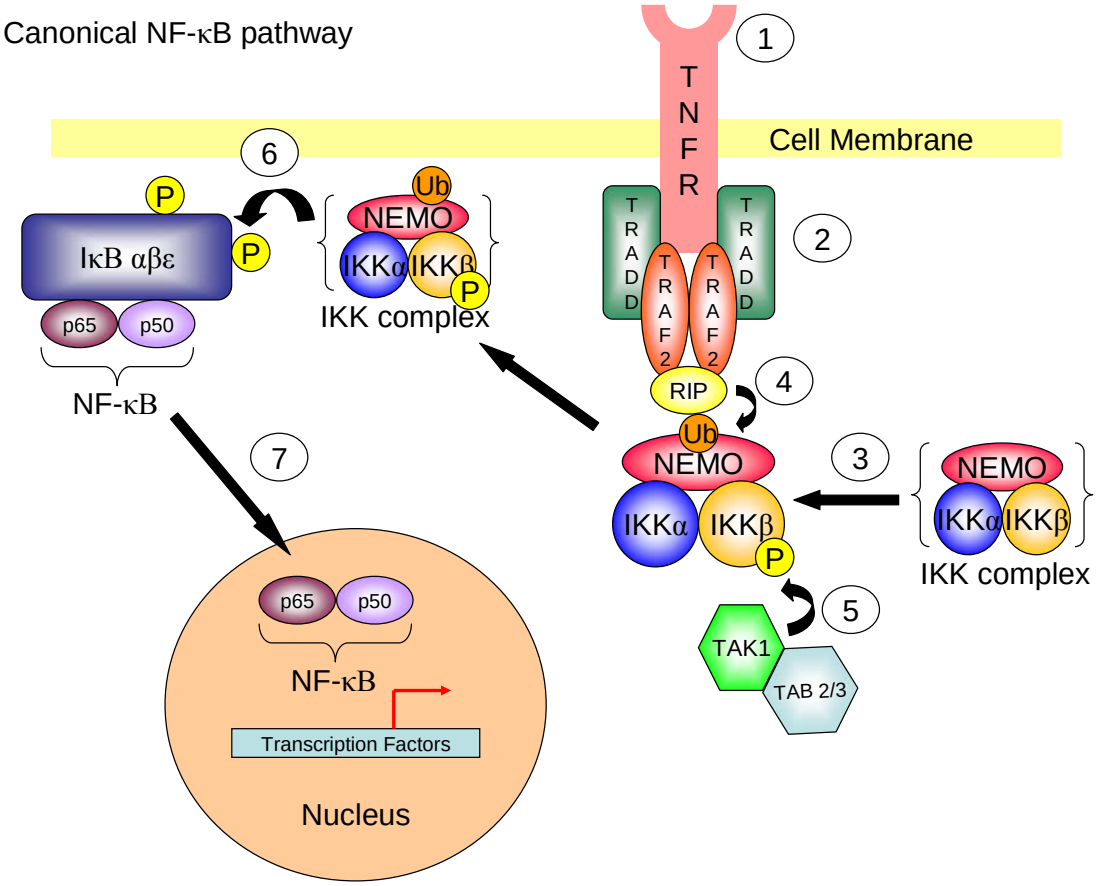


Figure 1.10: Schematic representation of the canonical NF-κB pathway. 1. Ligand interaction with TNF receptor. 2. Recruitment of adaptor proteins such as TRADD, TRAFs and RIP. 3. Mobilisation of the IKK complex to the activated receptor. 4. Ubiquitination of NEMO subunit of IKK complex. 5. Recruitment of TAK1/TAB2/3 and phosphorylation of IKKβ subunits by TAK1 and activation of IKK complex. 6. Phosphorylation of IκB subunits on NF-κB dimers, leading to liberation of NF-κB. 7. Translocation of NF-κB to nucleus to interact with transcriptional targets.

1.4.3.2 Non-canonical pathway

The non-canonical pathway is used by certain members of the TNF cytokine family and proteins of the Epstein-Barr virus. This pathway activates the NF-κB2/p100 subunit which preferentially dimerises with the RELB subunit [137, 138]. Ligand-receptor interaction recruits adaptor proteins such as TRAFs and the NF-κB inducing kinase (NIK), which in turn phosphorylates p100 on residues S866 and S870, and leads to ubiquitination of p100 [137-139]. Ubiquitin-dependent proteasome processing of p100 results in the liberation of a p52-RELB heterodimer which can translocate to the nucleus and regulate the expression of transcriptional targets [138].

NIK also phosphorylates the IKK complex. In this case, the IKK complex contains two IKKα subunits but no NEMO or IKKβ subunits. IKKα is crucial for the phosphorylation of the N- and C-terminal sites on p100 which are required for ubiquitination [137-139].

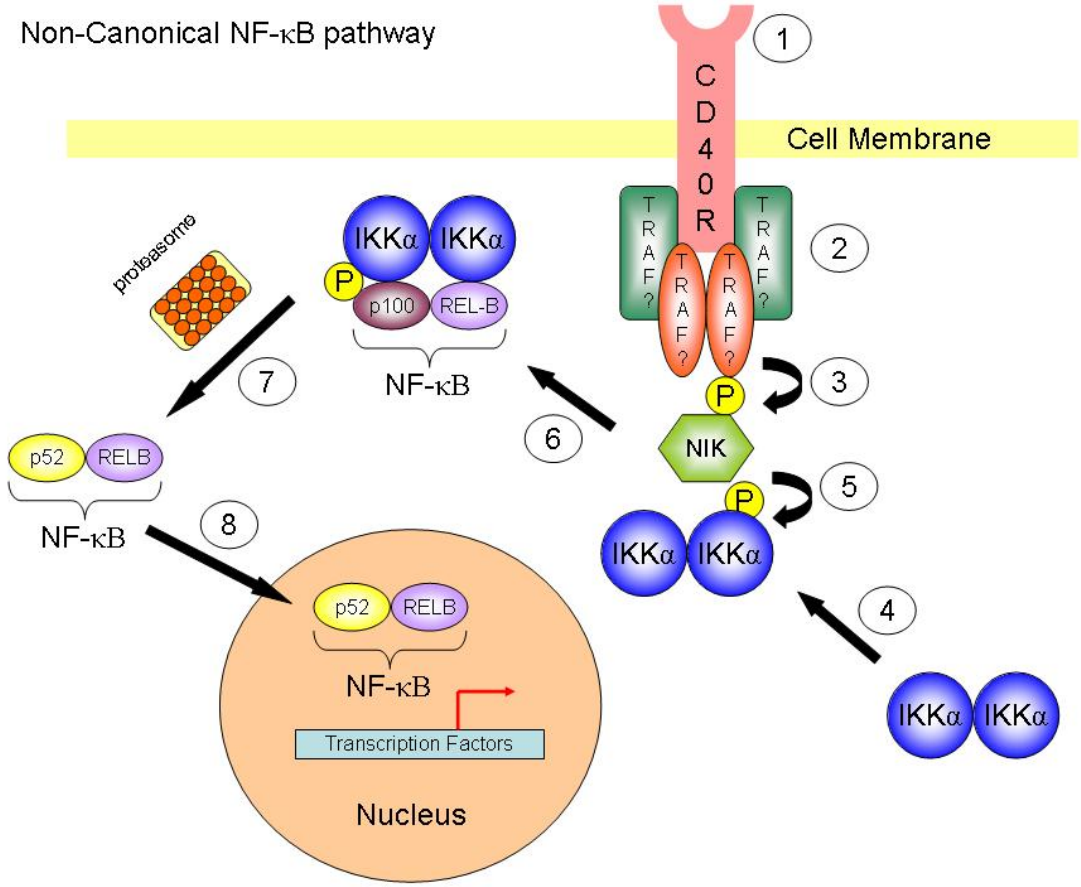


Figure 1.11: Schematic representation of the non-canonical NF-κB pathway. 1. Ligand interaction with specific TNF receptor. 2. Recruitment of adaptor proteins, possibly TRAFs. 3. Phosphorylation and activation of the kinase NIK. 4. Mobilisation of IKK complex containing two IKKα subunits to receptor complex. 5. Phosphorylation of IKK complex by NIK. 6. IKK complex interaction with unprocessed NF-κB dimers. 7. Processing of NF-κB dimers by proteasomal degradation resulting in the formation of the mature and activated heterodimer. 8. Translocation of NF-κB dimer to nucleus to interact with transcriptional targets.

1.4.3.3 NF-κB pathway summary

There are two distinct pathways that result in NF-κB activation, the canonical and non-canonical pathways. Signal transduction from the receptor to the activation of different

NF-κB homo- and heterodimers is thought to involve members of the TRAF family of adaptor proteins [23, 24, 26]. TRAF proteins signal downstream of the TNF superfamily and can regulate signalling to NF-κB [23, 24, 26].

NF-κB has over 150 potential transcriptional targets. Many of these are involved in the immune response, but they also include stress response proteins and apoptotic regulators [125]. Differential regulation of these target genes arise from the activation of different Rel dimers as a consequence of different stimuli, modes of receptor activation and/or the adaptor proteins involved in signalling [125].

Unravelling the regulation and activation of NF-κB pathways and the role of aberrant NF-κB in tumourigenesis is essential to understand the processes behind the formation of cancer, chemo-resistance and metastases formation.

1.5 Raf-1 kinase inhibitor protein (RKIP)

Raf-1 kinase inhibitor protein (RKIP) is an evolutionarily conserved member of the phosphatidlyethanolamine-binding protein (PEBP) family that was first discovered as an endogenous inhibitor of the Ras-Raf-MEK-ERK signalling cascade [140-144]. PEBP proteins are basic, cytosolic proteins with molecular weights that range between 21-23 kDa [145]. PEBP proteins possess a unique protein sequence, the PEB motif, that has no significant homology to any other protein family [140-142]. In addition, the PEBP family members display affinity for phosphatidylethanolamine (PE), a lipid that is found in biological membranes and from whom their name derives, and other molecules with hydrophobic regions including GTP and GDP, and G-proteins amongst others [140, 142, 146].

The physiological functions of the PEBP family include the inhibition of serine proteases [141] and the activation of G-proteins [140, 147]. Due to their high expression in differentiating cells, it is possible that these PEBP proteins may have a role in mammalian development and cell membrane formation [142].

The functions of RKIP have attracted significant attention due to its unique role in vital signalling cascades. As mentioned previously, RKIP was first discovered as an inhibitor of the ERK1/2 MAPK pathway [143, 144] but research has elucidated the roles that RKIP plays in the inhibition of the NF-κB pathway [148] and in the regulation of G-protein coupled receptor (GPCR) activation [147, 149, 150].

The crystal structure of human RKIP comprises of four α-helices and nine β-strands with unique folding into two almost identical chains, chain A has 180 amino acid residues and chain B has 185 amino acid residues [140, 145]. The bovine RKIP crystal structure shows that there are tryptophan residues arranged in a hydrophobic niche [151, 152], yet overall the RKIP molecule is hydrophilic [140]. However within the cell, RKIP exists in a monomeric form of 187 amino acids [140, 145]. RKIP has been found localised to the inner leaflet of the plasma membrane where it is ideally positioned to transfer and/or regulate signals from the extracellular environment into the cell [140, 152]. However, immunohistochemical studies have shown that the RKIP protein is also found in the

cytosol as well as in the nucleus [153, 154]. The ligand binding pocket of RKIP is believed to be crucial for its function as an inhibitor within signalling pathways.

1.5.1 Inhibition of the ERK pathway

The MAPK pathway is important in many cellular processes including cell growth and proliferation. This signal transduction system is utilised by many cytokines and growth factors for transferring signals into the intracellular environment [94, 102, 105, 109, 110, 114]. The ERK pathway has been described above but is summarised as follows - upon receptor-ligand binding, many adaptor and activating proteins are recruited to the cell membrane to mediate the activation of the small GTPase Ras [109-112, 114-117, 119, 155]. This is followed by the recruitment of Raf-1 to the membrane and its subsequent activation by Ras [118, 120]. The signal is then passed from Raf-1 to MEK and then to ERK1/2 [101-104, 114]. The final components ERK1 and ERK2 have over 150 substrates including nuclear transcription factors, structural proteins, receptors and other proteins involved in cell signalling [107].

RKIP binds both Raf-1 and MEK preventing their interaction and thereby interfering with the activation of MEK [143, 144]. RKIP binding to Raf-1 and MEK is mutually exclusive due to the overlapping binding domains for Raf-1 and MEK present on RKIP. Enzymatic analysis demonstrates that RKIP affects the Km, but not the Vmax, of the phosphorylation of MEK by Raf-1. This indicates a competitive inhibition of MEK activation [143]. It is likely that RKIP diminishes the binding affinity between Raf-1 and MEK by competing for the MEK binding sites [143]. The RKIP binding sites on Raf-1 include the phosphorylation sites S338 and Y341. For RKIP binding to Raf-1 and MEK, the ligand binding pocket of RKIP is thought to be crucial for the interaction [143, 156, 157]. With respect to the Raf proteins, RKIP is the only known endogenous inhibitor of the ERK pathway, and the functional implications of this repression are numerous and the consequences far-reaching [151, 158, 159].

1.5.2 Inhibition of the NF-κB pathway

The importance of RKIP is not limited to its inhibition of the ERK/MAPK pathway [143, 144]. This protein is also a negative regulator of the NF-κB pathway [148].
Extracellular ligands that initiate the NF-κB signal cascade include pro-inflammatory cytokines, like TNF-α and IL-1, and by-products of bacterial invasion [28]. NF-κB itself is a transcription factor involved in the regulation of genes associated with the immune response, apoptosis and cell proliferation [97, 127].
As described previously, NF-κB activation requires proteasomal degradation from inactive precursors or else it is retained in an inactive state in the cytoplasm by a family of inhibitor proteins called IκBs. Liberation and activation of NF-κB requires phosphorylation and degradation of IκB [126, 127, 129, 130] in the canonical pathway. In the non-canonical pathway, activation of NF-κB occurs *via* phosphorylation by an IKKα complex [129, 139, 160-162]. Upstream inducers of NF-κB include TAK1, NIK, MEK kinase 1 (MEKK1), protein kinase B (PKB) and protein kinase C (PKC) [126, 129, 130, 134, 135, 139, 160-162].

RKIP physically associates with the NIK and TAK1 kinases to inhibit activation of the NF-κB pathway [148]. Furthermore, RKIP also binds weakly to subunits of the IKK complex, including IKKα and IKKβ, and antagonises their activation upon TNF-α stimulation [148]. The role of the RKIP protein in the NF-κB pathway is independent of its role in the ERK cascade [148]. Finally, it is believed RKIP-dependent repression of the NF-κB pathway may be more complex than simple competitive inhibition. A recent article by the Yeung group discusses a scaffold-like response by RKIP with respect to NF-κB signalling [163]. Both up- and down-regulation of RKIP during IL1-β signalling leads to decreased phosphorylation of IκB. This biphasic response is usually indicative of scaffolding activity in a protein [163]. This scaffold-like effect of RKIP appears to involve TRAF6 and upstream kinases of the NF-κB pathway that are known to interact with RKIP [148, 163].

In summary, the mechanisms by which RKIP regulates NF-κB signalling and its downstream effects require further characterisation. However, what is certain is that the role of RKIP as an endogenous regulator requires consideration when analysing the NF-κB pathway.

1.5.3 Inhibition of G-protein coupled receptor kinase-2 (GRK2)

It was recently discovered that RKIP has a third role in signal transduction pathways within the cell. The internalisation of GPCRs plays an important role in regulating the cellular response to external stimuli [146]. RKIP prolongs activation of ERK *via* inhibition of the kinase GRK2 [147, 150]. This kinase is responsible for prompting the removal of GPCRs from the cell membrane and so preventing constant stimulation of the cell by external ligands [146]. RKIP binds to the N-terminus of GRK2 preventing the interaction between GRK2 and GPCRs [150]. This allows prolonged receptor activation and therefore increased activity of highly interacting kinases such as ERK [147]. Interestingly, the inhibition of GRK2 by RKIP is more potent than the inhibition of Raf-1 by RKIP [147]. This switch from RKIP–Raf-1 to RKIP–GRK2 is mediated by PKC-dependent phosphorylation of RKIP at S153 [150]. While this role has a significant effect in the cell, it is likely that the full involvement of RKIP has not yet been elucidated. There is however no doubt that RKIP is acting as a signal modifier between the cell survival and the growth pathways based on its interactions with Raf-1 and GRK2. This could have important implications in the formation and spread of cancerous tissues [150].

1.5.4 Other and unrelated functions of RKIP

Other studies have suggested roles of RKIP in the suppression of Alzheimer's disease (AD) and spermatogenesis. RKIP is thought to be a precursor for hippocampal neurostimulating peptide (HCNP) . HCNP maintains cholinergic signalling in the brain by maintaining the high levels of choline acetyltransferase in the hippocampus. Dysfunctional cholinergic signalling is a hallmark of AD. Further, RKIP expression is pervasive in the limbic areas of the brain such as the amygdala, olfactory nuclei and hypothalamic nuclei [164]. RKIP knock-out (KO) mice display learning difficulties and have problems with olfaction, which correlates to an increase in amyloid plaque formations in RKIP-deficient mice [164]. This evidence suggests that RKIP may have a role in cholinergic signalling and in the development of AD.

The role of RKIP in spermatogenesis has yet to be characterised. RKIP may be involved in membrane biogenesis and remodelling in spermatozoa. In mice and rats, RKIP-1 is ubiquitously expressed in seminiferous tubules, Leydig cells and testicular and epididymal

fluids [164]. In addition, RKIP knock-out mice do not display impaired spermatogenesis or fertility, but exhibit drastically decreased reproductive rates suggesting some abnormality in fertility [164].

1.5.5 RKIP and cancer

With respect to cancer, mutations leading to cancerous phenotypes in both the ERK and NF-κB pathways are well-documented [64, 94, 96-98, 100, 128]. In general, the biochemical effect of these abnormalities is an increase in the anti-apoptotic and proliferative response of cells, as well as a resistance of neoplastic tissues to cell death [94, 96, 98, 100, 165]. The role of RKIP as an endogenous inhibitor of both the ERK and NF-κB pathways [143, 144, 148] implies that it is likely to have an important role in the formation and/or progression of cancer.

Several studies have highlighted RKIP as a suppressor of metastases in colorectal, prostate, breast and hepatocellular carcinomas [153, 154, 159, 166-175]. The down-regulation of RKIP in these tumours correlates with a metastatic phenotype and a reduced overall survival rate. Ectopic expression of RKIP reduced the incidence of metastases and invasion in breast and prostate cells [170-172]. Furthermore, RKIP expression is negatively-correlated with the zinc finger-transcriptional repressor Snail. Snail is a key modulator of epithelial–mesenchymal transition (EMT) in normal and neoplastic tissues, and is strongly implicated in the formation of metastatic and invasive carcinomas [166-168].

In contrast to this metastatic suppressing role of RKIP, two studies have identified a functional role for RKIP in cell migration and in the regulation of cell adhesion [176-178]. The intracellular target of the cell migration inhibitor locostatin was found to be RKIP. The presence of locostatin disrupted/prevented RKIP binding to Raf-1 but the exact mechanism by which RKIP regulates cell migration is unknown [177, 178]. Moreover, over-expression of RKIP in Madin-Darby canine kidney (MDCK) epithelial cells increased adhesion of the cells to the substratum but decreased adhesion of the cells to each another [176]. The effect of RKIP on cell migration and its subsequent suppression by the migration inhibitor locostatin are controversial. A recent article highlighted the off-target effects of locostatin and suggested that the inhibition of cell migration observed in the presence of this

compound may be independent of RKIP [179]. In the same study, a precursor of locostatin was shown to bind directly to the ligand-binding pocket of RKIP, yet curiously, this binding had no detectable effect on RKIP-dependent inhibition of the MAPK pathway [179].

Another function of RKIP that suggests that it has an active role in the development of neoplastic tissues is the discovery that, in mitotic cells, phosphorylated RKIP (pRKIP) is localised to the centrosomes and kinetochores [180]. RKIP is phosphorylated on S153 and is thought to regulate mitotic progression [180]. Down-regulation of RKIP in HeLa cells led to a concomitant decrease in the activity of the "chromosomal passenger" protein Aurora B kinase. Aurora B kinase is essential for correct chromosomal alignment prior to cell division [180]. Other effects of RKIP depletion include the relaxation of the spindle checkpoint and resistance to mitotic arrest by spindle poisons such as paclitaxel; the combined effects of which can lead to chromosomal damage [180].

1.5.6 RKIP and chemotherapy

As well as evidence showing that RKIP has a role in metastatic suppression, other studies have unearthed a role for RKIP as an inducer of apoptosis and/or a sensitiser of cancer cells to chemotherapy. Down-regulation of RKIP in certain prostate and breast carcinomas conveyed chemotherapeutic resistance to treatment with 9-nitrocamptothecin (9-NC), cisplatin and etoposide. However, the reintroduction of RKIP into these cell lines resulted in apoptosis [181]. Furthermore, treatment of certain cell lines with agents such as rituximab, cisplatin and etoposide led to increased expression of RKIP, and subsequently the initiation of apoptosis/sensitisation of the cells to chemotherapy [181-183]. It is thought that these sensitizing effects of RKIP are a result of its inhibitory functions on the ERK and NF-κB pathways [181-183]. Upon treatment of cells with rituximab there was an increase in the association of RKIP to Raf-1 and an overall decrease in the activity of the ERK pathway and AP-1 transcription [182, 183]. Induction of RKIP upon rituximab therapy coincided with a reduction in the anti-apoptotic protein Bcl-xl, a downstream effector of the NF-κB pathway [182, 183]. The results of the rituximab study concurs with a recent study by Baritaki *et al.* [184] where cells displayed RKIP-mediated sensitivity to TRAIL treatment. Baritaki *et al.* suggested that RKIP inhibition of the NF-κB pathway led to the

modulation of the transcription factor YY1, the TRAIL receptor DR5 and anti-apoptotic molecules such as Bcl-xl and c-FLIP [184]. Moreover the proteasomal inhibitor NPI-0052 has also been shown to sensitise cells to TRAIL-induced apoptosis *via* Snail inhibition. In this report, a concomitant increase in the levels of RKIP was detected [185]. Finally, nitric oxide (NO) is a controversial molecule in the development of neoplasias, and there is a great deal of conflicting evidence about its involvement in apoptosis and cell proliferation [186-188]. However, with respect to RKIP, NO donors such as DETANONOate and SNAP have been shown to induce the expression of RKIP [189, 190]. RKIP-induction by DETANONOate also displayed a concomitant decrease in YY1 and NF-κB repression, resulting in sensitisation to treatment with FasL and TRAIL [189]. Additionally, the 3',5'-cyclic nucleotide phosphodiesterase (PDE) subtype 5 (PDE5) inhibitor PF-3717842 has been shown to bind RKIP. PDE5 selectively degrades 3',5'-cyclic guanosine monophosphate (cGMP) which is an important mediatior of NO signalling. The RKIP-PDE5 interaction is described and discussed in greater detail in Chapter 5.

1.6 Summary and Aims of PhD

In summary, there is much that has yet to be discovered concerning the roles of RKIP in normal tissues as well as in cancerous tissues. It is evident that RKIP has a central role in the regulation of a number of important signalling pathways. Moreover RKIP has been implicated in the suppression of cancerous phenotypes as well as in enhancing the sensitivity of these cells to chemotherapy.

With this evidence in mind, the aims of this PhD project are to characterise the role of RKIP in the cellular response to chemotherapy in a colon carcinoma cell line.
We hypothesise that the levels of RKIP are an important determinant of the response of cells to cytotoxic therapy as well as the acquisition of drug resistance.
Following on from this hypothesis, elucidation of the mechanisms of action behind the RKIP-dependent cellular changes in response to cytotoxic treatment would be imperative.

Understanding the role of RKIP in modulating drug response will not be limited to studies using chemotherapeutic drugs but will also include other potential RKIP interactors such as phosphodiesterase 5 (PDE5) inhibitors.

Elucidating the role of RKIP as an endogenous inhibitor of signalling pathways that are essential to cellular growth, proliferation and apoptosis will enhance our understanding of the mechanisms involved in tumourigenesis, chemo-resistance and metastasis.

Finally, characterising the involvement of RKIP, and/or the modulation of any of the steps involved in the formation of a cancerous or metastatic phenotype by RKIP, should provide new weapons for our armoury in the battle against cancer.

CHAPTER 2:
MATERIALS AND METHODS

2.1 Materials

2.1.1 General Reagents

All reagents were purchased from Sigma-Aldrich unless stated otherwise here.

BioRad:	Precision Plus dual colour standards
Biosource:	Alamar Blue Assay
Costar:	Tissue culture dishes
	6-well and 96-well plates
GE Healthcare:	Hybond paper
Gibco:	DMEM media
	RPMI-1640 media
	Tetracycline-free RPMI-1640 media
	L-glutamine
Invitrogen:	Foetal Bovine Serum
	Novex XCell SureLock Mini-Cell
	Novex XCell II Blot Module
	NuPage 4-12% Bis-Tris gels
	NuPage MOPS buffer
	NuPage transfer buffer
	Trypsin
PAA:	Tetracycline-system approved foetal bovine serum
Promega:	Caspase 3/7-Glo Assay
Promocell:	Cryo-Serum Free Media
Roche:	BM Chemiluminescence substrate POD
	Complete Protease Inhibitor cocktail tablet
	PhosSTOP Phosphatase Inhibitor cocktail tablet
ThermoFisher/Nunc:	Cryo Freezing containers
	Microplate 96 well optical flat bottom plate
	Tissue culture flasks
	West PICO Chemiluminescent substrate
Whatman:	Chromatography paper

2.1.2 Primary antibodies

Primary antibody	Type	Species	Dilution	Supplier
α - tubulin	Monoclonal	Mouse	1 : 2000	Santa Cruz Biotechnology
Bcl-xl	Monoclonal	Mouse	1 : 250	Santa Cruz Biotechnology
DR5	Polyclonal	Rabbit	1 : 500	Axxora
Phospho-ERK 1/2	Monoclonal	Mouse	1 : 5000	Sigma
ERK 1/2	Monoclonal	Rabbit	1 : 5000	Sigma
GST		Rabbit	1 : 1000	Invitrogen
Phospho-IκB α	Monoclonal	Mouse	1 : 1000	Cell Signaling
IκB α	Polyclonal	Rabbit	1 : 1000	Cell Signaling
Phospho-IKK α/β	Polyclonal	Rabbit	1 : 500	Santa Cruz Biotechnology
IKK α/β	Polyclonal	Rabbit	1 : 1000	Santa Cruz Biotechnology
NIK	Monoclonal	Mouse	1 :1000	Santa Cruz Biotechnology
RKIP	Polyclonal	Rabbit	1 : 1000	Upstate
TAK1	Monoclonal	Mouse	1 : 1000	Santa Cruz Biotechnology
TRAF2	Monoclonal	Mouse	1 : 1000	Santa Cruz Biotechnology
TRAF6	Monoclonal	Mouse	1 : 1000	Santa Cruz Biotechnology
YY1	Monoclonal	Mouse	1 : 1000	Santa Cruz Biotechnology

2.1.3 Chemotherapeutic agents

BIOMOL International:

Soluble TRAIL, Apo2L

MBL International:

Anti-FasL, CD95

Sigma-Aldrich:

5-fluorouracil

Cisplatin, *cis*-Platinum(II) diammine dichloride

Doxorubicin, Adriamycin® hydrochloride

Etoposide, 4′-Demethylepipodophyllotoxin 9-(4,6-O-ethylidene-β-D-glucopyranoside)

Paclitaxel

2.1.4 Pharmacological inhibitors

Merck Chemicals:

Locostatin, (4S)-3-[(E)-But-2-enoyl]-4-benzyl-2-oxazolidinone

Sequoia Research Products:

Sildenafil citrate, 1-[[3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo [4,3-d]pyrimidin-5-yl)-4-ethoxyphenyl]sulfony]-4-methylpiperazine

Vardenafil citrate, 4-[2-ethoxy-5-(4-ethylpiperazin-1-yl)sulfonyl-phenyl]-9-methyl-7-propyl- 3,5,6,8-tetrazabicyclo[4.3.0]nona-3,7,9-trien-2-one

2.1.5 Cell stimulants

Sigma-Aldrich:

EGF, Epidermal Growth Factor

TPA, Phorbol 12-myristate 13-acetate

2.1.6 Buffers

Lysis buffer:

50 mM TrisHCl, 15 mM EGTA, 100 mM NaCl, 0.1 % TRITON X-100, 1 mM DTT. (pH 7.5)

Prior to cell lysis, this buffer was supplemented with protease and phosphatase inhibitors from Roche.

Stripping buffer:

0.2 M Glycine**,** 1 % SDS**,** dH_2O and acidified to pH 2.5

NuPAGE Bis-Tris gels:

Bis-Tris-HCl buffer (pH 6.4), Acrylamide (4-12 %), Bis-acrylamide, Ammonium persulphate (APS), Ultrapure water

The separating gel operated at pH 7.0

NuPAGE LDS sample buffer:

106 mM Tris HCl, 141 mM Tris Base, 2 % LDS, 10 % glycerol, 0.51 mM EDTA, 0.22 mM SERVA Blue G250, 0.175 mM Phenol Red

(pH 8.5)

NuPAGE transfer buffer:

25 mM Bicine, 25 mM Bis-Tris (free base), 1 mM EDTA

(pH 7.2)

NuPAGE MOPS running buffer:

50 mM MOPS, 50 mM Tris base, 0.1 % SDS, 1 mM EDTA

(pH 7.7)

Antibody and blocking buffer:

5 % bovine serum albumin (BSA), 0.02 % Na azide

Washing buffer/TBST:

25 mM Tris base, 125 mM NaCl, 0.1 % Tween-20

(pH 7.6)

2.2 Cell Culture

Cell culture is a widely used technique where cells are grown under controlled conditions on a 2-dimensional surface. Cell culture permits high throughput analysis of multiple experimental conditions and the ability to transfect and manipulate a variety of cellular proteins. This allows researchers to more rapidly evaluate the cellular response of cells to external stimuli and the factors that may modulate this response. Furthermore, cell culture allows the production of consistent and reliable results which can then be used as a platform for the planning of future experiments. However within the body, cells live in a complex 3-dimensional environment therefore cell culture cannot always predict how cells

will respond in the real world and in real patients. Take drug studies as an example, when applied to cells in culture drugs do not face the complications of drug absorption, metabolism, distribution and excretion. Furthermore cells in culture may change their characteristics over time therefore stringent culture practice must be maintained to ensure the reliability of results. However despite these limitations, cell culture is a valuable first-stage test for the development of new drugs, and also for the understanding of cellular processes and how they can be manipulated in the treatment of disease. Establishment of responses within cell culture will also optimise conditions for more labour intensive processes such as animal models and other *in vivo* techniques.

2.2.1 Ls174T cells

The Ls174T epithelial human cell line is derived from a Dukes' stage B colorectal adenocarcinoma, and is positive for expression of c-myc, N-myc, H-ras, N-ras, Myb, and fos oncogenes [191] [192]. This cell line has a doubling time of 24 hours and the ability to form tumours when injected into mice, indicative of a metastatic potential in subpopulations of these cells [191]. This cell line has also been shown to display microsatellite instability, but this is discussed in more detail in later chapters. The Ls174T cells were cultured in tetracycline-free Roswell Park Memorial Institute (RPMI)-1640 media supplemented with 5 % foetal bovine serum, 2 mM L-glutamine and 400 ng/ml doxycycline. Cells were grown at 37 °C and 5 % CO_2. At 70-80 % confluency, the cells were trypsinised and passaged at a ratio of 1:3.
The cell line used for testing the effect of RKIP levels on the response of cancerous cells to chemotherapy was the Ls174T colon carcinoma cell line. Further, stable clones of this cell line were created by Kam Yeung and Oliver Rath and kindly gifted, which allowed the RKIP levels within this cell line to be manipulated and controlled using the tetracycline-ON (Tet-ON) gene expression systems from Invitrogen [193].

The low RKIP clones in the presence of tetracycline, or more commonly its derivative doxycycline, produced short hairpin RNAs (shRNAs) against endogenous RKIP within the cell leading to RKIP down-regulation.

When induced with either tetracycline or doxycycline, the high RKIP clones produced artificially inserted Flag-tagged RKIP leading to a large increase in the level of RKIP within the cell. The procedures for stable clone insertion into the Ls174T colon carcinoma

cell line can be found in detail in the following references [193-195], and more information can be found in the Appendix.

A wild type (WT) RKIP cell line containing an empty vector acted as a control cell line. In these cells, endogenous levels of RKIP were expressed.

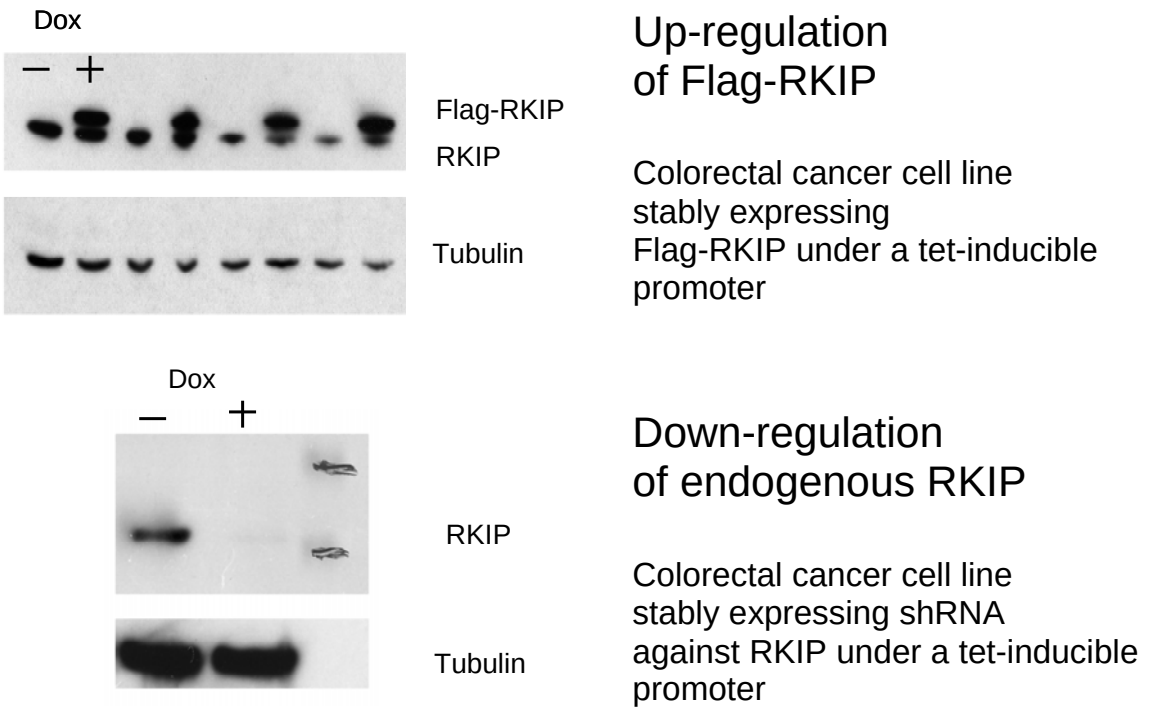


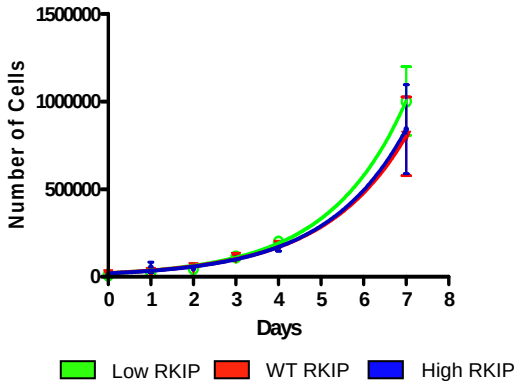
Figure 2.1: Controlled RKIP expression in Ls174T colon carcinoma cells. Western blots of low, WT and high RKIP-expressing colon carcinoma cells. From top to bottom; the stable induction of Flag-RKIP in four clones in the presence of doxycycline, alongside non-induced endogenous RKIP; tubulin controls for each of the four clones; stable down-regulation of endogenous RKIP by doxycycline-induced siRNAs; tubulin control for the downregulation of endogenous RKIP. All induced in the Ls174T colon carcinoma cell line. The induced cell lines western blots are courtesy of Oliver Rath.

Upon stimulation of high RKIP cells, up-regulation of Flag-RKIP in the presence of doxycycline was achieved in four sets of clones within the Ls174T colorectal carcinoma cell line. This stable up-regulation did not affect expression of endogenous RKIP or tubulin expression, indicating specificity for Flag-RKIP.

Stable down-regulation of endogenous RKIP levels in the Ls174T colorectal carcinoma cell line in the presence of doxycycline was observed when low RKIP cells were treated with doxycycline. All cell lines were maintained in doxycycline at all times.

Standard growth curves were generated for each of these cell lines to ensure that any differences that were observed during treatment with pharmaceutical agents were not due to variations in proliferation as a result of different levels of RKIP in the cells.

a)



b)

Statistical Analysis	Source of variation	p-value
Two-way ANOVA	RKIP levels	0.8327
	days	<0.0001
Wilcoxon signed-rank test	Low VS WT RKIP	N/A
	Low VS High RKIP	N/A
	WT VS High RKIP	N/A

Figure 2.2: The effect of RKIP levels on cell growth. a) Cell growth curve (n=4) plotted over a period of 7 days in a 6-well plate for each of low, WT and high RKIP expressing cell lines. Cell numbers were calculated using a Casyton cell counter and the original starting cell number for each cell line was 10000 cells per well. **b)** Table of statistics showing the effect of RKIP levels on cell growth.

Changing the RKIP levels within the Ls174T colorectal carcinoma cell line did not affect the growth of the Ls174T colon carcinoma cells. Two-way analysis of variance (ANOVA) further confirmed this biological observation (Figure 2.1). There was a slight increase in growth for the down-regulated RKIP cell line compared with WT and high-RKIP after 5 days, but this was not statistically significant.

2.2.2 HeLa cells

The HeLa epithelial human cell lines (ATCC) derived from an adenocarcinoma of the cervix, were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % foetal bovine serum and 2 mM L-glutamine. Cells were grown at 37 °C and 5 % CO_2. At 70-80 % confluency, the cells were trypsinised and passaged at a ratio of 1:3.

2.2.3 HCT 116 cells

The HCT 116 epithelial human cell lines (ATCC) derived from a colorectal carcinoma, were cultured in DMEM supplemented with 10 % foetal bovine serum and 2 mM L-glutamine. Cells were grown at 37 °C and 5 % CO_2. At 70-80 % confluency, the cells were trypsinised and passaged at a ratio of 1:3.

2.2.4 MCF7 cells

The MCF7 epithelial human cell lines (ATCC) derived from a breast adenocarcinoma, were cultured in DMEM supplemented with 10 % foetal bovine serum and 2 mM L-glutamine. Cells were grown at 37 °C and 5 % CO_2. At 70-80 % confluency, the cells were trypsinised and passaged at a ratio of 1:3.

2.3 Cell Counting

2.3.1 Casyton Counter

Cells were diluted to a total volume of 20 ml with appropriate media. 400 µl of this cell solution was transferred to a Casyton cup and diluted in phosphorylated buffer solution (PBS). The counter was pre-cleaned with PBS to ensure the number of counts were less than 15. Finally, samples were measured between 2-3 times to obtain an average number of cells per ml.

2.3.2 Haemocytometer

50 µl of a cell suspension was diluted with 50 µl Trypan Blue solution and 100 µl PBS. The solution was pipetted onto a haemocytometer. The numbers of cells were counted excluding blue cells (dead cells) and an average number of cells obtained using the following calculation;

$$\text{Average} \times 4 \times 10 = \text{number of cells } X10^3 \text{ /ml.}$$

2.4 Western Blotting

2.4.1 Cell preparation and treatment with chemotherapeutic agents for western blotting

For Costar 6-well plates, 250,000 cells per well was desired, thus a master mix containing 1.5 X 10^6 cells in 15 ml media was made and divided into X6 2.5 ml per well. For Costar 10 cm dishes, 1 X 10^6 cells per dish was desired, thus a master mix containing 6 X 10^6 cells in 48 ml was made and 8 ml cell solution transferred into each 10 cm dish.
Cells were then grown overnight at 37 ºC/ 5 % CO_2 before treatment.
Cytotoxic agents and their dilutions were prepared in Eppendorf tubes and added directly to the cell medium at required concentrations and incubated at 37 ºC/ 5 % CO_2 for the appropriate length of time.

2.4.2 Cell preparation and treatment with locostatin and PDE5 inhibitors for western blotting

Cells were plated as described in Section 2.4.1. Cells were grown overnight at 37 ºC/ 5% CO_2 and were ready for treatment (in case of growing cells) with sildenafil citrate *etc* or for media replacement with RPMI-1640 containing 0.1 % FBS (in case of starved cells) for a further 24 hours.
Locostatin, sildenafil citrate and vardenafil citrate were added directly to the cells from stock solutions to give the required concentrations. The previous agents were added 30 minutes prior to stimulation with EGF or TPA (30 ng/ml and 100 ng/ml respectively according to the procedures described in [194]). After treatment the cells were then incubated at 37 ºC/ 5 % CO_2 for a further 60 minutes to give a total incubation time of 90 minutes.

2.4.3 Preparation of cell lysates for western blotting

After the appropriate incubation, the cell medium was discarded and the cells were washed with ice-cold PBS. Lysis buffer containing protease and phosphatase inhibitors was added to the cells at a volume dependent on the size of the plate *e.g.* 250 µl per well in a 6-well plate. Cells were scraped using a cell lifter/scraper and transferred to Eppendorf tubes on ice. The Eppendorf tubes were centrifuged at 13200 rpm at 4 °C for 15 minutes, the supernatant transferred to a fresh Eppendorf tube and the pellet discarded. NuPage LDS Sample Buffer was added to the supernatant at a ratio of 1:4, and the solution heated for 7 minutes at 60 °C. The sample was centrifuged at 13200 rpm at 4 °C for 2 minutes, and stored at -20 °C until analysis.

2.4.4 Polyacrylamide Gel Electrophoresis (PAGE)

Proteins of interest were separated based on their molecular weight on Invitrogen NuPAGE Novex 4-12 % Bis-Tris gels in a Novex XCell SureLock Mini-Cell Module. This system offered advantages such as a neutral pH environment leading to increased protein stability and better resolution. The combination of MOPS running buffer further allowed separation of a wide range of protein molecular weights. The Precision Plus Dual Colour Protein standard from BioRad was used to estimate molecular weights of the proteins of interest. Gels were then electrophoresed for 30 minutes at 50 V, and then 150 V for 1 hour.

2.4.5 Transfer of proteins to nitrocellulose

After protein separation by PAGE as described above, the proteins were transferred to a nitrocellulose membrane (Hybond blotting paper from GE Healthcare**)** by electro-blotting. Electro-blotting was performed using the Novex XCell II Blot Module using NuPage Transfer buffer containing 10 % methanol. The nitrocellulose membrane was soaked in 100 % methanol for 1 minute prior to placement over the gel. Electro-blotting was performed for 2 hours at 30 V.

2.4.6 Blocking

The nitrocellulose membranes were blocked in either 5 % bovine serum albumin (BSA) + 0.02 % Na azide (dH_2O) or 5 % Marvel milk powder (dH_2O) for at least 1 hour with gentle rotation or shaking.

2.4.7 Antibody detection

After blocking, the membranes were washed for 5 minutes in 10 ml TBS containing tween 20 (TBST). Next, the primary antibody was added according to the manufacturer's guidelines and at the concentrations stated in section 2.1.2. The membranes were washed for 5 minutes in 10 ml TBST before the secondary antibody incubation. The secondary antibody employed was an appropriate horseradish peroxidase conjugate as described above and the membranes were incubated for 1 hour at room temperature. After secondary antibody incubation the membranes were washed for 5 minutes in 10 ml TBST and placed in West PICO Chemi-luminescent substrate for 5 minutes.
The membranes were then transferred to transparent sheets and placed in a cassette to be detected on photographic film, or analysed using the G-Box chemi-luminescence detection system.

2.5 96-well plate assays

2.5.1 Assay preparation for 96-well plates

For each 96-well assay, the same procedure was employed. Once cells had been counted and a value obtained for the number of cells per ml as described above, the volume required to make a 222,222 cells in 10 ml was calculated. The appropriate volume of cell solution was diluted to 10 ml total volume with appropriate media. Then 90 µl of cellular solution was added to each well of the 96-well plate, giving a total of 2000 cells per well. Cells were then incubated at 37 ºC/ 5% CO_2. Cells were plated in a standard Costar 96-well tissue culture plate for alamarBlue assays and Nunc Micro-plate 96-well optical flat bottom plates for Caspase-Glo 3/7 assays [196, 197].

2.5.2 Chemotherapeutic agent treatment for 96-well plate assays

Once the cells were plated and grown overnight, they were treated as follows. For treatment with chemotherapeutic agents in the 96-well assay, 9 different concentrations with a 1/3 dilution were added in triplicate.

Dilutions of stocks were made in a separate 96-well plate (dilution plate) and 10 µl added into the well containing the cells. The starting concentration of each agent was generally 100 µM, except paclitaxel 50 µM, TRAIL 100 nM and FasL 100 pM. Subsequent 1/3 dilutions were performed – 70 µl of previous concentration was diluted in in 140 µl medium. As mentioned previously, 10 µl of agent was added to each well containing the cells.

In the cell-containing plate there were always three wells for each concentration of drug treatment, and three control wells (no chemotherapeutic agent) thus there was 2 and ½ rows per chemotherapeutic on the 96-well plate. The last 6 wells on the plate were left free of cells and media, for use as the assay blank wells.

The cells were then incubated in the chemotherapeutics for the relevant incubation time at 37 ºC/ 5 % CO_2.

2.5.3 Chemotherapeutic agent time-courses

In order to optimise incubation times, several time-courses were performed. Cells were plated on Costar (for alamarBlue assay) or Nunc (for Caspase-Glo 3/7 assay) 96-well plates as described above, and treated with high concentrations of cytotoxics over a 72 hour time period and incubated at 37 ºC/ 5 % CO_2.

2.5.4 alamarBlue Assay

The alamarBlue assay from BioSource was used to measure cell proliferation [196]; alamar blue becomes reduced in the presence of cytochromes, nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide (reduced form) (NAD/NADH) *etc* that are associated with an observable and measurable colour change [196]. Proliferating cells have a more reduced environment than non-proliferating cells.

10% alamarBlue was added to the total well volume therefore in the case of cytotoxic assays 10 µl alamarBlue was added to 100 µl well. Some of the wells from the 96-well plate were filled with 100 µl media only, three of these were treated with alamarBlue, serving as the medium blank reading. The alamarBlue treated cells were incubated at 37 ºC/ 5% CO_2 for 2 ½ hours for Ls174T and HCT 116 colon carcinoma cells. HeLa and MCF7 cells were incubated for 4-5 hours.

The colour change in each well was measured at 570 nm and 600 nm using a spectrophotometer. In monitoring alamarBlueTM reduction spectrophotometrically, reduction is expressed as a percentage (% Reduced).

The calculation of % Reduced is as follows when the samples are read at

$\lambda 1 = 570$ nm

$\lambda 2 = 600$ nm

$$\% \text{ Reduced} = \frac{(\varepsilon ox\ \lambda 2)(A\ \lambda 1) - (\varepsilon ox\ \lambda 1)(A\ \lambda 2)}{(\varepsilon red\ \lambda 1)(A'\ \lambda 2) - (\varepsilon red\ \lambda 2)(A'\ \lambda 1)} \times 100$$

Where:

(εred λ1) = 155,677 (Molar extinction coefficient of reduced alamarBlueTM at 570 nm)

(εred λ2) = 14,652 (Molar extinction coefficient of reduced alamarBlueTM at 600 nm)

(εox λ1) = 80,586 (Molar extinction coefficient of oxidized alamarBlueTM at 570 nm)

(εox λ2) = 117,216 (Molar extinction coefficient of oxidized alamarBlueTM at 600 nm)

(A λ1) = Absorbance of test wells at 570 nm

(A λ2) = Absorbance of test wells at 600 nm

(A'λ1) = Absorbance of negative control wells which contain medium plus alamarBlueTM but to which no cells have been added at 570 nm.

(A'λ2) = Absorbance of negative control wells which contain medium plus alamarBlueTM but to which no cells have been added at 600 nm.

2.5.5 Caspase-Glo 3/7 Assay

The Caspase-Glo 3/7 assay from Promega was employed to measure cell death [197]. Both extrinsic and intrinsic apoptotic pathways converge on the activation of caspase 3 hence the selection of this particular caspase assay. The assay involves a luminogenic substrate containing the DEVD sequence recognized and cleaved by caspases, which upon degradation by said caspases forms amino-luciferin resulting in the luciferase reaction and the production of light [197]. The luminescent intensity is proportional to the caspase activity present in the cells.

50% caspase-Glo reagent was added to the total well volume therefore 50 µl caspase-Glo reagent was added to 100 µl well. Some of the wells of the 96-well plate were filled with medium only; three of these were treated with caspase-Glo 3/7 reagent serving as the negative control reading. The cells were incubated at 37 ºC/ 5% CO_2 for 2 hours for Ls174T colon carcinoma cells. The luminescence of the samples was measured using a luminometer.

The luminescent signal is directly proportional to the caspase activity of the cells.
Luminescent values are calculated by:

Luminescence = (luminescence of cell sample wells) – (luminescence of negative control wells which contain medium plus caspase 3/7-Glo reagent but to which no cells have been added)

2.6 Fluorescence activated cell sorting (FACS)

2.6.1 FACS preparation

A total of 2 x10^6 cells were diluted in 30 ml of desired medium. This master mix of cell solution was then divided between 10 Costar 6 cm cell culture dishes. The cells were then grown overnight at 37 ºC/ 5 % CO_2 prior to treatment. At treatment cells were approximately 70-80% confluent.

2.6.2 FACS time-course

After the overnight incubation, doxorubicin and 5-FU at various concentrations were added to cells over a 48 hour time period. Two plates were left untreated to serve as controls.

2.6.3 Cell Fixing

After treatment, the medium was removed from the cells and the cells removed from the plate surface using trypsin solution. Fresh medium was then added and the cells were harvested by centrifugation at 1000 rpm for 3-5 minutes. The supernatant was removed and the pellet washed in 4-5 ml PBS. The solution was then centrifuged at 1000 rpm for 3-5 minutes and the pellet re-suspended in 200 µl PBS. 2 ml of fridge-cold 70 % ethanol was then added with vortexing.

2.6.4 Propidium Iodide staining

After cell fixing, the cell-ethanol solution was centrifuged at 1000 rpm for 5 minutes. The ethanol supernatant was removed and the pellet re-suspended in 300 µl of master mix. Master Mix for 30 samples consisted of 9 ml PBS, 18 µl RNAseA (250 µg/ml) and 18 µl Propidium iodide (PI)(10 µg/ml).
The cell-master mix solutions were incubated at room temperature for 30 minutes.

2.7 Data and statistical analyses

All data analysis for alamarBlue and caspase-Glo 3/7 assays was performed using Microsoft Office Excel. Graphs of this data were created using GraphPad Prism 4 software.

Western blot images were created using Adobe Photoshop Elements 4.0 and Microsoft Office Powerpoint. The levels of protein on the western images were then quantified according to the integrated density of the image, using ImageJ analysis. The results from ImageJ analyses shown in this study were created using GraphPad Prism 4 software.

All statistical analyses were performed using GraphPad Prism 4 or by Dr Ronan Daly from the University of Glasgow, Department of Computer Science Inference Group. Two-way analysis of variance (ANOVA) was employed as a suitable test in all experiments with an n value greater than 1. Both RKIP levels and drug concentration were variables in all experiments hence the selection of two-way ANOVA. All drug concentrations were compared between the three cell lines and a mean plus standard error of the mean calculated for each drug concentration. Differences in the mean for both the different cell lines and between the drug concentrations were calculated. A p-value less than 0.05 was considered statistically significant. More details of the analysis can be found in the following reference [198]. If the p-value for the RKIP levels from the two-way ANOVA analysis was less than 0.05, a Wilcoxon signed-rank test was performed that compared the three RKIP-expressing cell lines. This was performed using the statistical programme R by Dr Ronan Daly. Again, for the post tests a p-value less than 0.05 was considered statistically significant.

CHAPTER 3:
RKIP MODULATION OF CHEMOTHERAPEUTIC DRUG RESPONSE

3.1 The effect of RKIP on cell proliferation after treatment with chemotherapeutic agents

Uncontrolled cell proliferation is a hallmark of cancer. Many neoplastic cells display mutations in major signalling pathways such as the ERK and NF-κB pathways, and relaxation of cell cycle checkpoints; all of which allow limitless growth and proliferation to occur [3].

RKIP is an important endogenous regulator of three major processes involved in cell growth and proliferation; the ERK and NF-κB signal transduction pathways, and the GRK2-mediated down-regulation of GPCRs [143, 144, 148, 150].
Following treatment with conventional chemotherapeutic agents such as DNA chelators and enzyme inhibitors, which can be cytotoxic, an initial cessation of proliferation is generally observed as the cellular mechanisms attempt to repair the damage inflicted by these agents [43, 199-201]. While RKIP has been shown to modulate the sensitivity of cells to chemotherapy-induced apoptosis as discussed in the introductory chapters [181, 183, 184, 189]; no interrogation has been made on the effect of RKIP on cell proliferation after the administration of chemotherapeutic agents. It is important to determine the effect of endogenous cell regulators such as RKIP on the growth and proliferative pathways, as they may provide a means of cellular manipulation, or act as a future target for cancer treatment and chemotherapy regimes.

In this chapter, studies that focus on the effect of different levels of RKIP on cell proliferation after cytotoxic therapy in the Ls174T colon carcinoma cell line will be described. Although the effects will be observed over a period of days, whereas cancer treatments can span several weeks or months, the identification of potentially resistant cells at an early stage may help us understand how cancer develops and mutates throughout treatment [58, 59, 202, 203].

Chemotherapeutic agents have varying modes of action. In this study, chemotherapeutic agents with diverse characteristics were selected to encompass a range of drug mechanistic action. The drugs selected included TRAIL, 5-FU, doxorubicin and etoposide. As RKIP may affect all or a specific subtype of cytotoxic therapy; these studies will provide

valuable insights into the workings of this cryptic protein and how it can influence chemo-sensitivity.

TRAIL is a death receptor agonist that is subject to modulation by RKIP [184]. Baritaki *et al.* demonstrated that cells expressing high levels of RKIP displayed increased sensitivity to TRAIL-mediated apoptosis in prostate and melanoma cell lines [184], thus it would be interesting to observe whether the differences in the levels of RKIP exerted any effect on cell proliferation in a colon carcinoma cell line.

The anti-metabolite 5-FU was selected based upon its routine use in colorectal carcinomas [204]. As described in greater detail in the Introduction, 5-FU inhibits the enzyme thymidylate synthase and incorporates into the growing DNA strand. These two actions result in impaired DNA replication and repair resulting in profound cellular damage [70]. As the cell attempts to overcome treatment with 5-FU by initiating DNA damage and repair responses, halting of growth and proliferation should be observed before the cell succumbs to apoptosis [43, 199-201, 205]. As a modulator of proliferative pathways, RKIP may alter these expected patterns of drug treatment on cancer cells.

The choice of doxorubicin was due to its multifarious mechanistic action and its use as a chemotherapeutic agent in a wide variety of tumour types [76]. Doxorubicin is thought to induce cell death by inhibiting enzyme activity, generating free radicals and by intercalating with DNA [76, 77]. One or a combination of these effects will result in substantial cell stress, hence doxorubicin shows great efficacy as an anti-tumour agent. Doxorubicin-induced cell stress should result in subsequent effects on growth and proliferative pathways; which in turn may be affected by altered levels of the RKIP protein.

Etoposide is an inhibitor of the enzyme topoisomerase II [54]. Topoisomerase II is essential for DNA replication and transcription [53, 54]. Etoposide-based blockage of DNA replication results in the activation of the mismatch repair (MMR) complex as the cell attempts to overcome the DNA damage [54]. Cancer cells may have a range of defects in the MMR complex that result in a resistance to chemotherapeutic drugs like etoposide. In contrast, these mutations may also make cancer cells more susceptible to treatments with chemotherapeutic drugs (*e.g.* etoposide) [206]. The inactivation of the MMR complex is particularly relevant to this study as it is common in colorectal carcinomas [206], like the Ls174T cell line chosen as the focus of this study. In addition, etoposide has been shown to

up-regulate RKIP expression in a prostate cancer cell line [181], making this an attractive chemotherapeutic agent to examine. It would be interesting to see if RKIP, which affects major growth pathways, affects the stress and repair processes of the cells in a colorectal carcinoma cell line after etoposide treatment, or whether modulation of etoposide-induced apoptosis by RKIP is cell-type specific.
Treatment of the Ls174T cells with the chemotherapeutic agents selected above should display an initial halting of growth and proliferation as the cell attempts to overcome the damage inflicted by the cytotoxic agents.

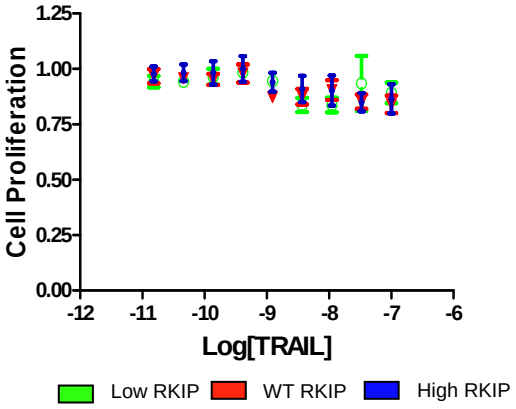
The alamarBlue assay (BioSource), a commercially available and validated enzyme assay, has been developed to quantify the proliferative capacity of cells. The alamarBlue assay measures the reducing environment of cells based on the fact that proliferating cells are more reduced than non-proliferating cells [207]. This highly reduced environment is a result of the enhanced production of metabolic intermediates such as NADPH/NADP, FADH/FAD and NADH/NAD that are produced in the mitochondria of proliferating cells. This highly reducing environment leads to the subsequent reduction of the alamarBlue dye and a detectable colour change that can be measured spectrophotometrically at two wavelengths, 570 and 600 nm [207]. The alamarBlue assay has a number of advantages over some of the alternative assays that measure cell proliferation. First, alamarBlue is a stable, non-toxic dye which makes this safe to use. Secondly, and equally importantly, alamarBlue does not affect the viability of the cells being tested, which is a problem associated with other methods of measuring cell proliferation such as Trypan Blue exclusion [207]. This makes the alamarBlue assay the assay of choice compared to some of the other assays, for example the MTT assay [207].
The alamarBlue assay was used to measure the colour change for untreated Ls174T colon carcinoma control cells and for the same cells that had been treated with cytotoxic agents. The treated cells were normalised to the untreated cell control measurement, with the untreated value for all three cell lines set at 1. This was possible due to the three cell lines displaying no significance differences in proliferation for the untreated controls. This normalisation means that the data represents the fold decrease in the proliferation of the cells after treatment with the cytotoxic agent of interest.

3.1.1 The effect of RKIP on cell proliferation in Ls174T colon carcinoma cells treated with death receptor agonists

3.1.1.1 Tumour Necrosis Factor-related Apoptosis-Inducing Ligand (TRAIL)

RKIP has been shown to modulate TRAIL-induced apoptosis [184], but the effect of RKIP on cell proliferation following the administration of cytotoxic therapy has not yet been examined. Therefore Ls174T colon carcinoma cells with low, WT and high RKIP expression levels were treated with TRAIL over a range of concentrations for 48 hours, after which cellular proliferation was measured using the alamarBlue assay.

a)



b)

Statistical Analysis	Source of variation	p-value
Two-way ANOVA	RKIP levels	0.5668
	[TRAIL]	0.0030
Wilcoxon signed-rank test	Low VS WT RKIP	N/A
	Low VS High RKIP	N/A
	WT VS High RKIP	N/A

Figure 3.1: The effect of RKIP on the cell proliferation of TRAIL-treated colon carcinoma cells. a) Ls174T colon carcinoma cells with low, WT and high RKIP expression were treated with TRAIL (n=4) with a concentration range of 100 nM (log -7) to 10 pM (log -11) for 48 hours; after which the proliferative activity of the cells was measured using the alamarBlue assay. **b)** Table of statistical analyses displaying effect of RKIP on cell proliferation of colon cancer cells treated with TRAIL.

The data in figure 3.1 above showed that TRAIL therapy did not dramatically decrease cell proliferation. All three RKIP-expressing cell lines sustained some proliferation even at the highest concentrations of TRAIL. The largest decrease in cell proliferation after TRAIL

treatment was 20% and all three RKIP cell lines appeared to respond in a similar manner. This difference of 20% would likely not display a noticeable effect on cell numbers. Moreover, two-way analysis of variance (ANOVA) showed that the effect of RKIP levels on the cell proliferative response of Ls174T colon carcinoma cells after TRAIL treatment were not significant. Since the RKIP levels within the cell did not result in a significant response, multiple comparisons between the low, WT and high RKIP-expressing cells were not performed. In contrast, the concentration of TRAIL had a significant effect on the cellular proliferation; demonstrated by the decrease in proliferation observed in Figure 3.1 after treatment with 1 nM TRAIL (log -9). This may have been a consequence of drug toxicity or secondary signalling complexes being activated from the TRAIL receptor.

TRAIL is a highly specific death receptor agonist, thus it is likely that any cells expressing these receptors on their surface will initiate the extracellular death pathway, without any need for initial growth arrest or the cessation of proliferation. Halting of proliferation in a dose-dependent manner is generally seen in DNA-damaging agents [43, 199-201] as the cell attempts to repair itself and deal with the stresses that have been imposed upon it. The significant decrease in cell proliferation observed as a result of increasing TRAIL concentration, could be a consequence of secondary complexes being formed by the receptor, leading to the modulation of signalling pathways. FADD which initiates the apoptotic machinery can also mediate non-apoptotic functions and interact with the adaptor proteins RIP and TRAFs [20-22, 25, 29, 208]. These adaptors link other TNF family members to signalling pathways such as the NF-κB pathway, which in turn can regulate the growth and proliferative responses of the cell [208-211].

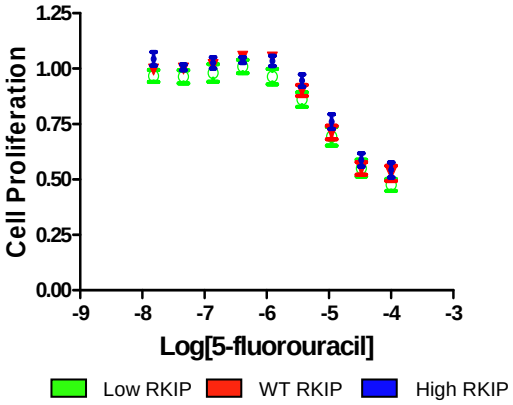
RKIP has been shown to modulate the apoptotic response of cells post-TRAIL administration [184]. In these cell proliferation studies, we observed that there was no significant change in the response of colon carcinoma cells to TRAIL treatment in these experiments regardless of the levels of RKIP within the cell. Previous studies have analysed the effect of RKIP on chemotherapy-induced apoptosis not proliferation, thus investigation into the effect of RKIP levels on TRAIL-induced apoptosis in this cell line would be required to confirm a possible modulation of TRAIL treatment by RKIP in a colon carcinoma cell line. Therefore the selection of an apoptosis assay may be more suitable for analysing the effect of RKIP following treatment with TRAIL in the Ls174T cell line. This study is described in a later section.

3.1.2 The effect of RKIP on cell proliferation in Ls174T colon carcinoma cells treated with DNA damage-inducing agents

3.1.2.1 5-fluorouracil (5-FU)

5-FU is routinely employed in the treatment of colon carcinomas where it has been consistently efficacious, and is still a front-line defence in colorectal carcinomas although various regimes have been employed [204]. To determine the effect of 5-FU treatment on colon carcinoma cells expressing different levels of RKIP; low, WT and high RKIP-expressing cells were treated with various concentrations of 5-FU for 48 hours and the alamarBlue assay was used to quantify cellular proliferation.

a)



b)

Statistical Analysis	Source of variation	p-value
Two-way ANOVA	RKIP levels	<0.0001
	[5-FU]	0.003
Wilcoxon signed-rank test	Low VS WT RKIP	0.00356
	Low VS High RKIP	<0.0001
	WT VS High RKIP	0.382

Figure 3.2: The effect of RKIP on 5-FU-treated colon carcinoma cell proliferation. a) 5-FU, 100 μM (log -4) to 10 nM (log -8), was administered to Ls174T colon carcinoma cells with low, WT and high RKIP expression for 48 hours; after 48 hours the proliferative activity of the cells was then measured using the alamarBlue assay (n=13). **b)** Table of statistical analyses on the effect of RKIP on the cell proliferation of Ls174T cells administered 5-FU.

Ls174T colon carcinoma cells exhibited a dose-dependent decrease in cell proliferation after 48 hours of treatment with 5-FU. The proliferation of all three RKIP-expressing cell lines decreased when 1 μM (log -6) 5-FU was used to treat the cells. In addition, all three

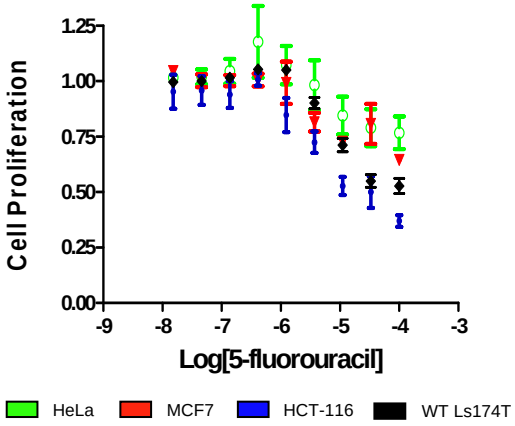
cell lines showed a similar pattern of behaviour upon treatment with 5-FU (Figure 3.2). Two-way ANOVA analysis showed the effect of RKIP levels within the cell had a significant effect on the proliferative response of 5-FU-treated cells (Figure 3.2). In addition, the concentration of 5-FU had a significant effect on the cell proliferative response of colon carcinoma cells after 5-FU administration. Since the two-way ANOVA showed that the RKIP levels had a significant effect on cellular proliferation, Wilcoxon signed-rank tests were performed between the three cell lines that expressed different levels of RKIP. The post tests showed that the low RKIP cell line had significantly different proliferative responses after 5-FU treatment compared to the WT and the high RKIP-expressing cell lines (Figure 3.2). This result would confirm other studies that have demonstrated the chemo-sensitising effect of RKIP [181, 183, 184, 189]. Although statistically significant, this difference may not be biologically significant and as such further validation perhaps through proliferative markers such as Ki-67 and clonogenic assays would be required.

In this study, the role of RKIP on cell proliferation after 5-FU treatment has been demonstrated. However, the chemo-sensitising effect of RKIP has been shown in previous studies to be involved in cell death induced by cytotoxic agents [181, 183, 184]. Therefore examination of the effect of RKIP on 5-FU-induced cell death would be required to further establish a role for RKIP in chemo-sensitisation in this colorectal carcinoma cell line after treatment with 5-FU.

Treatment of RKIP-expressing Ls174T cells with 5-FU resulted in an interesting pattern of behaviour. To determine whether the effect of 5-FU treatment on Ls174T cells was cell type specific, three other cancer cell lines – HeLa, MCF7 and HCT-116 – were targeted for analysis. The HeLa cell line is a cervical carcinoma cell line; MCF7 is a breast cancer cell line and the HCT-116 cell line is another colon carcinoma cell line. All three cell lines are routinely used in scientific research. Further, since the HCT-116 cell line is also a colon carcinoma cell line akin to the Ls174T cell line, the HCT-116 cell line would confirm whether the Ls174T colon carcinoma cell line was responding reliably to treatment and was not adversely affected by the stable incorporation of RKIP-expressing vectors.

HeLa, MCF7 and HCT-116 cells were treated with 5-FU at a range of different concentrations for 48 hours, after which the proliferative capacity of the cells were measured using the alamarBlue assay. The results from these three cell lines were compared to the WT RKIP-expressing Ls174T colon carcinoma cell line.

a)



b)

Statistical Analysis	Source of variation	p-value
Two-way ANOVA	Cell line	<0.0001
	[5-FU]	<0.0001
Wilcoxon signed-rank test	HeLa VS MCF7	0.124
	HeLa VS HCT-116	0.0001
	MCF7 VS HCT-116	0.0298
	HeLa VS Ls174T	0.0104
	HCT-116 VS Ls174T	0.00515
	MCF7 VS Ls174T	0.780

Figure 3.3: HeLa, MCF7 and HCT-116 cellular proliferation after treatment with 5-FU in comparison to WT RKIP-expressing Ls174T colon carcinoma cells. a) HeLa, MCF7 and HCT-116 cell lines were treated with 5FU (n=4),100 μM (log -4) to 10 nM (log -8) for 48 hours. After 48 hours the alamarBlue assay was used to measure the proliferative activity of the cells. The results from alamarBlue assay of the HeLa, MCF7 and HCT-116 post 5-FU-treatment were compared with the WT RKIP Ls174T colon carcinoma cell line data from Figure 3.2. **b)** Table of statistics showing the effect of 5-FU administration in different cell lines in comparison to the WT Ls174T colon carcinoma cell line.

All three cell lines selected for analysis – HeLa, MCF7 and HCT-116 - exhibited a decrease in cellular proliferation after treatment with 5-FU in a similar manner to the WT RKIP Ls174T colon carcinoma cell line (Figure 3.3). In addition, all three cells lines were sensitive to 5-FU treatment in a dose-dependent fashion when concentrations of 5-FU exceeded 1 μM (log -6), as was observed for the WT RKIP Ls174T colon carcinoma cell line (Figure 3.3). The responses of the HeLa, MCF7 and HCT-116 cell lines displayed a higher degree of variation (as shown by the larger error bars in Figure 3.3 compared with Figure 3.2), both within experiments and between different experiments when compared to the WT RKIP-expressing Ls174T colon carcinoma cell line. It was likely that due to the RKIP-expressing Ls174T colon carcinoma cell lines being clones; the population of cells were more homogenous compared with the HeLa, MCF7 and HCT-116 cell lines.

Furthermore the dose-dependent decrease in cell proliferation was more pronounced in the HCT-116 colon carcinoma cell line when compared to the HeLa and MCF7 cell lines. Indeed, multiple comparisons showed a greater significant difference in the response of the HCT-116 cell line to both HeLa and MCF7 cell lines following 5-FU treatment, compared

to the HeLa versus MCF7 cell lines (Figure 3.3). This justifies the routine use of 5-FU in the treatment of colorectal carcinomas [204]. Attempts were made to analyse the results using IC50 values, however as can be seen in later chapters, many of the drugs failed to produce sigmoidal responses which were difficult to analyse using non-linear regression.

Two-way ANOVA showed that the 5-FU concentration had a significant effect on cell proliferation of the three cell lines (Figure 3.3). Upon treatment with 5-FU the Ls174T colorectal carcinoma cell line displayed a dose-dependent decrease in cell proliferation (Figure 3.2). This was expected since 5-FU disrupts DNA replication *via* thymidylate synthase inhibition and false incorporation into DNA [50, 70]. For this reason, 5-FU is most effective in the S phase of the cell cycle where the cell increases DNA replication and synthesis to prepare for protein synthesis and cell division [205]. Aberrations in the S phase, such as treatment with 5-FU, results in the activation of the DNA damage response and cell cycle checkpoints subsequently leading to the halting of proliferation [205]. This corroborates the data presented above (Figures 3.2 and 3.3).

The sequence of events between DNA damage by 5-FU and observable cessation of cell proliferation are likely to be as follows.

The DNA damage response network is activated during genotoxic stress such as treatment with chemotherapeutic agents, and is a highly complex and intricate process. At the most basic level, double-strand and single-strand breakages in the DNA activate two phosphatidylinositol-3-related kinases (PI3-kinases), ATM (ataxia telangiectasia mutated) and ATR (ATM-Rad3-related) respectively; these two kinases are central to the DNA damage response signal [212]. ATM and ATR phosphorylate two related serine/threonine checkpoint kinases (Chk) – Chk1 and Chk2; the general consensus being that Chk1 is phosphorylated by ATR and Chk2 by ATM, however there is now thought to be cross-talk and overlap between the two pathways [74, 213]. Chk2 is activated solely upon DNA damage and is prevalent throughout the cell cycle stages; Chk1 is restricted to the S and G2-phases and is active without genotoxic stress [213].

The downstream effectors of the four core DNA damage kinases - Chk1, Chk2, ATM and ATR - are many and varied and can lead to cell cycle arrest within different stages of the cell cycle; these are reviewed by Dasika *et al.* in the following reference [201]. Furthermore, activation of checkpoint and DNA damage kinases recruits the omnipresent p53 protein family. The protein p53 and its associated family members are some of the best

characterised examples of cell fate decisions during DNA damage and are reviewed extensively by Harms *et al.* [214].

The culmination of all the above effects would primarily arrest the cell whilst DNA repair is initiated; if this was unsuccessful the signal would then be given to induce apoptosis *via* the mitochondrial death pathway [43, 200]. Some of the 5-FU treated cells may have already undergone this stress-induced apoptosis thus it would be interesting to investigate the effect of RKIP on 5-FU-induced apoptosis; particularly since RKIP has been implicated in the modulation of chemotherapy-induced apoptosis.

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Treatment of other neoplastic cell lines – HeLa, MCF7 and HCT-116 – with 5-FU also displayed this dose-dependent decrease in cell proliferation (Figure 3.3). Thus treatment with 5-FU resulted in all cell lines, including the RKIP clone cell lines, acting in a manner consistent with the expected pattern of behaviour.

The HCT-116 and Ls174T colon carcinoma cell lines both exhibited a dose-dependent decrease in cell proliferation upon 5-FU administration (Figures 3.2 and 3.3), thus 5-FU was behaving in a manner consistent with its known functions in colorectal carcinomas. Moreover, the similarity in response between the HCT-116 and Ls174T colon carcinoma cell lines was evidence that the stable transfection of RKIP-expressing plasmids into the Ls174T colon carcinoma cell line had not adversely affected the response of this cell line to drug treatment.

The level of variation between 5-FU treatments was higher in the HeLa, MCF7 and HCT-116 cell lines (Figure 3.3) compared to the Ls174T clone cell lines (Figure 3.2); thus the Ls174T colon carcinoma cell line was better suited for observing the effects of 5-FU treatment and for the subsequent statistical interpretation of the biological data. It was surprising that RKIP levels in Ls174T cells did not lead to a more pronounced effect on the proliferative response of cells after chemotherapy administration, particularly because of the role of RKIP in cell cycle progression.

During mitosis, phosphorylated RKIP (pRKIP) levels increase relative to unphosphorylated RKIP [180]. pRKIP localises to the kinetochores and centrosomes and regulates the number of mitotic cells, particularly in metaphase. Loss of RKIP in these cells leads to a concomitant decrease in Aurora B kinase; this kinase is crucial for ensuring correct alignment of the chromosomes along the mitotic spindle prior to cytokinesis [180]. Moreover, treatment with the taxane drug paclitaxel in RKIP-depleted H19-7, HeLa, or Rat-1 cells resulted in overriding of the mitotic checkpoint, and a subsequent increase in

chromosomal damage. Paclitaxel stabilises microtubules in the polymerised form leading to an inflexible cytoskeleton; a dynamic cytoskeleton is essential for mitosis, therefore paclitaxel is highly effective at preventing cancer cell division and hence is known as a mitotic inhibitor [55, 57]. Cells with depleted RKIP did not arrest at the mitotic checkpoint although paclitaxel had adversely affected the cytoskeleton, leading to the formation of incomplete and damaged daughter cells [180]. Due to the involvement of RKIP in cell cycle progression [180] it was thought that altering the levels of RKIP in Ls174T colon carcinoma cells would affect the cell proliferative activity of the cells after 5-FU administration.

There are a number of possible explanations as to why RKIP levels did not profoundly alter the proliferative response of the Ls174T colon carcinoma cells after treatment with 5-FU.

Firstly, 5-FU is most effective during the S-phase of the cell cycle [215]. The role of RKIP within the cell cycle has been shown only in mitotic cells [180], and particularly metaphasic cells, thus it was possible that RKIP regulation of cell cycle processes may not be more pronounced until the G2-M phases of the cell cycle. It is possible that after treatment with taxane derivatives cells may show RKIP-dependent changes in cell proliferation, since RKIP depletion has been shown to override the mitotic checkpoint in cells treated with paclitaxel. Increasing the levels of RKIP would theoretically have the opposite effect, thus investigation of the effect of RKIP on paclitaxel-treated Ls174T colon carcinoma cells would be of interest. This was performed in a later section.

Secondly, the effect of RKIP on the cell cycle requires the phosphorylation of RKIP at serine 153 [180]. This phosphorylation is dependent on protein kinase C (PKC). Classical PKC isoforms such as α, βI, βII and γ phosphorylate RKIP, but not the novel δ, ε, η and θ isoforms [149, 150]. PKC isoforms can be activated during DNA-damage but of the novel variety that have not yet been shown to phosphorylate RKIP; the role of PKCδ in DNA damage has been demonstrated repeatedly in DNA damage and has been reviewed extensively by Yoshida *et al.* [216]. Since the classic isoforms induce RKIP phosphorylation [149], DNA damage caused by agents such as 5-FU, was not likely to induce phopshorylation of RKIP. Perhaps other PKC isoforms will prove to be involved in modulation of the apoptotic response, and if so, pre-treatment with a PKC inducer may display changes in the response of the three Ls174T colon carcinoma cell lines to treatment

with 5-FU. Furthermore, after induction of PKC, the presence of phosphorylated RKIP could be analysed using mass spectrometry or 2-dimensional gel electrophoresis.

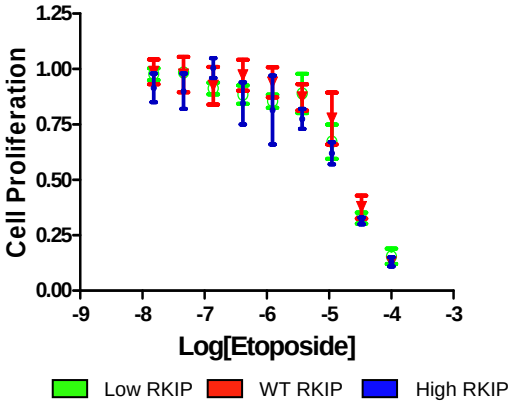
This evidence indicated that it was likely that RKIP was not in the correct phosphorylation state to exert prominent effects on the cell proliferative response of colon carcinoma cells undergoing treatment with 5-FU. In addition, the RKIP protein and 5-FU mechanistic action do not overlap at the appropriate point in the cell cycle, hence the lack of changes observed after 5-FU administration in the RKIP-expressing cell lines.

Since there were statistically significant differences between the low, WT and high RKIP-expressing Ls174T cell lines, this suggested that there was some degree of modulation following 5-FU treatment in the colon carcinoma cell line. It is possible that over a longer period, perhaps several weeks or months as can occur in patients undergoing chemotherapy, these small differences may lead to the development of a small population of potentially resistant cells. More stringent analysis of changes in cell proliferation between the three RKIP cell lines during 5-FU treatment *e.g.* clonogenic assays would determine whether these changes could lead to a clinically meaningful difference in cell proliferation. Since RKIP has been shown to modulate chemotherapy-induced apoptosis, investigation into the effect of RKIP levels on 5-FU-induced apoptosis is crucial. This is examined in a later section.

3.1.2.2 Etoposide

Chatterjee *et al.* have previously demonstrated that RKIP modulates etoposide-induced apoptosis in prostate cancer cells, however no investigations have been conducted on the effect of RKIP on cellular proliferation post-etoposide treatment [181]. To determine whether etoposide had any effect on proliferation, the topoisomerase II inhibitor etoposide was administered to Ls174T colon carcinoma cells with low, WT and high RKIP expression levels and incubated for 48 hours; after treatment with etoposide for 48 hours, the proliferative ability of the Ls174T cells was quantified using the alamarBlue assay.

a)



b)

Statistical Analysis	Source of variation	p-value
Two-way ANOVA	RKIP levels	0.1239
	[etoposide]	<0.0001
Wilcoxon signed-rank test	Low VS WT RKIP	N/A
	Low VS High RKIP	N/A
	WT VS High RKIP	N/A

Figure 3.4: The effect of RKIP on cell proliferation of etoposide-treated Ls174T colon carcinoma cells. a) Etoposide, 100 μM (log -4) to 10 nM (log -8), was administered to Ls174T colon carcinoma cells with low, WT and high RKIP expression for 48 hours; post-incubation the proliferative activity of the cells were measured using the alamarBlue assay (n=4). **b)** Table of statistical analyses displaying the effect of RKIP on etoposide-administered colon carcinoma cell proliferation.

Both the alamarBlue assay and two-way ANOVA showed that the concentration of etoposide significantly affected the proliferative responses of Ls174T cells; resulting in a dose-dependent decrease in cell proliferation for all three RKIP expressing cell lines with increasing etoposide concentration (Figure 3.4). In contrast, the RKIP levels had no significant effect on the cell proliferation of colon carcinoma cells treated with etoposide. Since the levels of RKIP had no significant effect on the response of the Ls174T cells to etoposide treatment, multiple comparisons were not performed on the three RKIP cell lines (Figure 3.4b).

Treatment with etoposide for 48 hours resulted in a slow decrease in cell proliferation after which the cell proliferation decreased sharply between 3 – 100 μM (log -5.5 – log -4) (Figure 3.4). This cessation of proliferation as a consequence of etoposide administration was to be expected. Inhibition of topoisomerase II function by etoposide results in the generation of double-strand breaks in the DNA. This would lead to the blockage of DNA replication and transcription [217, 218]. Activation of the MMR complex then occurs as the cell attempts to repair this DNA damage and this leads to the cessation of growth and proliferative cascades. Etoposide-induced activation of DNA damage and checkpoint

kinases have been described previously and the end result is the cessation of proliferation observed in Figure 3.4. The decrease in cellular proliferation did not occur until the concentration of etoposide was 3 μM or higher suggesting that although working effectively in accordance with its known function, etoposide may not be the best agent for inducing a strong anti-proliferative response in this cell line. Furthermore, etoposide requires higher doses clinically than 5-FU, and is not usually prescribed for the treatment of colon cancer. Also, although the concentrations used throughout the study are comparable, clinical dosing regimes would differ between the agents therefore greater clinical effects may be observed.

There was no change in the cell proliferative response in the three Ls174T colon carcinoma cell lines treated with etoposide, regardless of the levels of RKIP protein that the cells were expressing (Figure 3.4). This is interesting since etoposide has been shown to induce RKIP expression in a prostate cancer cell line [181]. This suggested that the etoposide-directed increase in RKIP expression may be a cell-type specific response. This was supported by the fact that a breast cancer cell line did not display such RKIP induction following etoposide treatment [181]. It is also possible that RKIP may only modulate the induction of apoptosis but not the cessation of cell proliferation after etoposide administration.

The reasons as to why the RKIP levels had a negligible effect on the response of etoposide-treated Ls174T colon carcinoma cells may be similar to those discussed previously for 5-FU. Like 5-FU, etoposide induces DNA damage resulting in the activation of the DNA damage and cell stress responses; this explains the observed dose-dependent decrease in proliferation.
This also means that etoposide was most effective on cells that were in the S-phase of the cell cycle. At present, RKIP activity has only been shown in mitotic cells [180]. Similar to the 5-FU treated cells, the effects of RKIP in modulation of cell proliferative responses may be restricted to mitotic cells and not cells treated with S-phase effective agents.
Furthermore, the effect of RKIP on mitotic cells requires phosphorylation by PKC [180], thus RKIP may not have been in the correct phosphorylation state to exert any cell cycle effects on Ls174T cells administered etoposide.

However it is still plausible that RKIP modulates the response of Ls174T colon carcinoma cells to etoposide treatment. The etoposide-induced apoptotic response is known to be affected by the levels of RKIP in prostate cancer cells [181], thus examination of the effect

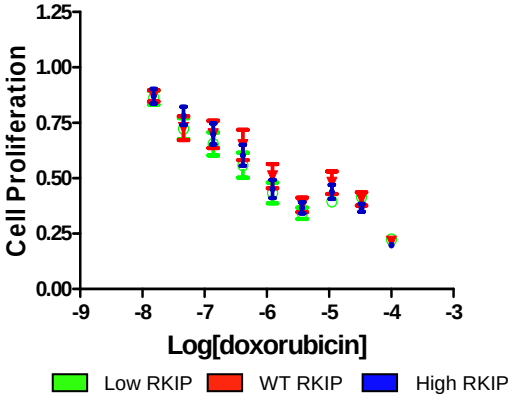
of RKIP etoposide-induced apoptosis in the Ls174T colon carcinoma cell line would be interesting, and is performed in a later section.

3.1.3 The effect of RKIP on cell proliferation in Ls174T colon carcinoma cells treated with cell stress-inducing chemotherapeutic agents

3.1.3.1 Doxorubicin

The chemotherapeutic drug doxorubicin inflicts extensive cell stress *via* DNA damage, free radical formation and enzyme inhibition [76, 77]. RKIP has been shown to affect chemotherapy-induced apoptosis [181, 183, 184, 189], however no investigation has been performed on the modulation of cell proliferation after doxorubicin treatment in the presence of different levels of RKIP. Therefore, low, WT and high RKIP-expressing Ls174T colon carcinoma cells were treated with doxorubicin for 48 hours. Thereafter, cellular proliferation was measured using the alamarBlue assay.

a)



b)

Statistical Analysis	Source of variation	p-value
Two-way ANOVA	RKIP levels	0.1651
	[doxorubicin]	<0.0001
Wilcoxon signed-rank test	Low VS WT RKIP	N/A
	Low VS High RKIP	N/A
	WT VS High RKIP	N/A

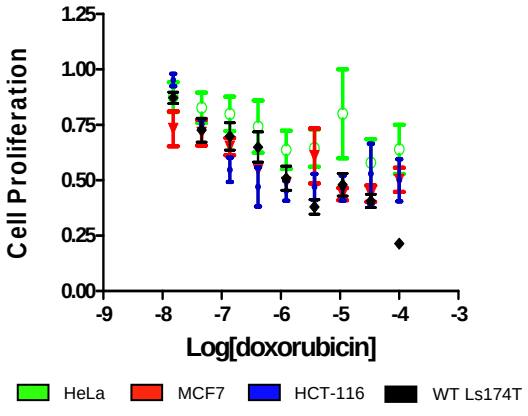
Figure 3.5: The effect of RKIP on doxorubicin-treated Ls174T colon carcinoma cell proliferation. a) Ls174T colon carcinoma cells with low, WT and high RKIP expression were treated with a range of doxorubicin concentrations, 100 µM (log -4) to 10 nM (log -8) for 48 hours; after 48 hours the proliferative activity of the cells were measured using the alamarBlue assay (n=16). **b)** Table of statistical analyses on the effect of RKIP on cell proliferation of Ls174T cells treated with doxorubicin.

The treatment of Ls174T cells with varying concentrations of doxorubicin for 48 hours resulted in a sharp decrease in cell proliferation as the concentration of doxorubicin increased (Figure 3.5). There was a small increase in proliferation at log -5 but this is not likely to be biologically significant. This was confirmed by the two-way ANOVA which showed that the concentration of doxorubicin had a significant effect on the proliferative response of colon carcinoma cells. In contrast, the levels of RKIP had no significant effect on cellular proliferation. Again, this was corroborated by the two-way ANOVA (Figure 3.5b).

To determine if the decrease in cellular proliferation after treatment with doxorubicin was cell type-specific, three different cancer cell lines (HeLa, MCF7 and HCT-116) were used in comparative experiments with Ls174T cells. In addition to determining the efficacy of doxorubicin treatment on Ls174T cells, the use of alternative cells lines allowed the validation of the low, WT and high RKIP-expressing cell lines and the determination that the response of the Ls174T cells to drug treatment was not adversely affected by the stable transfection of the Ls174T cells.

As before, three different cancer cell lines were treated for with varying concentrations of doxorubicin for 48 hours, after which the cell proliferative capacity of each cell line was measured using the alamarBlue assay.

a)



b)

Statistical Analysis	Source of variation	p-value
Two-way ANOVA	Cell line	<0.0001
	[doxorubicin]	<0.0001
Wilcoxon signed-rank test	HeLa VS MCF7	<0.0001
	HeLa VS HCT-116	0.00072
	MCF7 VS HCT-116	0.512
	HeLa VS Ls174T	<0.0001
	HCT-116 VS Ls174T	0.300
	MCF7 VS Ls174T	0.125

Figure 3.6: Cell Proliferative capacity of HeLa, MCF7 and HCT-116 cell lines after treatment with doxorubicin in comparison to the WT RKIP-expressing Ls174T cell line. a) Doxorubicin with a range of concentrations, 100 µM (log -4) to 10 nM (log -8),

was administered to HeLa, MCF7 and HCT-116 cell lines for 48 hours; afterwards the proliferative activity of the cells were measured using the alamarBlue assay (n=4). The results from the three cell lines – HeLa, MCF7 and HCT-116 – were compared with the WT RKIP Ls174T colon carcinoma cell line. **b)** Table of statistical analyses showing the effect of doxorubicin on cell proliferation in HeLa, MCF7 and HCT-116 cell lines in comparison to the WT Ls174T cells line.

Both two-way ANOVA and the alamarBlue assay showed the three cell lines had significantly different responses to treatment with doxorubicin, but that the decreased cell proliferation occurred in a similar manner to the WT Ls174T colon carcinoma cells (Figure 3.6). Multiple comparisons using the Wilcoxon signed-rank tests confirmed a significant difference in cell proliferation between HeLa and the MCF7, HCT-116 and Ls174T cell lines, but not MCF7 cells and HCT-116 (Figure 3.6). There was no significant difference in the response of HCT-116 and MCF7 cell lines in comparison to the Ls174T cells. In addition, the concentration of doxorubicin itself was shown to have a significant effect on cell proliferation; confirming the dose-dependent decrease in cell proliferation was observed after treatment of HeLa, MCF7 and HCT-116 cells with increasing concentrations of doxorubicin (Figure 3.6).

Furthermore, the HeLa, HCT-116 and MCF7 cell lines displayed larger variation in their responses, as demonstrated by the larger error bars, compared to the Ls174T colon carcinoma cell line post-doxorubicin administration (Figures 3.5 and 3.6). This was observed before when these cell lines were treated with 5-FU (Figure 3.2 versus Figure 3.3). This is most likely due to the Ls174T cell populations being more homogenous since they are clone cell lines, compared to the more heterogenous populations of the HeLa, HCT-116 and MCF7 cell lines. Therefore the Ls174T colon carcinoma cell line appears to be a more reliable cell line in which to study the effect of cellular treatments with chemotherapeutic agents, and to statistically analyse the biological data.

The results for Ls174T colon carcinoma cells treated doxorubicin were similar to those for Ls174T cells treated with etoposide and 5-FU. The treatment of cells with doxorubicin displayed an inversely proportional relationship between the drug concentration and cellular proliferation. Interestingly, the decline in cellular proliferation was much sharper for doxorubicin than for 5-FU and etoposide thus it was possible that doxorubicin was more potent at inducing the cessation of cell proliferation in the Ls174T cell line than 5-FU and etoposide.

Doxorubicin induces a variety of cell stress responses as a result of DNA intercalation, protein inhibition and free radical formation; these have been extensively reviewed in the following references [76, 77]. The combined effects of these stress-induced responses explain the halting of proliferation that was observed [43, 199-201] and may account for the greater potency of doxorubicin over both etoposide and 5-FU. Finally, the induction of the cell stress response by doxorubicin treatment led to the halting of proliferation in all the cell lines employed demonstrating that doxorubicin was behaving in a manner consistent with its characterised functions. Cessation of proliferation as a result of genotoxic stress has been described previously in the discussion of the 5-FU data. Similarly, in this experiment, the administration of doxorubicin resulted in the activation of the DNA damage response network [76] and a resultant dose-dependent decrease in cell proliferation. This was the pattern of behaviour expected for an agent that induced cell stress and damaged DNA [43, 199-201].

There was no observable modulation of the dose-dependent decrease in cell proliferation by RKIP in the Ls174T colon carcinoma cell lines, after treatment with doxorubicin (Figure 3.5). No studies have examined the effect of doxorubicin treatment on cells expressing altered levels of RKIP thus further investigation is required.
RKIP modulation is related to chemotherapy-induced apoptosis thus RKIP may modulate the apoptotic response induced by doxorubicin more prominently than cell proliferation. Therefore the examination of the response of colon carcinoma cells expressing different levels of RKIP to doxorubicin treatment using apoptosis assays, as opposed to cell proliferation assays, would be very interesting. The effect of RKIP on doxorubicin-induced apoptosis is examined in a later section.

The reasons why the cellular levels of RKIP did not have a significant observable effect on cell proliferation after treatment with doxorubicin are likely to be similar to those for Ls174T cells treated with 5-FU and etoposide. RKIP may not interfere with pre-mitotic stages of the cell cycle where doxorubicin is most effective [77]. Additionally, RKIP may not have been in the appropriate phosphorylation state to affect cell cycle progression [180]. It is also possible that the modulation of chemotherapeutic drug response by RKIP may only involve the apoptotic machinery, and not cell proliferation and growth pathways.

3.1.4 Discussion of Cell Proliferation results

Ls174T colon carcinoma cell lines that expressed low, WT or high levels of RKIP and treated with 5-FU, doxorubicin or etoposide all displayed a dose-dependent decrease in cell proliferation. This was the expected of agents that activate the DNA-damage response network in cells [43, 199-201].
In contrast, TRAIL showed only a 20% decrease in cell proliferation at the highest concentrations. This result can be explained; since TRAIL is a death receptor agonist it was unlikely to display initial cessation of proliferation as the cell attempted to repair the damage that was inflicted by the TRAIL treatment.

Ls174T colon carcinoma cell lines treated with doxorubicin, etoposide, 5-FU or TRAIL showed no significant observable differences in the patterns of cellular proliferation behaviour regardless of whether the cells expressed low, WT or high levels of RKIP. There were statistical differences suggesting that RKIP may modulate the proliferative response of colon carcinoma cells treated with chemotherapeutic drugs. It is possible that this effect may be a by-product of RKIP-based modulation of chemotherapy-induced apoptosis. Thus examination of the apoptotic response of the low, WT and high RKIP-expressing colon carcinoma cells after cytotoxic therapy would be very interesting, and is investigated in a later section. Furthermore, as mentioned previously, these small differences may lead to a larger difference in a clinical setting over a period of weeks or months. Further cell proliferative analysis using clonogenic assays may help determine how meaningful these differences in proliferation are.

Although RKIP inhibits two major signalling pathways involved in growth and proliferation, the NF-κB and ERK pathways [143, 144, 148], growth curves for the three RKIP clones (low, WT and high) were not significantly different (Chapter 2, Figure 2.2). A small acceleration of growth was detected in the low RKIP-expressing Ls174T colon carcinoma cell line after 5 days but this was not significant. If RKIP was involved in the modulation of primary tumourigenesis, a substantial difference in growth would have been observed between the three RKIP-expressing cells. Further, RKIP KO mice do not display enhanced tumourigenesis or any other proliferative defects [164].

It is possible that RKIP may act as a regulator in terms of cell growth and proliferation; as opposed to being an outright inhibitor, and preventing cell growth by the ERK and NF-κB pathways. Indeed, many of the studies into the role of RKIP as a metastases suppressor have demonstrated that RKIP has no effect on primary tumourigenesis and supports a more modulatory function for RKIP in the initial stages of cell growth, proliferation [159, 169, 170, 173]. In terms of cell proliferation, it is likely that RKIP plays a more prominent role in the later stages of tumour invasion and metastases [173]. This is supported by evidence of RKIP down-regulation by Snail [167]; Snail is a transcription factor, and one of the main culprits in the epithelial-mesenchymal transition (EMT) [167], that is generally associated with the formation of aggressive and invasive carcinomas.

The results from the HCT-116, HeLa and MCF7 cell lines treated with 5-FU and doxorubicin also displayed a dose-dependent decrease in cell proliferation. The responses of these cell lines displayed high errors within the biological experiments; this was probably due to the population of cells being heterogenous. Compared with our Ls174T clone cell lines which are more homogenous, this result verified that the Ls174T RKIP-expressing cell lines were responding reliably and consistently to treatment with chemotherapeutic agents. Further, due to the lower level of standard error in the response to cytotoxic therapy displayed in the Ls174T colon carcinoma cell line, any changes in the patterns of behaviour would be easier to detect and analyse.

Several conclusions can be drawn from this study.

1. The Ls174T colon carcinoma cell line with low, WT and high RKIP expression was a good choice for studying chemotherapeutic drug responses in comparison to the MCF7, HeLa and HCT-116 cell lines. The Ls174T cell lines showed consistent and reliable results compared to the MCF7, HeLa and HCT-116 cell lines. Furthermore, this Ls174T colon carcinoma cell line served as a good system upon which to test the effects of RKIP on different cytotoxic agents. In addition, these RKIP - low, WT and high - cell lines could be used to test different drugs and their possible modulation by RKIP.

2. The chemotherapeutic agents selected for analysis in this study behaved effectively in the low, WT and high RKIP-expressing cell lines and also in a manner consistent with their known actions. Therefore, these studies allowed the validation of the Ls174T cell line selected for analysis as well as the chemotherapeutic agents.

3. Ls174T cell lines with varying levels of RKIP expression showed very small changes in cellular proliferation after treatment with chemotherapeutic agents. This may lead to clinically meaningful differences however over a period of weeks or months, thus further analysis of cell proliferation over longer time periods and/or using clonogenic assays may determine the clinical significance of these changes. However it was possible that the alamarBlue assay was inappropriate for the analysis of the chemotherapeutic agents selected for investigation. The ultimate goal of chemotherapy is to induce cell death, and RKIP has been shown to modulate this apoptotic response, particularly after treatment with etoposide and TRAIL [181, 184]. Therefore, in future studies, the effect of the levels of RKIP on chemotherapy-induced apoptosis will be examined. The caspase-Glo 3/7 assay (Promega) was selected to analyse apoptosis induced by both the internal and external apoptotic pathways. In addition to the convenience of using a validated, commercially available, and high throughput assay system, this assay required no changes in the experimental protocol designed and optimised for the alamarBlue assay.

3.2 The effect of RKIP on the cell cycle after treatment with chemotherapy

The previous study on cell proliferation showed that proliferation of the cell halted as the cell attempted to repair the damage inflicted after cytotoxic therapy. The process of cell proliferation is controlled and regulated by the cell cycle machinery.

In order to proliferate and divide in the correct manner, the cell has to ensure all of its components are ready and available, hence the necessity for the cell cycle machinery. The cell cycle comprises five main stages; quiescence (G0), Gap1 (G1), synthesis (S), Gap2 (G2) and mitosis (M) phase which are driven and regulated by protein complexes containing cyclins and cyclin-dependent kinases (CDKs) [219]. Upon stimulation by growth factors, quiescent cells (G0) enter a period of growth as the cells prepare for the DNA synthesis stage; this period is known as the G1 stage. Before transition to the second stage the cell must first pass a restriction point known as the G1-S checkpoint [220]. Here, the cell mechanisms monitor the integrity of the DNA and signalling prior to entrance into the S-phase [220]. Once the cell has passed this checkpoint it is committed to the continuation of the cell cycle process unless a cataclysmic internal or external event occurs [221].

The second stage is known as the S-phase; during this stage rapid DNA synthesis occurs as the cell duplicates the chromosomes in order for the daughter cells to receive a full DNA complement [222]. After DNA synthesis, another period of growth ensues as the cell prepares for cell division; this period is known as the G2-phase. The final phase, the M-phase, results in mitosis/cell division [222]. Prior to the transition from G2- to M-phase there is another checkpoint known as the G2-M checkpoint; this checkpoint ensures correct DNA replication has occurred and that there is no evidence of DNA damage during S-phase before cell division [223].

Tumour cells display various mutations within the cell cycle machinery and its regulatory components; one of the most common being loss or relaxation of the G1-S checkpoint, as a result of p53 mutations [220]. The p53 protein is often coined “the guardian of the genome” and belongs to a family of proteins (p53 family) that are extensively involved in maintaining DNA and cellular integrity [214]. Amongst its myriad of functions, p53 is crucial for determining whether a cell arrests or undergoes apoptosis upon DNA damage

[214]. Loss of p53 function, leading to cell cycle aberrations, allow the development and evolution of cancer cells, and contribute to drug insensitivity, evasion of death and unlimited proliferation; all hallmarks of cancer [214, 223].
The influence of MAPK and NF-κB signalling on cell cycle progression [222, 224] is well documented. The role of RKIP in these pathways suggested there may be a role for RKIP in the modulation of cell cycle progression *via* its effects on the ERK and NF-κB pathway. Furthermore, RKIP itself has been shown to regulate mitotic progression [180]; thus it was hypothesised that there may be differences in the cell cycle dynamics following chemotherapy treatment as a consequence of changes in RKIP levels within the cell.

Fluorescence activated cell sorting (FACS) is a technique that has been employed in numerous applications, most notably in cell population studies that determine the distribution of cells in the various stages of the cell cycle, and in studies that measure the cellular DNA content [225, 226]. FACS has advantages such as its high sensitivity, precision and the speed at which large amounts of biological data can be collected [225]. Propidium iodide FACS analysis was employed to study the cell cycle of the Ls174T colon carcinoma cells after treatment with various chemotherapeutic drugs. Propidium iodide intercalates and labels the DNA within the cell allowing the determination of the stage of the cell cycle, of each cell in a population, to be quantified by the measurement of the fluorescence intensity [227].
Cells in the G0 or G1 states will contain X1 copy of DNA, cells in the G2-M phase will have X2 copies of DNA and finally, cells undergoing S-phase will contain DNA content between X1 and X2 copies. As the DNA concentration inside the cell increases, the propidium iodide label bound to the DNA increases and thus the fluorescent signal measured by the flow cytometer increases [226].

Serum-starved Ls174T colon carcinoma cells treated with foetal bovine serum (FBS), to determine the effect of the RKIP on cell cycle distribution in the absence of all cytotoxic agents, were selected as a baseline experiment. The cells were analysed over a 48 hour period to allow the comparison of this data to the cytotoxic-administered cell FACS data. In addition to providing a baseline for these studies, these FBS-treated Ls174T colon carcinoma cells served as a further control and means of comparison for cells treated with chemotherapeutic agents to untreated cells. All cells were harvested at the end of the 48 hour time point and the cells described a “0” are untreated control cells. Cell treatment times ran backwards therefore for the 1 hour treatment time, the drug was added 1 hour

prior to harvest, for the 2 hour treatment time the drug was added 2 hours prior to harvest *etc*.

Initially it was thought that cell proliferation was affected by RKIP levels following the administration of doxorubicin thus this agent was selected for cell cycle investigation. Doxorubicin exerts strong effects on the cell cycle, and is thought to be more potent at certain stages such as the S-phase [77, 228]. In addition, doxorubicin displayed a strong dose-dependent decrease in cell proliferation, an indication that the cell could be attempting to repair the damage inflicted by doxorubicin treatment; thus these cell populations may display alterations in the cell cycle [43, 199-201]. It would therefore be interesting to determine whether the levels of RKIP had any effect on the distribution of cells in the cell cycle after doxorubicin treatment.

5-FU is routinely employed in the treatment of colorectal carcinomas [204], and like doxorubicin, 5-FU has displayed effects on the cell cycle during treatment in other cell lines [205, 229]. Further, in our previous studies, 5-FU activates the DNA damage response akin to doxorubicin and displayed a dose-dependent decrease in cell proliferation in the Ls174T colon carcinoma cell line. Moreover, statistical analyses showed a difference in the cell proliferative responses of the three RKIP expressing cell lines following 5-FU treatment. Therefore 5-FU was selected as a positive control for this set of experiments.

3.2.1 The effect of RKIP on the cell cycle in Ls174T colon carcinoma cells treated with 5-fluorouracil (5-FU)

Due to its routine employment in the treatment of colorectal carcinomas [204] and its role in the modulation of cell proliferation, 5-FU was chosen as a positive control for FACS analysis. Ls174T colorectal carcinoma cells with low, WT and high RKIP expression levels as described previously were treated with 25 μM 5-FU. Cell replication states within the cell population were measured using a BD FACSCalibur™ and analysed with the CellQuest Pro software.

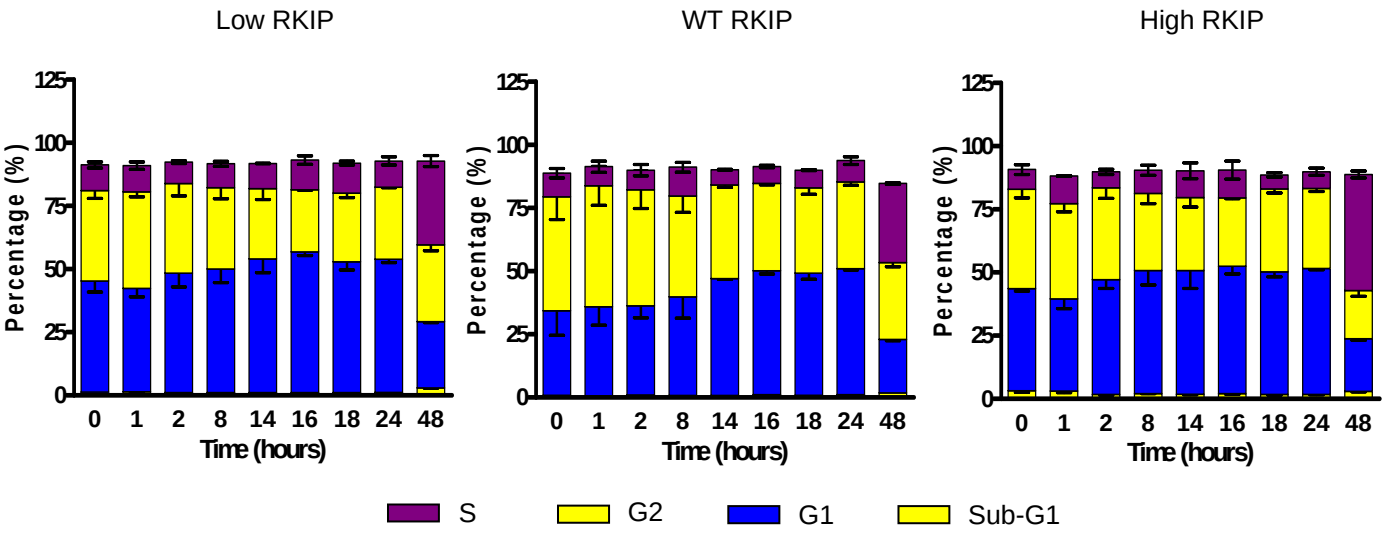


Figure 3.7: The effect of RKIP levels on cell cycle distribution of 5-FU-treated colon carcinoma cells. Ls174T colon carcinoma cells with low, WT and high RKIP expression were treated with 25 μM 5-FU over a period of 48 hours, the cells were then prepared and labelled with propidium iodide for FACS analysis and the cell cycle stage of each population determined, (n = 2). Cells were grouped into four specific stages in the cell cycle (S, G2, G1, sub-G1) based on the level of fluorescence scattered.

Treatment of Ls174T cells expressing varying levels of RKIP with 5-FU over a period of 48 hours showed few changes in the cell cycle dynamics. The low and high RKIP cells did show slightly different proportion of cells in the S-, G1- and G2-phase compared with WT RKIP cells (*e.g.* at 24 hours; low RKIP S average 10.3 %, G1 average 52.8 %, G2 average 28.5 %; WT RKIP S average 8.4 %, G1 average 49.9 %, G2 average 34.3 %; high RKIP S average 6.6 %, G1 average 49.9 %, G2 average 31.7 %). In addition, WT RKIP-expressing

cells appeared to have a slightly larger percentage of cells in the G2-phase and lower percentage of cells in the S- and G1-phases than low and high RKIP-expressing cells (Figure 3.7). Additionally, low and high RKIP cells appeared to plateau at G1 from about 2-8 hours onwards, in comparison to WT RKIP cells which displayed a plateau from 14 hours. This suggests that the low and high RKIP cells have similar cell cycle kinetics, why this is so is discussed as follows.

MAPK/ERK, and to some extent, NF-κB signalling has many effects on the cell cycle from checkpoint signalling to cell cycle arrest thus an endogenous inhibitor of these pathways, such as RKIP, may affect this regulation. ERK signalling in the cell cycle is multifaceted and highly complex (reviewed in [230, 231]); NF-κB appears to exert effects on cell cycle progression by acting at the G1-S phase transition through its actions on cyclins D1 and D2 [232, 233].

In terms of the results observed, activation of ERK is sometimes necessary for the G1-S phase transition [231, 234, 235]. ERK requirement for G1-S progression may explain the increase in G1-phase of cells with high RKIP-expressing cells; if RKIP inhibits ERK activity then the cells may have been unable to progress to the S-phase hence the accumulation of high RKIP-expressing cells in the G1-phase. Further, NF-κB activity is required for G1-S phase transition *via* its activation of cyclin D1 [232, 233], and inhibition of NF-κB by RKIP may explain the increase in high RKIP-expressing with higher G1 percentages.
In contrast, for the low RKIP-expressing cells there was likely to be MAPK pathway hyper-activation due to the lack of RKIP inhibition. MAPK hyper-activation can result in cell cycle arrest *via* induction of the cyclin dependent kinase (CDK) inhibitor p21 [236-238].
Thus it is possible that MAPK signalling is a double-edged sword in cell cycle progression; RKIP may act to keep this balance in place, and thus affect cell cycle progression through its effects on the MAPK pathway. There may also have been some NF-κB modulation, although this is unlikely due to the overwhelming MAPK effect.

There also appeared to be a sharp increase in the percentage of cells in S-phase at 48 hours, particularly in the high RKIP-expressing cells. Due to false incorporation of 5-FU into the DNA, the cells were beginning to arrest and activate the DNA repair machinery, hence the accumulation of cells in S-phase at the 48 hour time-point – this is a characteristic of 5-FU

treated cells [205]. Moreover, induction of the DNA damage response concurred with the dose-dependent decrease observed in the cell proliferation assay for 5-FU-treated cells. High RKIP-expressing cells displayed a larger proportion of cells in the S-phase at the 48 hour timepoint thus there may have been an effect of RKIP on the DNA damage response. The reasons as to why this occurred are speculative.

As mentioned previously, MAPK/ERK signalling has many cell cycle effects from checkpoint signalling to cell cycle arrest, thus an endogenous inhibitor of ERK signalling such as RKIP may affect this regulation. In terms of the results observed, activation of ERK is sometimes necessary for the G2-M phase transition [231, 234, 235]. ERK requirement for G2-M progression may explain the increase in S-phase arrest in cells with high RKIP-expressing cells; if RKIP inhibits ERK activity then the cells may have been unable to progress to the G2-M phase hence the accumulation of high RKIP-expressing cells in the S-phase and the decrease in G2 cells. Indeed, Chapter 5 which displays phosphorylated ERK western blots show that increasing RKIP leads to a decrease in phosphorylated ERK.

However a study by Zhu *et al.* found that modulation of the anti-apoptotic protein Bcl-xl led to changes in the sensitivity of DLD1 colon cancer cells to 5-FU treatment [239]. Bcl-xl has been found to be down-regulated in cells sensitised to chemotherapy as a result of RKIP reintroduction [183, 184], thus there is already evidence of a Bcl-xl and RKIP interaction. It is possible that this interaction is mediated *via* RKIP inhibition of the NF-κB pathway [183, 184]. It is possible that a similar event may have occurred in this study.

Unfortunately, in this study, the Ls174T colon carcinoma cells were not synchronised prior to treatment therefore the variations observed may in part be explained by the cells being in different stages of the cell cycle. Further investigation is essential in order to elucidate the functions of RKIP on the cell cycle under normal conditions, before strong conclusions can be made on RKIP effects under cell stress conditions.

3.2.2 The effect of RKIP on the cell cycle in Ls174T colon carcinoma cells treated with doxorubicin

Cells treated with doxorubicin displayed a strong dose-dependent decrease in cell proliferation. Furthermore, initial cell proliferation assays showed that altering the levels of RKIP affected the dose-dependent decrease in cell proliferation following doxorubicin treatment; therefore cells treated with this agent were selected for investigation by FACS analysis. Ls174T colon carcinoma cells with low, WT and high RKIP expression levels were treated with doxorubicin and cell replication states within the populations measured as described previously.

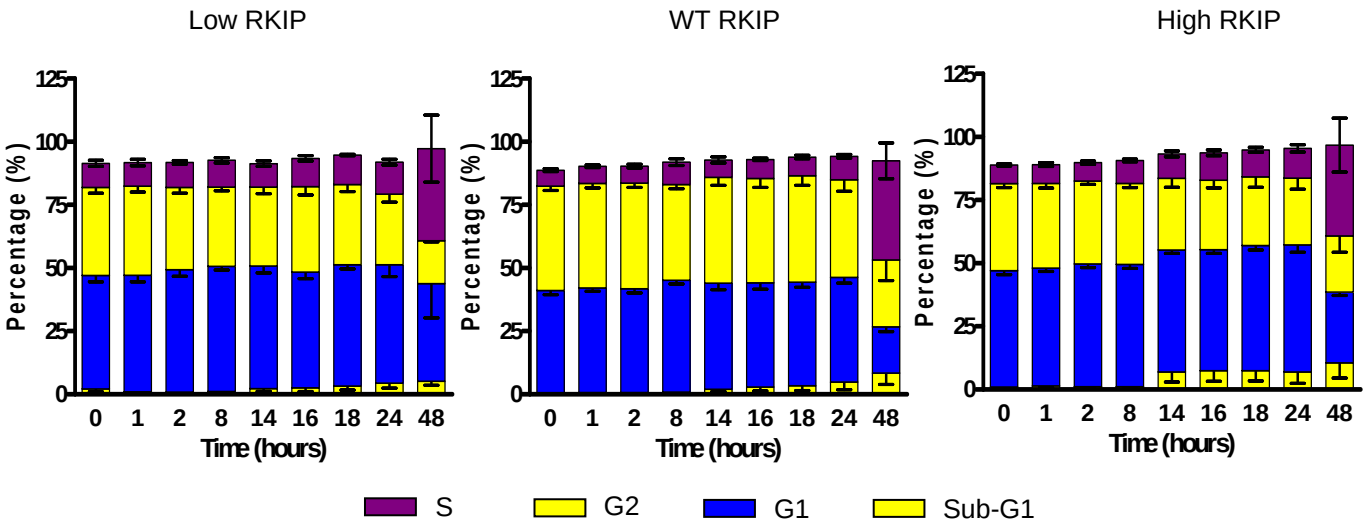


Figure 3.8: The effect of RKIP on cell cycle distribution of doxorubicin-treated Ls174T colon carcinoma cells. Ls174T colon carcinoma cell lines with low, WT and high RKIP expression were treated with 5 μM doxorubicin over a period of 48 hours were labelled with propidium iodide and subjected to FACS analysis (n = 6). Cells were grouped into four specific stages in the cell cycle (S, G2, G1, sub-G1) based on the level of fluorescence scattered.

The three Ls174T colon carcinoma cell lines with low, WT and high RKIP expression treated with doxorubicin showed very small differences in the distributions of cell populations in the stages of the cell cycle that were selected for analysis (Figure 3.8). There appearred to be slight differences in the proportion of cells in the S-, G2- and G1-phases; WT RKIP-expressing cells had a higher percentage of cells in G2- (average 38.6 %) and lower in the G1- (average 41.4 %) and S-phases (average 9.33 %) compared to the

low (average G2 27.9 %, G1 46.9 %, S 12.7 %) and high RKIP-expressing (average G2 26.5 %, G1 50.3 %, S 11.7 %) cells. A similar result was observed in the 5-FU treated cells (Figure 3.7).

As discussed previously, the reasons for this were likely to be due to the effects of the ERK and NF-κB pathways on cell cycle progression [230-235, 237] - most notably the ERK pathway - and RKIP modulates both these signalling cascades [143, 144, 148]. The low and high RKIP-expressing cells displayed similar cell cycle profiles suggesting that; yet again, that ERK signalling requires a delicate balance and that this balance must be maintained. Hyper-activation of ERK signalling as a result of low RKIP levels within the cells leads to cell cycle arrest *via* p21 [236, 238]; too little ERK as a consequence of over-expressed RKIP leads to failure of the G1-S transition [231, 234, 235]. Furthermore, high RKIP cells may also inhibit NF-κB mediated G1-S transition [232, 233].
A sharp increase in the proportion of cells in the S-phase was observed in all three RKIP-expressing cells after 48 hours doxorubicin treatment (Figure 3.8). As with the observations made for Ls174T cells treated with 5-FU (Figure 3.7), this was probably due to the actions of the doxorubicin itself. Doxorubicin is a multifarious cytotoxic agent with particular efficacy in the S-phase [240, 241], thus disruption of DNA synthesis would lead to an accumulation of cells in this cell cycle stage as the cells attempt to overcome the damage inflicted by the chemotherapeutic treatment. This cell cycle arrest in the S-phase concurs with the dose-dependent decrease in cell proliferation observed previously for Ls174T colon carcinoma cells treated with doxorubicin (Figure 3.5).

3.2.3 The effect of RKIP on the cell cycle in serum-starved Ls174T colon carcinoma cells treated with foetal bovine serum

A control experiment was set up whereby the three low, WT and high RKIP-expressing cell lines were starved in 0.1 % foetal bovine serum (FBS) for 24 hours then stimulated using 20% FBS. The cell population was analysed using FACS as before.

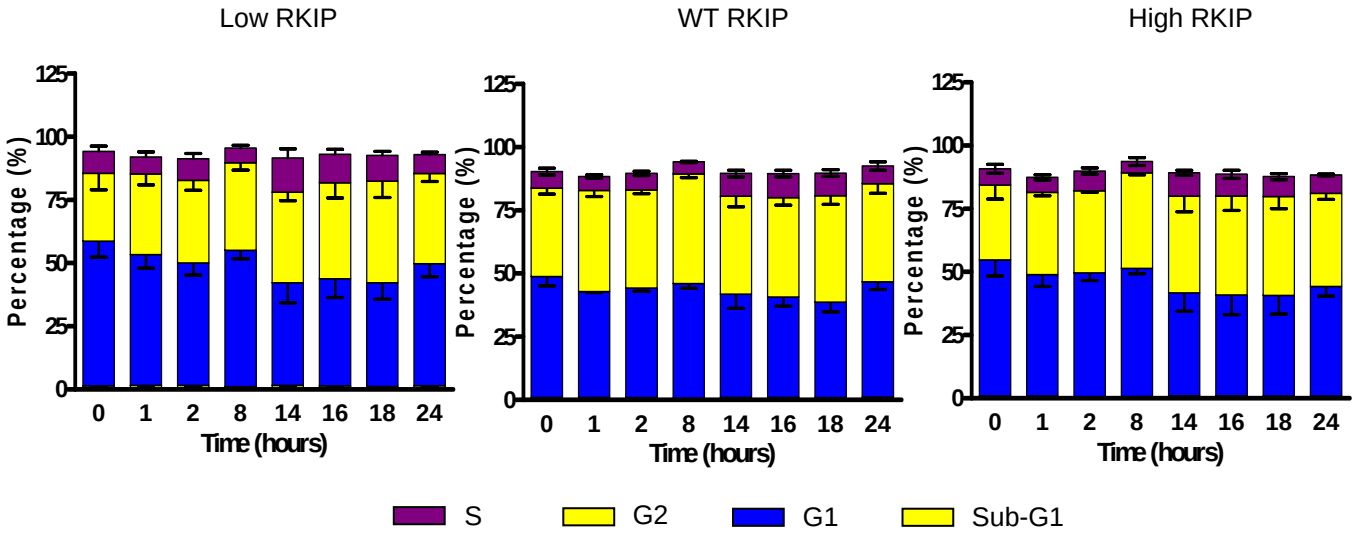


Figure 3.9: The effect of RKIP on cell cycle distribution of Ls174T colon carcinoma cells treated with foetal bovine serum. with low, WT and high RKIP expression were starved for 24 hours in 0.1% serum then treated with 20% FBS over a period of 48 hours, the cells were then prepared and labelled with propidium iodide for FACS analysis and the cell cycle stage of each population determined, according to fluorescence intensity (n = 3).

Treatment of low, WT and high RKIP-expressing cell lines with FBS resulted in very similar results. There were no prominent changes throughout the 48 hour time-course experiment, excepting a small difference in G1 and S phase at 8 and 24 hours that was observed in all three cell lines, and is likely to be a consequence of the cell synchronisation. It is possible that RKIP did not exert any cell cycle related effects under normal growth conditions within this Ls174T colon carcinoma cell line. Therefore, it is likely that any changes observed after chemotherapeutic treatment would have been a result of the drug administration, and not due to the effect of the level of RKIP on the cell cycle.

The lack of changes observed for cell cycle dynamics in the three RKIP-expressing cell lines – low, WT and high – may have been due to RKIP being unphosphorylated within these cells. RKIP is phosphorylated by protein kinase C (PKC) on serine 153 [149]; this has been shown to change RKIP function from inhibition of Raf-1 to inhibition of GRK2 [150]. This phosphorylation is is also required for RKIP localisation to centrosomes and kinetichores in mitotic cells [180]. This switch does not occur under basal conditions thus the use of TPA or another PKC inducer may result in a clearer response of the cell to the levels of RKIP. On the other hand, it is possible that the effect of RKIP on the cell cycle

may be limited to cells undergoing mitosis, be restricted to cells subject to treatment with paclitaxel or the response may be be cell-type specific.

3.2.4 Discussion of FACS Results

From the results above it could be seen that changing the RKIP levels did not noticeably affect the cell cycle dynamics of the Ls174T colon carcinoma cell line after treatment with doxorubicin or 5-FU over 48 hours. This contradicted our proposed hypothesis.
Moreover, serum-starved cells treated with FBS over a 48 hour time-course did not display any changes in cell cycle distribution as a consequence of the levels of RKIP in the Ls174T colon carcinoma cells. This too was in disagreement with our hypothesis.

There may have been small changes in the proportions of cells in the G1, S and G2 stages; with the high and low RKIP-expressing cells behaving in a similar manner compared to the WT RKIP-expressing cells. The data suggests that the maintenance of the balance of ERK signalling (and possibly to a lesser extent NF-κB) was optimal for cell cycle progression. Down-regulation of RKIP resulting in the hyper-activation of ERK signalling can cause cell cycle arrest *via* a p21-mediated mechanism [237, 238]. Over-expression of RKIP resulting in decreased MAPK/ERK signalling can prevent G1-S phase transition; this is also the case for RKIP inhibition of the NF-κB pathway that is also required for G1-S transition [232, 233]. These effects are best displayed during 5-FU treatment therefore RKIP effects on the cell cycle may be more prominent *in vivo* or during periods of cell stress.

Both 5-FU- and doxorubicin-treated Ls174T colon carcinoma cells displayed an increase in the proportion of cells in the S-phase after 48 hours treatment. This may be due to the cell cycle arrest as the cell attempted to repair the damage inflicted by the chemotherapeutic agents 5-FU and doxorubicin; both of which are highly effective in the S-phase of the cell cycle [205, 228, 229, 242]. Activation of the DNA damage response and cell cycle arrest by these agents corroborated with the dose-dependent decrease in cell proliferation observed previously with the alamarBlue assay (Figures 3.2 and 3.5).
On the other hand, investigation of the proportion of cells in S-phase using the technique above may have been adversely affected by the treatments employed, and thus may not have been accurate [226]. FACS analysis allowed a snap-shot of the cell cycle distribution

of the cells in a population; the cells may have arrested in S-phase or were still synthesising DNA [243]. Therefore future experiments would should perhaps employ bromo deoxyuridine (BrdU) staining to allow more detailed analysis of S-phase [244].

Unfortunately, there may also have been a number of possible experimental issues that may explain the lack of observable changes in this study.

1. From the sample preparation perspective, attempts were made to optimise the experimental procedure using different drug concentrations. It is possible that if the concentrations were too low there would be no observable changes or if the drug concentration too high, that the cell number was insufficient for complete analysis.

2. Aside from the serum-starved cells treated with FBS, there was no synchronisation of the cells to a particular stage in the cell cycle; this may account for the large variation between samples with cells undergoing analysis at different stages of the cell cycle. Cell synchronisation is often employed within FACS analysis in order to measure progression through the cell cycle [244]. However synchronisation was deemed unsuitable prior to chemotherapeutic treatment as it was thought that this may stress the cells too much and produce erroneous results. Further, the FBS-treated "control" cells were synchronised, and did not display changes in the cell cycle profile as a consequence of changing the RKIP levels within the cells.

3. Heterogenous uptake of propidium iodide by the different samples may also have led to varying results [226]. However, since clone populations of cells were used it was likely that was not as much an issue as with other cell lines *e.g.* MCF7, HCT-116 and HeLa that displayed higher variations in response following cell proliferative assays.

4. Finally loss of samples due to aggregates interfering with uptake and measuring within the instrument may have contributed to the variation within the data set and the lack of changes observed; particularly in the later time points as many of the cells began to experience cell arrest and possibly, cell death.

However despite experimental issues with FACS analysis and the lack of changes, the data we did observe corroborated with the cell proliferation studies, that drug administration resulted in cellular arrest. In addition, the RKIP levels did not appear to change the

response of the cells to the cytotoxic treatment. Using this experimental data, it is not possible to determine whether RKIP has a role in the modulation of cell proliferation. This could be due to the lack of RKIP involvement within these stages of cell proliferation. The cytotoxic agents employed are most effective in the DNA synthesis/S-phase of the cell cycle and RKIP has been shown only to affect mitotic cells [180].
Finally, the control experiments with serum-starved cells did not show any observable changes in the cell cycle dynamics with varying RKIP expression; even in the G2-M phase. This could be explained by the requirement of RKIP to be phosphorylated before it could localise to the centrosomes and kintechores in mitotic cells and thus regulate their progression [180].

In summary the FACS analysis concurred with the data shown for cell proliferation that small changes may be occurring as a consequence of the levels of RKIP within the cell. However this may be a by-product of a greater effect on cell death. It is important that apoptotic assays be used to study whether the levels of RKIP are involved in the modulation of the cell death response of Ls174T cells after treatment with cytotoxic agents.

3.3 The effect of RKIP on chemotherapy-induced cell death

As the levels of RKIP in Ls174T cells did not show a response in the FACS analysis and the cell proliferative assays, it was hypothesised that the cellular response being monitored was inappropriate. The aim of cytotoxic therapy is to induce cell death; all cytotoxic agents from the conventional to the targeted are specifically designed to initiate apoptosis. Further, previous studies in other cell lines have shown sensitisation of cells to chemotherapy-induced apoptosis in the presence of RKIP. Therefore, the aim of this study was to investigate the modulation of chemotherapy-induced apoptosis by RKIP in the Ls174T colon carcinoma cell line using a variety of chemotherapeutic agents.

The cytotoxic agents employed in the previous study - 5-FU, doxorubicin, etoposide and TRAIL – were investigated, in addition to cisplatin, FasL and paclitaxel.

Cisplatin is one of the oldest chemotherapeutic agents and is a classic DNA cross-linker [48]. It is also strongly associated with activation of DNA damage proteins and the MMR complex [72]. Cisplatin resistance is becoming increasingly common in clinical studies, thus novel ways to overcome this resistance are required. Cisplatin has also been shown to induce the expression of RKIP in a prostate cell line but not in a breast cancer cell line [181]. Hence better understanding the role of RKIP in apoptosis modulation, and determining whether this response is cell-type specific, has important consequences for anti-cancer regimes.

Fas ligand (FasL) is a strong death receptor agonist from the same TNF family as TRAIL. Unlike TRAIL, FasL does not show selectivity for cancer cells [67, 79]. Since RKIP has been shown to modulate TRAIL-mediated apoptosis in cancer cells [184], a comparison with between FasL and TRAIL treatments would be highly interesting.

Paclitaxel (or Taxol) is an anti-mitotic drug; paclitaxel stabilises microtubules thereby preventing the cytoskeleton restructuring, a process essential for mitosis and cell division [55]. RKIP has been shown to regulate mitotic progression and depletion of RKIP overrides the checkpoint induced by spindle poisons such as paclitaxel [180]. Furthermore,

RKIP-induction by rituximab is thought to sensitise Ramos and Daudi B-cells to paclitaxel treatment [182], hence the selection of this cytotoxic agent for investigation in the Ls174T colon carcinoma cell line.

All chemotherapeutic agents employed in this study initiate apoptosis in some manner – either by the external apoptotic pathway or by the mitochondrial cell death pathway. Both of these pathways have been described in detail in the Introduction. FasL and TRAIL activate the external apoptotic machinery as they both act upon death receptors present on the surface of cells. The other cytotoxic agents induce cell stress and/or DNA damage which, after failed repair attempts, will initiate the mitochondrial programmed cell death machinery.

The Caspase-Glo 3/7 assay from Promega was used as an indicator of apoptosis in the Ls174T colon carcinoma cell lines after treatment with various chemotherapeutic drugs. Both extrinsic and intrinsic apoptotic pathways converge on the activation of caspase 3 hence the selection of this caspase assay. The assay involves a luminogenic substrate containing the DEVD sequence recognized and cleaved by caspases, which upon degradation by said caspases forms amino-luciferin resulting in the luciferase reaction and the production of light. As the luminescent intensity is proportional to the caspase activity present in the cells, apoptosis can be quantified and compared between biological samples.

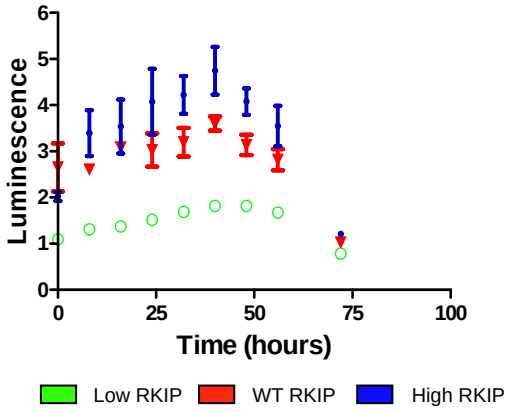
In the following results, the level of luminescence, as measured using a luminometer, after administration of cytotoxic agents to the Ls174T colon carcinoma cells was compared to the untreated cell control, with the untreated value for all three cell lines set at 1. This was possible due to the three cell lines displaying no significance differences in cell death for the untreated controls. The normalisation of the treated cells to the untreated cell control allowed collation of multiple data sets in a single graph.

3.3.1 The effect of RKIP on cell death in Ls174T colon carcinoma cells treated with death receptor agonists

3.3.1.1 Tumour Necrosis Factor-related Apoptosis-Inducing Ligand (TRAIL)

TRAIL-induced apoptosis has been shown to be modulated by RKIP in prostate and melanoma cell lines [184], therefore Ls174T colon carcinoma cells with low, WT and high RKIP expression levels were treated with TRAIL over a period of 72 hours in order to determine the optimum incubation time to achieve maximum caspase activation. The caspase 3/7 activity of the cells was measured using the luminescent assay described above.

a)



b)

Statistical Analysis	Source of variation	p-value
Two-way ANOVA	RKIP levels	<0.0001
	Time (hours)	<0.0001
Wilcoxon signed-rank test	Low VS WT RKIP	<0.0001
	Low VS High RKIP	<0.0001
	WT VS High RKIP	0.0001

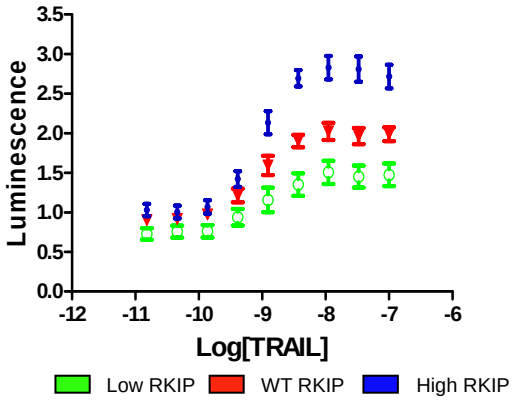
Figure 3.10: The effect of RKIP on caspase 3/7 activity of TRAIL-treated colon carcinoma cells over 72 hours. a) Ls174T colon carcinoma cells with low, WT and high RKIP expression were treated with 100 nM TRAIL (n=3) over a period of 72 hours, after which the caspase activity of the cells were then measured using the caspase 3/7-Glo assay. **b)** Table of statistics displaying the effect of RKIP on caspase 3/7 activity in Ls174T cells over a 72 hour time-course of TRAIL administration.

Maximum experimental caspase activity was achieved after 48 hours of TRAIL treatment (Figure 3.10). In this preliminary study, there appeared to be a difference in sensitivity to

TRAIL treatment; the high RKIP-expressing cells were more sensitive to TRAIL treatment followed by the WT and finally the low RKIP-expressing cells (Figure 3.10). Two-way ANOVA analysis of the biological data showed that both the RKIP levels and the TRAIL incubation time had a significant effect on cell death. Moreover, multiple comparisons showed all three RKIP cell lines to have significantly different cell death responses over the time-course treatment with TRAIL (Figure 3.10).

Based on the time-course experiments the RKIP-expressing Ls174T colon carcinoma cells were treated with a range of TRAIL concentrations for 48 hours to generate log dose-response curves.

a)



b)

Statistical Analysis	Source of variation	p-value
Two-way ANOVA	RKIP levels	<0.0001
	[TRAIL]	<0.0001
Wilcoxon signed-rank test	Low VS WT RKIP	<0.0001
	Low VS High RKIP	<0.0001
	WT VS High RKIP	<0.0001

Figure 3.11: The effect of RKIP on the caspase 3/7 activity of TRAIL-treated cells.
a) TRAIL (n=6), with a concentration range of 100 nM (log -7) to 10 pM (log -11), was administered to Ls174T colon carcinoma cells with low, WT and high RKIP expression for 48 hours; the caspase activity of the cells was then measured using the caspase 3/7-Glo assay. **b)** Table of statistics displaying the effect of RKIP on TRAIL-induced apoptosis in colon cancer cells.

When RKIP-expressing cells were treated with more than 1 nM (log –9) TRAIL, the high RKIP-expressing cells were more sensitive to the drug followed by WT and finally low RKIP-expressing cells (Figure 3.11). Analysis of the biological data with a two-way ANOVA showed that both the RKIP levels and the TRAIL concentration had a significant effect on the cell death response of colon carcinoma cells (Figure 3.11). Wilcoxon signed-rank tests showed that the cell death responses of all three RKIP-expressing cell lines after

TRAIL treatment were significantly different to each other (Figure 3.11). As the only differences between the three cell lines were the levels of RKIP, it implied that the levels of this protein were responsible for the different patterns of behaviour observed in Figure 3.11.

Ls174T cells with down-regulated RKIP displayed increased resistance to TRAIL compared with high and WT RKIP-expressing cells. This was in accordance with the literature [151, 181, 182, 184], particularly the research by Chatterjee *et al.* [181] who found that prostate and breast carcinomas with RKIP down-regulated were insensitive to chemotherapy due to hyper-activation of both the Raf-1/ERK and NF-κB pathways. This is the first time that this insensitivity to chemotherapeutic treatment as a consequence of RKIP levels has been shown for a colorectal carcinoma cell line; which is particularly important as RKIP has been found to be down-regulated in many colorectal neoplasias [154, 245].

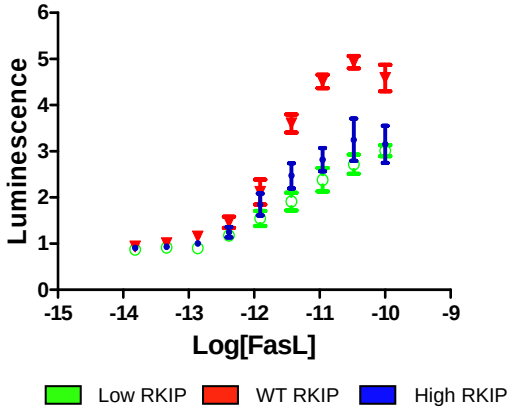
Furthermore, Ls174T treatment with TRAIL was most effective when cells expressed high levels of RKIP. This is understandable, considering that low levels of RKIP result in resistance to drug treatment [181]. This data also concurs with reviews that show that the reintroduction of RKIP into low RKIP-expressing and resistant cell lines results in an increased sensitivity of these cells to cytotoxic agents [181]. Moreover, a recent study by Baritaki *et al.* [184] displayed this susceptibility of high RKIP-expressing cells to TRAIL treatment in melanoma and prostate cancer cell lines. The mechanism by which increased RKIP conveyed sensitivity in these cell lines was *via* up-regulation of the death receptor DR5 and inhibition of the transcription repressor Yin Yang 1 (YY1) [184]. There were also negative effects on anti-apoptotic cell death effectors such as X-linked inhibitor of apoptosis (XIAP), c-FLICE inhibitory protein (c-FLIP) long, and Bcl-xL [184]. The mechanism by which over-expressed RKIP increased the efficacy of TRAIL treatment in the colorectal cell line could occur through similar pathways suggesting further avenues of research.

3.3.1.2 FasL

The result from TRAIL treatment was compared with another member of the tumour necrosis factor (TNF) family – FasL. Ls174T colon carcinoma cells with low, WT and

high RKIP expression were treated with a range of different FasL concentrations for 24 hours to generate log dose-response curves.

a)



b)

Statistical Analysis	Source of variation	p-value
Two-way ANOVA	RKIP levels	<0.0001
	[FasL]	<0.0001
Wilcoxon signed-rank test	Low VS WT RKIP	<0.0001
	Low VS High RKIP	0.00844
	WT VS High RKIP	<0.0001

Figure 3.12: The effect of RKIP on FasL-induced cell death in colon carcinoma cells. **a)** A range of FasL concentrations (100 pM (log -10) to 10 fM (log -14)) were administered to low, WT and high RKIP colon carcinoma cells (n=5) for 24 hours; thereafter the caspase activity of the cells were then measured using the caspase 3/7-Glo assay. **b)** Table of statistics showing the effect of RKIP on cell death in FasL-treated Ls174T cells.

Ls174T cells expressing WT levels of RKIP were more sensitive to treatment with FasL than either the low or the high RKIP expressing cells (Figure 3.12). Two-way ANOVA analysis showed that both the levels of RKIP and the concentration of FasL had significant biological effects on the Ls174T cells. In addition, multiple comparisons showed the cells expressing different levels of RKIP resulted in different cell death responses in the three cell lines (Figure 3.12). This is the first demonstration of RKIP-modulation of FasL-induced apoptosis in a neoplastic cell line.

The TRAIL and FasL-treated cells displayed conflicting sensitivity to RKIP treatment although they come from the same TNF family. High RKIP-expressing cells were most sensitive to TRAIL-induced apoptosis whereas WT RKIP cells were most sensitive to FasL-induced apoptosis.

FasL is a member of the same family of ligands as TRAIL – the TNF-α family; therefore the differing results are highly unexpected. The observed increase in sensitivity of WT RKIP cells, compared to low RKIP cells, following FasL treatment concurred with studies that showed that reintroduction of ectopic RKIP in neoplasias with down-regulated RKIP showed increased sensitivity to chemotherapy when previously resistant [181]. Low RKIP-expressing Ls174T cells would possess hyper-activated NF-κB pathways leading to chemo-resistance [96-98, 246, 247], and the decreased sensitivity to FasL observed in Figure 3.12. It would be of interest to see whether the increased expression of RKIP (by means of transiently transfected RKIP expressing plasmids) in low RKIP-expressing cells treated with FasL reinstates the apoptotic phenotype. This would confirm the RKIP-dependent phenotype in these cells.

High RKIP-expressing cells treated with FasL displayed a curious result. Previous studies have shown that introduction of RKIP into low RKIP-expressing cells resulted in a sensitivity to chemotherapy-induced apoptosis [181]; and in this study, high RKIP-expressing cells were more sensitive to TRAIL-induced apoptosis [184]. Why then, in this study do WT RKIP cells show a higher susceptibility to FasL treatment and high RKIP-expressing cells to TRAIL treatment? This question could be partly explained by Tang *et al.*[163]; this group demonstrated a scaffold protein role for RKIP in the activation of the NF-κB pathway. Both the over-expression and the knockdown of RKIP resulted in decreased phosphorylation of IκB. Further, RKIP was shown to associate with the tumour necrosis factor receptor-associated factor 6 (TRAF6) [163]. TRAF6 belongs to a class of adaptor proteins called TRAFs of which there are six proteins (TRAF1-TRAF6); the function of these proteins are to couple receptors of the TNF-α family to intracellular signalling cascades, reviewed extensively in the following references [23, 24, 26, 248].

Both Fas and TRAIL receptor signalling recruit the adaptor protein Fas-associated protein with death domain (FADD) which induces the death response; however FADD can also interact with RIP, TRAFs and TRADD that can signal to the NF-κB pathway [29, 208-211, 249]. In TRAIL signalling, a secondary complex of FADD, TRAF2 and RIP has been shown [249]. On the other hand, Fas signalling to TRAF6 has been shown *via* Fas-associated factor 1 (FAF1), an adaptor similar to FADD [250]; Fas can also interact with RIP and other less well characterised adaptors [22, 29].

The differences in TRAF adaptor proteins recruited to the individual receptors and their effects on downstream signalling pathways, most notably the NF-κB pathway, could explain the contrasting response. Indeed, the scaffold-like response of RKIP shown in

these results after FasL treatment (Figure 3.12) may be due to TRAF6 and NF- κB pathway interactions and warrants further investigation.

On a similar note, the NF-κB pathways are renowned for their anti-apoptotic function. However more and more studies are unearthing a pro-apoptotic function for this pathway – reviewed in the following articles [251, 252]. NF-κB activation is essential for apoptosis induced by certain DNA-damaging agents [253-255]; which in turn can lead to up-regulation of the Fas ligand [255, 256]. Furthermore, up-regulation of the Fas receptor by NF-κB has been demonstrated post-adenoviral infection in mice [257].
There are two NF-κB binding sites on the Fas ligand promoter [255, 258]. In addition, binding sites on the Fas receptor have been shown to bind p65-p50 subunits leading to up-regulation of the Fas receptor [259]. Additionally, Fas-mediated cell death as a result of NF-κB up-regulation of Fas ligand expression has been demonstrated in a colon carcinoma cell during cell stress [253]. The Ls174T cells are of colorectal origin thus NF-κB activation could be essential for Fas-mediated cell death in this cell line.

NF-κB activation following doxorubicin treatment has also been shown to be dependent on TRAF6 expression [256] therefore investigation of the TRAF proteins after FasL and TRAIL treatment will be of high priority.

In summary, if NF-κB activation is required for FasL-induced apoptosis, down-regulation of NF-κB as a consequence of RKIP over-expression would explain the decrease in sensitivity to FasL-mediated apoptosis in the high RKIP-expressing Ls174T colon carcinoma cell lines. Why NF-κB activation would be required for FasL-induced apoptosis but not TRAIL-mediated apoptosis in the same cell line supports an observation by Lin *et al.* [260], that NF-κB activation can be both pro- and anti-apoptotic within a single cell type dependent upon the cell stimulus employed.

Further investigation of these cytotoxic compounds would explore differences in the expression of TRAF subtypes between FasL and TRAIL-treated cells. Differential utilisation of TRAF subtypes by death receptors may be responsible for the difference in sensitivity of Ls174T cells to treatment with FasL and TRAIL. Moreover, analysis of components of the NF-κB pathway following treatment with both drugs may help to unravel the mysteries behind their contrasting responses; and whether both drugs activate this pathway, and if they activate the NF-κB pathway in contrasting manners leading to

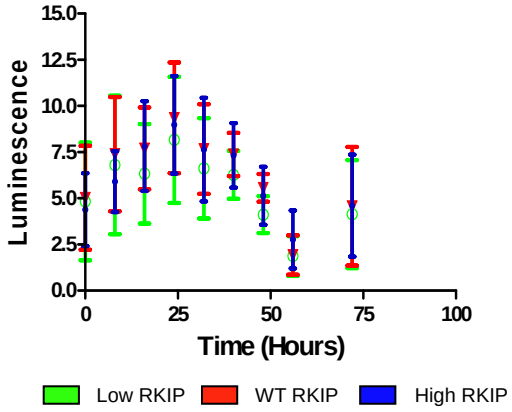
different cellular outcomes. Elucidation of the mechanism behind this response is crucial as this could have important implications in the treatment of cancer patients; if individuals have different RKIP protein expression levels this could influence the choice of drug administered.

3.3.2 The effect of RKIP on cell death in Ls174T colon carcinoma cells treated with cell stress-inducing chemotherapeutic agents

3.3.2.1 Doxorubicin

In a previous cell proliferation study of Ls174T cells after treatment with doxorubicin, we observed that the proliferative response of doxorubicin-treated Ls174T cells was not affected by the levels of RKIP levels in the cell (Figure 3.5). It is possible that the effect of RKIP may be limited to regulation of the apoptotic response, as previous studies have only linked RKIP with regulation of the apoptotic response during chemotherapy treatment. Thus Ls174T colon carcinoma cells with low, WT and high RKIP expression levels were treated with doxorubicin over a period of 72 hours, in order to determine the optimum incubation time to achieve maximum caspase activation. The caspase 3/7 activity of the cells was measured using the luminescence assay described above.

a)



b)

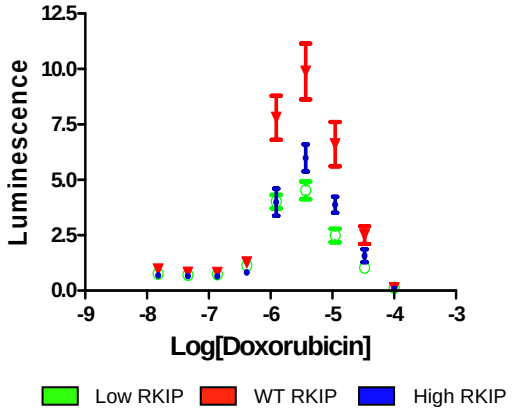
Statistical Analysis	Source of variation	p-value
Two-way ANOVA	RKIP levels	0.7745
	Time (hours)	0.0830
Wilcoxon signed-rank test	Low VS WT RKIP	N/A
	Low VS High RKIP	N/A
	WT VS High RKIP	N/A

Figure 3.13: Modulation of caspase 3/7 activity over 72 hours by RKIP in doxorubicin-treated colon carcinoma cells. a) Ls174T colon carcinoma cells with low, WT and high RKIP expression were treated with 3 μM doxorubicin (n=5) over a period of 72 hours, post-timecourse the caspase activity of the cells were measured using the caspase 3/7-Glo assay. **b)** Table of statistics displaying the effect of RKIP on doxorubicin-administered Ls174T cells over a 72 hour time-course.

The caspase activity in Ls174T cells treated with doxorubicin increased in the first 24 hours, reaching maximal activity at 24 hours and then decreasing again to 52 hours (Figure 3.13). The luminescence values were particularly high during treatment with doxorubicin indicating that this drug is more potent for the induction of cell death in comparison to many of the other treatments employed. A substantial number (≥75%) of cells visably were rounded up during treatment with this agent. Furthermore Figure 3.8 shows an increase in the proportion of sub-G1 cells from 16 to 48 hours during the treatment period. There was no significant difference between the cell lines expressing different levels of RKIP. This was corroborated by the two-way ANOVA analysis which did not show a significant difference between Ls174T cells expressing different levels of RKIP or between different times of doxorubicin treatment (Figure 3.13).

Following from the time-course study above; Ls174T colon carcinoma cells with low, WT and high RKIP expression were then treated with a range of doxorubicin concentrations for 24 hours to generate log dose-response curves.

a)



b)

Statistical Analysis	Source of variation	p-value
Two-way ANOVA	RKIP levels	<0.0001
	[doxorubicin]	<0.0001
Wilcoxon signed-rank test	Low VS WT RKIP	<0.0001
	Low VS High RKIP	0.688
	WT VS High RKIP	<0.0001

Figure 3.14: Modulation of caspase 3/7 activity by RKIP in doxorubicin-treated cells. **a)** Doxorubicin (n=5) (100 μM (log -4) to 10 nM (log -8)) was administered to Ls174T colon carcinoma cells with low, WT and high RKIP for 24 hours; after which the caspase activity of the cells was measured using the caspase 3/7-Glo assay. **b)** Table of statistical analyses showing the effect of RKIP on doxorubicin-induced apoptosis in colon cancer cells.

Doxorubicin treatment of Ls174T cells resulted in an increase in sensitivity between 1 μM and 30 μM (log -6 and log -4.5). The apoptotic activity was highest for the WT RKIP-expressing cells when compared to the low and high RKIP-expressing cells. (Figure 3.14). Two-way ANOVA showed both RKIP levels and doxorubicin concentration had a significant effect on cell death. Moreover, Wilcoxon signed-rank tests displayed significant differences in the cell death responses of WT RKIP-expressing cells compared to both low and high RKIP-expressing cells (Figure 3.14). There was no significant difference in the cell death response of the low versus the high RKIP-expressing cells.

In this experiment, we demonstrated that the RKIP levels within a neoplastic cell line modulated the response of colon cancer cells to doxorubicin treatment.

In comparison to the low RKIP-expressing cells, the WT RKIP cells were behaving in a manner consistent with previous studies that showed that the reintroduction of RKIP into cells with down-regulated RKIP resulted in the sensitisation of those cells to chemotherapy-induced apoptosis [181].The apoptotic response of the low RKIP-expressing cells to treatment with doxorubicin was lower than that of the WT cells and was likely due to over-activation of the ERK and NF-κB pathways as mentioned previously [181]. NF-κB

hyper-activation has been implicated in chemo-resistance to many drugs, including doxorubicin [97, 98, 165].

Akin to the FasL-administered cells, high RKIP-expressing cells were less sensitive to apoptosis in comparison to the WT RKIP-expressing cells. This result from the high RKIP-expressing cells was unexpected. The reasons as to why increased RKIP should convey resistance to doxorubicin are highly speculative.
One theory proposes that higher RKIP levels in the cells means that more protein is available for phosphorylation by PKC [149], resulting in RKIP-mediated GRK2 inhibition [150] and prolonged stimulation of ERK and other signalling pathways *via* cAMP. Indeed, many studies have shown that PKA can lead to activation of the ERK cascade in certain cell lines; not only *via* Raf-1 activation but also the other Raf isoforms, in particular B-Raf [261, 262]. However, if the Raf-1 isoform was activated in a manner that prevent its inhibition by RKIP this could also explain the observed resistance to doxorubicin; over-activation of Raf-1 kinase has been shown to confer a resistant phenotype to doxorubicin in MCF7 breast cells [263]. Thus, high RKIP levels could have conferred drug resistance to doxorubicin by preventing ERK pathway hyper-activation.

In contrast, the pro-apoptotic face of NF-κB and the involvement of Fas signalling could be responsible for the decreased sensitivity of high RKIP-expressing cells to doxorubicin-induced apoptosis. Doxorubicin treatment has been shown to induce IκB degradation leading to NF-κB activation resulting in, and being a requirement for, cell death [254, 264-268]. It is thought that this activation of the NF-κB pathway may occur *via* phosphorylation by PKC [254]. Moreover the p65 subunit activated upon doxorubicin treatment is transcriptionally inactive as a consequence of deficient post-translational modifications such as phosphorylation and acetylation [267]. This leads to down-regulation of anti-apoptotic genes commonly implicated in the role of NF-κB as anti-apoptotic, and confounds expectations that activation of NF-κB during chemotherapy treatment results in the up-regulation of pro-apoptotic genes [267].
This reliance of doxorubicin on NF-κB activation could explain the lack of cell death observed in the high RKIP-expressing Ls174T colon carcinoma cells in comparison to the WT RKIP-expressing cells. The high RKIP-expressing cells would have down-regulated NF-κB activity as a consequence of RKIP inhibition of this pathway.

Another possible explanation for the decreased sensitivity of high RKIP-expressing cells could be the reliance of doxorubicin on Fas signalling in order to exert its cytotoxic effects. Doxorubicin up-regulates the expression of the Fas receptor (CD95) and in some cases, the mRNA and protein expression of Fas ligand; these results have been displayed in several cell lines [83-86]. Induction of Fas would result in DISC formation and apoptosis by way of the external death pathway described in the Introduction. In our study, FasL administration displayed a similar increase in sensitivity of WT RKIP cells compared to low and high RKIP-expressing cells. Thus if doxorubicin utilised the Fas signalling pathway to exert its cytotoxic effects in the Ls174T colon carcinoma cell line, it is not surprising that a similar response was observed between doxorubicin and FasL-treated cells. If Fas signalling was employed by doxorubicin, it is possible that adaptor proteins which signal to the NF-κB pathway from the Fas receptor [21, 22, 29, 269] were responsible for the differences in sensitivity observed, as discussed previously.

It is important to note that the effect of RKIP modulation of doxorubicin-induced apoptosis was observed within a very narrow concentration range. In this colorectal cell line doxorubicin appeared to work within a narrow window, thus further analysis of doxorubicin modulation of RKIP would concentrate upon this therapeutic window.

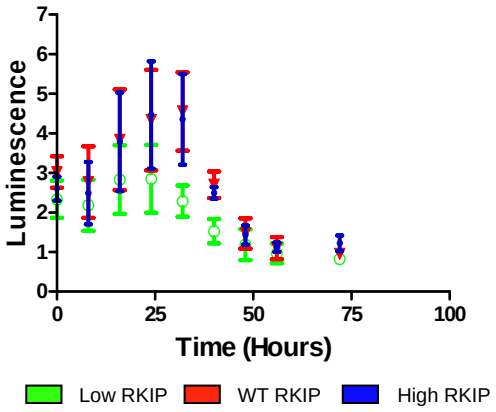
Realising that one protein has the potential to modulate the entire output of a drug's response is very exciting. Unravelling the mechanism behind the response of these cells to chemotherapy, in relation to RKIP, may shed light on the processes behind drug resistance and explain why some chemotherapy regimes fail in some individuals and not in others. Moreover, interrogation of the Fas-mediated cell death pathway may also provide answers to this puzzle since doxorubicin is thought to employ Fas signalling to exert its apoptotic effects.

3.3.2.2 Paclitaxel

Apoptosis-induced by the taxane drug paclitaxel has been shown to be modulated by RKIP [182]. In addition, RKIP depletion is thought to override mitotic checkpoint during paclitaxel administration [180]. Thus Ls174T colon carcinoma cells with low, WT and high RKIP expression levels as described above were treated with paclitaxel over a period

of 72 hours in order to determine the optimum incubation time to achieve drug-induced apoptosis, as measured by the caspase 3/7 activity of the cells.

a)



b)

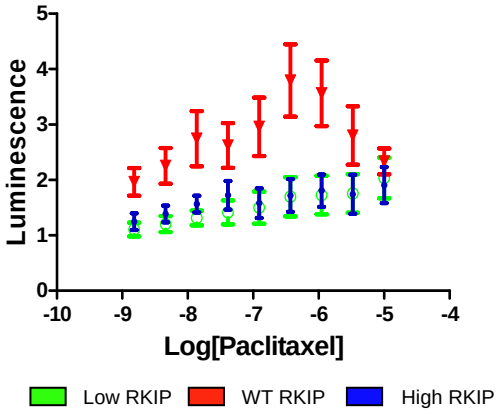
Statistical Analysis	Source of variation	p-value
Two-way ANOVA	RKIP levels	0.0221
	Time (hours)	<0.0001
Wilcoxon signed-rank test	Low VS WT RKIP	0.0758
	Low VS High RKIP	0.0933
	WT VS High RKIP	0.9550

Figure 3.15: The effect of RKIP on caspase 3/7 activity of paclitaxel-treated colon carcinoma cells over 72 hours. a) 10 μM paclitaxel was administered over a period of 72 hours (n=4) to low, WT and high RKIP-expressing colon carcinoma cells, thereafter the caspase activity of the cells was measured using the caspase 3/7-Glo assay. **b)** Table of statistics displaying the effect of RKIP on caspase activity in paclitaxel-treated Ls174T cells over a 72 hour time-course.

Paclitaxel treatment of Ls174T cells resulted in maximum caspase activity between 16 and 40 hours of treatment (Figure 3.15). The 36 hour time point was selected as an appropriate incubation time for future experiments. Statistical analyses showed that both the incubation time and the levels of RKIP in the cell significantly affected the cell death response of the colon carcinoma cells. Throughout the time-course it appeared that the response of the low RKIP-expressing cells was close to being significantly different to both WT and high RKIP-expressing cells (Figure 3.15a). This corroborated with other studies that showed that the reintroduction of RKIP into cells with down-regulated RKIP re-sensitised the cells to chemotherapy-induced apoptosis [181].

Ls174T cells with low, WT and high RKIP expression were then treated with a range of concentrations of paclitaxel for 36 hours to generate log dose-response curves.

a)



b)

Statistical Analysis	Source of variation	p-value
Two-way ANOVA	RKIP levels	<0.0001
	[paclitaxel]	0.0121
Wilcoxon signed-rank test	Low VS WT RKIP	<0.0001
	Low VS High RKIP	0.106
	WT VS High RKIP	<0.0001

Figure 3.16: RKIP modulation of paclitaxel-induced cell death in colon carcinoma cells. a) Ls174T colon carcinoma cells with low, WT and high RKIP expression were treated with paclitaxel (n=7) (10 μM (log -5) to 1 nM (log -9)) for 36 hours; after which the caspase activity of the cells was measured using the caspase 3/7-Glo assay. **b)** Table of statistics showing the effect of RKIP on paclitaxel-induced apoptosis in colon cancer cells.

Analysis of the biological data with a two-way ANOVA showed that the RKIP levels and the concentration of paclitaxel both had significant effects on the apoptotic response of the Ls174T cells (Figure 3.16). In addition, multiple comparisons showed that the cell death response of WT RKIP-expressing cells was significantly different from both low and high RKIP-expressing cells (Figure 3.16). The response of the low RKIP-expressing cells to treatment with paclitaxel was lower than that of the WT RKIP cells and would concur with previous reports suggesting over-activation of the ERK and NF-κB pathways are responsible [181]. Also hyper-stimulation of the NF-κB pathway can result in chemo-resistance to paclitaxel [165]; and also in the up-regulation of the Bcl-xl gene product, which leads to paclitaxel resistance in pancreatic cell lines [270]. Furthermore, depletion of RKIP has also been shown to override the mitotic checkpoint induced by paclitaxel [180] and results in the cellular resistance to this drug.

The chemo-sensitisation to paclitaxel-induced apoptosis observed in the study by Jazirehi *et al.* occurred after the restoration of down-regulated RKIP to WT levels [182]. Therefore, the paclitaxel-treated WT RKIP Ls174T colon carcinoma cells were behaving in a manner comparable to that observed by Jazirehi *et al.* [182].

In a method similar to doxorubicin and FasL-treated cells, the high RKIP-expressing cells were less sensitive to paclitaxel-induced apoptosis compared to WT RKIP-expressing cells. Why this was so, is very interesting.

RKIP sensitisation of Ramos and Daudi cells treated with paclitaxel was thought to be *via* down-regulation of the Bcl-xl gene product, as a consequence of NF-κB inhibition [182]. If this also occurred in the Ls174T colorectal cell line, over-expression of RKIP could actually lead to the opposite effect. A recent study by Tang *et al.* has shown that both down-regulation and up-regulation of RKIP result in decreased IκB phosphorylation and the resultant activation of NF-κB [163]. Thus the high RKIP cells could allow the up-regulation of the anti-apoptotic Bcl-xl protein by NF-κB activation. In short, chemo-sensitisation by RKIP as shown here for the first time in a colorectal cancer cell may be a double-edged sword.

On the other hand, RKIP effects on the ERK pathway may have been responsible for the results observed. Raf-1 suppressed chemotherapy-induced apoptosis in cervical neoplasias leading to paclitaxel insensitivity [271]. The Raf-1 isoform is subject to complex phosphorylation and regulation; thus if Raf-1 was activated in such a way that RKIP was unable to inhibit its function, this could explain why the high RKIP cells exhibited a decreased sensitivity to paclitaxel-induced apoptosis. Furthermore, as mentioned earlier, the excess RKIP may be phosphorylated by PKC, thus switching its function from inhibition of Raf-1 to inhibition of GRK2, leading to prolonged ERK pathway activation.

Alternatively, the hyper-activation of the ERK pathway is necessary for the induction of apoptosis in response to some chemotherapeutic drugs including cisplatin [272] and taxol [273]. Thus, high RKIP levels could have conferred drug resistance by preventing ERK pathway hyper-activation.

Similarly NF-κB activation has been shown to mediate paclitaxel-induced apoptosis [254, 274-277], thus high RKIP-expressing cells could prevent apoptosis as a result of NF-κB pathway inhibition; if RKIP was behaving as an NF-κB inhibitor originally described by Yeung *et al* [148] and not in a scaffold-like manner. As discussed in the doxorubicin and Fas sections, NF-κB activation has been shown to be a requirement for cell death induced by a variety of chemotherapeutic agents [253-255]. Similar to other cytotoxic drugs, paclitaxel is thought to activate the NF-κB pathway by inducing the degradation of IκB [254, 274, 275, 277]. As seen with doxorubicin-treated colon carcinoma cells, the activation of NF-κB by paclitaxel may also involve phosphorylation by PKC [254].

Investigation of NF-κB signalling in Ls174T colon carcinoma cells would be of interest to determine the involvement of this pathway in paclitaxel-induced apoptosis.

While there is some evidence supporting paclitaxel-based induction of the Fas/CD95 system, it is hardly overwhelming [82, 87, 278]. Therefore in a manner similar to doxorubicin, Fas-induction may be a further explanation for the curious results displayed in the Ls174T colon carcinoma cell line treated with paclitaxel. As shown here, Fas signalling led to increased sensitivity of WT cells to chemotherapy-induced apoptosis compared to low and high RKIP cells. This is thought to be a result of secondary complexes, *via* TRAF adaptor proteins, being formed from the DISC resulting in modulation of the NF-κB pathway [21, 22, 29, 269]. Further understanding of Fas-mediated signalling and indeed its induction by paclitaxel, must be characterised first before conclusions can be drawn.

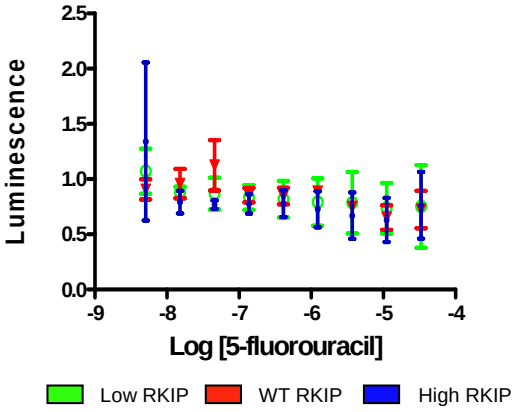
This was the first time that paclitaxel-induced apoptosis has been shown to be modulated by RKIP in a colon cancer cell line. Since this drug is known to be affected by RKIP in other cell lines [180, 182], it is imperative to understand the mechanisms behind this process. This may provide insight into cell-type specific functions of many drugs and also reveal common mechanisms that can be exploited to enhance cancer care and treatment.

3.3.3 The effect of RKIP on cell death in Ls174T colon carcinoma cells treated with DNA-damaging agents

3.3.3.1 5-fluorouracil (5-FU)

Cell proliferation studies with 5-FU showed a possible modulation of the cell proliferative response by RKIP after 5-FU treatment. To determine whether 5-FU treatment resulted in the apoptotic pathways, Ls174T colon carcinoma cells with low, WT and high RKIP expression were treated with a range of concentrations of 5-FU for 48 hours.

a)



b)

Statistical Analysis	Source of variation	p-value
Two-way ANOVA	RKIP levels	0.8804
	[5-FU]	0.4605
Wilcoxon signed-rank test	Low VS WT RKIP	N/A
	Low VS High RKIP	N/A
	WT VS High RKIP	N/A

Figure 3.17: The effect of RKIP on caspase 3/7 activity of cells treated with 5-fluorouracil. a) A range of 5-FU concentrations (n=2) (100 µM (log -4) to 10 nM (log-8)) were administered to low, WT and high RKIP-expressing Ls174T cells for 48 hours; post-incubation the caspase activity of the cells was then measured using the caspase 3/7-Glo assay. **b)** Table of statistical analyses showing the effect of RKIP on 5-FU-induced cell death in colon cancer cells.

All three RKIP-expressing cell lines did not show any differences in the caspase 3/7 activity nor any significant changes in the cell death response regardless of the levels of RKIP within the cell. This was corroborated by the statistical analysis of the biological data (Figure 3.17).

Ls174T colon carcinoma cell lines did not appear to undergo apoptosis when administered with 5-FU and this response was similar for all three RKIP – low, WT and high – cell lines. This was particularly worrying since 5-FU is routinely employed in the treatment of colorectal carcinomas [204]. However, when treated with 5-FU the Ls174T colon carcinoma cells displayed a dose-dependent decrease in cell proliferation. A dose-dependent decrease in cell proliferation with no observable effect on cell death suggests there may be drug-induced senescence occurring in this cell line during treatment with 5-FU.

It is becoming more and more apparent that many drugs act to arrest the cell cycle and/or halt proliferation of cells, instead of causing acute apoptosis (reviewed in [279-281]). This is particularly the case for DNA chelators and anti-metabolites such as 5FU and cisplatin

and could explain the lack of apoptosis displayed by the Ls174T colorectal cell line after treatment with this agent.

Senescence is still poorly understood. It is thought to be instigated by the p53 and p16 proteins [282] in response to activation of the DNA damage response network described previously. From the dose-dependent decrease in cell proliferation observed after 5-FU treatment it is likely that the Ls174T colon carcinoma cells have activated the DNA damage response. How and why the cells determined senescence, as opposed to apoptosis, as their fate is highly speculative. Senescence, like apoptosis, is thought to be a fail-safe mechanism when DNA and cellular repair cannot rectify damage [279, 281]. If the apoptotic response is compromised – for example, in a cancer cell – senescence is thought to be the next cellular option.

The Ls174T colon carcinoma cell line has been shown to induce senescence upon DNA damage with other agents [283], thus it is possible that 5-FU was inducing a similar response.

Moreover, the lack of cell death displayed by the Ls174T colon carcinoma cell line to treatment with 5-FU could also be explained in part by the unstable nature of microsatellites in the DNA of this cell line [284].

Microsatellites are short repetitive sequences present in large numbers throughout the human genome [285, 286]. During DNA replication microsatellites may form insertion-deletion loops (IDLs) leading to what is recognised as microsatellite instability (MI), usually formed from an underlying problem with the DNA MMR system [285, 286]. Normally the role of MMR is to rectify these errors of DNA synthesis alongside base-base mismatches; there are at least six MMR proteins required for efficient repair and a defect in even just one MMR protein can generate large MI leading to mutagenesis and eventually cancer [286-288].

Many colorectal carcinomas are MMR-deficient and display high frequencies of MI [173, 285, 288, 289]. In addition, cells with inefficient repair systems are more resistant to cytotoxic therapy than neoplasias with efficient repair pathways [288]. Moreover these cells may have accumulated additional mutations due to the lack of MMR, making them more transformed. Cancers that are MMR-deficient become accustomed or tolerant to DNA damage because they do not attempt to repair errors in their DNA; 5-FU works at the level of DNA synthesis and initiates DNA damage responses within the cell. Thus cells that are already DNA-damage tolerant are highly likely to be insensitive to these therapies.

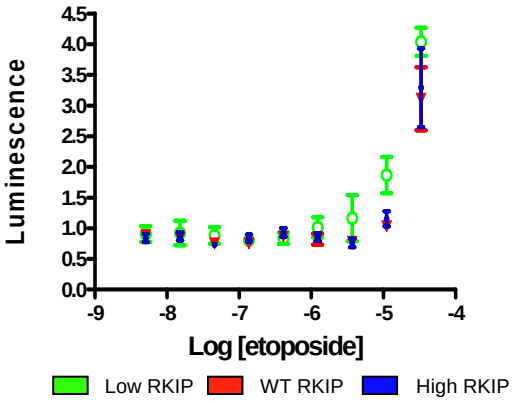
Indeed, studies have shown that the efficacy of 5-FU treatment is drastically reduced in microsatellite unstable cell lines [75, 245, 285, 289]. Hence the insensitivity of the Ls174T colorectal carcinoma cell line used in this project to 5-FU treatment may also be due to DNA-damage tolerance as a consequence of MMR-deficiency. Indeed the Ls174T colon carcinoma cell line has been shown to display microsatellite instability [284].

The discussion above has yielded some interesting routes for future investigation. Further characterisation of the Ls174T colon carcinoma cell line itself is essential to understand the effect of chemotherapeutic administration and more so, the effect of RKIP, following this treatment. Understanding the activation of the DNA damage response and subsequent determination of cell fate by proteins such as p53 will hopefully provide more answers to the results observed. Therefore an effect of RKIP on 5-FU treated Ls174T colon carcinoma cells cannot be ruled out.

3.3.3.2 Etoposide

Etoposide has been shown to induce RKIP expression [181] thus the investigation of RKIP modulation of etoposide-induced apoptosis in a colon carcinoma cell line was of significant interest. For this reason, Ls174T colon carcinoma cells with low, WT and high levels of RKIP expression were treated with a range of concentrations of etoposide for 48 hours to generate log dose-response curves.

a)



b)

Statistical Analysis	Source of variation	p-value
Two-way ANOVA	RKIP levels	0.0100
	[etoposide]	<0.0001
Wilcoxon signed-rank test	Low VS WT RKIP	0.01053
	Low VS High RKIP	0.03884
	WT VS High RKIP	0.87447

Figure 3.18: The effect of RKIP levels on etoposide-induced apoptosis in colon carcinoma cells. a) Colon carcinoma cells with low, WT and high RKIP expression were treated with etoposide (n=3), with a concentration range of 100 µM (log -4) to 10 nM (log -8), for 48 hours; post-incubation the caspase activity of the cells were then measured using the caspase 3/7-Glo assay. b) Table of statistics showing the effect of RKIP on the caspase activity of Ls174T cells administered etoposide.

The treatment of this cell line with a range of etoposide concentrations for 48 hours displayed no significant increase in caspase 3/7 activity unless overly high concentrations of the drug were administered (Figure 3.18). Two-way ANOVA however showed that the RKIP levels had a significant effect on the apoptotic response of the colon carcinoma cells, as did the concentration of etoposide, following etoposide administration. Finally, Wilcoxon signed-rank tests showed that low RKIP-expressing cell line had a significantly different cell death response following etoposide treatment to both the WT and high RKIP-expressing cells (Figure 3.18). However despite statistical significance of the biological caspase 3/7 data, etoposide treatment displayed few apoptotic effects unless very high drug concentrations were used (Figure 3.18). This corresponded with a dose-dependent decrease in cell proliferation observed with the alamarBlue assays (Figure 3.4). Since etoposide is a DNA damaging agent like 5-FU, it is likely that drug-induced cell senescence occurred in the Ls174T colon carcinoma cell line after administration of etoposide. Indeed, a study by te Poele *et al.* showed etoposide-induced senescence in the Ls174T colorectal carcinoma cell line, the cell line employed in this study [283].

On the other hand, etoposide activates the DNA damage response that is often inefficient or impaired in cancer cells, and the Ls174T colon carcinoma cell line has been shown to display microsatellite instability [284], which may explain the lack of cell death observed.

Etoposide has been shown to induce RKIP expression leading to cell death in a prostate cancer cell line but not in a breast cancer cell line [181]. Due to the less prominent effects of RKIP on etoposide-induced cell death modulation (seen in Figure 3.18) in the Ls174T colon carcinoma cell line, it was possible that other factors were affecting this response, *e.g.* the induction of senescence [283]. Thus far, RKIP has not been shown to have a role in senescence or senescence-related effects. The induction of senescence as a result of etoposide treatment may be an RKIP-dependent factor since the levels of RKIP were shown to have a statistically significant effect on the caspase 3/7 activity. An investigation into the effects of RKIP on cell senescence would be of interest. Particularly since research into drug-induced senescence and the RKIP protein is still poorly understood.

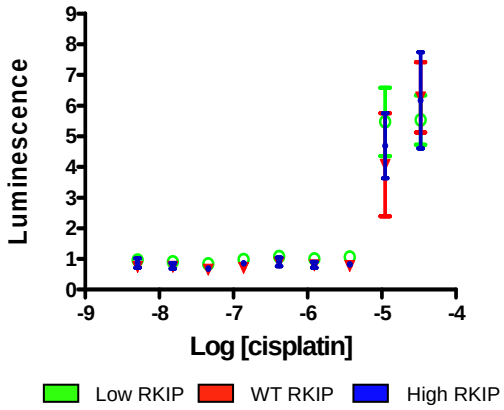
Finally, as discussed in the 5-FU results that showed a similar lack of apoptosis; etoposide may have reduced efficacy in the Ls174T colon carcinoma cell line due to aberrations in the MMR system resulting in the poor apoptotic response observed (Figure 3.18).

In summary, further elucidation of the mechanisms which govern cell fate and activate the DNA damage machinery are required before strong assertions can be made into the effect of RKIP on etoposide-treated Ls174T colon carcinoma cells. As yet, the role of RKIP in the fate of etoposide-treated cells is still open to investigation.

3.3.3.3 Cisplatin

Cisplatin is another DNA-damaging agent that has been shown to induce RKIP expression in a prostate cancer cell line but not a breast cancer cell line [181]. The investigation of the effect of RKIP on cisplatin-induced apoptosis in a colorectal cancer cell line was of interest. Ls174T colon carcinoma cells with low, WT and high RKIP expression were treated with a range of concentrations of cisplatin for 48 hours to generate log dose-response curves.

a)



b)

Statistical Analysis	Source of variation	p-value
Two-way ANOVA	RKIP levels	0.6924
	[cisplatin]	<0.0001
Wilcoxon signed-rank test	Low VS WT RKIP	N/A
	Low VS High RKIP	N/A
	WT VS High RKIP	N/A

Figure 3.19: The effect of RKIP on caspase 3/7 activity of Ls174T cells treated with cisplatin. a) Low, WT and high RKIP-expressing colon carcinoma cells were treated with a range of cisplatin concentrations (n=3), 100 µM (log -4) to 10 nM (log -8), for 48 hours; after which the caspase activity of the cells was measured using the caspase 3/7-Glo assay. **b)** Table of statistics showing the effect of RKIP on cisplatin-induced cell death in colon cancer cells.

From the biological data and the statistical analyses of the biological data, it was clear that for this colon carcinoma cell line, the levels of RKIP within the cell had no significant effect on the cell death response of cells (Figure 3.19). Although the two-way ANOVA showed the concentration of cisplatin to have a significant effect on the apoptotic response; the biological data demonstrates that the increase in caspase 3/7 activity did not occur until very high concentrations of the drug were administered (Figure 3.19).

In a prostate cancer cell line, cisplatin has been shown to induce RKIP expression [181], yet the results shown here are very similar to the data from the 5-FU and etoposide-treated Ls174T colon carcinoma cells. This brings to the forefront a number of explanations and hypotheses that have already been raised. Cisplatin-induced RKIP expression occurred in a prostate but not a breast cancer cell line [181] thus modulation of cisplatin-induced apoptosis by RKIP may be cell-type specific. Although examination of the effect of cisplatin on cell proliferation in the Ls174T colon carcinoma cell line did not occur, cisplatin treatment may also lead to drug-induced senescence in this cell line. Finally, cisplatin efficacy has been shown to be reduced in MI unstable cell lines [75], thus the

Ls174T colon carcinoma cell line may have an MMR deficiency resulting in DNA-damage tolerance.

As with the sections on 5-FU and etoposide-treated cells, further investigation into the response of the Ls174T cells themselves to DNA-damage is crucial before the effect of RKIP can be fully characterised.

3.3.4 Discussion of Cell Death Results

In summary, treatment with doxorubicin, FasL, paclitaxel and TRAIL suggested Ls174T colon carcinoma cells were affected by RKIP levels within the cell in terms of cell death; supporting our hypothesis.
In contrast, treatment with 5FU, cisplatin and etoposide suggested Ls174T colon carcinoma cells were not greatly affected by RKIP levels within the cell in terms of cell death; contradicting our hypothesis.

Multiple conclusions can be drawn from this study.

1. The modulation of TRAIL-induced apoptosis by RKIP occurred in the Ls174T colorectal carcinoma cell line in a manner similar to those reported in previous studies in other cell lines [151, 181, 182]. This is however the first report of TRAIL modulation of apoptosis by RKIP in a colorectal carcinoma. This opens up a number of avenues for future work, but the most important would be to investigate if treatment with TRAIL in Ls174T cells results in the activation of similar pathways to those observed in other cancer cell lines; namely DR5 up-regulation and Bcl-xl down-regulation [184, 189].

2. Apoptosis as a result of paclitaxel administration was affected by RKIP levels in this colon carcinoma cell line. Low levels of RKIP displayed a similar drug resistant phenotype as shown in previous studies [181, 182], which was not seen in the WT RKIP colorectal cells. It appears that RKIP sensitised these cells to chemotherapy-induced apoptosis. However, over-expression of RKIP in the colorectal cells behaved similarly to the low RKIP cells; it seems that RKIP modulation of chemotherapy-induced apoptosis may be a double-edged sword. This initially appeared to be a conflicting and unexpected result; but a

recent study by Tang *et al.* [163], has displayed a scaffold function for RKIP in relation to the NF-κB pathway. Paclitaxel-induced sensitivity to RKIP levels was thought to be mediated *via* RKIP interaction with the NF-κB pathway in other cell lines [182]. Paclitaxel-induced cell death modulation by RKIP has never been shown in a colorectal carcinoma thus examination of the effect of RKIP on NF-κB components in this colorectal cell line would be a strong candidate for further investigation.

3. We have shown, for the first time, that doxorubicin-induced apoptosis was subject to regulation by RKIP. Whether this is specific for the Ls174T colorectal carcinoma remains to be seen. As with the paclitaxel-treated cells, low and high RKIP-expressing cells treated with doxorubicin were less sensitive to apoptosis when compared to the WT RKIP cells. Since doxorubicin and paclitaxel both induce cell stress resulting in the initiation of apoptosis, it is likely that doxorubicin-induced cell death is being modulated by RKIP in a manner similar to paclitaxel. However it is also possible that these two drugs may be affected differentially. Determining the mechanism of drug modulation by RKIP is of great importance for cancer therapy and gives weight to the need for individualised chemotherapy regimes.

4. The death receptor agonist FasL was selected for analysis because it belonged to the same receptor family as TRAIL, and was expected to behave in a similar manner in Ls174 cells expressing different levels of RKIP. Curiously, cells treated with FasL exhibited a response analogous to the cell stress-inducing cytotoxic drugs doxorubicin and paclitaxel.
It is possible that the difference between the TRAIL and Fas responses may be due to the adaptor proteins that couple the death receptors to NF-κB signalling; RKIP associates with TRAF6 but not the other TRAFs [163]. This would be a highly exciting and novel subject for investigation into the functions of RKIP and secondary signalling by death receptors.

5. 5-FU, cisplatin and etoposide did not induce substantial cell death in this Ls175T colorectal carcinoma cell line. This is curious, particularly as 5-FU is routinely employed as a treatment for colorectal carcinomas [204]. It was possible that the RKIP–expressing Ls174T colorectal cells were undergoing drug-induced senescence as a consequence of 5-FU, cisplatin or etoposide administration since this cell line has previously displayed drug-induced senescence [283]. On the other hand, aberrations in the MMR response of the Ls174T colon carcinoma cell line [284] may have led to inefficient apoptotic responses to these DNA-damaging agents.

This would require a study focused on cellular senescence and the unravelling of treatments that induce this state in the cells. Of particular interest would be the pathways that were activated following treatment with these chemotherapeutic agents.

Overall, because much has yet to be learned about the functions and interactions of RKIP - particularly within cancerous tissues undergoing treatment – there could be unidentified binding partners of RKIP that only bind during specific cellular stresses, leading to unexpected phenotypes and responses to external stimuli.

Moreover, the data displaying the modulation of TRAIL and FasL-induced apoptosis by RKIP were distinct, highly contrasting and displayed less variation when compared to the other cytotoxic therapies. Any exploration of the mechanism behind RKIP regulation of chemotherapeutic agents would concentrate on these two compounds.

Modulation of the cell death response of the colon carcinoma cell line to chemotherapeutic agent treatment as a consequence of the levels of RKIP within the cell corroborated with previous studies showing a chemo-sensitising effect of RKIP. However not all of the agents tested were subject to apoptosis-modulation by RKIP, and if they were the response was different between different agents. This varied response of cytotoxic agents to differing RKIP levels within neoplastic cell lines emphasises the need for individualised treatment regimes and a better understanding of the modulation of chemotherapeutic agents.

3.4 Chapter Discussion

RKIP is well known as an endogenous inhibitor of the ERK, NF-κB and GRK2 pathways [143, 144, 147, 148]. An increasing body of evidence is revealing a tumour-suppressive and chemo-sensitising role for this protein in cancer cell lines [153, 166, 168, 169, 171, 173, 181-185, 189, 290]. However the functional implications of RKIP on these pathways, its anti-tumour role and the conditions under which RKIP modulates cellular fate are all still poorly understood.

Why the RKIP protein has been selected for investigation in cancer and chemotherapy regimes is not limited to the role of the protein itself in chemo-sensitivity and tumour suppression. MEK and NF-κB inhibitors are becoming widespread in the treatment of cancer [64, 94, 97, 99, 108, 128, 165, 291-294] and RKIP is essentially an endogenous MEK and NF-κB inhibitor. Thus understanding how this protein affects and alters the chemotherapeutic drug response will help shed light on the interaction of MEK and NF-κB inhibitors with current chemotherapeutic agents. In addition, it would open new avenues of how these inhibitors can be used in combination to create synergistic effects or alternatively, the conditions under which combination therapies may be inappropriate.

To date, RKIP has been shown to modulate the apoptotic response of cells to treatment with 9-nitrocamptothecin (9-NC), cisplatin, etoposide, paclitaxel and TRAIL [180, 181, 184]. RKIP expression has also been shown to be induced after treatment with the proteasome inhibitor NPI-0052, nitric oxide donors, rituximab and silbinin as well as the agents above [182, 183, 185, 189, 295]. These studies have used a variety of different cell lines, from breast and prostate cells to melanoma and HeLa cells, however no real interrogation of the different effects of RKIP in a single cell line have been made.
Moreover, RKIP expression has been shown to be down-regulated in colorectal carcinomas leading to an increase in the incidence of metastases [154, 173, 245], yet investigation into the effects of RKIP on colorectal carcinomas post-treatment with chemotherapeutic agents has not yet been performed.

This study shows for the first time that the response of a single colon carcinoma cell line to a variety of different chemotherapeutic agents can be modulated by the levels of RKIP within the cell. Moreover, the data from this study shows that this RKIP modulation of

chemotherapeutic drug response can have different cellular outcomes depending upon the treatment employed. There was no discernable effect of RKIP on the cellular proliferation response in colon carcinoma cell line. In addition, the levels of RKIP resulted in very small changes in cell cycle distribution following cytotoxic therapy. Although RKIP has been shown to regulate the ERK and NF-κB pathways which are important for the cell cycle, growth and proliferation, it is possible that the role of RKIP in these processes following chemotherapy administration and cancer formation is restricted to the modulation of the apoptotic machinery and/or metastases suppression. Indeed previous studies have highlighted a role for RKIP only in chemotherapy-induced apoptosis; furthermore RKIP is not thought to be involved in primary tumourigenesis but rather the later stages of tumour suppression [173].
This is the first time however that it has been shown that RKIP modulation of the cellular response to chemotherapeutic agents may be limited to the apoptotic machinery and not involve significant proliferative or cell cycle-related effects.

Cisplatin- and etoposide-induced apoptosis have been shown to be modulated by RKIP in other cell lines [181]; however in the Ls174T colon carcinoma cell line the cells did not appear to undergo apoptosis after treatment with either of these agents. Furthermore the apoptotic response remained the same regardless of the levels of RKIP within the cell. Etoposide-treated Ls174T colon carcinoma cells did display a dose-dependent decrease in cell proliferation after treatment but this was not affected by the levels of RKIP within the cell. Therefore the results of this study suggest that the modulation of the response of cancer cells to cisplatin and etoposide treatment by RKIP may be cell-specific responses. It is also possible that the colon carcinoma cells were undergoing drug-induced senescence following treatment with these compounds.

The effect of RKIP on TRAIL-induced apoptosis in the colon carcinoma cell line corroborated data from previous studies that showed the sensitisation of cells to TRAIL by RKIP [184, 185, 189]. This study demonstrates that the modulation of TRAIL-induced apoptosis by RKIP can occur in a colon carcinoma cell line. Analysis of the mechanisms behind this response would be of particular interest since TRAIL has been shown to be highly selective for cancer cells [67]. Furthermore, TRAIL is one of the latest anti-cancer agents in treatment and already tumours are beginning to display drug resistance, thus understanding the processes behind apoptosis-induction by TRAIL is crucial as is the

understanding of whether these processes can be manipulated, in our studies by RKIP, to have therapeutic benefits.

This is also the first study to demonstrate RKIP modulation of FasL and doxorubicin-induced apoptosis in a cancer cell line. The response of low, WT and high RKIP-expressing cells to treatment with both of these agents was different to the response elicited by TRAIL-treated colon carcinoma cells. The effect of paclitaxel, which has been shown to be modulated by RKIP in another cell line [180], was also subject to cell death modulation by RKIP in the Ls174T colon carcinoma cell line. The apoptotic response of paclitaxel-treated cells was similar to that of FasL and doxorubicin-treated cells but in contrast to TRAIL-treated cells. Why TRAIL should elicit a different cell death response to doxorubicin, FasL and paclitaxel as a consequence of RKIP levels within the cell is very interesting, and implies that the induction of death within cancer cells, and that the proteins involved in this process, could be more complex than previously imagined. In short, cellular signalling may be a double-edged sword in the treatment of cancer.

On another note, the synergistic effects of these treatments have not been examined therefore future work could study the effect of TRAIL in combination with Fas, and various other drug combinations. Moreover how this drug combination could be affected by the level of RKIP within the cell would also be an interesting route of investigation. In particular, it would be interesting to see if the addition of a MEK or NF-κB inhibitor to any of the drug treatments would affect the cell death modulation by RKIP.

No previous study has shown a differing response in chemotherapy-induced apoptosis as a consequence of the levels of RKIP within a cancer cell.

The results of the TRAIL-treated cells versus the FasL-treated cells were the most intriguing since both ligands belong to the TNF super-family and induce cell death by initiating the external apoptotic pathway. These results are highly exciting and prompt further investigation. Understanding the differential modulation of chemotherapeutic agents by RKIP, particularly within the same family, may open up new avenues for manipulation of cancers and indeed, anti-cancer care.

Finally, this study highlights that the levels of RKIP within a tumour could literally mean the difference between life and death of the cell in terms of chemotherapeutic regimes.

CHAPTER 4:
MODULATION OF TRAIL AND FAS-INDUCED CELL DEATH BY RKIP

4.1 The RKIP and TRAIL story

Treatment of low, WT and high RKIP-expressing Ls174T colon carcimona cells with TRAIL displayed an increased caspase-3/7 activity in the high RKIP-expressing cell line compared to both the WT and the low RKIP-expressing cells. This sensitivity of the high RKIP cells to TRAIL concurred with studies in melanoma and prostate cancer cell lines conducted by Baritaki *et al.* [184]. These authors stated that the mechanism of this sensitivity was *via* up-regulation of the death receptor DR5 and inhibition of the transcription repressor YY1. They also reported down-regulation of anti-apoptotic cell death effectors such as XIAP, c-FLIP long, and Bcl-xl [184].

The increased activation of the death receptor pathway would explain the sensitivities to TRAIL-induced apoptosis observed in the Baritaki study [184]. The up-regulation of DR5 causing an increased sensitivity to TRAIL is logical; one of the main receptors for TRAIL is DR5. Increasing the number of DR5 receptors should increase the number of TRAIL-receptor interactions, resulting in the increased activation of the death receptor pathway.

Inhibition of anti-apoptotic molecules by RKIP, whether by direct or indirect mechanisms, allowed an increase in levels of pro-apoptotic effectors such as the Bcl-2 family members Bid, Bad, Bax *etc* by preventing their inhibition by Bcl-xl. An increase in pro-apoptotic members of the Bcl-2 family leads to increased permeability of the mitochondrial outer membrane and release of cytochrome c into the cytosol [38, 39, 41, 296]. Cytochrome c and other toxic mitochondrial proteins allow formation of the apoptosome and thus initiation of the internal cell death pathway [34, 35].

Down-regulation of XIAP, which limits the levels of caspase 3, results in the increased activation of this caspase [297]. Both the external and the internal apoptotic pathways converge on the activation of caspase 3 [11, 298]; hence this caspase is named the executioner caspase as it results in the activation of the final stages of the apoptotic pathways and in the morphological changes that are associated with apoptosis.

The RKIP-mediated effects described above in the Baritaki *et al.* [184] study were attributed to the RKIP-dependent inhibition of the NFκB pathway. RKIP prevents the

activation of upstream kinases in the NF-κB pathways resulting in the inhibition of both the activation and the nuclear translocation of NF-κB dimers [148].
Bcl-xl, XIAP and c-FLIP expression are impeded upon treatment with the NF-κB inhibitor DHMEQ [184]. Indeed, Bcl-xl and XIAP are subject to NF-κB regulation at the transcriptional level [299-301]. Therefore, RKIP-based modulation of the NF-κB pathway would explain the down-regulation of these anti-apoptotic molecules after TRAIL treatment.

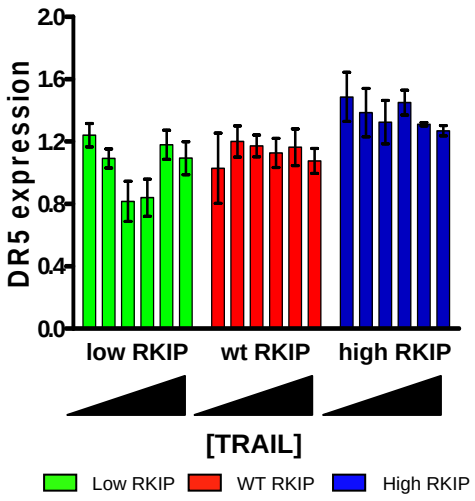
YY1 is a multi-functional zinc-finger transcription factor that can both activate and inhibit transcription; making it distinct from the usual transcription factors. YY1 plays pivotal roles in the cell cycle, growth, proliferation and differentiation and is reviewed extensively in the following references [302-304]. Most importantly, expression of YY1 correlates with the levels of NF-κB, and repression of NF-κB results in the concomitant decrease in the levels of YY1 [184, 305, 306]. Thus RKIP inhibition of NF-κB, *via* upstream kinases, could lead to down-regulation of YY1.
DR5 contains an YY1 binding site on its promoter; this site serves to repress DR5 expression. Indeed, deletion or mutation of this binding site leads to the up-regulation of DR5. Thus, if RKIP inhibited YY1 *via* the NF-κB pathway, this would result in an increase in the expression of DR5, as shown by Baritaki *et al.* [184].

We therefore decided to investigate whether the sensitivity displayed by the high RKIP-expressing Ls174T colon carcinoma cells to TRAIL treatment was through the same mechanisms as those described by Baritaki *et al.* [184].

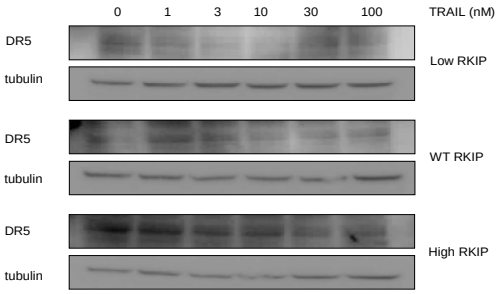
4.1.1 Death Receptor 5 (DR5)

Since RKIP has been shown to up-regulate the expression of the TRAIL receptor DR5 in melanoma and prostate cancer cell lines *via* NF-κB-related effects [184], the expression of DR5 was tested in the three RKIP-expressing Ls174T cell lines after treatment with TRAIL for 48 hours. The data from three western blots were collated and DR5 levels quantified using ImageJ analysis.

a)



b)



c)

Statistical Analysis	Source of variation	p-value
Two-way ANOVA	RKIP levels	<0.0001
	[TRAIL]	0.0659
Wilcoxon signed-rank test	Low VS WT RKIP	0.263
	Low VS High RKIP	<0.0001
	WT VS High RKIP	<0.0001

Figure 4.1: The effect of RKIP on DR5 expression in TRAIL-treated colon carcinoma cells. a) ImageJ analysis of three DR5 western blots of low, WT and high RKIP Ls174T cells treated with TRAIL. DR5 expression was normalised to tubulin (n=3). **b)** Ls174T colon carcinoma cells with low, WT and high RKIP expression were treated with TRAIL for 48 hours (0 – 100 nM) after which the changes in DR5 expression were monitored by western blot. All cells were grown for 24 hours in 5 % foetal calf serum prior to treatment. Tubulin was employed as a loading control. (n=3 with sample experiment shown). **c)** Table of statistical analyses showing the effect of RKIP on DR5 expression after TRAIL treatment.

Analysis of DR5 expression in low, WT and high RKIP-expressing colon carcinoma cells after treatment with TRAIL showed an increased expression of DR5 in cells expressing

higher levels of RKIP (Figure 4.1). In addition, the concentration of TRAIL appeared to have a slight affect on the expression of DR5; the levels of DR5 decreased as the concentration of TRAIL used for the treatment increased. This was particularly evident in the high RKIP-expressing cells. Two-way ANOVA showed that the levels of RKIP protein had a significant affect on the expression of DR5; however the effect of TRAIL concentration on DR5 expression was not significant (Figure 4.1c). The elevated level of DR5 expression in cells expressing high levels of RKIP when compared to WT and low RKIP-expressing cells could also be determined from the multiple comparisons conducted on the biological data. These analyses showed that the level of DR5 was significantly different in high RKIP-expressing cells compared to both low and WT RKIP-expressing cells (Figure 4.1).

The increased in DR5 expression in cells expressing higher levels of RKIP levels would concur with the study by Baritaki *et al.* [184], who showed that elevated levels of RKIP resulted in the up-regulation of DR5. Inhibition of the NF-κB pathway by RKIP resulted in the up-regulation of DR5 expression in the melanoma and prostate cancer cell lines [184]. In this colon cancer cell line, high RKIP-expressing cells possessed more DR5 receptors than WT and low RKIP-expressing cells hence the increased in sensitivity of this cell line to TRAIL-induced apoptosis. This RKIP-mediated up-regulation of the DR5 receptor leading to chemo-sensitivity in the Ls174T cells may also be a consequence of RKIP inhibition of the NF-κB as described by Baritaki *et al.* [184].

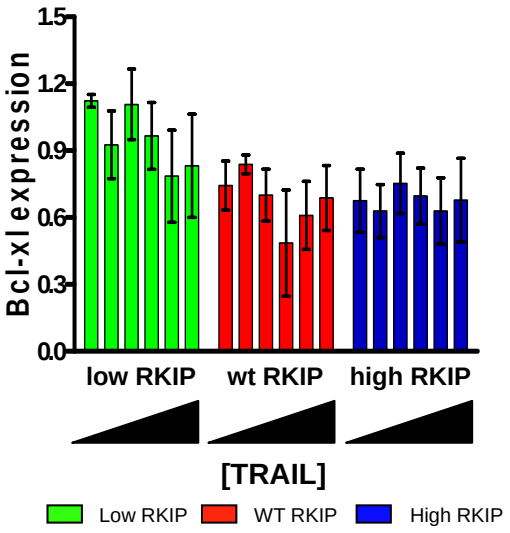
Chemo-sensitivity due to RKIP levels within the cell has also been shown previously with NO donators such as DETANONOate [189] and the monoclonal antibody rituximab [183]; both drugs show startling similarities in the modulation of apoptotic molecules as described by Baritaki *et al.* [184]. NF-κB inhibition was evident following treatment with DETANONOate [189]. This inactivation of NF-κB resulted in YY1 inhibition, and also displayed a decrease in anti-apoptotic effectors such as Bcl-xl, and an up-regulation of the DR5 receptor [189]. The overall effects of such modulation led to sensitisation of previously resistant cell lines to chemotherapy-induced apoptosis. This inhibition of NF-κB was a result of RKIP induction by DETANONOate [189], the full mechanism of which remains unclear. It is however, believed to involve the relief of RKIP down-regulation as a result of the inhibition of Snail [189]; a transcription factor whose expression is inversely proportional to that of RKIP [166, 167, 185].

In the Baritaki *et al.* study [184], the changes in DR5 expression were mediated by the down-regulation of YY1. DR5 contains binding sites in its promoter that have the potential to negatively regulate DR5 expression, by NF-κB or by negative regulation of DR5 by YY1 [307]. Investigation of the levels of expression of YY1 in the TRAIL-treated Ls174T colon carcinoma cells with low, WT and high RKIP levels would be interesting to determine if DR5 up-regulation was a consequence of YY1 inhibition. Furthermore, it would be exciting to examine if Bcl-xl expression was also affected by the RKIP levels in these TRAIL-treated colon carcinoma cells.

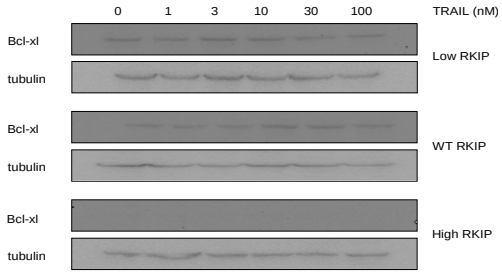
4.1.2 The anti-apoptotic protein Bcl-xl

The expression of the anti-apoptotic molecule Bcl-xl has been shown to be down-regulated by RKIP in prostate and melanoma cancer cell lines after treatment with TRAIL [184]. Thus, the three Ls174T cell lines with low, WT and high RKIP expression were treated with TRAIL for 48 hours. After treatment, proteins extracted from these samples were separated by SDS-PAGE and the protein of interest, Bcl-xl detected by using Bcl-xl specific antibodies. Bcl-xl levels from three western blot experiments were collated and analysed using ImageJ analysis.

a)



b)



c)

Statistical Analysis	Source of variation	p-value
Two- way ANOVA	RKIP levels	0.0035
	[TRAIL]	0.6293
Wilcoxon signed-rank test	Low VS WT RKIP	0.0241
	Low VS High RKIP	0.0177
	WT VS High RKIP	0.669

Figure 4.2: The effect of RKIP on Bcl-xl expression in TRAIL-treated colon carcinoma cells. a) ImageJ analysis of three western blots showing the Bcl-xl expression as a consequence of RKIP levels in TRAIL treated colon carcinoma cells (n=3). Bcl-xl levels were normalised to tubulin. **b)** Western blot analysis of Bcl-xl expression after 48 hours in Ls174T colon carcinoma cells with low, WT and high RKIP levels treated with TRAIL (0 – 100 nM). All cells were grown for 24 hours in 5 % foetal calf serum prior to treatment (n=3 with sample experiment shown). Alpha-tubulin was used as a loading control. **c)** Table of statistical analyses showing the effect of RKIP levels on Bcl-xl expression in TRAIL-treated colon carcinoma cells.

Western blot analysis of proteins isolated from TRAIL-treated cells showed that an increase in the levels of RKIP in Ls174T cells resulted in decreased levels of the Bcl-xl protein; suggesting the down-regulation of Bcl-xl (Figure 4.2). Treatment with increasing concentrations of TRAIL did not appear to have a visual effect on the levels of Bcl-xl protein detected on the western blots. Two-way ANOVA of the biological data showed that the levels of RKIP within the cell had a significant effect on the levels of Bcl-xl protein expression. Indeed Wilcoxon signed-rank tests showed that the expression of Bcl-xl in the low RKIP-expressing cell line to be significantly different to both the WT and high RKIP-expressing cells. In contrast, the concentration of TRAIL used in the treatment had no effect on the levels of the Bcl-xl protein (Figure 4.2).

The down-regulation of Bcl-xl by the increased RKIP levels would be consistent with previous studies that showed increased chemo-sensitivity of cells as a consequence of RKIP down-regulation of the anti-apoptotic molecule Bcl-xl [182, 184]. Again, inhibition of the NF-κB pathway by RKIP is thought to be involved in this down-regulation of Bcl-xl.

There are two binding sites for NF-κB on the Bcl-xl promoter. In addition, NF-κB is required for activation of Bcl-xl [299-301]. In this study, the low RKIP-expressing Ls174T cells displayed higher levels of Bcl-xl compared to the WT cells. In addition, the high RKIP cells displayed very little detectable levels of Bcl-xl protein. Again, this data supports that reported by Baritaki *et al.*[184]; it suggests that RKIP-based inhibition of the NF-κB pathway results in the down-regulation of Bcl-xl following treatment with TRAIL. Since Bcl-xl is an anti-apoptotic member of the Bcl-2 family, down-regulation of the Bcl-xl gene product shifts the apoptotic equilibrium in favour of apoptosis. Thus, high RKIP-expressing cells with lower Bcl-xl levels compared to WT and low RKIP cells exhibited a higher sensitivity to cell death induced by TRAIL. Whether inhibition of NF-κB is the mechanism by which the down-regulation of Bcl-xl exerts its effect requires further investigation.

Evidence in other cell lines and with other chemotherapeutic agents suggests that RKIP inhibition of NF-κB is the most likely scenario by which the changes observed in the cells can be explained [182, 183, 189]. For example, NF-κB inhibition due to RKIP induction by rituximab leads to the down-regulation of anti-apoptotic effectors [182, 183]. Again, the mechanism of this drug-induced up-regulation of RKIP is currently unknown, but like DETANONOate [189], it may involve abrogation of Snail repression on RKIP [166, 167, 185].

On the other hand, the effect on MAPK as a result of RKIP over-expression was displayed in the rituximab-treated cells, which included a decrease in AP-1 transcription due to ERK pathway inhibition [182, 183]. The Bcl-xl gene product is activated by both NF-κB and AP-1 transcription, thus inhibition of both transcription factors as a consequence of over-expression of RKIP would result in the down-regulation of Bcl-xl [182, 183].

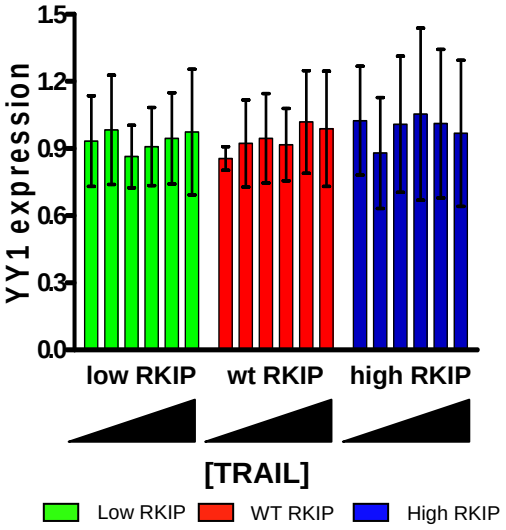
Therefore in Ls174T cells, Bcl-xl down-regulation may not be due to RKIP-dependent inhibition of the NF-κB pathway alone. As mentioned above, AP-1 which is activated by the ERK pathway also activates Bcl-xl [182, 183]. RKIP inhibition of the ERK pathway could lead to the down-regulation of Bcl-xl expression by preventing activation of AP-1.

Analysis of protein components of the ERK and NF-κB pathways as well as the analysis of AP-1 and NF-κB transcriptional activity, after TRAIL treatment should elucidate the pathways affected by modulation of RKIP expression as well as how this subsequently affects chemotherapy-induced apoptosis..

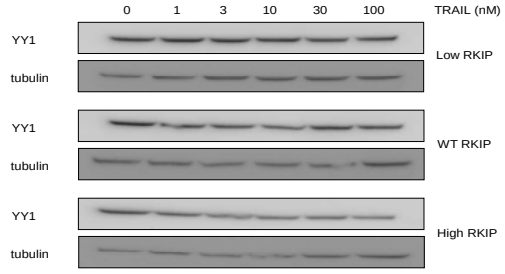
4.1.3 Yin Yang 1

Baritaki *et al.* [184] reported that the changes in DR5 and Bcl-xl expression were due to the RKIP-dependent inhibition of the transcriptional regulator YY1, through the RKIP-based inhibition of the NF-κB pathway. To determine if this was the case in the Ls174T cells, the expression of YY1 was monitored in the three RKIP cell lines after TRAIL treatment. YY1 expression in the three RKIP cell lines was quantified for three western blot experiments using ImageJ analysis.

a)



b)



c)

Statistical Analysis	Source of variation	p-value
Two-way ANOVA	RKIP levels	0.9095
	[TRAIL]	0.9995
Wilcoxon signed-rank test	Low VS WT RKIP	N/A
	Low VS High RKIP	N/A
	WT VS High RKIP	N/A

Figure 4.3: The effect of RKIP on YY1 expression in TRAIL-treated colon carcinoma cells. a) ImageJ analysis of three western blot results showing YY1 expression in low, WT and high RKIP Ls174T colon carcinoma cells treated with TRAIL (n=3). YY1 was normalised to tubulin loading control. **b)** Ls174T colon carcinoma cells with low, WT and RKIP expression were treated with TRAIL (0- 100 nM) for 48 hours, and the changes in Yin Yang 1 expression monitored by western blot. All cells were grown for 24 hours in 5% foetal calf serum prior to treatment (n=3 with sample set shown). Tubulin was employed as

a loading control. **c)** Table of statistical analyses showing the changes in YY1 as a consequence of RKIP levels within TRAIL-treated Ls174T cells.

Analysis of the collated western blots (Figure 4.3a) demonstrated that neither the RKIP levels in the Ls174T cells nor the concentration of TRAIL used to treat the cells altered the expression of the YY1 protein. Moreover, two-way ANOVA showed YY1 expression to be unchanged despite changes in the RKIP levels within the cells, or the TRAIL concentration administered (Figure 4.3c).

From the western blots and the statistical analyses of the biological data (Figure 4.3), it is apparent that the different levels of RKIP had no effect on the levels of YY1 protein after TRAIL treatment. The lack of changes observed in YY1 expression after TRAIL treatment was in disagreement with our hypothesis. Furthermore, the study by Baritaki *et al.* on TRAIL administration and the studies with nitric oxide (NO) donors had both implicated YY1 inhibition as the cause of anti-apoptotic molecule down-regulation and/or DR5 up-regulation [184, 189]. Baritaki *et al.* [184] reported that RKIP-based repression of the NF-κB pathways resulted in the down-regulation of the levels of the YY1 transcription factor protein. Since the expression of YY1 in the Ls174T colon carcinoma cells was unchanged for all three RKIP-expressing cell lines and for increasing TRAIL concentrations, this would contradict the previous reports on RKIP-related chemo-sensitivity [184, 189].

One possible explanation for the lack of changes in the expression of YY1 could be the 48 hour incubation time. YY1 is a dynamic cellular protein; YY1 is activated by phosphorylation and has a half-life of 3.5 hours [308]. Down-regulation of YY1 is thought to occur as early as 6 hours into drug treatment [184] and up-regulation of DR5 at 18-24 hours post-treatment [184]. The maximum length of TRAIL incubation in the prostate and melanoma studies conducted by Baritaki *et al.* was 24 hours [184]. Therefore it is possible that any changes in YY1 protein expression due to drug treatment would have occurred before the 48 hour end-point employed in this study. A TRAIL timecourse was performed with treatments with 30 nM TRAIL for between 0 - 48 hours to test this hypothesis. Unexpectedly, the western blot of YY1 expression showed no detectable differences between any of the time points (data not shown).

Although, the YY1 antibody employed in this study was the same as the one employed in the Baritaki article [184], it is possible that the role of YY1 in the Ls174T cell line is

different to the role this protein plays in the prostate and melanoma cells lines used by Baritaki *et al.* [184]. As mentioned previously, YY1 is a multi-functional transcriptional activator, repressor and regulator. It has at least 8 protein isoforms produced by alternative splicing and can potentially interact with at least 2540 vertebrate genes; the full functions of which have yet to be elucidated [304]. It is a significant character in many cellular processes, ranging from apoptosis and development, to cell migration and the regulation of viral genes, yet it remains largely misunderstood and difficult to discern; the archetypical scarlet pimpernel of the cell [302-304].

Furthermore YY1 is subject to complex regulation and post-translational modifications which may explain the lack of changes observed in this study. Like many transcription factors YY1 is phosphorylated with an apparent half-life of only 3.5 hours [308]. The functional consequences of this phosphorylation are as yet, unknown. Another modification this protein undergoes is O-glycosylation, which leads to the disruption of YY1 binding to retinoblastoma protein (Rb) in favour of DNA [309]; again the full implications of this alteration are yet to be described.

In summary, it is likely that such modifications of YY1 may alter the protein binding partners and affect protein stability. There was no phosphorylated YY1 antibody available at the time these experiments were conducted. In the future, with the availability of antibodies directed towards the active YY1 protein, a better understanding for the role of this protein in the chemotherapeutic drug responses may be clearer. Therefore, due to this incomplete evidence about the function of YY1, we cannot exclude the involvement of this protein in the modulation of DR5 expression displayed in this cell line as a result of changes in RKIP expression.

On a different note, it is possible that in the Ls174T colon carcinoma cell line DR5 up-regulation is not mediated by YY1 inhibition or that it does not rely solely upon the inhibition of YY1.

C/EBP homologous protein (CHOP), also known as growth arrest- and DNA damage-inducible gene 153 (GADD153), is yet another curiosity in the cell. CHOP is member of the CCAAT/enhancer binding proteins (C/EBPs) and also serves as a negative regulator of these proteins [310]. CHOP is predominantly involved in the cell stress response, particularly that of the endoplasmic reticulum (ER) [310], and is poorly expressed during non-stressed conditions. Conditions such as oxidative damage, nutrient deprivation and genotoxic stress contribute to the induction of the expression of this protein [310].

With relevance to this study, CHOP has been shown to up-regulate DR5 expression following treatment with various anti-tumour compounds such as dipyrimadole [311], fenretinide [312] and thapsigargin [313] in colon cancer cell lines; thus making these cells sensitive to treatment with TRAIL. In other cell lines, capsaicin [314] and withaferin A [315] also displayed down-regulation of anti-apoptotic molecules such as Bcl-xl and c-FLIP as well as the CHOP-mediated up-regulation of DR5. Although very poorly understood as yet, there appears to be a CHOP-binding site on the DR5 promoter that may be responsible for the effects described [310-312, 314, 315]; how CHOP would interact with RKIP is currently unknown.

Furthermore, RKIP effects on the ERK pathway may be involved in the modulation of chemo-sensitivity. The anti-apoptotic molecule Bcl-xl can be regulated by the AP-1 transcription factor [182, 183]. Therefore investigation of other proteins and transcription factors known to modulate DR5 and Bcl-xl such as CHOP and AP-1 would be essential to determine the mechanisms by which RKIP influences and modulates chemo-sensitivity *via* the inhibition of the NF-κB and ERK pathways.

4.1.4 Discussion

In the Ls174T colon carcinoma cell line after treatment with TRAIL, high RKIP-expressing cells up-regulated DR5 expression and down-regulated expression of Bcl-xl. In contrast, the low RKIP cell line displayed decreased expression of DR5 and increased expression of Bcl-xl. These results were in agreement with those reported by Baritaki *et al.* [184], based on a study conducted in prostate and melanoma cell lines. In addition, the results from Ls174T cells provided further support to the hypothesis that these cells lines may have similar mechanisms for the regulation of sensitivity to TRAIL treatment in high RKIP cells.
In contrast to the Baritaki *et al.* study [184], the expression of YY1 was not altered by the levels of RKIP in the cells.

The alteration of Bcl-xl and DR5 levels in the Baritaki *et al.* study [184], was through the RKIP-dependent inhibition of the NF-κB pathway. It is likely that this was also the case for the results displayed in the Ls174T colon carcinoma cell line. The effect of RKIP on the NF-κB pathway is to inhibit upstream kinases, namely NIK and TAK1, and also

members of the IKK complex [148]. RKIP inhibition of these molecules prevents NF-κB activation by both the canonical and non-canonical pathways mentioned in the Introduction. Decreased NF-κB activation results in the decreased translocation of NF-κB to the nucleus and the subsequent preventing of the transcription of NF-κB-dependent gene products.

Examination of the downstream effects of NF-κB inhibition and the modulation of NF-κB gene products as a consequence of RKIP inhibition of NF-κB [148] would be necessary to better understand the effects of RKIP on the modulation of TRAIL signalling. Furthermore, the analysis of AP-1 and CHOP transcription factor protein levels following TRAIL treatment in low, WT and high RKIP cells would be interesting. It would allow the elucidation of whether RKIP also modulates the transcriptional activity of these proteins. Since both these transcription factors, AP-1 and CHOP, have been shown to affect Bcl-xl and DR5 expression respectively [182, 183, 310-315] it is an interesting possibility. These protein studies would help to unravel the effect of RKIP levels within this cell line and to better understand some of the mysteries surrounding this enigmatic protein.

4.2 Differences in TRAIL and Fas modulation by RKIP

An investigation into the effect of RKIP levels on the response of Ls174T colon carcinoma cells to TRAIL-induced apoptosis demonstrated that high RKIP-expressing cells displayed an increased susceptibility to apoptosis than the WT and low RKIP cell lines (Chapter 3, Section 3.3.1, Figure 3.11). This pattern of behaviour was in stark contrast to that obtained when the same cell lines were treated with FasL (Chapter 3, Section 3.3.1, Figure 3.12). After FasL treatment, WT RKIP-expressing cells were more sensitive to FasL-induced apoptosis compared to low and high RKIP-expressing cells.
The differences in the responses to TRAIL and FasL administration between the cell lines were a direct consequence of the RKIP levels within the cell. This data was unexpected; TRAIL and FasL are members of the same family of receptors – the TNF family - and both are involved in the initiation of the external apoptotic machinery. The fact that cells treated with FasL and TRAIL elicited such different responses in the cell lines makes unravelling the mechanisms behind these responses very interesting.

The mechanism by which high RKIP-expressing cells exhibited sensitivity to TRAIL treatment in the Ls174T colon carcinoma cell line was *via* up-regulation of the death receptor DR5 and down-regulation of the anti-apoptotic molecule Bcl-xl. This data corroborated the findings of Baritaki *et al.* [184] in prostate and melanoma cell lines. The modulation of DR5 and Bcl-xl proteins was hypothesised to be *via* RKIP inhibition of the NF-κB pathway [148]. Since we observed differences in the sensitivities of Ls174T cells, to TRAIL and FasL treatment, that were a consequence of the RKIP levels in the cells, we thought that RKIP-dependent inhibition of the NF-κB pathway may account for the changes in the sensitivity of RKIP-expressing cells to these drug treatments.

The modulated sensitivity of cells to chemotherapy-induced apoptosis as a consequence of RKIP levels within the cell has been shown to involve members of the NF-κB pathway [184, 189]. Moreover, it is suspected that members of the TRAF family of adaptor proteins that link receptors of the TNF-family to signalling pathways, such as the NF-κB, p38 and JNK pathways, are involved [23, 24, 26, 316]. There are six TRAF proteins and all contain a C-terminal TRAF domain that is essential for homo- and hetero-dimerisation of TRAF proteins, and also for the interaction of the TRAF proteins with receptors and other adaptor proteins [23, 24, 26, 316]. The amino terminus of all TRAF proteins, except TRAF1,

contains a RING finger and several zinc finger motifs that are thought to be involved in mediating downstream signalling events [23, 24, 26, 316]. RKIP has been shown only to interact with the TRAF6 subtype [163], thus preferential employment of one TRAF or several TRAF subtypes by Fas or TRAIL receptors may explain the differences in the responses to drug administration that we observed.
Furthermore, investigation of components of the NF-κB pathway such as IKK was deemed critical since the TRAF proteins signal through this pathway [23, 24, 26, 316]; RKIP has been shown to modulate the NF-κB pathway [148, 163] and this modulation has been implicated in RKIP-related sensitivity to chemotherapy-induced apoptosis [184, 189].

In order to differentiate between the signalling pathways activated by RKIP-dependent modulation of TRAIL and FasL treatment, three potentially pivotal proteins were selected for analysis; phosphorylated IKK α/β, TRAF2 and TRAF6.

Phosphorylated IKK α/β was selected based on its role in both the canonical and non-canonical NF-κB pathways, and is reviewed in the following references [127, 129, 133]. RKIP-based inhibition of kinases has been demonstrated in both pathways; NIK in the non-canonical and TAK1 in the canonical [127, 148]. Furthermore, RKIP has been shown to interact with the IKK α and β subunits themselves [148]. The NF-κB pathway has been shown to have both pro- and anti-apoptotic functions depending on the cell-type and stimulus employed [251, 252, 260, 317]. It is therefore possible that the differences in the response of Ls174T cells with different RKIP levels to TRAIL and FasL treatment could be due to the role of NF-κB in downstream signalling from their respective receptors. Due to the insensitivity of high RKIP-expressing cells to FasL treatment it is possible that NF-κB may have an apoptotic function during FasL stimulation. However the high RKIP-expressing cells were sensitive to TRAIL treatment thus it is possible that NF-κB inhibition may be a requirement for TRAIL-induced apoptosis.

Although the adaptor proteins involved in secondary signalling from TRAIL and Fas death receptors are currently unknown; evidence has highlighted a possible role for TRAF proteins in both TRAIL and Fas signalling. TNF receptor coupling to TRAF adaptors is well documented in non-death receptors [23, 24, 26, 316] however secondary signalling from death receptors (such as DR4, DR5) and the Fas receptor are thought to activate NF-κB and JNK pathways [21, 22, 29, 208-211, 269, 318, 319]. The adaptor FADD and caspase 8, that are responsible for propagating the TRAIL- and Fas-directed apoptotic

response, have been shown to interact with TRADD and RIP adaptors *via* their death domains (DDs). The receptors can then recruit TRAFs, in particular the TRAF2 subtype [20-22, 25, 29, 208-211, 249, 269]. RKIP has been shown only to interact with the TRAF6 subtype [163]. This interaction was observed during interleukin-1 (IL-1) administration so it is possible than an RKIP-dependent effect on TRAF2 may occur after apoptotic stimulation. Since both Fas and TRAIL death receptors have been linked to this TRAF subtype, TRAF2 expression was selected for analysis following FasL and TRAIL treatment in low, WT and high RKIP-expressing Ls174T cells.

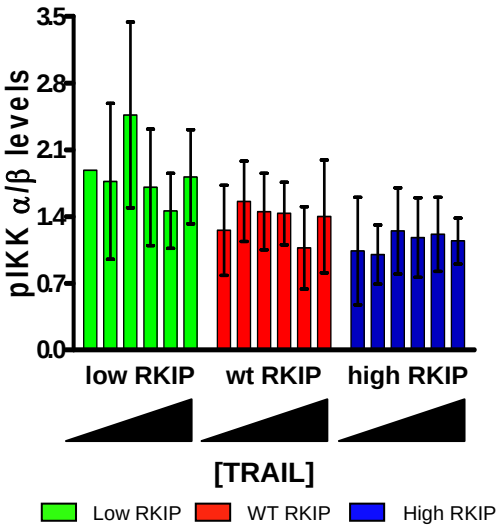
The interaction of TRAF6 with RKIP led to the selection of this protein for investigation [163]. TRAF6 has a different receptor recognition motif from the other TRAF family members and is the only TRAF subtype that has a role in both TNF and IL-1 signalling [23, 24, 26, 316]. Unfortunately, the adaptors that link the death receptors to the JNK and NF-κB pathways are unknown. Since the stimulus determines the behaviour of NF-κB [260] it is possible that TRAIL and Fas receptors may utilise different TRAF subtypes to modulate NF-κB function. In addition, it is possible that RKIP may interact with TRAF6, but not TRAF2 subtypes. If this is the case, it may help to unravel the mechanisms underlying the differences observed in the responses of low, WT and high RKIP-expressing Ls174T colon carcinoma cells to treatment with TRAIL and FasL.

4.2.1 Modulation of TRAIL-induced apoptosis by RKIP in Ls174T colon carcinoma cells

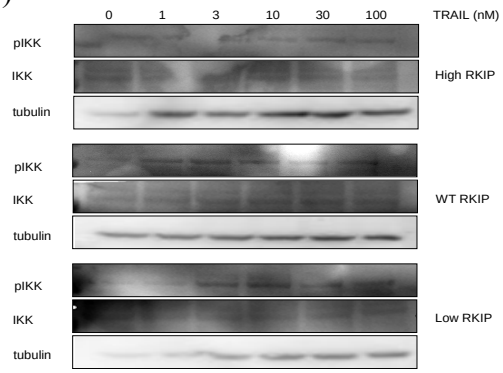
4.2.1.1 Phosphorylated IκB kinase α/β (pIKK α/β)

Ls174T cells expressing low, WT and high levels of RKIP were treated with a range of TRAIL concentrations for 48 hours. Proteins extracted from these samples were analysed for the expression of the phosphorylated (active) and unphosphorylated (inactive) alpha (α) and beta (β) subunits of IκK kinase (IKK). The activation of pIKK taken from three western blot experiments were collated and quantified using ImageJ analysis.

a)



b)



c)

Statistical Analysis	Source of variation	p-value
Two-way ANOVA	RKIP levels	0.0858
	[TRAIL]	0.9251
Wilcoxon signed-rank test	Low VS WT RKIP	0.471
	Low VS High RKIP	0.0902
	WT VS High RKIP	0.471

Figure 4.4; pIKK α/β levels in TRAIL-treated colon carcinoma cells expressing low, WT and high RKIP levels. a) The results from three individual western blots were analysed with ImageJ and collated to analyse the overall effect of RKIP on pIKK expression as a result of treatment with TRAIL. The pIKK value from each experiment was normalised to the tubulin control from the same experiment (n=3). **b)** The three RKIP-expressing (low, WT and high) Ls174T colon carcinoma cell lines were incubated with various TRAIL concentrations (0 – 2 μg/ml) for 48 hours, after which samples were prepared and monitored by western blot analysis. The samples were then blotted for changes in phosphorylated IKK (pIKK) levels. IKK and tubulin controls were also

performed. (All experiments were repeated three times and one example of the experiment is shown above). **c)** Table of statistical analyses for pIKK α/β levels as a consequence of RKIP levels in TRAIL-treated cells.

The levels of the proteins of interest were difficult to quantify based on the quality of the western blot. It is possible that the levels of pIKK increased in the cells as the levels of RKIP decreased (Figure 4.4a); since pIKK leads to activation of the NF-κB, this would concur with the known function of RKIP as an inhibitor of the NF-κB pathway. There was no change in pIKK activation regardless of the TRAIL concentrations used for the treatment (Figure 4.4a). Also, the expression of IKK was very low over the range of TRAIL concentrations used in the treatments but appeared to remain unchanged (Figure 4.4b). Moreover, the levels of IKK appeared higher in the high RKIP-expressing cells compared to the low RKIP-expressing cells and the expression of IKK decreased slightly as the expression of pIKK increased (Figure 4.4b). The inactivation of pIKK to IKK is poorly understood, however auto-phosphorylation or phosphorylation by protein phosphatases are strong candidates for pIKK inactivation [127, 129, 133]. It is not known if IKK is degraded in a manner similar to IκB. Furthermore, a nuclear export role for IKK has also been shown, therefore normalisation of pIKK levels to α-tubulin would help to eliminate discrepancies due to pIKK inactivation and/or IKK degradation [127, 129, 133].

Statistical analysis of the biological data using a two-way ANOVA showed that the concentration of TRAIL used in the study did not significantly affect the level of phosphorylated IKK in the cells (Figure 4.4c). In contrast, the levels of the RKIP protein in the cells had an effect close to significance on the level of the active IKK protein (Figure 4.4c). Therefore Wilcoxon signed-rank tests performed on the biological data, the tests demonstrated that the levels of pIKK in the high RKIP cell lines were close to being significantly different to the levels of pIKK in the low RKIP-expressing cell line.
The statistical analyses exhibited a lower degree of significance than expected. This may be due to the 48 hour incubation time. pIKK exists in various protein complexes and is an extremely dynamic cellular protein; these events occur over various time scales from minutes to hours. Therefore the 48 hour TRAIL treatment may have been too long to observe the rapid changes associated with IKK activation. This is supported by the low level of signal detected for pIKK and IKK in all three RKIP cell lines. Future experimental work could include a time course study to observe the level of IKK phosphorylation in the

three RKIP cells lines after treatment with TRAIL. This experiment is more likely to display more overt changes in the levels of pIKK in the cell.
Overall the data trend suggests that increased RKIP levels corresponded to a decrease in IKK activation to pIKK; this corroborated data from previous studies which showed that RKIP inhibited the NF-κB pathway *via* inhibition of upstream kinases such as the subunits of the IKK complex [148].

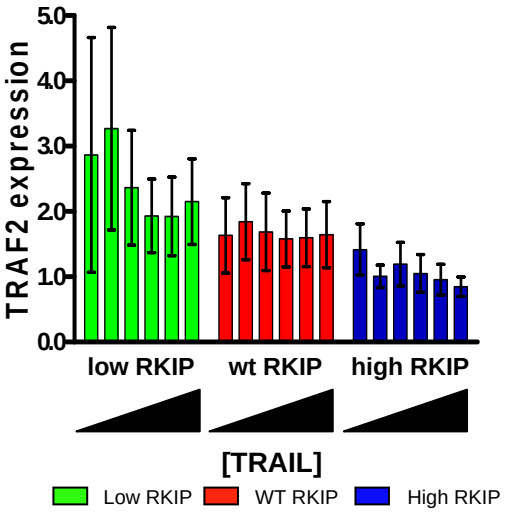
Furthermore, the behaviour observed in Ls174T cells agreed with the observation that NF-κB inhibition was required for TRAIL-mediated apoptosis [184, 189], especially since NF-κB activation has been shown to confer resistance to TRAIL-induced cell death [91, 320-324]. Previous studies on the caspase activity in the Ls174T colon carcinoma cell line displayed an increased sensitivity of high RKIP-expressing cells to TRAIL-induced apoptosis; *via* the up-regulation of DR5 and the down-regulation of Bcl-xl. In the study by Baritaki *et al.* [184] this was mediated through RKIP-dependent inhibition of the NF-κB pathway. Down-regulation of pIKK with increasing levels of RKIP, as seen in Figure 4.4 suggests that the same mechanism takes place in the Ls174T colon carcinoma cell line.

Further experimentation with pIKK time-courses and other NF-κB pathway components would be required to confirm an RKIP-dependent inhibition of the NF-κB pathway as the mechanism for the up-regulation and down-regulation of members of the apoptotic machinery.

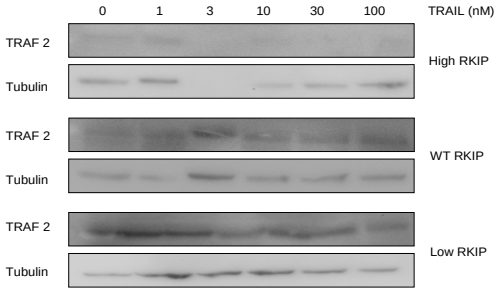
4.2.1.2 Tumour necrosis factor receptor associated factor 2 (TRAF2)

The adaptor protein TRAF2 is known to interact with TRAIL receptors; this interaction results in the activation of the JNK and NF-κB pathways [208, 209, 249]. For this reason, the levels of TRAF2 adaptor protein were monitored in low, WT and high RKIP-expressing Ls174T cells after treatment with TRAIL. Protein samples were separated on an SDS-PAGE gel and proteins of interest detected on western blots using specific antibodies. Protein expression was quantified using ImageJ analysis and the data from three western blot experiments collated.

a)



b)



c)

Statistical Analysis	Source of variation	p-value
Two-way ANOVA	RKIP levels	0.0055
	[TRAIL]	0.8519
Wilcoxon signed-rank test	Low VS WT RKIP	0.144
	Low VS High RKIP	0.00582
	WT VS High RKIP	0.0805

Figure 4.5: The effect of RKIP levels on TRAF2 expression in TRAIL-treated colon carcinoma cells. a) The results from three separate western blot experiments were analysed with ImageJ and collated. This allowed analysis of TRAF2 expression after TRAIL treatment in Ls174T colon carcinoma cell lines with different RKIP levels – low, WT and high. TRAF2 expression was normalised to the tubulin control for each experiment (n=3). **b)** Low, WT and high RKIP Ls174T colon carcinoma cells were treated with TRAIL (0 – 100 nM) for 48 hours, then the samples prepared and analysed by western blot to detect changes in the expression of TRAF2. An alpha-tubulin control blot was performed to ensure changes were not due to error in loading. (n=3 with sample western blot displayed). **c)** Table of statistical analyses showing the effect of RKIP levels on TRAF2 expression following TRAIL administration.

Analysis of the western blot data suggested that cells with lower levels of RKIP displayed increased expression of TRAF2 (Figure 4.5a). In addition, the concentration of TRAIL used in the treatment did not appear to influence the level of TRAF2 protein detected (Figure 4.5). A two-way ANOVA analysis of the biological data further confirmed that TRAF2 expression was independent of the TRAIL concentration administered to the cell and expression of TRAF2 was significantly affected by the level of RKIP present in the cell (Figure 4.5c). Comparison of the levels of TRAF2 expression in the low RKIP-expressing cell line was significantly different compared to the high RKIP-expressing cell lines; and close to significance for the WT RKIP cells versus high RKIP-expressing cells (Figure 4.5c).

Caspase assays conducted on TRAIL-treated RKIP-expressing cells showed that high RKIP-expressing Ls174T cells displayed an increased sensitivity to TRAIL-induced apoptosis (Chapter 3, Section 3.3.1, Figure 3.11). The increased expression of TRAF2 in the presence of lower levels of RKIP would explain the decreased sensitivity of low RKIP-expressing cell lines to TRAIL-induced apoptosis. The lack of TRAF2 expression in the high RKIP cells may explain the increased sensitivity of these cells to TRAIL-induced apoptosis. High RKIP Ls174T cells treated with TRAIL have been shown to up-regulate TRAIL receptor (DR5) expression and down-regulate the expression of the anti-apoptotic protein Bcl-xl. Both these actions are thought to be a result of the RKIP-dependent inhibition of the NF-κB pathway [148].

The TRAIL receptor is a very efficient apoptosis inducer and the function of TRAF2 in TRAIL receptor signalling is to relay signals to pathways such as the NF-κB and JNK pathways [208, 209, 249]. The NF-κB pathway has been shown to induce proliferative and anti-apoptotic responses during TRAIL signalling. Thus the lack of TRAF2 – which may induce anti-apoptotic responses *via* NF-κB activation [91, 320-324] – in the high RKIP-expressing cells supports the increased sensitivity of these cells to TRAIL-induced apoptosis.

Whether RKIP interacts directly with TRAF2 requires further investigation. It is possible that RKIP levels may indirectly affect TRAF2 expression *via* its effects on the NF-κB pathway [148]. A study by Tang *et al.* [163] demonstrated the interaction between TRAF6 and RKIP, but none of the other TRAF subtypes. Therefore immunoprecipitation and binding studies would be required to investigate the interaction between TRAF2 and RKIP following TRAIL administration.

There is some controversy about whether adaptors from the DR5 family are involved in secondary signalling complexes. TRAF2 has been found in complexes containing FADD, RIP and procaspase 8 [20, 22, 25, 29, 208-210, 249]. FADD and caspase 8 are essential for apoptosis induced by the death receptors [211]. However recent studies have shown that these proteins are essential for the formation of secondary signalling complexes that result in the activation of signalling pathways such as the JNK, p38 and NF-κB pathways [20, 22, 29, 208, 211]. Therefore the examination of the effect of RKIP levels on the expression of these proteins post-TRAIL treatment would be required to fully understand how TRAIL receptor-dependent secondary activation of pathways is occurring. Finally, investigation of RKIP binding to other death receptor adaptor proteins is crucial to better

understand and confirm the link between RKIP and chemotherapy-induced apoptosis. RKIP has already been shown to interact with the TRAF6 subtype of TRAF adaptors [163]. Due to the range of RKIP interacting proteins, it is likely that TRAF6's interaction with RKIP is not unique.

4.2.1.3 Tumour necrosis factor receptor associated factor 6 (TRAF6)

In addition to TRAF2, the expression of another TRAF subtype - TRAF6 - was analysed in TRAIL-treated Ls174T cells expressing low, WT and high levels of RKIP. TRAF6 has been shown to interact with RKIP [163] thus the expression of TRAF6 in the low, WT and high RKIP Ls174T colon carcinoma cell lines would be interesting.

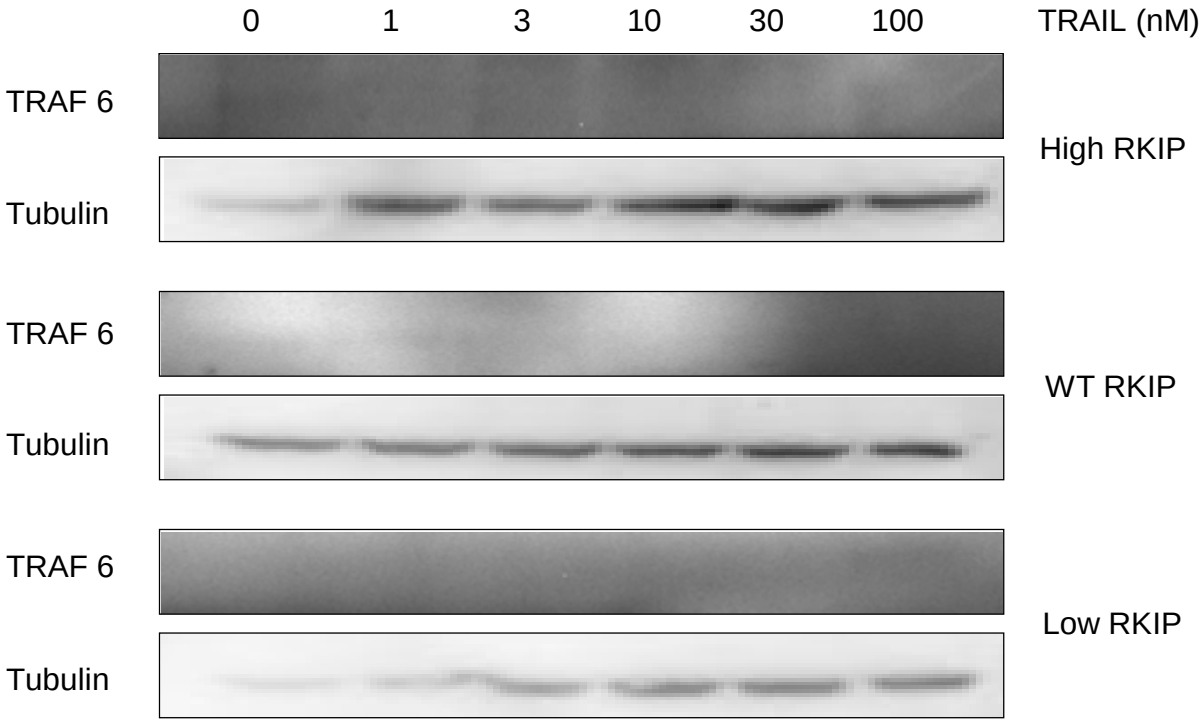


Figure 4.6: The effect of RKIP levels of TRAF6 expression in TRAIL-administered colon carcinoma cells. Western blot displaying the expression of TRAF6 after 48 hours of treatment with TRAIL (0 – 100 nM) in the low, WT and high RKIP expressing Ls174T colon carcinoma cell lines. Tubulin was employed as a loading control. (n=3 with sample experiment shown).

Analysis of proteins isolated from TRAIL-treated cells showed no detectable levels of TRAF6 after 48 hours of treatment in any of the three RKIP-expressing (low, WT and

high) Ls174T cell lines (Figure 4.6). This experiment was repeated twice more with the same result (data not shown). Due to the lack of TRAF6 expression, protein quantification using ImageJ analysis was not performed.

While RKIP has been shown to interact with TRAF6 [163], we observed no detectable expression of TRAF6 protein in our biological experiments. It is possible that in Ls174T cells, TRAF6 did not interact with TRAIL receptors or that TRAF6 was not involved in the formation of secondary complexes upon TRAIL receptor activation. Indeed, it has been TRAF2 and other adaptors such as RIP that have been mainly implicated in TRAIL receptor activation of signalling pathways such as the JNK and NF-κB pathways [208-210, 249]. This gives weight to the hypothesis that differential employment of adaptor proteins by the TRAIL and Fas receptors may be responsible for the RKIP-dependent changes in chemo-sensitivity. Furthermore the RKIP-TRAF6 interaction was observed during IL-1 stimulation thus this interaction may be stimulus-dependent.

To conclusively demonstrate that TRAF6 is not involved in TRAIL-dependent induction of apoptosis in Ls174T cells, further examination of the secondary complexes formed from the TRAIL receptor would be required. Finally, it is possible that in Ls174T cells, other TRAF subtypes may be involved in TRAIL signalling as well as adaptor proteins such as FADD and RIP; all of which may interact with RKIP and provide insight into the chemo-sensitivity of high RKIP-expressing colon carcinoma cells to TRAIL treatment.

4.2.1.4 Discussion

TRAIL-treated high RKIP-expressing Ls174T colon carcinoma cells exhibited decreased pIKK levels and TRAF2 expression compared to cells expressing WT and low levels of RKIP protein. No expression of TRAF6 was detected in TRAIL-treated cells regardless of the levels of RKIP within the cell.

pIKK levels were used as a measure of NF-κB activity thus high RKIP-expressing cells appeared to have lower levels of NF-κB activity than low or WT RKIP-expressing cells. This would concur with the increased sensitivity of high RKIP-expressing cells to TRAIL-induced apoptosis observed when the caspase activity was measured in TRAIL-treated high RKIP-expressing cells. Increased NF-κB activity is thought to confer resistance to

TRAIL treatment [91, 320-323] thus cells with low levels of NF-κB should be more susceptible to TRAIL treatment.

The expression of TRAF2 was also higher in low RKIP-expressing cells compared to the level of expression in WT RKIP and high RKIP-expressing cells. TRAF2 is thought to mediate secondary signalling from death receptors to pathways such as the NF-κB pathway [23-26, 208, 209, 211, 249]. The decrease in TRAF2 expression as RKIP levels were increased would corroborate with the decrease levels of pIKK observed in previous experiments. It would also explain the increased sensitivity of high RKIP-expressing cells to TRAIL-induced apoptosis. Decreased TRAF2 in the high RKIP cells would imply there was less activation of potentially anti-apoptotic pathways such as the NF-κB pathway, leading to the increase in cell death observed in the high RKIP-expressing cells after TRAIL administration.
There appeared to be no expression of TRAF6 in any of the RKIP cell lines after TRAIL treatment with a range of TRAIL concentrations. This may be due to the preferential use of other adaptors for TRAIL signalling to NF-κB pathways and other signalling cascades in Ls174T cells.

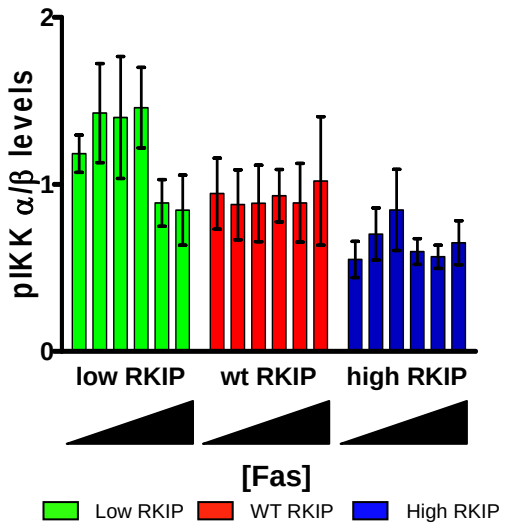
In summary, secondary signalling from the TRAIL receptor resulting in the activation of the NF-κB pathway appeared to be partly responsible for the decreased sensitivity of low and WT RKIP-expressing cells to TRAIL-induced apoptosis compared to high RKIP cells. Further examination of other adaptor proteins, the complexes formed and the direct binding of RKIP to these components would be essential to unravel the mechanism behind the RKIP-dependent chemo-sensitivity of Ls174T cells to TRAIL-induced apoptosis.

4.2.2 Modulation of Fas-induced apoptosis by RKIP in Ls174T colon carcinoma cells

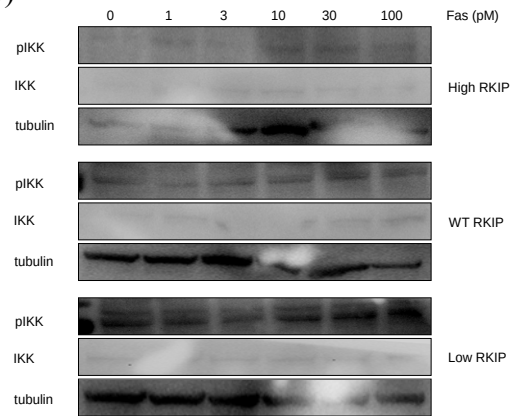
4.2.2.1 Phosphorylated IκB kinase α/β (pIKK α/β)

In previous experiments, we observed that FasL and TRAIL-treated Ls174T cells, expressing different levels of RKIP, exhibited different patterns of behaviour after treatment. These differential sensitivities were attributed to differences in the activation of NF-κB pathway components. To better understand the mechanisms by which FasL treatment of Ls174T colon carcinoma cells expressing low, WT and high RKIP activated apoptosis of the cells, proteins of interest were analysed within treated cells.

a)



b)



c)

Statistical Analysis	Source of variation	p-value
Two-way ANOVA	RKIP levels	0.0004
	[FasL]	0.9227
Wilcoxon signed-rank test	Low VS WT RKIP	0.0799
	Low VS High RKIP	<0.0001
	WT VS High RKIP	0.0608

Figure 4.7: The effect of RKIP on pIKK α/β levels in FasL-treated colon carcinoma cells. a) ImageJ analysis of three separate western blots of pIKK expression normalised to tubulin control, after 24 hours treatment with various concentrations of FasL in Ls174T colon carcinoma cells with low, WT and high RKIP levels. (n=3). **b)** Western blot analysis of pIKK and IKK levels in FasL-treated (0 – 100 pM) low, WT and high RKIP-expressing Ls174T colon carcinoma cells after 24 hours FasL incubation. Alpha-tubulin was used as a

loading control. (n=3 with example western blot displayed). **c)** Table of statistical analyses showing the effect of RKIP on pIKK α/β levels in Ls174T colon cells administered FasL.

The levels of pIKK after FasL treatment in Ls174T cells increased as the level of RKIP expression decreased. Unfortunately, the levels of IKK expression were low in all three RKIP cell lines (Figure 4.7b). By using the levels of the tubulin protein as a control, the levels of pIKK and unphosphorylated IKK in each protein sample could be quantified using ImageJ analysis. From the data presented in Figure 4.7a, it can be clearly observed that increasing levels of RKIP within the cell lead to a concomitant decrease in expression of active IKK protein after 24 hours FasL administration. Moreover, two-way ANOVA analysis of the biological data showed that the level of pIKK was unaffected by the FasL concentration used to treat the cells. In contrast, the levels of pIKK were significantly affected by the levels of RKIP in the cell. Further statistical analyses showed that the levels of pIKK in the low RKIP cell lines to be significantly different to the high RKIP-expressing cells; the levels of pIKK in the WT RKIP cells versus low and high RKIP cell lines were close to being significantly different (Figure 4.7c).

The increased pIKK levels in cells expressing low levels of RKIP supported evidence that over-expression of NF-κB pathway could convey chemotherapy drug resistance, including FasL resistance [96-98, 165, 181, 247], as shown by the decreased sensitivity of these cells to FasL-induced apoptosis (Chapter 3, Section 3.3.1, Figure 3.12). WT RKIP-expressing Ls174T colon carcinoma cells exhibited increased sensitivity to FasL-induced apoptosis compared with low RKIP-expressing cells (Chapter 3, Section 3.3.1, Figure 3.12). In addition, in FasL-treated cells the levels of pIKK decreased as the RKIP levels in the cells increased. This behaviour concurs with the anti-apoptotic function of NF-κB. The increased activation of the NF-κB pathway as a result of RKIP depletion has been shown to convey chemotherapy drug resistance [181, 183, 184, 189]; NF-κB pathway activation independent of RKIP has also been shown to confer drug resistance, reviewed in the following references [96-98, 165]. Furthermore, the decrease in pIKK levels as a consequence of over-expression of RKIP concurred with the known functions of RKIP as an endogenous inhibitor of the NF-κB pathway [148, 163].

However the anti-apoptotic functions of the NF-κB and RKIP-based inhibition of this pathway implied that high RKIP-expressing cells should have been more sensitive to apoptosis, as seen in the TRAIL results shown previously.

Instead, FasL-treated cells that expressed high levels of RKIP exhibited a similar cell death response to the low RKIP-expressing cell line. This could be explained by recent studies that have revealed a pro-apoptotic function for the NF-κB pathway. This pro-death face of NF-κB is dependent on both the cell-type and the stimulus employed [251, 252, 254, 255, 260, 317]. NF-κB activation is essential for apoptosis induced by various agents and can involve regulation and expression of the Fas ligand (FasL) and receptor [251-253, 255-257, 317, 325]. Moreover the FasL promoter contains putative binding sites for several transcription factors including AP-1, NFAT and NF-kB [255, 258, 259]. This regulation of FasL by NF-κB has been demonstrated in colon carcinoma cell lines [253, 325] and the Ls174T cell line used in this study is a colon carcinoma cell line. The pro-apoptotic nature of NF-κB occurs during genotoxic stress such as treatment with DNA damaging agents, and can lead to induction of FasL as a consequence of NF-κB activation [255, 256, 264]. Since NF- κB is thought to regulate the Fas receptor [326], inhibition of the pro-apoptotic functions of NF-κB, for example by over-expression of RKIP, could lead to a decrease in Fas-induced apoptosis as seen in the response of the FasL-treated high RKIP-expressing Ls174T cells.

Additionally, doxorubicin and paclitaxel, both of which activate the DNA damage response and have been shown to employ Fas as a means of inducing apoptosis [82-87, 278], displayed the same scaffold-like response as FasL-treated Ls174T colon carcinoma cells with low, WT and high levels of RKIP. Thus high RKIP-expressing cells may be preventing the pro-apoptotic functions of NF-κB in chemotherapy-induced cell death.

Although NF-κB inhibition appeared to be essential for TRAIL-induced apoptosis in Ls174T cells, FasL-treatment appeared to require a balance in the pro- and anti-apoptotic functions of NF-κB [260]. It is possible that NF-κB exerts different effects in the Ls174T cells depending on the stimulus employed. In this study, the use of TRAIL, FasL and RKIP may involve the preferential employment and expression of certain TRAF subtypes. Overall this data suggests that NF-κB signalling could be a double-edged sword in Fas-mediated apoptosis.

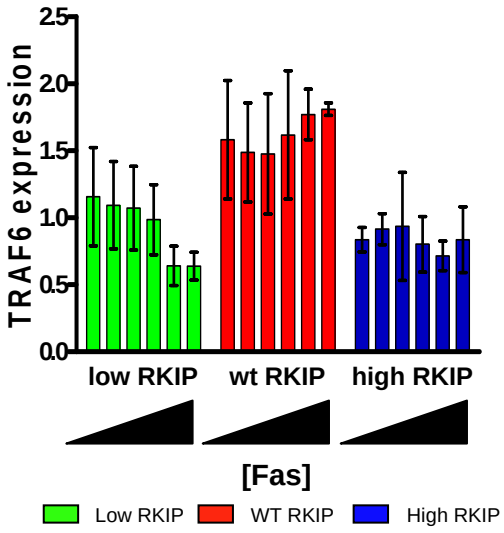
However, examination of other NF-κB components would be essential to confirm the potential pro-apoptotic function of NF-κB in this cell line and under the various treatment conditions employed. Furthermore, the investigation of the expression of adaptor proteins that are thought to mediate secondary signalling from death receptors to the signal

cascades, including the NF-κB pathway, would be interesting. In particular, how different stimuli and receptors can modulate the functionality of proteins and transcription factors, resulting in different cell fates. Finally, the involvement of the RKIP protein in modulating this switch-like behaviour could have important clinical consequences, particularly since RKIP is an inhibitor of the ERK and NF-κB pathways; both of which are manipulated in the treatment of cancers.

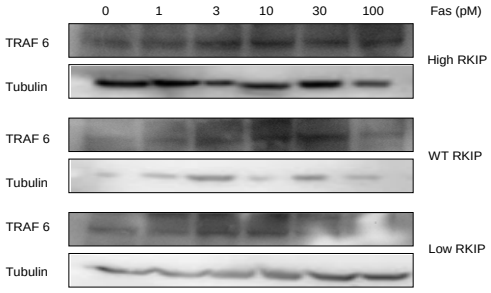
4.2.2.2 Tumour necrosis factor receptor associated factor 6 (TRAF6)

The interaction of RKIP and TRAF6 has been demonstrated by Tang *et al.* [163]. In the previous section, we examined the levels of TRAF6 in TRAIL-treated Ls174T cells expressing different levels of RKIP. Interestingly, TRAF6 was not expressed in any of the three RKIP-expressing cell lines. It would be interesting to determine whether this protein played a role in FasL-induced apoptosis in Ls174T cells. Ls174T cells expressing different levels of RKIP were treated with a range of FasL concentrations for 24 hours. Proteins were isolated from these cells, separated on an SDS-PAGE gel and probed for the presence of the TRAF6 protein. ImageJ analysis was used to quantify the expression of TRAF6.

a)



b)



c)

Statistical Analysis	Source of variation	p-value
Two-way ANOVA	RKIP levels	0.0002
	[FasL]	0.9938
Wilcoxon signed-rank test	Low VS WT RKIP	0.0164
	Low VS High RKIP	0.853
	WT VS High RKIP	0.0025

Figure 4.8: The effect of RKIP on TRAF6 expression in FasL-treated colon carcinoma cells. a) ImageJ of TRAF6 expression in Ls174T colon carcinoma cells with low, WT and high RKIP treated with FasL over a range of concentrations for 24 hours. TRAF6 was normalised to tubulin loading control. (n=3). **b)** TRAF6 expression after 24 hours in low, WT and high RKIP Ls174T colon carcinoma cells treated with various FasL concentrations. The loading control used was alpha-tubulin. (n=3 with sample experiment displayed). **c)** Table of statistical analyses showing TRAF6 expression as a consequence of RKIP levels in FasL-administered Ls174T cells.

Analysis of western blots with TRAF6-specific antibodies showed that TRAF6 expression appeared to be lowest in both the low and high RKIP-expressing cells and highest in the WT RKIP-expressing cells (Figure 4.8). It is interesting that both low and high RKIP Ls174T cells appear to have lower levels of TRAF6 than WT RKIP-expressing cells. In addition, ImageJ analysis and a two-way ANOVA analysis confirmed the trend that was observed in the western blot data (Figures 4.8a and 4.8c). Both the low and high RKIP-expressing colon carcinoma cells had lower levels of TRAF6 expression when compared to the WT RKIP-expressing cells (Figure 4.8c). The expression of TRAF6 was not affected by the concentration of FasL used to treat the cells. When the levels of TRAF6 protein expression were compared between the different cell lines, it was apparent that the levels of TRAF6 expression in WT RKIP-expressing colon carcinoma cells were significantly different to both low and high RKIP-expressing cells (Figure 4.8c).

Both low and high RKIP-expressing Ls174T cells displayed a decreased sensitivity to FasL-induced apoptosis in comparison to the WT RKIP-expressing cells (Chapter 3, Section 3.3.1, Figure 3.12). This scaffold-like effect of RKIP on TRAF6 expression can be seen clearly in Figure 4.8, where FasL-treated cells expressing WT levels of RKIP showed higher TRAF6 expression than both low and high RKIP-expressing cell lines. TRAF6 has been previously shown to immunoprecipitate with RKIP in complexes by Tang *et al.* [163]. Since TRAF6 is thought to link death receptors to signalling pathways such as the NF-κB pathway; over-expression of RKIP may have prevented TRAF6-NF-κB signalling by direct inhibition of NF-κB pathway components and/or binding to the individual components of the complexes in a scaffold-like manner. Tang *et al.* [163] also observed that both low and high RKIP levels can lead to a decrease in the phosphorylation of IκB and thus decreased NF-κB activity. This suggests that RKIP may have a scaffold-like function with respect to the levels of NF-κB inhibition [163]. If TRAF6 is responsible for the activation of the NF-κB pathway in FasL-treated Ls174T cells, then the behaviour observed in Figure 4.8 would lend further weight to the argument that RKIP can behave as a scaffold-like protein.

Unfortunately the phosphorylation of IKK (Figure 4.7) did not display this interesting scaffold-like behaviour for RKIP in Ls174T cells treated with FasL. FasL-treated high RKIP-expressing cells displayed less TRAF6 and less pIKK protein when compared to the WT RKIP cells, and suggested that over-expression of RKIP could lead to increased resistance to FasL by preventing the pro-apoptotic functions of NF-κB (summarised in the following references [251, 252, 254, 255, 260, 317, 326]). Whether the decrease in NF-κB activity is a direct consequence of TRAF6-RKIP interactions [163] or a result of RKIP-directed inhibition of the NF-κB pathway [148] requires further investigation.

Additionally, TRAF6 has also been shown to have a pro-apoptotic role [327]; which it does by recruiting caspase 8 [327, 328]. The general assumption is that secondary signalling from death receptors would be anti-apoptotic; and this appeared to be the case for the TRAIL-treated Ls174T cells which showed increased cell death with decreased expression of TRAF2. However TRAF6 and NF-κB could be acting in a pro-apoptotic manner during FasL stimulation in the high RKIP-expressing cells, thus their down-regulation by RKIP could explain the decreased sensitivity of high RKIP-expressing cells when compared to the WT RKIP-expressing cells. Future experiments that examine the interaction of TRAF6 and RKIP would help to address this hypothesis.

Doxorubicin and paclitaxel-treated high RKIP-expressing cells also displayed a decrease in sensitivity to apoptosis when compared to the WT RKIP cells (Chapter 3, Section 3.3.2, Figures 3.14 and 3.16). Again the Janus-like properties of NF-κB in apoptosis regulation are thought to be the reason behind the increased susceptibility of WT RKIP-expressing cells to chemotherapy-induced apoptosis. It would be of interest to determine whether doxorubicin and paclitaxel-treated cells also displayed similar scaffold-like responses in the expression of adaptor proteins, such as TRAF6, and NF-κB components, such as pIKK.

The low RKIP-expressing cells exhibited decreased TRAF6 yet increased pIKK levels after FasL treatment which suggested that the lack of RKIP resulted in the hyper-activation of the NF-κB pathway such that the pathway exhibited an anti-apoptotic activity. Since TRAF6 can behave in a pro-apoptotic manner [327], it may also be pro-apoptotic in this cell line. The lack of TRAF6 in the low RKIP-expressing cell line would corroborate with the decreased sensitivity of the low RKIP cells to FasL-induced apoptosis. Further experiments could interrogate the TRAF6-NF-κB interaction and start to piece together how this relates to RKIP modulation of chemotherapy-induced apoptosis.
In summary, it is possible that the recruitment of TRAF6 and/or components of the NF- κB pathway by RKIP resulted in an alteration in the response and function of the NF- κB pathway; these changes induced the formation of complexes that initiated cell death. However over-expression of RKIP resulted in RKIP-binding to components individually, as opposed to facilitating the formation of a complex leading to apoptosis, leading to survival.

A diagrammatic representation of what may be happening with RKIP, TRAF6 and NF-κB components following FasL stimulation is shown below (Figure 4.9).

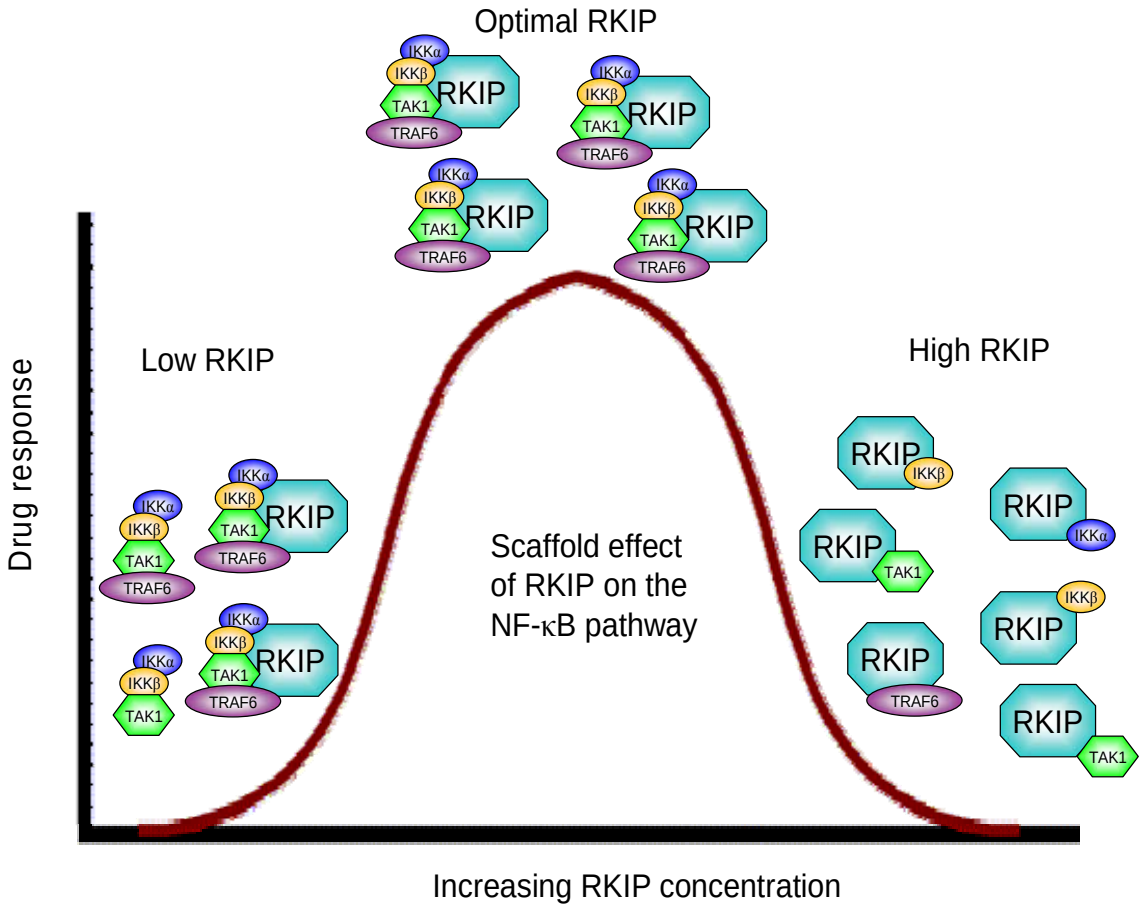


Figure 4.9: Diagrammatic representation of the potential scaffold-like effect of RKIP on chemotherapy-induced apoptosis. The scaffold-like activity displayed by RKIP on TRAF6 and NF-κB components – TAK1, IKKα and IKKβ - resulting in the modulation of the chemotherapeutic-drug response. Optimal RKIP concentrations would promote the interaction of pro-apoptotic TRAF6 and NF-κB pathway leading to induction of apoptosis in the case of Fas, and potentially doxorubicin and paclitaxel.

Theoretically, at low RKIP levels there would be insufficient RKIP to facilitate complex formation hence the lack of drug response. As RKIP levels increase to optimum concentrations a concomitant increase in drug response is observed as RKIP would promote the interaction of pro-apoptotic mediators such as TRAF6 and NF-κB pathway components. However at high levels of RKIP, RKIP would bind to each of the components individually and decrease the number of complexes and/or interactions; and so lead to a decrease or modulation of the drug response. Future work to investigate this hypothesis could employ co-immunoprecipitation and mass spectrometry techniques to determine complex formation. The use of protein arrays to investigate other protein interactors of RKIP that may be involved in this complex would also be interesting.

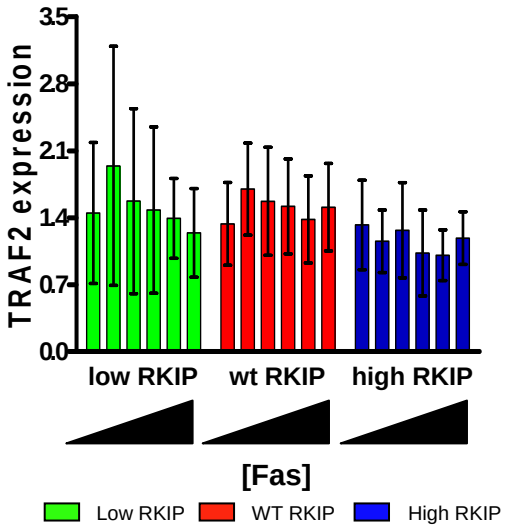
TRAF6 protein expression was not detected in TRAIL-treated Ls174T cells regardless of the levels of RKIP the cells expressed. The unique scaffold-like response of FasL-treated cells to both cell death and TRAF6 expression as a consequence of different RKIP levels, supports the hypothesis that the preferential employment of different TRAF subtypes in death receptor signalling were responsible for the differences observed between Fas and TRAIL-induced apoptosis. This was in agreement with the work reported by Lin *et al.* that described that the functions of the NF-κB pathway were dependent on the stimulus used as well as the cell type tested [260].

Future experimental work would examine the expression of other components of the NF-κB pathway as well as the proteins involved in DISC formation. It would be interesting to observe whether the expression of these proteins was altered by the treatment (FasL or TRAIL) or by the level of RKIP present in the cell. In addition, the use of immunoprecipitation experiments would allow the examination of the different complexes formed based upon the extracellular stimulus selected for use (*e.g.* FasL versus TRAIL). The data from these experiments would allow a more detailed characterization of the differential modulation of Fas and TRAIL sensitivity by RKIP in a colon carcinoma cell line.

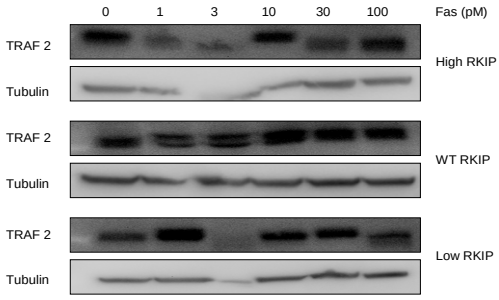
4.2.2.3 Tumour necrosis factor receptor associated factor 2 (TRAF2)

The levels of expression of TRAF2 protein in TRAIL-treated Ls174T cells indicated that this protein was involved in TRAIL signalling (Figure 4.5). It would be interesting to compare expression of TRAF2 in TRAIL-treated cells to the levels of protein expression in FasL-treated cells. Ls174T cells expressing low, WT or high levels of RKIP were treated with a range of FasL concentrations for 24 hours. Proteins extracted from these cells were separated on SDS-PAGE gels and the presence of the protein of interest targeted using TRAF2 specific antibodies. TRAF2 expression was then quantified using ImageJ analysis.

a)



b)



c)

Statistical Analysis	Source of variation	p-value
Two-way ANOVA	RKIP levels	0.5225
	[FasL]	0.9858
Wilcoxon signed-rank test	Low VS WT RKIP	N/A
	Low VS High RKIP	N/A
	WT VS High RKIP	N/A

Figure 4.10: The effect of RKIP levels on TRAF2 expression in colon carcinoma cells treated with FasL. a) ImageJ analysis of western blots (n=3) displaying TRAF2 expression after 24 hours treatment with various concentrations of FasL in Ls174T colon carcinoma cells with low, WT and high RKIP levels. TRAF2 expression was normalised to the tubulin control. **b)** Low, WT and high RKIP Ls174T colon carcinoma cell TRAF2 expression was monitored by western blot analysis after 24 hours treatment with various FasL concentrations (0 – 100 pM). Tubulin was employed as a loading control. (n=3 with sample western blot shown). **c)** Table of statistics showing the effect of RKIP on TRAF2 expression in FasL-treated Ls174T colon carcinoma cells.

TRAF2 protein was detected in FasL-treated Ls174T cells expressing low, WT and high levels of RKIP. The analysis of the western blots did not indicate a FasL concentration dependent or RKIP-dependent change in the level of TRAF2 expression in Ls174T cells (Figure 4.10). Two-way ANOVA analysis demonstrated that neither RKIP nor the FasL concentration had any significant effect on the level of TRAF2 protien expression (Figure 4.10c). This concurred with the data presented by Tang *et al.* [163] that indicated that RKIP did not bind TRAF2 but did bind TRAF6.

TRAF2 has been shown to bind adaptors downstream of death receptor signalling and link death receptors to signalling pathways such as the NF-κB and JNK pathways [20, 22, 25, 29, 208-210, 249]. The adaptors involved in Fas signalling are less characterised than those

involved in TRAIL signalling. Although signal transduction has been better characterised for TRAIL-dependent signal stimulation, some similarities between the two stimuli have been reported. TRAIL and Fas both employ FADD and caspase 8 for their apoptotic signalling thus it is possible that these stimuli may employ similar mechanisms for the formation of secondary signalling complexes from their receptors. FADD and caspase 8 can recruit RIP and TRADD, and through this recruitment the TRAF adaptors can interact including TRAF2 [20-22, 25, 29, 210, 249]. Furthermore, if TRAF6 was involved in Fas signalling, TRAF2 could be recruited through hetero-dimerisation of TRAF2 with TRAF6 [329-331]. A TRAF6-TRAF2 interaction *via* the TRAF domains [329-331] could have resulted in the expression of TRAF2 as observed in the low, WT and high RKIP-expressing cells after Fas stimulation (Figure 4.10). Since TRAF6 has been shown to interact with RKIP [163], TRAF2 levels could have been indirectly affected due to interaction with TRAF6. If indirect binding of TRAF2 occurred, this may explain the lack of detectable changes in TRAF2 expression as a consequence of RKIP levels.

Whether TRAF2 levels are directly affected by RKIP as a consequence of RKIP-binding or *via* interaction with an RKIP-modulated complex formation requires further examination.

TRAIL-treated cells appeared to employ only the TRAF2 subtype following stimulation whereas the FasL-treated cells appeared to employ both TRAF2 and TRAF6 proteins. Furthermore, TRAIL-treated cells exhibited a decrease in TRAF2 expression as the RKIP levels were increased whereas FasL-treated cells displayed a potentially scaffold-like expression of TRAF6, and an involvement of TRAF2, upon increasing RKIP levels.

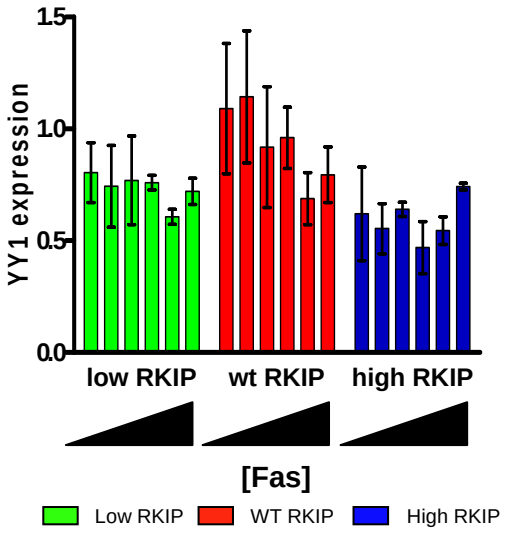
These initial studies suggest that differential employment of TRAF subtypes and their interaction with RKIP, whether directly or indirectly, contributed to the different responses of the low, WT and high RKIP-expressing Ls174T colon carcinoma cells to treatment with TRAIL and FasL.

4.2.2.4 Yin Yang 1

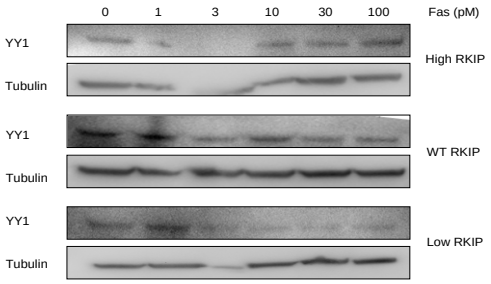
The expression of YY1 was monitored in FasL-treated Ls174T cells expressing low, WT and high RKIP levels. TRAIL-treated cells did not display any detectable changes in YY1 protein expression however, due to the differential activation of the TRAF subtypes and/or

the NF-κB pathway, it is possible that altered YY1 expression may be observed in FasL-treated cells. The expression of YY1 in RKIP cell lines were analysed by SDS-PAGE and the resulting western blots were quantified by ImageJ analysis.

a)



b)



c)

Statistical Analysis	Source of variation	p-value
Two-way ANOVA	RKIP levels	0.0037
	[FasL]	0.6613
Wilcoxon signed-rank test	Low VS WT RKIP	0.150
	Low VS High RKIP	0.119
	WT VS High RKIP	0.00951

Figure 4.11: The effect of RKIP levels on YY1 expression after FasL stimulation in colon carcinoma cells. a) ImageJ analysis of YY1 expression nomalised to tubulin loading control after 24 hours in low, WT and high RKIP-expressing cells treated with FasL. (n=3). **b)** YY1 expression monitored by western blot analysis after 24 hours in low, WT and high RKIP-expressing Ls174T colon carcinoma cells treated with 0-100 pM FasL. Tubulin was employed as a loading control. (n=3 with sample western blot shown). This membrane was stripped from Figure 4.10. **c)** Table of statistical analyses on the expression of YY1 following FasL stimulation as a consequence of RKIP levels within colon carcinoma cells.

Analysis of the western blots suggested that the expression of YY1 was higher in the WT RKIP-expressing cells compared to the levels of YY1 in the high and low RKIP-expressing colon carcinoma cells. This was confirmed by analysis of the quantified protein levels (Figure 4.11a) and indicated that the trends observed in the western blots was accurate (Figure 4.11b); both high and low RKIP-expressing cells had a negative effect on YY1 protein expression. Statistical analysis of the biological data using a two-way

ANOVA showed that the levels of RKIP had a significant effect on the YY1 protein expression (Figure 4.11c). Comparison of the YY1 protein levels in the three cell lines using Wilcoxon signed-rank tests showed that the WT RKIP-expressing cell lines were significantly different to high RKIP cells. Finally, the expression of the YY1 protein was not affected by the concentration of FasL used to treat the cells (Figure 4.11c).

The decrease in the level of YY1 expression observed in low and high RKIP-expressing cells (Figure 4.11) concurred with the data presented by Tang *et al.* that showed that both low and high RKIP levels resulted in the decreased activation of the NF-κB pathway [163]. Since YY1 expression is thought to correlate with NF-κB activity [302, 303, 332, 333], this adds weight to the argument that RKIP behaves as a scaffold-like protein. Furthermore, RKIP-dependent inhibition of the NF-κB pathway has been shown to decrease the YY1 activity in cells treated with chemotherapeutic agents [184, 189]. If YY1 expression is dependent on NF-κB activity in the Ls174T cell line, then TRAF activation of the NF-κB pathway could explain the increased expression of YY1 protein. The TRAF6 subtype displayed a similar expression profile (Figure 4.8) to the YY1 results above (Figure 4.11); both low and high RKIP-expressing cells appeared to exhibit lower TRAF6 protein levels than the WT RKIP-expressing cells. In addition, there appeared to be the involvement of the TRAF2 protein in Fas signalling however the expression of this protein was not affected by the cellular levels of RKIP (Figure 4.10). Despite this, the FasL-treated low and high RKIP-expressing cells displayed a decreased sensitivity to FasL-induced apoptosis (Chapter 3, Section 3.3.1, Figure 3.12).

In terms of FasL stimulation, all of the proteins thought to confer anti-apoptotic functionality to the cell have behaved in a contradictory manner in this study. This investigation suggests that NF-κB, TRAF6, TRAF2 and YY1 may have pro-apoptotic functions.

Indeed, a truncated version of YY1 has been produced by capsase-dependent cleavage in response to various stimuli, including FasL. This truncated form of YY1 enhances the apoptotic response [334]. Additionally, YY1 expression has been associated with a good prognosis in ovarian cancer and YY1 over-expression confers sensitivity to paclitaxel-induced apoptosis [335]. The effect of the taxane drug paclitaxel on colon carcinoma cells was studied in previous experiments and the cell death response of the paclitaxel-treated cells displayed a similar response to the FasL-treated cells (Chapter 3, Section 3.3.1 Figure 3.16). In these experiments, the WT RKIP-expressing cells were more sensitive to drug

treatment than the low and high RKIP-expressing cells. Furthermore, doxorubicin-treated cells also displayed this scaffold-like response after treatment (Chapter 3, Section 3.3.1, Figure 3.14). It is interesting to note doxorubicin and paclitaxel-treated cells showed a similar pattern of behaviour to FasL-treated cells. The examination of YY1 protein expression in Ls174T cells treated with these chemotherapeutic drugs would be very interesting.

It is however evident that differential expression of YY1 after TRAIL and FasL stimulation of Ls174T cells contributes to the modulation of the cell death response by RKIP. Further analysis would require the examination of the interaction between RKIP and YY1 to determine if they do indeed interact, or whether they form a complex; and finally whether this interaction is dependent on the stimulus or the cell-type, or indeed both variables.

4.2.2.5 Discussion

Expression of TRAF6 and YY1 in FasL-treated Ls174T cells exhibited a scaffold-like response dependent on the levels of RKIP present in the cells. WT RKIP-expressing cells treated with FasL appeared to have higher levels of these proteins compared to the low and high RKIP-expressing cells. This corroborated with the scaffold-like cell death response of the low, WT and high RKIP colon carcinoma cells to treatment with FasL; with WT RKIP-expressing cells demonstrating a higher sensitivity to FasL treatment than low or high RKIP cells.
The levels of pIKK increased as the levels of RKIP within the cell decreased. Again, this agreed with the known function of RKIP as an inhibitor of the NF-κB pathway. In contrast, it contradicted the cell death response observed after treatment with FasL. Additionally, there appeared to be the involvement of TRAF2 in Fas signalling but this involvement was independent of the levels of RKIP within the cell.

pIKK levels were used as an indicator of NF-κB activity; lower pIKK levels would correspond to lower NF-κB activation. NF-κB activation generally results in an anti-apoptotic response within the cell [96-98, 165, 251, 292]; since both high and low RKIP-expressing cells resulted in insensitivity to FasL administration, these results suggest that NF-κB was behaving in a pro-apoptotic manner.

Depending upon the stimulus, NF-κB can be both pro- and anti-apoptotic within a single cell-type [260]. It is possible that NF-κB activity during FasL stimulation was a delicate balance between the pro- and anti-apoptotic functions and that the cells expressing high levels of RKIP may have conferred resistance to the cells by preventing NF-κB dependent FasL-induced apoptosis [253, 255, 257-259, 325, 326]. Further examination of the transcriptional activity of NF-κB after FasL stimulation would be required to confirm this pro-apoptotic role of NF-κB.

The expression of TRAF6 and YY1 displayed a scaffold-like response with increasing RKIP levels following FasL stimulation. Both the low and high RKIP-expressing cells had lower levels of both proteins than the WT RKIP-expressing cells. For the high RKIP cells, it is possible that RKIP over-expression prevented the pro-apoptotic functions of these proteins [327, 328, 334, 335]. In addition, TRAF6 and YY1 have both been shown to interact with the NF-κB pathway, so it is possible that these proteins interact so as to induce a change in function; this function would be dependent upon the stimulus.
The adaptor protein TRAF2 was prevalent throughout FasL stimulation but its expression did not appear to change between the low, WT and high RKIP-expressing cells. TRAF2 could have been recruited to a protein complex through its interaction with TRAF6 [329-331] and/or other as yet unknown adaptors. However, the expression of TRAF2 was not subject to modulation by RKIP levels within the cell. Further studies would be required to examine the role of TRAF2 in Fas signalling, to determine what proteins TRAF2 interacts with, and finally, to determine whether RKIP can bind TRAF2 in FasL stimulated Ls174T cells.

Overall it appears that the classic anti-apoptotic proteins may behave in a pro-apoptotic manner should the need for this arise within the cell. This may occur *via* the recruitment of certain adaptor proteins to the death receptor; resulting in alternative cellular outcomes. Further examination of the key players involved in Fas signalling and how they can be modulated by RKIP would be required to better understand the mechanisms behind the increased sensitivity of WT RKIP-expressing cells to FasL-induced apoptosis.

4.2.3 Discussion of TRAIL versus Fas modulation by RKIP

The western blot technique used in this Chapter to examine the proteins potentially involved in RKIP modulation of chemosensitivity has some limitations. Firstly, many of the antibodies employed in this study are, as yet, of relatively poor quality and attempts were made to optimise antibody concentrations and incubations. Secondly, quantification of the western blot images using ImageJ and subsequent statistical analyses was difficult given the quality of some of the western blot results. As a consequence, the trends described in this Chapter require further characterisation *via* co-immunoprecipation and 2-dimensional gel electrophoresis before they can be described as conclusive. Despite these limitations, these exploratory results have generated some interesting theories.

TRAIL and FasL-treated colon carcinoma cells appeared to display differential expression of TRAF subtypes and the transcriptional regulator YY1 as a consequence of RKIP levels within the cells. This agreed with our hypothesis.
RKIP levels appeared to affect the levels of pIKK in a manner consistent with its function as an inhibitor of the NF-κB pathway [148] in both FasL and TRAIL-treated cells. However, this action was in disagreement with a potential scaffold-like function for the RKIP protein [163].

Two TRAF proteins were analysed in this study, TRAF2 and TRAF6. Only TRAF2 expression was evident in Ls174T cells following TRAIL stimulation and both TRAF2 and TRAF6 were present in the same cell line when cells were treated with FasL.
The expression of TRAF6 in FasL-treated cells displayed a scaffold-like response that was dependent on the RKIP levels in the cells. In the same cells, the levels of the TRAF2 protein were unchanged. In contrast, in TRAIL-treated cells the levels of TRAF2 protein decreased as the RKIP levels increased. This confirmed the differential behaviour of the same TRAF proteins by death receptors in the low, WT and high RKIP-expressing Ls174T cells. It also confirmed our hypothesis that differences in TRAF expression, and their possible modulation by RKIP, accounted for the differences in response of these cells to treatment with TRAIL and FasL.

Differential employment of adaptor proteins by Fas and TRAIL receptors supported evidence reported by Thomas *et al.* [336] that showed that the Fas and TRAIL death

receptors can recruit FADD in different manners dependent upon their C-terminal tails. Also, different agonists binding to these receptors can recruit different adaptors due to agonist-receptor binding resulting in conformational changes in the intracellular domain [336]. The involvement of TRAF6 after FasL stimulation appears to be pro-apoptotic as the highest expression was found in WT RKIP cells which experienced the highest level of cell death. Further investigation would be required to confirm RKIP and TRAF subtype binding in this cell line. In addition, the expression of other adaptors (such as FADD) would need to be tested.

The transcriptional regulator YY1 was affected by the levels of RKIP within the cell after FasL stimulation but not after TRAIL treatment. It is possible that YY1 may have undergone post-translational modifications and exerted cellular effects after treatment with TRAIL prior to the analysis at 48 hours. Thus far, the involvement of YY1 in TRAIL-induced apoptosis has not been demonstrated. In contrast, YY1 expression displayed a scaffold-like response after FasL treatment, with the highest level of YY1 expression present in the WT RKIP-expressing cells. These are the same cells that are more susceptible to FasL-induced apoptosis. In a manner similar to that for TRAF6 after FasL stimulation, it appears that the involvement of YY1 is pro-apoptotic in this FasL-treated cell line. YY1 is assumed to be an anti-death protein but it has been shown to behave in a pro-apoptotic manner [334, 335]. These studies suggest that the switch in YY1 function from anti- to pro-apoptotic may be cell-type and stimulus-dependent. Further studies would need to examine the downstream transcriptional targets of YY1 after FasL treatment of low, WT and high RKIP-expressing cells to confirm the pro-apoptotic functions of this protein. Finally, a future experiment would need to examine the potential interaction of the RKIP-YY1 proteins and the conditions under which this interaction may occur; this information would help better understand the involvement of YY1 in RKIP-related chemo-sensitivity.

Activation of the NF-κB pathway was measured by the phosphorylated IKK levels, and both FasL and TRAIL-treated colon carcinoma cells displayed a decrease in pIKK levels with increasing RKIP levels. This confirms that RKIP acts as an inhibitor of the NF-κB pathway [148]. In particular, NF-κB inhibition by RKIP in TRAIL-treated colon carcinoma cells, which resulted in increased sensitivity to TRAIL-induced apoptosis in the high RKIP-expressing cells, concurred with previous studies that showed that RKIP sensitised

cells to chemotherapy-induced apoptosis as a result of NF-κB inhibition [181, 182, 184, 189].

The effect of RKIP on the NF-κB pathway in FasL-treated colon carcinoma cells was more complex. An increasing body of evidence suggests that the NF-κB pathway has a pro-apoptotic function [251, 252, 254, 260, 317]. This function is necessary for chemotherapy-induced apoptosis and can involve the up-regulation of both the Fas ligand and the Fas receptor [251-253, 255-257, 317, 325]. So it is possible that Ls174T cells expressing high levels of RKIP may have prevented FasL-induced apoptosis by preventing the pro-apoptotic functions of NF-κB. However colon carcinoma cells with depleted RKIP showed decreased sensitivity to FasL-induced apoptosis despite the fact that there was hyper-activation of the NF-κB pathway; thus there may be a switch modulating the change of NF-κB function from anti-apoptotic to pro-apoptotic. Since TRAF subtypes are thought to be involved in linking death receptors to signalling pathways [21-25, 208-211, 249, 269, 316], and TRAF6 and YY1 displayed a similar scaffold-like response in expression as a consequence of RKIP levels following FasL stimulation. These adaptor proteins and the transcriptional regulator YY1 could be responsible for changing the functionality of NF-κB. Investigation into the expression of other NF-κB components, complex formation and transcriptional activity would be essential to determine the changes in the NF-κB pathway after TRAIL and FasL treatment.

This study focused on RKIP-dependent inhibition of the NF-κB pathway as a mechanism for the modulation of chemo-sensitivity. This was based on the observations described by Baritaki *et al.* [184]. However, it is also possible that the RKIP-related effects observed after FasL and TRAIL treatment may have been a result of RKIP inhibition of the ERK pathway. RKIP is a known inhibitor of the ERK pathway and acts at the Raf-1-MEK interface [143, 144]. The ERK pathway has been shown to affect both TRAIL- and FasL-induced apoptosis.

Evidence from TRAIL-treated HeLa, glioma and T-cells suggests that inhibition of the ERK pathway sensitises these cells to TRAIL-induced apoptosis [337-339]. This would corroborate with the data generated in the Ls174T cells; that increased RKIP resulted in an increased TRAIL-sensitivity if ERK inhibition was involved in this RKIP-related effect. Further studies would need to study the expression and activation of the ERK pathway components after TRAIL treatment in low, WT and high RKIP-expressing Ls174T cells.

The effect of the ERK pathway on Fas signalling is more complex; ERK can be either anti-apoptotic or pro-apoptotic depending on the cell line. In HeLa, T cells and glioma cells, ERK activation is anti-apoptotic, and conveys resistance to FasL-induced cell death [339-343]. In the HeLa and T cells, no negative effects were reported for ERK/MAPK on DISC assembly or on Fas receptor expression; however the overall activation of caspase 8 was impaired [339-342]. Inhibition of MAPK restored sensitivity to FasL-induced apoptosis in these cells [339-342]. In contrast, neuroblastoma cells required ERK for FasL-mediated apoptosis and appeared to induce expression of the Fas receptor [344]. These studies concurred with our results; that signal transduction can be a double-edged sword in Fas-mediated apoptosis. The fact that both low and high RKIP-expressing colon carcinoma cells displayed resistance to FasL-induced cell death was attributed to the balance between pro- and anti-apoptotic functions of the NF-κB pathway. Since RKIP can affect the ERK pathway, analysis of the expression of MAPK proteins would be interesting to determine if ERK signalling is also responsible for the contrasting results displayed in the colon carcinoma cell line following treatment with FasL.

In summary, FasL and TRAIL signalling differed in Ls174T colon carcinoma cells. Also, RKIP was able to modulate the response of Ls174T cells under both FasL and TRAIL stimulation. The data generated in this study point to the complexity of the role of the same proteins within the cells. The observation that different stresses may induce functional changes in proteins within cells, resulting in unexpected cellular responses has great implications for our understanding of cellular processes. Finally, the involvement of RKIP in the different responses of Ls174T cells to TRAIL and FasL stimulation emphasises the adaptability, flexibility and importance of the RKIP protein in determining cell fate.

4.3 Chapter Discussion

This study has shown for the first time how RKIP levels within a cancer cell modulate the apoptotic response of two death receptor ligands in two different manners. WT RKIP colon carcinoma cells were more sensitive to treatment with FasL, compared to high RKIP cells which were more sensitive to TRAIL administration.
The mechanism behind this difference involved members of the TRAF family of adaptor proteins, components of the NF-κB pathway and the transcriptional regulator YY1. Whether RKIP directly interacts with each of these proteins or *via* a complex with known RKIP interactors such as NIK, TAK and IKK [148] still needs to be clarified; however the functional implications of the modulatory effect of RKIP are very exciting.

TRAIL and FasL have already been shown to behave differently in other carcinoma cell lines – breast, colon, thyroid and paediatric rhabdomyosarcoma [345-348]. These cells were either resistant to TRAIL but not FasL or *vice versa*. Our results corroborate this data; we demonstrate that FasL and TRAIL treatment results in different physiological outcomes in the cell. Differences between the TRAIL and the FasL-treated cells were thought to be a result of differential receptor expression and differential recruitment of caspase 8 by these receptors [345-348]. The expression of $cFLIP_L$ and $cFLIP_S$ also appeared to affect the response of FasL and TRAIL treatments in the breast and paediatric rhabdomyosarcoma cell lines; however it is unclear whether the expression of these proteins resulted in resistance or sensitivity to treatment [345, 348]. The C-terminal tails of the Fas and TRAIL receptors have been shown to recruit the adaptor FADD in different manners [336]. This may explain the differential employment of TRAF adaptors in this study leading to the different responses observed. It may also explain the differences in the expression of cFLIP in the breast carcinoma and paediatric rhabdomyosarcoma cell lines [345, 348].

Moreover, RKIP may interfere or promote binding of proteins to DISC, and enhance or inhibit secondary signalling resulting in the activation of the NF-κB pathway. In fact, RKIP has been shown to bind the TRAF6 subtype [163] thus other DISC components may also bind and interact with RKIP leading to changes in the apoptotic response. This hypothesis is extremely exciting as it may further explain the role of RKIP in chemo-sensitivity.

The increased sensitivity of high RKIP-expressing colon carcinoma cells to TRAIL treatment involved up-regulation of the DR5 receptor. This mechanism of increased TRAIL sensitivity has been consistent throughout many studies on TRAIL-treated cells [91, 184, 323, 349]. How RKIP would lead to increased DR5 expression requires further investigation, however we can state that it is unlikely to involve the transcriptional factor YY1. The decreased activation of the NF-κB pathway is essential for the increased sensitivity to TRAIL in this study. However a review of the literature shows that the effect of NF-κB on TRAIL sensitivity is conflicting and appears to be cell-type specific; some cell lines have shown a requirement for NF-κB activity for TRAIL-induced apoptosis whereas other studies have shown NF-κB activation to contribute to TRAIL resistance [91, 184, 321-324, 349, 350]. The results of this study add weight to the argument that the effect of NF-κB on TRAIL-induced apoptosis is cell-type specific.

In contrast, up-regulation of the Fas receptor in the presence of FasL may have been impeded by RKIP, due to the RKIP-directed inhibition of the NF-κB pathway. The pro-apoptotic function of NF-κB may be required for FasL-induced apoptosis in this colon carcinoma cell line. This study has strengthened previous studies that have shown that NF-κB can be both pro- and anti-apoptotic depending on the stimulus employed, even within a single cell type [251-253, 255, 257-260, 317, 325, 326, 351].
Furthermore, the YY1 protein also appeared to be pro-apoptotic following treatment with FasL in this cell line, and in a stimulus-dependent manner. The YY1 protein was modulated by RKIP in FasL-treated cell but not in TRAIL-treated cells. It is interesting to speculate that the difference in YY1 expression in these two treatments is a consequence of the different TRAF adaptor proteins recruited by the TRAIL and Fas receptors respectively. Differential recruitment of TRAF proteins to the receptors could also be affected by the levels of RKIP within the cell. Therefore, proteins within the cell may be able to alter their function depending on the stimulus employed, leading to different cellular outcomes. This is an important observation in understanding and elucidating the functions of proteins within the cell, and how they could be potentially manipulated for the treatment of cancer.

The involvement of the ERK pathway may also have be responsible for the differences observed between the TRAIL and FasL-treated cells because RKIP is an endogenous inhibitor of the ERK pathway [143, 144]. ERK has been shown to modulate both FasL and TRAIL-induced apoptosis [337-344, 347, 348]. Unfortunately due to time constraints, we

were unable to perform studies on the effects of TRAIL and FasL treatments on the MAPK proteins within the cells. This investigation may provide some new mechanistic insights into the differences in the responses of the Ls174T cells to treatment with FasL and TRAIL.

From the observations made in this study; what could the consequences of the differences in the cellular response to FasL and TRAIL treatment be for the treatment of colon cancer?
Over-expression of RKIP within a cell would mimic treatment with a MEK or NF-κB inhibitor; molecules that are becoming more prevalent in anti-cancer treatment [64, 94, 97-99, 108, 128, 165, 291-294]. These MEK or NF-κB inhibitors are being used in conjunction with other agents to overcome particularly resistant strains of cancer. This study highlights the importance of understanding the type of cancer being treated and the implications of different drug regimes. Since NF-κB appears to have a role in the RKIP modulation of TRAIL- and Fas-induced apoptosis, the use of an NF-κB inhibitor in combination therapy will be taken as an example.
TRAIL in combination with an NF-κB inhibitor would be an excellent therapy for an individual with WT levels of RKIP present in their tumour. In contrast, treatment of the same individual with FasL, (or paclitaxel or doxorubicin) and an NF-κB inhibitor and the tumour could be resistant to such therapy. The latter treatment would be ideal for tumours with down-regulated RKIP, as the NF-κB inhibitor would mimic or restore WT RKIP levels within the tumour; WT RKIP expressing tumours are more susceptible to treatment with FasL, doxorubicin and paclitaxel.

The studies shown here are clinically important as they provide a glimpse into the reasons behind differing responses to chemotherapeutic agents, and how they may succeed or fail when used in combination with other agents. Further understanding of these differences in response to FasL and TRAIL treatment may also provide clues as to why some cancers become resistant to particular drug treatments. Finally, the involvement of RKIP in modulating these responses adds weight to the use of NF-κB and MEK inhibitors in the clinic, and the importance of this protein in determining and modulating cellular fate.
There is no doubt that this protein will have an integral part to play in new targeted therapies, and will provide a fresh area for manipulation, in order to optimise cancer care and treatment.

CHAPTER 5:
PDE5 INHIBITORS
AND RKIP FUNCTION

5.1 G-protein coupled receptors and cyclic nucleotide signalling

G-protein coupled receptors (GPCRs) form the largest family of cell surface receptors. They are transmembrane surface receptors that are characterised by seven membrane-spanning regions which have an extracellular N domain and intracellular cytoplasmic tail [352]. As their name suggests, GPCRs couple to G-proteins of which there are two types; monomeric G-proteins (such as Ras, Rho and Rac) and heterotrimeric G-proteins (*e.g.* $G_{\alpha i}$ and $G_{\alpha s}$). GPCRs use the heterotrimeric G-proteins to transduce signals from a range of diverse stimuli to multiple signalling pathways [353]. The heterotrimeric GPCR genes consist of 16 Gα, 5 Gβ and 14 Gγ subunits that in combination form the G-protein complex [354]. In the inactive state, Gα is bound to guanosine diphosphate (GDP) which together with the Gβ and Gγ subunits form the inactive heterotrimer [353, 355]. Upon GPCR-ligand interaction, the GPCR itself acts as a guanine nucleotide exchange factor (GEF) to transform GDP to GTP resulting in the activation of the Gα subunit and the liberation of the Gβ and Gγ subunits; both of which can activate downstream signalling pathways [355].

One of the most prevalent signalling molecules activated by GPCRs is the 3',5'-cyclic adenosine monophosphate (cAMP) molecule and this is excellently reviewed in the following reference [356]. Activation of certain Gα subunits results in the stimulation of adenyl cyclase (AC), another large transmembrane protein. AC generates cAMP from adenosine-5'-triphosphate (ATP). The implications of cAMP generation are extensive [356]. Most of the downstream effects of cAMP are mediated *via* cAMP-dependent protein kinase (PKA) and A kinase anchoring proteins (AKAPs); which phosphorylate numerous substrates involved in the cardiovascular system, glycogenolysis, neurotransmitter signalling and transcription factor activation (including cAMP response element-binding protein (CREB)) [357].

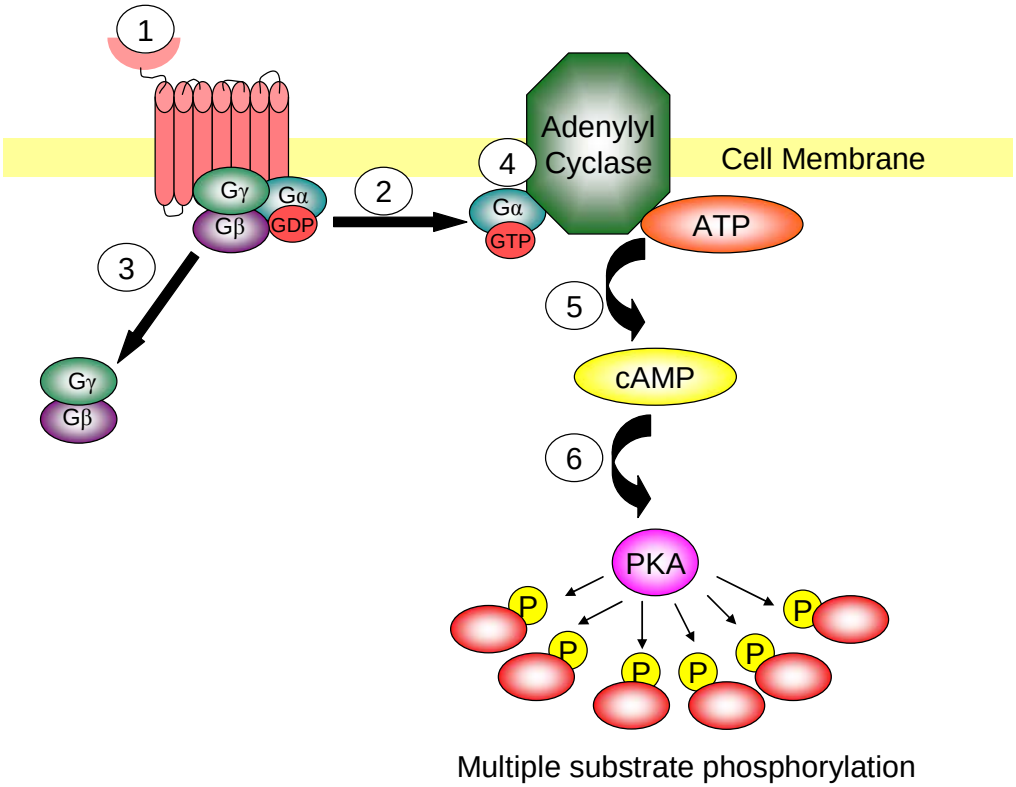


Figure 5.1: Diagrammatic representation of GPCR activation and cAMP signalling. 1. Ligand interaction with GPCR. 2. Guanine nucleotide exchange by GPCR resulting in the exchange of GDP on the Gα subunit for GTP. 3. Liberation of Gβγ subunits from the Gα subunit. 4. Activation of the membrane bound adenyl cyclase by Gα subunit 5. Generation of cAMP from ATP by adenyl cyclase. 6. Activation of PKA by cAMP results in the phosphorylation of multiple substrates.

A sister molecule of cAMP called 3',5'-cyclic guanosine monophosphate (cGMP) is an important mediator in NO signalling [358]. NO signalling is critical within the cardiovascular and central nervous systems; indeed aberrant signalling is linked to a number of serious conditions including hypertension, Alzheimer's disease (AD) and Huntington's disease, reviewed extensively by Moncada *et al.* [359]. NO is synthesised alongside citrulline from L-arginine by the enzyme nitric oxide synthase (NOS), of which there are three isoforms (endothelial, neuronal and inducible). The process also requires nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH) and molecular oxygen [359-362]. NO, in turn, is a ligand for soluble guanylate cyclase (sGC) [358] which catalyses the conversion of GTP into cGMP [363, 364]. Increased intracellular levels of cGMP result in the activation of cGMP-dependent protein kinases (PKGs) [365] which have many phosphorylation targets. The phosphorylation of these targets ultimately leads

to the relaxation of vascular smooth muscle and increased blood flow within the cardiovascular system [360, 366]. The functions of cGMP and NO within the central nervous are less well characterised but these molecules are thought to have a role in synaptic plasticity.

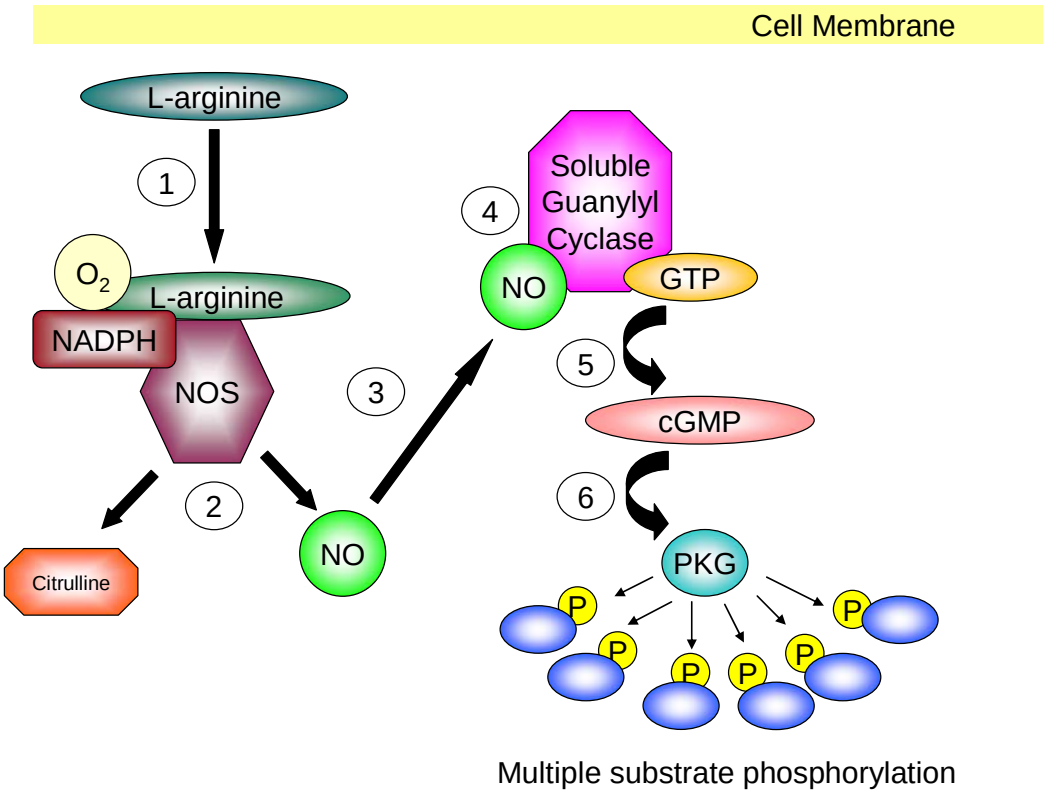


Figure 5.2: Schematic diagram of NO synthesis and cGMP signalling. 1. L-arginine, NADPH and molecular oxygen are recruited by nitric oxide synthase (NOS). 2. Production of NO and citrulline. 3. NO interacts with soluble guanylyl cyclase. 4. Activation of soluble guanylyl cyclase by NO. 5. Generation of cGMP from GTP. 6. cGMP activates PKG leading to phosphorylation of multiple substrates.

5.1.1 The phosphodiesterase (PDE) family

The 3',5'-cyclic nucleotide phosphodiesterase (PDE) family is a large superfamily of enzymes involved in the degradation of the 3',5'-cyclic nucleotide second messenger molecules cAMP and cGMP [367, 368]. These phospho-hydrolases catalyse the hydrolysis of cAMP and/or cGMP 3'-cyclic phosphate bonds in a highly specific manner. Depending on their substrate specificity, they are grouped into class I PDEs to distinguish from the

less selective class II PDEs. In this report, all references to PDEs refer to members of the class I PDEs unless otherwise stated [368].

To date, 11 subfamilies have been identified within the PDE superfamily and each has their own specific functions, including isoform-specific functions, however all PDEs degrade cyclic nucleotides [367]. The degradation of cAMP and cGMP is crucial for the transduction of signals from the exterior to the intracellular environment. In addition, hydrolysis of cyclic nucleotides serves as a modulator of both signal amplitude and duration [367, 368]. The effects of the PDE superfamily are seen in a number of different tissues and processes throughout the body. The effects of the PDEs and their effects on the vascular system are perhaps the best publicised due to availability of inhibitors such as Viagra (sildenafil citrate), however these PDEs have other important functions such as activation of T-cells and related immune responses as well as signalling within cardiac and neural systems [367, 368].

5.1.2 Sildenafil citrate

Sildenafil citrate (also known as Viagra) was developed by the pharmaceutical company Pfizer as part of a drug discovery programme looking for new agents for the treatment of angina pectoris [369]. Sildenafil citrate acts as a potent inhibitor of PDE5 and it was then observed to have an intriguing side effect. Originally, the rationale for the creation of potent PDE subtype 5 inhibitors for use in angina lay in the actions of cGMP and the nitric oxide (NO) pathway in the cardiovascular system.

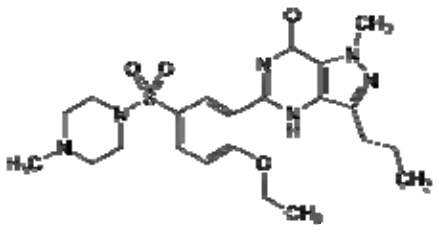


Figure 5.3: Structure of sildenafil citrate. Sildenafil citrate is a potent inhibitor of PDE5 and was originally created for the treatment of angina pectoris.

As described previously, the NO pathway results in an increase in the intracellular levels of cGMP which in turn stimulate PKGs. These PKGs have many phosphorylation targets and ultimately lead to the relaxation of vascular smooth muscle and increased blood flow within the cardiovascular system [360, 366]. The PDEs belonging to Group 5 (PDE5) are highly expressed in the vascular smooth muscle cells of the penis [368, 369]. The inhibition of PDE5 by sildenafil citrate [370, 371] results in an increase in the cGMP levels and subsequent relaxation of corpus cavernosum smooth muscle [370]. This unexpected side effect of sildenafil citrate has led to its notoriety as the drug of choice in the treatment of erectile dysfunction.

5.1.3 Vardenafil citrate

Vardenafil citrate (Levitra) is a structural analogue of sildenafil citrate. Vardenafil citrate inhibits PDE5 by an order of magnitude more than sildenafil citrate [372, 373]. This is probably due to structural variations in the double ring of the molecule including a nitrogen atom that is present in position 5 of the double ring that is present in vardenafil citrate but absent in sildenafil citrate [368, 372].

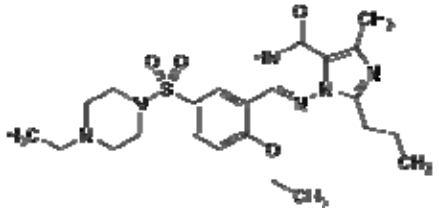


Figure 5.4: Structure of vardenafil citrate. Vardenafil citrate is a more potent inhibitor of PDE5 than sildenafil citrate. This is likely due to the nitrogen atom present in the double ring which is absent in sildenafil citrate.

Vardenafil citrate is also prescribed in the treatment of erectile dysfunction but the possible side-effects of treatment with this drug are less well known.

5.1.4 RKIP and interactions

In a recently published article, members of the Heck Laboratory in the Netherlands used an immobilized PDE5 inhibitor PF-3717842 in protein pull-down experiments to identify interacting protein molecules [374]. In HeLa cells, they identified the known target, PDE5 bound to PF-3717842, but they also identified RKIP [374].
We have been collaborating with the Heck group to validate RKIP as target for PDE5 inhibitors and to characterise the impact of the drugs on RKIP function. The experiments performed in this chapter attempt; (1) to understand the role that RKIP plays within the cell in the presence of sildenafil citrate and its analogue vardenafil citrate, and (2) to determine whether these drugs have any physiological effect on the known functions of RKIP. In the presence of the drugs sildenafil citrate or vardenafil citrate, we hypothesise that the function of RKIP within the cell will be affected. This would be very interesting, particularly in the development of new drug targets.

RKIP inhibition of the ERK pathway is well characterised. In fact, more research has focused on this aspect than on RKIP-dependent inhibition of the GRK2 and the NF-κB pathways [143, 144, 148, 150]. This study examines the effects of the drugs sildenafil citrate and vardenafil citrate on the RKIP-dependent inhibition of the ERK pathway.

Epidermal growth factor (EGF) and phorbol 12-myristate 13-acetate (TPA) were selected as inducers and used to stimulate serum-starved cells. EGF and TPA both strongly induce ERK pathway activation and are frequently used to study the interactions in this pathway [375-377].

EGF binds to the EGF receptor (EGFR), a receptor tyrosine kinase, and results in the activation of ERK as described in the Introduction [376]. EGF (30 ng/ml) induction was conducted as previously described by Rath *et al.* [156].
PKC contains two phorbol ester binding domains; thus TPA-activation results in the phosphorylation and activation of PKC [378], which in turn leads to activation the ERK pathway [377]. PKC activation of the ERK pathway can occur via Ras or Raf activation, or through the release of the negative effect of RKIP [94, 149]. In addition, PKC has been shown to phosphorylate RKIP on serine 153 switching RKIP function from Raf-1 inhibition to GRK2 kinase. The GRK2 kinase is involved in the down-regulation of

GPCRs [147, 149, 150]. TPA (100 ng/ml) induction was also conducted as described by Rath *et al.* [156].

The involvement of RKIP in the inhibition of the ERK pathway is well known [143, 144]. We planned to study the effect of sildenafil citrate and vardenafil citrate on RKIP-dependent inhibition of the ERK pathway. To ensure that the experiments were correctly designed and conducted, the RKIP inhibitor locostatin was used as a positive control; allowing the characterisation of the effects of the drugs sildenafil citrate and vardenafil citrate on RKIP function. Activation of the ERK pathway was measured by the expression of phosphorylated ERK 1/2.

5.2 The effect of locostatin on RKIP inhibition of the ERK pathway in a colon carcinoma cell line

Locostatin is an inhibitor of cell migration. The intracellular target for locostatin has been shown to be RKIP [178] although recent studies have suggested that locostatin may display substantial off-target effects [179]. As the only known chemical inhibitor of RKIP function, locostatin served as a positive control for analysing the effects of vardenafil citrate and sildenafil citrate on RKIP function in the ERK pathway.

5.2.1 The effect of locostatin and TPA stimulation on pERK levels in RKIP-expressing Ls174T cells

Three Ls174T cell lines expressing low, WT and high levels of RKIP were serum-starved for 24 hours then treated with TPA in the presence and absence of a range of locostatin concentrations. The ability of TPA to activate PKC [378] which in turn phosphorylates RKIP on serine 153 [150], may modulate RKIP-dependent inhibition of the ERK pathway.

It would be interesting to determine whether locostatin could affect RKIP function under these conditions. The proteins were separated on SDS-PAGE gels, proteins transferred to a nylon membrane and the presence of phosphorylated ERK 1/2 (pERK) and ERK detected using protein specific antibodies. The levels of protein were quantified using ImageJ analysis and the levels of pERK normalized to the control protein (ERK).

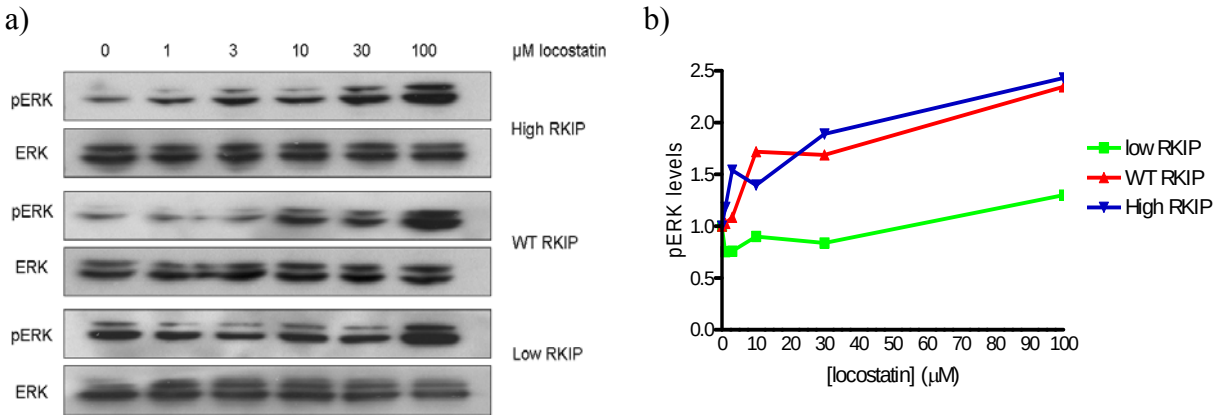


Figure 5.5: The effect of locostatin and stimulation with TPA on pERK activation in colon carcinoma cells expressing RKIP. a) Western Blots for pERK and ERK expression from top to bottom of; serum-starved Ls174T colorectal carcinoma high RKIP-expressing cells treated for 90 minutes with TPA alone, TPA + locostatin (0-100 µM); WT RKIP-expressing cells treated as above and low RKIP-expressing cells treated as above (n=1). **b)** ImageJ analysis of western blot (Figure 5.5a) showing pERK levels normalised to ERK loading controls, in RKIP-expressing Ls174T cells administered locostatin.

The western blot data image showed that the levels of pERK protein increased in the cell as the levels of RKIP protein within the cell were decreased (Figure 5.5a). The ERK loading control was comparable for all three Ls174T RKIP-expressing cell lines thus the changes in pERK observed were not due to experimental variation (*e.g.* protein loading), but to a biological phenomemon. This behaviour is seen most clearly in the untreated control samples (0 µM locostatin), suggesting that the RKIP-expressing Ls174T colon carcinoma cell lines were behaving in a manner consistent with the known functions of RKIP. RKIP is a known endogenous inhibitor of the ERK pathway and thus increased RKIP would lead to a decrease in ERK phosphorylation and activation [143, 144].

In addition, the levels of pERK were observed to increase as the concentration of locostatin was increased in all three Ls174T RKIP-expressing cell lines. This effect was most apparent in the high and WT RKIP-expressing cell lines compared to the low RKIP-expressing Ls174T colon carcinoma cell line (Figure 5.5). This data indicated that locostatin was acting in a manner consistent with its known function as an inhibitor of RKIP function [178]. Locostatin inhibition of RKIP would relieve the RKIP-based inhibition of the ERK pathway leading to the increase in phosphorylated ERK levels, as was observed above (Figure 5.5).

The small increase in pERK observed in the low RKIP cell line was likely due to TPA-directed stimulation of the ERK pathway (Figure 5.5). High RKIP-expressing cells displayed a similar increase in pERK levels to the WT RKIP-expressing Ls174T cells. Since locostatin is an inhibitor of RKIP function, it was hypothesised that locostatin would affect the high RKIP cells more than the low and WT cells. Due to the use of TPA, an inducer of PKC [378], it is possible that RKIP was phosphorylated on serine 153 by PKC. This would switch RKIP function from ERK inhibition to GRK2 inhibition [150]. Phosphorylation of RKIP, particularly in the high RKIP-expressing cells may have impeded locostatin-binding hence the similarity in response of the high and WT RKIP-expressing cells.

On the other hand, there may have been too much RKIP protein present in the high RKIP-expressing cells. It is possible that locostatin was unable to inhibit the large intracellular protein content of RKIP. The high RKIP expressing cells are thought to express approximately twice the amount of RKIP, in the form of FLAG-RKIP, than WT RKIP-expressing Ls174T colon carcinoma cells [194], this can also be seen in Chapter 2, Figure 2.1.

In summary, these initial results show locostatin to be behaving in a manner expected of an RKIP inhibitor, in terms of low RKIP-expressing cells versus both WT and high RKIP-expressing cells. Thus far, locostatin would be a good positive control upon which to observe the effects of PDE5 inhibitors on RKIP inhibition of the ERK pathway.

5.2.2 The effect of locostatin and EGF stimulation on pERK levels of colon cancer cells with low, WT and high RKIP

The experiment performed in Section 5.2.1 was repeated using EGF as an inducer instead of TPA. EGF activates the ERK pathway differently to TPA. EGF signals through the receptor tyrosine kinase as opposed to PKC-based phosphorylation of substrates [376]. EGF signalling should not result in the phosphorylation of RKIP. It would be interesting to see if EGF and locostatin elicited the same response at the RKIP level that was observed in pERK levels when Ls174T cells were treated with TPA and locostatin.

The low, WT and high RKIP Ls14T colon carcinoma cells were treated with EGF in the presence or absence of locostatin. Proteins were extracted from these samples and

separated on SDS-PAGE gels. The proteins were transferred to membranes and probed for the levels of pERK and ERK using protein specific antibodies. ImageJ analysis was used to quantify the levels of pERK, normalised to ERK loading controls.

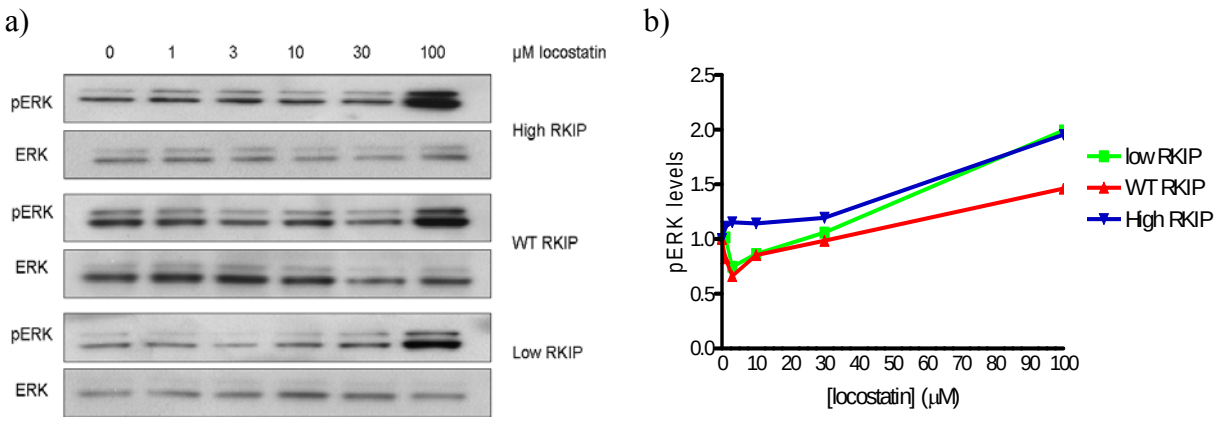


Figure 5.6: The effect of locostatin on the phosphorylation of ERK in EGF-stimulated Ls174T cells expressing low, WT and high RKIP levels. a) pERK and ERK western blots from top to bottom of; serum-starved Ls174T colorectal carcinoma high RKIP-expressing cells treated for 90 minutes with EGF alone, EGF + locostatin – 0, 1, 3, 10, 30 and 100 µM; WT RKIP-expressing cells treated as above and low RKIP-expressing cells treated as above (n=1). **b)** ImageJ analysis of pERK levels (normalised to ERK controls) in low, WT and high RKIP-expressing cells treated with locostatin, from western blot (Figure 5.6a).

The levels of pERK protein in the untreated control samples (0 µM locostatin) did not display changes in pERK according to the levels of RKIP within the cell (Figure 5.6a), as seen in the pERK levels from the untreated controls in Figure 5.5a. The low and high RKIP-expressing cells displayed the same levels of pERK, these were slightly lower than the levels of pERK expressed by WT RKIP-expressing cells. It is possible that the use of EGF as a stimulant, which strongly activates the ERK pathway, over-shadowed the subtle inhibitory effects of RKIP. Furthermore, EGF stimulation activates other Raf isoforms, in particular B-Raf, that are not subject to inhibition by RKIP [379]. RKIP has only been shown to interact with Raf-1 [143, 144]. This may explain the presence of pERK despite the levels of RKIP present within the cell.

Locostatin treatment between 0 - 30 µM had little effect on the levels of pERK in Ls174T cells expressing low or high levels of RKIP protein. Thereafter there was a dramatic

increase in the pERK levels in both cell lines (Figure 5.6). In the WT cells, there was an initial decrease in the levels of pERK (between 0-3 μM locostatin) followed by a marked increase in the levels of pERK (Figure 5.6).

In summary, locostatin-based inhibition was not observed until the drug concentrations were raised above 30 μM. At this point, elevated levels of pERK were observed. It is likely that the EGF-directed induction of ERK dampened the subtle effects of the locostatin treatment. Few changes were observed in the levels of pERK in untreated cells expressing low, WT and high levels of RKIP suggesting that the strong induction of the ERK pathway, perhaps through other Raf isoforms [121], was also masking the RKIP-based inhibition of this pathway.
Further experimentation with a variety of EGF concentrations would be required before conclusions can be made on the effect of locostatin on RKIP inhibition of the ERK pathway.

5.2.3 The effect of locostatin treatment under growth conditions in RKIP-expressing Ls174T cells

Previous experiments with serum-starved Ls174T colon carcinoma cells treated with TPA or EGF allowed the observation that locostatin had an effect on the levels of pERK and ERK in low, WT and high RKIP-expressing cells. We decided to look at whether locostatin had an effect on RKIP-directed modulation of the ERK pathway following normal cell culture growth conditions. The levels of pERK were quantified (normalised to ERK) using ImageJ analysis.

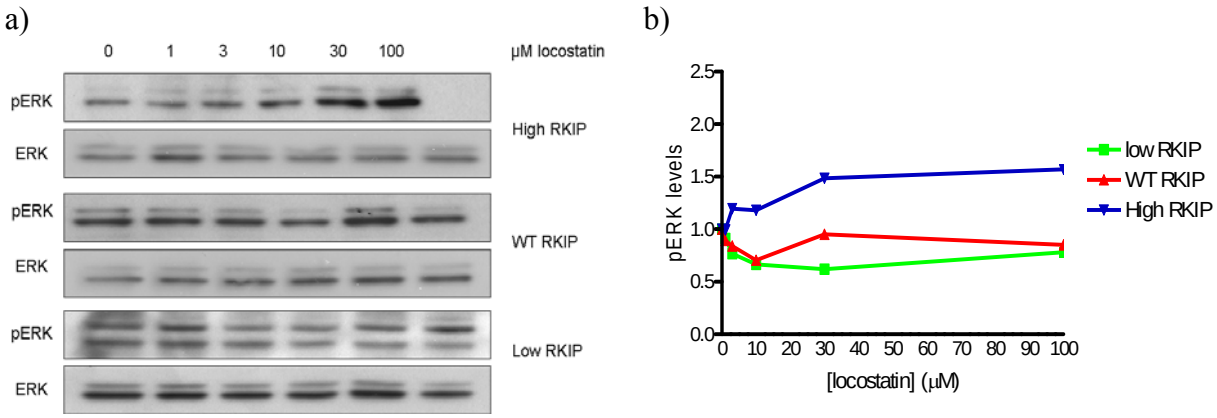


Figure 5.7: pERK activation after treatment with locostatin in growing colon carcinoma cells expressing RKIP. a) Western Blots for pERK and ERK expression from top to bottom; growing Ls174T colorectal carcinoma high RKIP-expressing cells treated for 90 minutes with locostatin – 0, 1, 3, 10, 30 and 100 µM; WT RKIP-expressing cells treated as above and low RKIP-expressing cells treated as above. All cells were grown for 24 hours in 5 % foetal calf serum prior to treatment (n=1). **b)** ImageJ analysis of pERK levels from Figure 5.7a (pERK levels normalised to ERK loading controls), in growing colon carcinoma cells expressing RKIP treated with locostatin.

pERK levels in the untreated low, WT and high RKIP-expressing Ls174T colon carcinoma cells (0 µM locostatin) correlated to the known effect of RKIP-based inhibition of the ERK pathway [143, 144]. The pERK protein levels decreased as RKIP levels within the cells increased (Figure 5.7a). As the ERK loading controls were consistent between all the locostatin-treated samples and similar for each of the RKIP-expressing Ls174T cell lines, it suggests that the pattern observed was a biological phenotype.

Treatment of Ls174T cells with locostatin in resulted in an increase in the pERK levels in the high RKIP-expressing cells but not the low or the WT RKIP-expressing Ls174T colon cacrcinoma cells (Figure 5.7). The treatment of Ls174T cells with increasing concentrations of the drug locostatin resulted in increasing levels of pERK in cells expressing high levels of RKIP protein. In contrast, the low RKIP cells displayed a decrease in pERK levels that correlated to treatment with increasing concentrations of locostatin. The pERK levels in the WT RKIP cells decreased between 0 - 10 µM locostatin but then recovered to basal levels of pERK protein (Figure 5.7). This suggested that locostatin was behaving as an RKIP inhibitor in the colon carcinoma cells. This corroborated previous studies that have reported that locostatin inhibits RKIP function [178].

Locostatin acted as an RKIP inhibitor in the high RKIP-expressing growing cells. This effect was not as dramatic as those observed when serum-starved Ls174T cells were treated with specific inducers (Figures 5.5 and 5.6). Furthermore, in the low and WT RKIP-expressing cells, the effect of locostatin concentration resulted in a slight decrease in pERK levels. It is possible that the locostatin was exhibiting off-target effects in these cell lines [179], which would account for the variation displayed in the results.

It is possible that any subtle locostatin-induced changes on the levels of pERK, as a consequence of RKIP inhibition in these low, WT and high RKIP Ls174T colon carcinoma cells, was over-shadowed by the growth and proliferative cascades within the cells. Moreover, the discrepancies in the pERK levels of growing cells may also be due to the off-target effects of locostatin [179]. Locostatin is known to modulate cell migration, motility and adhesion [176-178]. Locostatin may also interact with other growth and proliferative pathways resulting in the unusual response observed in pERK levels *via* cross-talk between pathways, particularly for the low and WT RKIP-expressing cells.

5.2.4 Locostatin treatment discussion

Locostatin was used as a positive control in these experiments because it is a known inhibitor of RKIP [178].

Changes in the pERK levels after locostatin treatment in the TPA- and EGF-stimulated cells were consistent with locostatin's known function as an RKIP inhibitor [178]. Therefore locostatin will serve as a good control for the validation of the effects of sildenafil citrate and vardenafil citrate treatment on RKIP-directed inhibition of ERK phosphorylation after stimulation with EGF and TPA. However it is possible that the EGF-based induction of the MAPK pathway is too strong such that the subtle modulation of pERK by RKIP cannot be detected. Thus experimental optimisation of EGF concentrations would be required before further interrogation of the effect of locostatin on RKIP function in these cells.

In growing cells, locostatin-treated cells exhibited pERK levels in a manner contradictory to its known function as an RKIP inhibitor [178]. The unusual effects observed may be due to off-target effects of the locostatin drug [179]. Further investigation of locostatin-directed inhibition of RKIP would be required before any clear conclusions can be drawn about the effect of this drug in growing cells.

The levels of pERK were influenced by the levels of RKIP present in the Ls174T colon carcinoma cells. Thus, overall the Ls174T colon carcinoma cell lines with low, WT and high RKIP appear to be reliable cell lines that can be used to study the effect of sildenafil citrate and vardenafil citrate on modulation of RKIP activity.

5.3 The effect of sildenafil citrate on RKIP inhibition of the ERK pathway in colon carcinoma cells

Sildenafil citrate is one of the best known and characterised PDE5 inhibitors [370] but the long-term effects of its use are still unknown. When it was reported that an analogue of sildenafil citrate was able to bind RKIP [374], an investigation into the effect of sildenafil citrate on RKIP-dependent inhibition of the ERK pathway was started. In previous experiments, we generated a pattern of behaviour for locostatin-treated Ls174T cells expressing RKIP. In this section, we treated the same cells lines with sildenafil citrate and then examined the levels of pERK/ERK in the cells.

5.3.1 The effect of sildenafil citrate and TPA stimulation on pERK levels in low, WT and high RKIP colon carcinoma cells

Ls174T cells expressing low, WT and high levels of RKIP were serum-starved for 24 hours before they were treated with TPA in the presence or absence of sildenafil citrate. The samples were then subjected to western blot analysis and the western blots probed with ERK and pERK specific antibodies. Protein levels were quantified using ImageJ analysis and pERK levels were normalised to ERK loading controls were. ImageJ analysis of the pERK levels of sildenafil citrate-treated cells were then compared with those of locostatin-treated Ls174T colon cells.

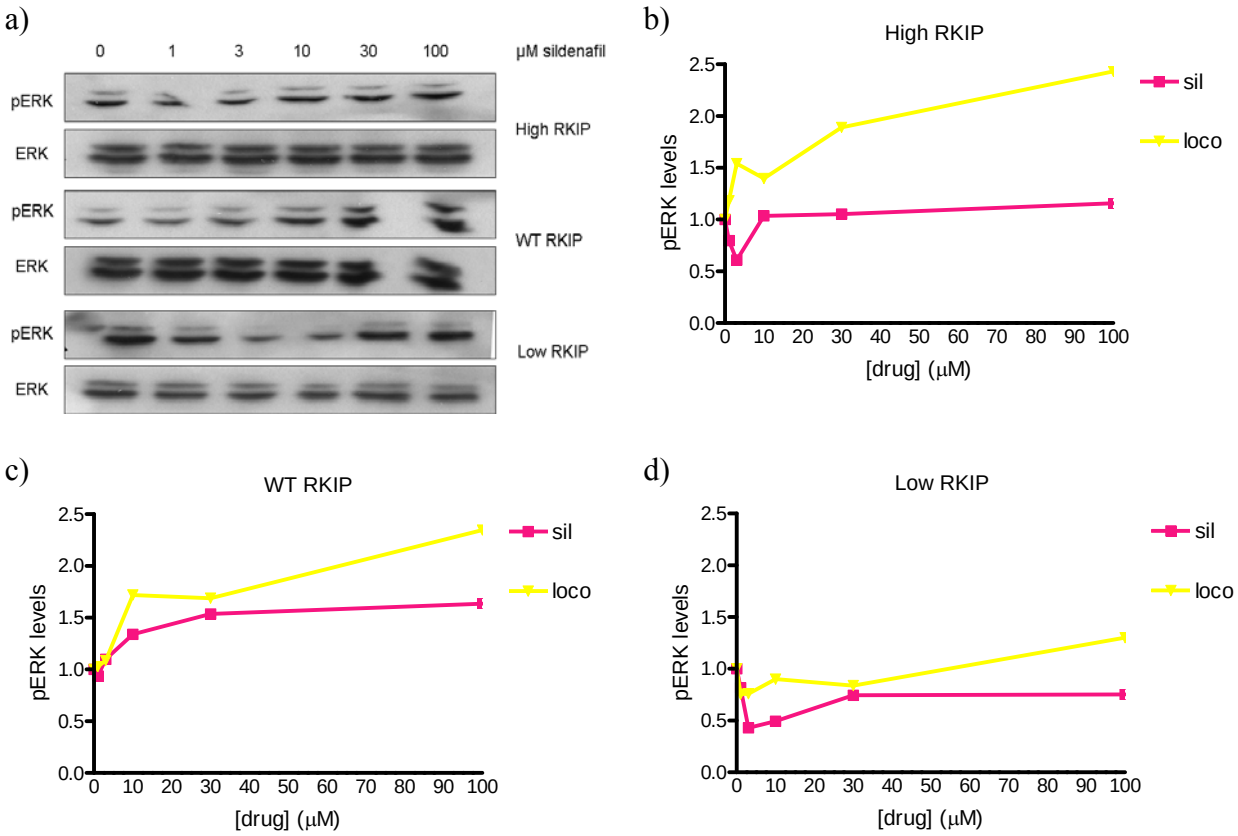


Figure 5.8: Comparison of the effect of sildenafil citrate versus locostatin on pERK activation of TPA-stimulated RKIP-expressing Ls174T cells. a) Western blots for pERK and ERK levels from top to bottom; serum-starved Ls174T colorectal carcinoma high RKIP-expressing cells treated for 90 minutes with TPA alone, TPA + sildenafil citrate – 0, 1, 3, 10, 30 and 100 μM; serum-starved Ls174T colorectal carcinoma WT RKIP-expressing cells treated as above and serum-starved Ls174T colorectal carcinoma low RKIP-expressing cells treated as above (n=1). **b)** ImageJ analysis of pERK levels of high RKIP-expressing cells from Figures 5.5a and 5.8a (pERK levels normalised to ERK levels). **c)** ImageJ analysis of pERK levels of WT RKIP colon cells, analysed from Figures 5.3a and 5.6a (pERK normalised to ERK). **d)** pERK levels normalised to ERK levels for low RKIP-expressing cells using ImageJ analysis, taken from Figures 5.5a and 5.8a.

The levels of pERK protein in the untreated Ls174T cells (0 μM sildenafil citrate) increased as the levels of RKIP within the cells decreased (Figure 5.8a). This corroborated the data presented in Figures 5.5a and 5.7a where Ls174T cells expressing RKIP showed a similar trend; highlighting that RKIP was responsible for inhibition of the ERK pathway [143, 144]. In order to gauge the significance of the sildenafil citrate treatment on the levels of pERK, the data for the sildenafil citrate-treated low, WT and high RKIP-expressing cells were compared to that of locostatin-treated cells. As locostatin is known to bind and inhibit RKIP [178] it serves as a positive control in this experiment. Treatment of Ls174T cells expressing low and high levels of RKIP with increasing concentrations of

sildenafil citrate resulted in a marked decrease in pERK levels (Figure 5.8). The levels of the pERK protein then increased gradually. In contrast, treatment of WT RKIP Ls174T cells with 3 - 100 μM sildenafil citrate resulted in an increase in pERK levels. The effect of sildenafil citrate treatment on pERK protein levels in Ls174T cells was not as dramatic as the effect of locostatin treatment. The pattern of behaviour exhibited by cells treated with sildenafil citrate was comparable to that of locostatin-treated cells; namely that the overall levels of pERK increased as the concentration of sildenafil citrate used in the treatment increased (Figure 5.8).

On the other hand, it is possible that the fluctuations observed in the three RKIP-expressing cell lines following sildenafil citrate treatment may have been due to the stimulation of the ERK pathway by TPA [375, 377], and not a result of the RKIP levels or the sildenafil citrate treatment. Furthermore, the use of TPA as a stimulant results in the phosphorylation of RKIP on serine 153 [149, 150, 378]. This switches the function of RKIP from the inhibition of Raf-1 to GRK2 inhibition [150]. Thus the observed increase in pERK levels may have been a consequemce of the relief of RKIP inhibition of the ERK pathway.

Further experiments would be required before any conclusions can be drawn about the effect of sildenafil citrate treatment on the pERK levels in the three TPA-stimulated RKIP cell lines. In addition, the phosphorylation of RKIP [149] as a result of the PKC-dependent phosphorylation [378] may have been preventing a potential sildenafil citrate-RKIP interaction; or leading to an increase in ERK activation due to the switch in RKIP function.

5.3.2 pERK levels after sildenafil citrate treatment and EGF stimulation in RKIP-expressing Ls174T cells

The effect of sildenafil citrate treatment on the levels of pERK in the three RKIP expressing cell lines was analysed after stimulation of serum-starved cells with EGF. The experiment was performed as stated in Section 5.3.1 but with EGF in place of TPA as a stimulant. ImageJ analysis was used to quantify pERK levels normalised to ERK loading controls, and the data overlapped with the data from the locostatin-treated cells.

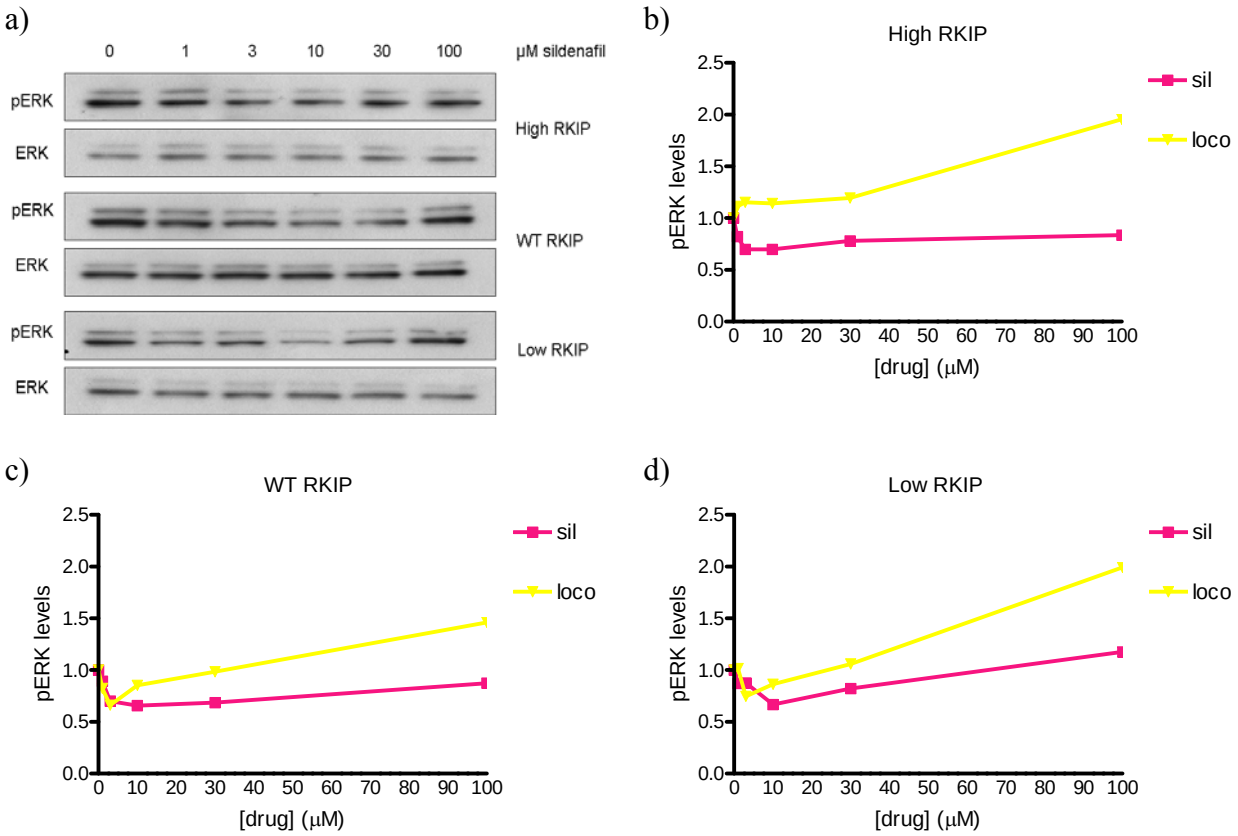


Figure 5.9: The effect of sildenafil citrate compared to locostatin on pERK levels of EGF-stimulated Ls174T cells with low, WT and high RKIP. a) pERK and ERK western blots of; serum-starved Ls174T colorectal carcinoma high RKIP-expressing cells treated for 90 minutes with EGF alone, EGF + sildenafil citrate – (0 - 100 μM); WT RKIP-expressing cells treated as above and low RKIP-expressing cells treated as above (n=1). **b)** ImageJ analysis of western blots (Figures 5.6a and 5.9a) showing pERK levels normalised to ERK levels for high RKIP cells for sildenafil citrate and locostatin-treated cells. **c)** ImageJ analysis of pERK levels (normalised to ERK loading controls) from WT RKIP colon cells, again analysed from Figures 5.6a and 5.9a. **d)** pERK levels normalised to ERK levels for low RKIP-expressing cells using ImageJ analysis, also taken from Figures 5.6a and 5.9a.

There was no great change in the levels of pERK in the EGF-stimulated cells, in the absence of sildenafil citrate, regardless of the levels of RKIP present in the cell (Figure 5.9a). The high and WT RKIP-expressing cells exhibited higher levels of pERK than the low RKIP-expressing cells, with the highest pERK levels observed in the high RKIP cells (Figure 5.9a). We expected the opposite to occur; increasing RKIP levels should have resulted in decreased levels of pERK [143, 144]. Since a similar pattern was observed in EGF-treated cell lines previously (Figure 5.6a), it is possible that the effect of RKIP on pERK levels was over-shadowed by the use of EGF as a stimulant.

Moreover, the behaviour observed for RKIP-expressing Ls174T cells treated with EGF in the presence of varying sildenafil citrate concentrations was similar to those observed with the same cells treated with EGF in the presence of varying locostatin concentrations (Figure 5.9b). Increasing concentrations of sildenafil citrate resulted in a small initial decrease in the levels of pERK in all three RKIP-expressing cell lines. Thereafter, in the WT and low RKIP cells there was a marked increase in the levels of pERK when cells were treated with more that 10 μM sildenafil citrate, following the trend of the RKIP inhibitor locostatin (Figure 5.9b). The pERK levels of the high RKIP cells returned to basal levels but did not display as sharp an increase as the low and WT RKIP-expressing cells. Overall, the administration of sildenafil citrate to the Ls174T cells displayed a pERK response similar to that observed when Ls174T cells expressing RKIP were treated with locostatin (Figure 5.9b). However the increase in pERK levels was not as prominent for sildenafil citrate-treated cells compared to cells administered locostatin.

This data suggests that sildenafil citrate treatment results in a small increase in the levels of pERK, thus sildenafil citrate may be a weak inhibitor of RKIP function. Since the low RKIP-expressing cell lines displayed a larger increase in pERK levels after sildenafil citrate treatment than the high RKIP-expressing cell lines, it is also possible that the increase in pERK levels may be an RKIP-independent effect.

On the other hand, the increase in the levels of pERK may be unrelated to the sildenafil citrate treatment. The stimulant in this experiment is EGF, a strong inducer of the ERK pathway [376]. It is possible that the lack of RKIP in the low RKIP-expressing cells would result in increased levels of Raf-1. This Raf-1 when activated could result in the increase in the levels of pERK that we observed in the low RKIP cells. It is also possible that EGF stimulation resulted in the activation of B-Raf. B-Raf has higher kinase activities compared to the other two Raf isoforms [121]. This may result in the elevated levels of pERK observed in the low RKIP-expressing cell line. It is interesting to note however, if the increase in pERK levels was a result of the stimulation employed, the effects were more pronounced in the low RKIP cells. This supports evidence that suggests a modulatory or rheostat role for RKIP in MAPK signalling [194].

Overall, the levels of pERK in sildenafil citrate-treated cells followed a similar trend to the locostatin-treated cells. The data suggests that sildenafil citrate may be acting in a similar

manner to locostatin [178] in the Ls174T colon carcinoma cell line. This is very exciting and further experimentation would be required to confirm this hypothesis.

5.3.3 The effect of sildenafil citrate administration under growth conditions in RKIP-expressing colon carcinoma cells

Understanding the effect of sildenafil citrate treatment on RKIP-based inhibition of the ERK pathway in non-stimulated cells was of interest particularly because the stimulants previously tested appeared to overshadow the subtle effects of the drugs selected for analysis. To address this, the three RKIP-expressing cells were grown for 24 hours in 5 % FBS then treated with sildenafil citrate at a range of different concentrations. The protein samples were then analysed for the presence of the proteins of interest on western blots using ERK and phosphorylated ERK antibodies. pERK levels were normalised to ERK levels and quantified using ImageJ analysis; this data was then compared to the pERK data from the locostatin-administered colon carcinoma cells.

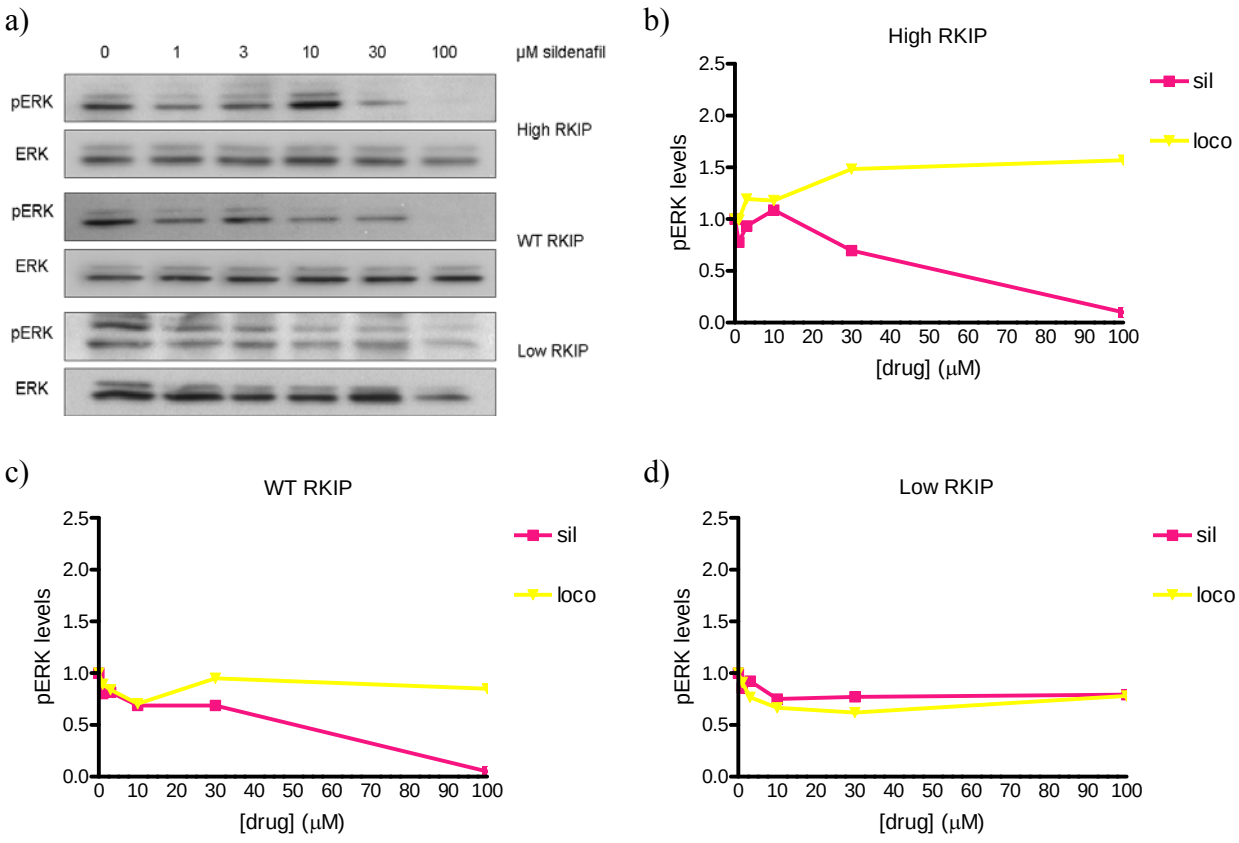


Figure 5.10: Activation of pERK in RKIP-expressing growing colon cancer cells treated with sildenafil citrate compared to locostatin-treated cells. a) Western blots analysis displayed the pERK and ERK levels from top to bottom of; Ls174T colorectal carcinoma high RKIP-expressing cells treated for 90 minutes with sildenafil citrate (0 - 100 μM); WT RKIP-expressing cells treated as above and low RKIP-expressing cells treated as above. All cells were grown for 24 hours in 5 % foetal calf serum prior to treatment (n=1). **b)** ImageJ analysis of sildenafil citrate and locostatin-treated high RKIP-expressing cells western blots (Figures 5.7a and 5.10a) showing pERK levels normalised to ERK levels for high RKIP cells. **c)** ImageJ analysis of pERK levels (normalised to ERK loading controls) of WT RKIP colon cells, also analysed from Figures 5.7a and 5.10a. **d)** pERK levels normalised to ERK levels for low RKIP-expressing cells from ImageJ analysis of Figures 5.7a and 5.10a.

The levels of pERK in the untreated control (0 μM sildenafil citrate) cells increased as the levels of RKIP within the cell decreased. The data in Figure 5.10a suggests that the RKIP-expressing cell lines were behaving in a manner consistent with the known functions of RKIP. This implied that RKIP-dependent inhibition of the ERK pathway was occurring.

The levels of pERK in Ls174T cells decreased as the concentration of sildenafil citrate used in the treatment increased. This decrease occurred in all three RKIP cell lines. Furthermore, these results contradicted our previous data (Figures 5.8 and 5.9). Here we observed an overall decrease in the levels of pERK protein as the concentration of the sildenafil citrate in the treatment increased, particularly in the WT and high RKIP cells where the pERK levels sharply dropped at 100 μM sildenafil. The most marked drop in pERK levels was seen in the high RKIP-expressing cells at concentrations higher than 10 μM. The sildenafil citrate-treated WT and low RKIP-expressing cells data generated the same trends that were observed in the locostatin-treated cells.
However, the locostatin-treated cells did not exhibit the patterns of behaviour expected from administration of an RKIP inhibitor [178] in the growing cells. Therefore it is possible that the growth and proliferative cascades within the cell were overshadowing the subtle effects of the sildenafil citrate and locostatin treatments.

On the other hand, since the high RKIP-expressing cells treated with sildenafil citrate behaved in a different manner to the locostatin-treated cells, it is possible that in growing cells, the effects of sildenafil citrate treatment were not dependent on the RKIP levels present in the cell.

Overall, due to the positive control locostatin behaving in an unexpected manner in the growing cells it is difficult to discern the effect of sildenafil citrate on RKIP function. It does appear that the induction of various proliferation and growth pathways as a result of the FBS present may be over-shadowing the effects of drug administration, and leading to off-target effects of sildenafil citrate. Further experimentation would be required before strong assertions can be made on the sildenafil citrate-RKIP interaction.

5.3.4 Sildenafil citrate treatment discussion

Treatment of Ls174T cells with sildenafil citrate resulted in virtually no change to the levels of pERK in the high RKIP-expressing cell lines. In contrast, low RKIP cells exhibited a varied response to treatment with sildenafil citrate. A marked decrease in the pERK levels was initially observed but this was followed by an increase in the levels of the protein. The WT RKIP cell line displayed an increase in the levels of pERK as the concentration of sildenafil citrate used in the treatment was increased. In summary, the effect of sildenafil citrate treatment on Ls174T cells was similar to that of the RKIP inhibitor locostatin [178]; albeit to a lesser degree.

It is also possible that in Ls174T cells, sildenafil citrate treatment did not exerting dramatic effects on pERK *via* RKIP. It is important to bear in mind that the inducers TPA and EGF were used in these experiments.
TPA strongly induces PKC [378] which in turn phosphorylates RKIP at serine 153 resulting in a switch in RKIP function from the inhibition of the Raf-MEK interaction to GRK2 inhibition [149, 150]. This could explanation the lack of response that was observed after treatment of the Ls174T cells with sildenafil citrate, particularly the high RKIP-expressing cells. It is likely that sildenafil citrate may have been unable to interact with phosphorylated RKIP. It is also possible that the actions of B-Raf over-shadowed the effects of the sildenafil citrate in the EGF-stimulated cells. B-Raf is not subject to direct regulation by RKIP [379] and is a more potent inducer of the ERK pathway than the other Raf isoforms [121].

Despite the strong effects of TPA and EGF-stimulation, there was a small modulation of RKIP function by sildenafil citrate resulting in effects in the MAPK pathway (Figures 5.8 and 5.9). This effect was comparable to that of locostatin treatment. Interestingly, both

locostatin and sildenafil citrate affected pERK levels in a contradictory manner in growing cells when compared to TPA and EGF-stimulated cells. It is possible that the drugs were exerting RKIP-independent effects and acting on other cellular targets, due to the activation of growth and proliferative cascades. Sildenafil citrate is a potent inhibitor of PDE5 and it is involved in cGMP signalling [370, 371]. Locostatin has also been shown to affect targets involved in cell migration [178, 179].

In summary, treatment of cells with sildenafil citrate resulted in changes in the pERK protein levels that showed a similar, but weaker, pattern as those observed when Ls174T cells were treated with locostatin. These results are promising, and further optimisation of these experiments may result in clearer patterns of behaviour being observed. This could include examining the expression of the proteins of interest at a variety of time points after treatment with the drug of interest. The data in this chapter would be confirmed if the interaction of RKIP and the PDE5 sildenafil citrate could be demonstrated. Future experimental work should prioritise protein-drug binding studies.

5.4 The effect of vardenafil citrate on RKIP inhibition of the ERK pathway in colon carcinoma cells

The PDE5 inhibitor vardenafil citrate is a structural analogue of sildenafil citrate [372, 373]. It is however, not as well characterised as its better known counterpart. The effect of vardenafil citrate on RKIP function was evaluated in a series of experiments in an attempt to better understand this drug.

5.4.1 pERK levels after vardenafil citrate treatment and TPA stimulation in RKIP-expressing colon carcinoma cells

Low, WT and high RKIP colon carcinoma cells were serum-starved for 24 hours prior to stimulation. These cells were treated with a range of increasing concentrations of vardenafil citrate. The phorbol ester TPA was used to stimulate cells and the levels of pERK protein in these samples visualised using western blot analysis. The pERK levels were normalised to ERK loading controls and quantified by ImageJ analysis; the data from the vardenafil citrate-treated cells was then compared to the data from the locostatin-administered cells.

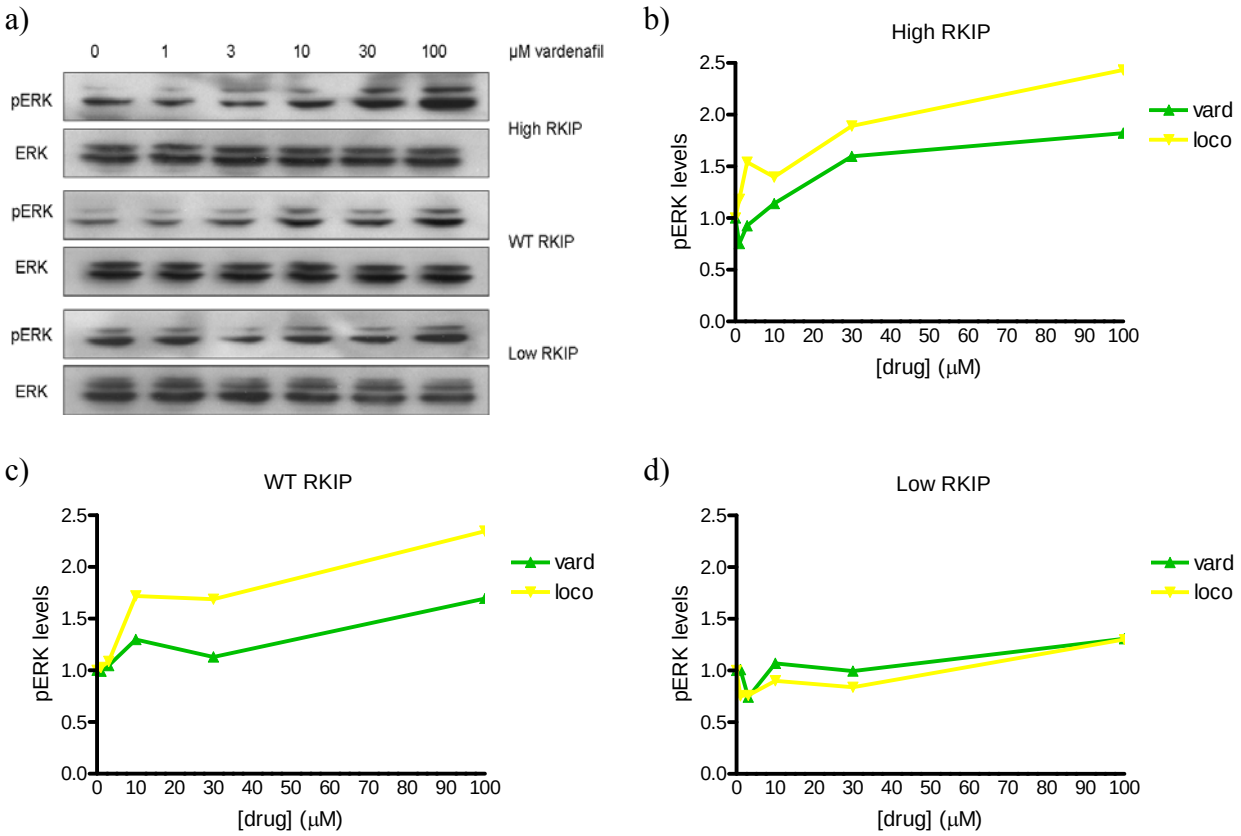


Figure 5.11: The effect of vardenafil citrate compared to locostatin on pERK levels in RKIP-expressing colon carcinoma cells stimulated with TPA. a) Western blot analysis displaying the pERK and ERK levels from top to bottom of; serum-starved Ls174T colorectal carcinoma high RKIP-expressing cells treated for 90 minutes with TPA alone, TPA + vardenafil citrate (0 - 100 μM); WT RKIP-expressing cells treated as above and low RKIP-expressing cells treated as above (n=1). b) ImageJ analysis of pERK levels (normalised to ERK loading controls) from high RKIP cells treated with vardenafil citrate and locostatin (Figures 5.5a and 5.11a). c) pERK levels normalised to ERK loading controls using ImageJ analysis of western blots showing WT RKIP cells treated with vardenafil citrate and locostatin (Figures 5.5a and 5.11a). d) pERK levels of low RKIP colon cells treated with vardenafil citrate and locostatin, normalised to ERK levels by ImageJ analysis of Figures 5.5a and 5.11a.

The levels of pERK in the untreated samples decreased as the levels of RKIP in the cell increased (Figure 5.11a). This correlated to the expected pattern of behaviour as RKIP as an inhibitor of the ERK pathway [143, 144].

Treatment with Ls174T cells with vardenafil citrate appeared to result in an increase in the levels of pERK in all three RKIP-expressing cells after 3 μM vardenafil citrate. This effect was more pronounced in high RKIP cells than in the low and WT RKIP-expressing cells

(Figure 5.11). These results were similar to the locostatin studies and suggested that vardenafil citrate had a potential RKIP inhibitory function [178]. Since the increase observed in the levels of pERK were higher in the high RKIP-expressing cells than in the WT and low RKIP cells, this suggests that the effect of vardenafil citrate on pERK levels is RKIP-dependent.

Vardenafil citrate-treated cells appeared to follow the same trend in pERK levels that was observed for the locostatin-treated cells (Figure 5.11), more so than the sildenafil citrate-treated cells (Figure 5.8) after TPA stimulation. In terms of structure, vardenafil citrate and sildenafil citrate have two major differences. The first is in the core ring that is used to mimic the structure of cGMP. The second difference is in the piperazine ring [372, 373]. These structural differences allow vardenafil citrate to have a 20-fold greater potency for PDE5 compared to sildenafil citrate [373]. Therefore it is possible that these differences could convey a similar potency in terms of their effect on RKIP function.

In summary the initial results from vardenafil citrate-treated cells are promising, and suggest an RKIP inhibitory function of vardenafil citrate in a manner comparable to locostatin. Further, the vardenafil citrate effects on RKIP appear to be more potent than its counterpart sildenafil citrate; this may be a consequence of structural differences between the molecules. Further replication of these results and protein-drug binding studies would be a priority to confirm this interaction.

5.4.2 The effect of vardenafil citrate and EGF stimulation on pERK levels in low, WT and high RKIP Ls174T cells

The levels of pERK obtained in Ls174T cells expressing varying levels of RKIP were performed on cells pre-treated with vardenafil citrate and stimulated with EGF. The proteins were analysed on western blots and the proteins of interest detected using pERK and ERK specific antibodies. ImageJ analysis was used to quantify pERK levels normalised to ERK, and the vardenafil citrate-treated pERK data was compared to the pERK data from locostatin-treated cells.

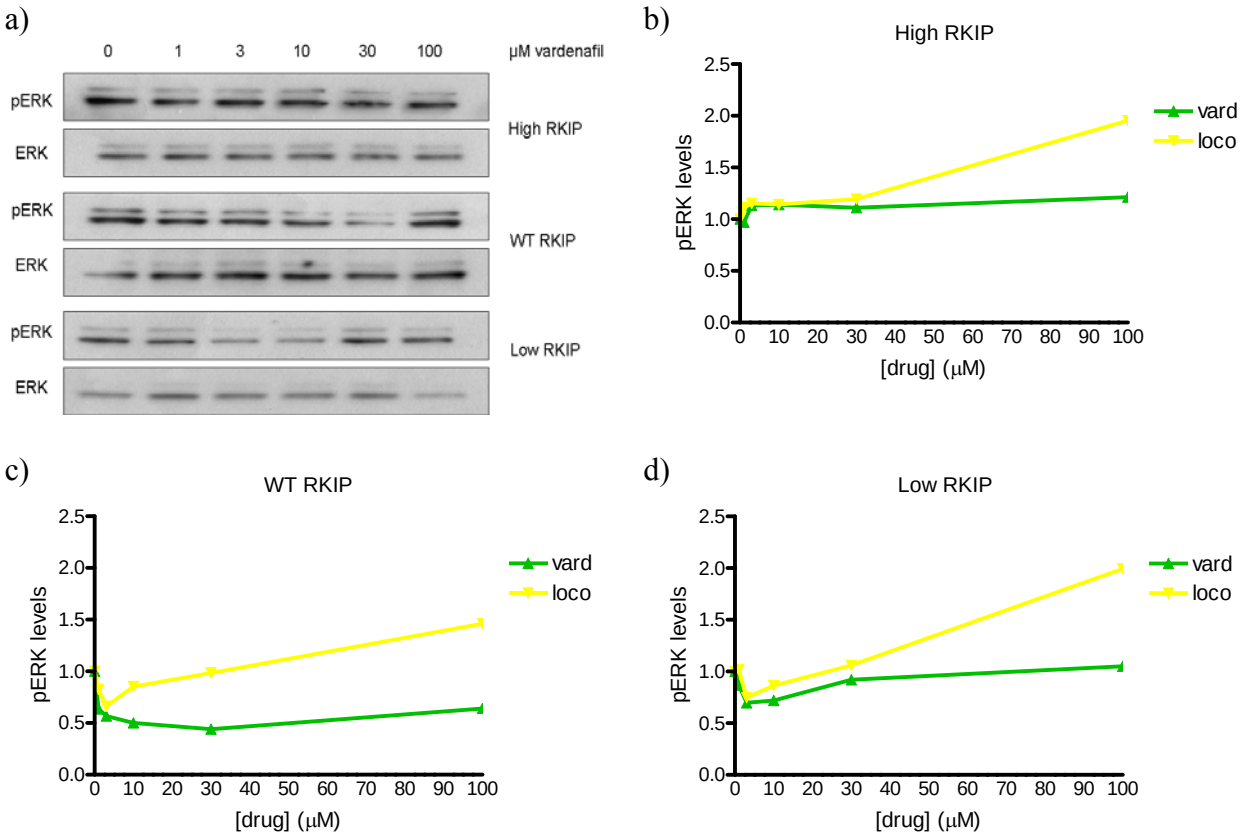


Figure 5.12: Vardenafil citrate versus locostatin effects on ERK phosphorylation in RKIP expressing colon cancer cells stimulated with EGF. a) Western blots for pERK and ERK from top to bottom of; serum-starved Ls174T colorectal carcinoma high RKIP-expressing cells treated for 90 minutes with EGF alone, EGF + vardenafil citrate (0 - 100 µM); WT RKIP-expressing cells treated as above and low RKIP-expressing cells treated as above (n=1). **b)** ImageJ analysis of pERK levels after vardenafil citrate or locostatin administration from Figures 5.6a and 5.12a in high RKIP-expressing colon cells, pERK was normalised to ERK loading controls. **c)** ImageJ analysis of the pERK levels (normalised to ERK) of WT RKIP-expressing cells treated with vardenafil citrate or locostatin, western blots analysed are shown in Figures 5.6a and 5.12a. **d)** pERK levels, from ImageJ analysis of Figures 5.6a and 5.12a , in low RKIP-expressing Ls174T cells treated with vardenafil citrate or locostatin. pERK normalised to ERK.

There was little difference between the levels of pERK in the three RKIP-expressing cell lines (Figure 5.12a). As in previous experiments that used EGF as a stimulant (Figures 5.6 and 5.9), the levels of pERK in the untreated samples did not behave in the manner expected [143, 144]; the levels of RKIP did not affect the levels of pERK present in the cell. The levels of the control protein ERK were comparable between the samples, regardless of the cell line or the vardenafil citrate concentration used to treat the cells.

When Ls174T cells expressing RKIP were treated with vardenafil citrate, increasing concentrations of vardenafil citrate had no effect on the levels of pERK in the high RKIP-expressing cells (Figure 5.12). In contrast, in the low and WT RKIP cells, vardenafil citrate treatment between 10-30 μM appeared to decrease the levels of pERK in the cells but concentrations between 10-30 μM resulted in the gradual increase in the levels of pERK (Figure 5.12). A similar result was seen in the pERK levels for low and WT RKIP cells treated with locostatin (Figure 5.12). It appears that the stimulation of EGF could have been too strong to observe the effects of vardenafil citrate on the levels of pERK, particularly since locostatin did not display any increases in pERK levels until 30 μM was administered. The optimisation of the parameters of this experiment would be required to better understand the effect of vardenafil citrate on pERK expression in low, WT and high RKIP-expressing Ls174T colon carcinoma cells.

Moreover, due to the independence of the pERK levels from the RKIP levels, we suggest that it is possible that the stimulation of the ERK pathway with EGF resulted in the activation of other Raf isoforms [121] which activate the MAPK pathway, and overshadowed the effect of both the levels of RKIP and the drug treatments. This result has been consistently observed throughout the three drug treatments during EGF stimulation. Therefore optimisation of experimental conditions would be required before further analysis of the effect of these compounds on RKIP inhibition of the ERK pathway.

5.4.3 The effect of vardenafil citrate treatment on pERK levels under growth conditions in RKIP-expressing colon cancer cells

The effect of vardenafil citrate on the levels of pERK in low, WT and high RKIP-expressing cells under normal cell culture growth conditions was also tested. Proteins from these samples were analysed on western blots using pERK and ERK specific antibodies. To better understand the levels of pERK protein in the samples, the levels of the proteins present on the western blots were analysed with ImageJ analysis. This data was compared to that of the locostatin-treated Ls174T cells.

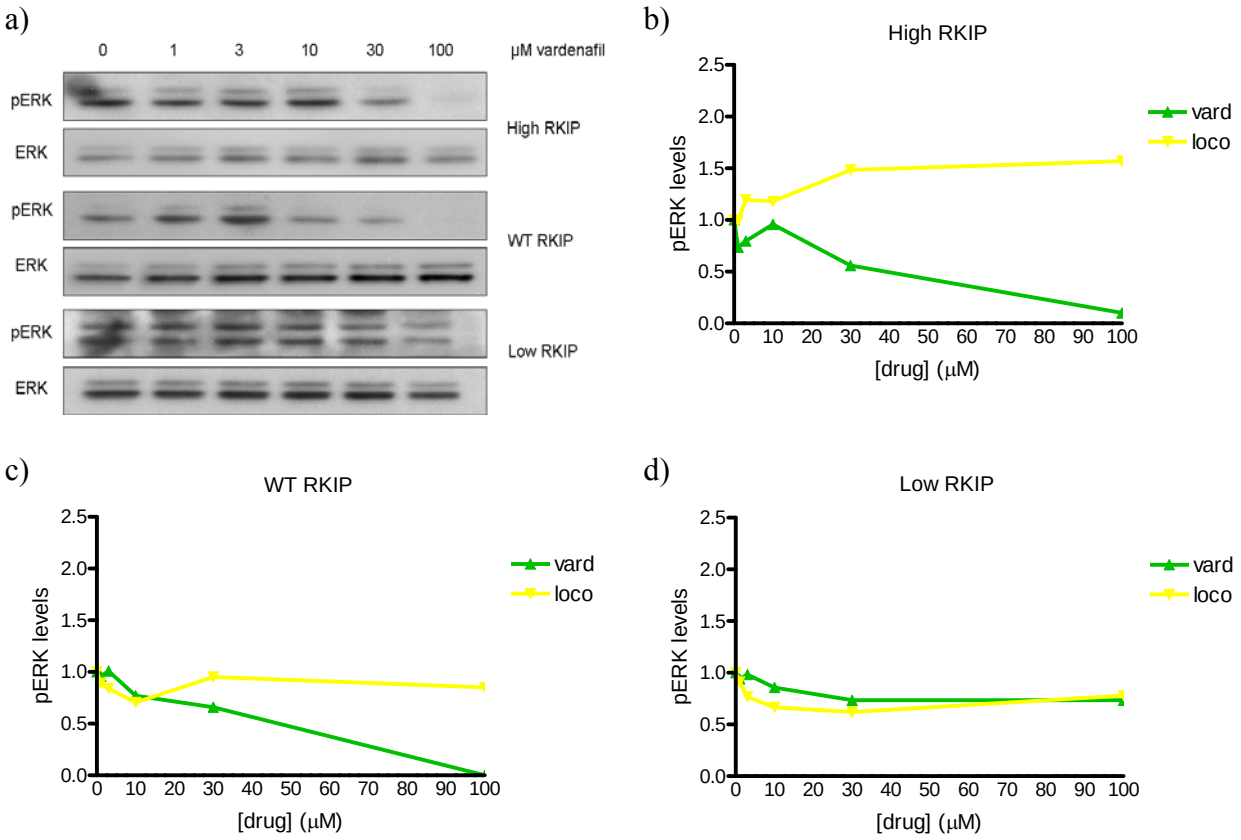


Figure 5.13: The effect of vardenafil citrate on pERK levels in growing colon carcinoma cells with low, WT and high RKIP, in comparison to locostatin. a) pERK and ERK levels from western blot analysis in, from top to bottom; growing high RKIP-expressing cells treated for 90 minutes with vardenafil citrate (0 - 100 μM); WT RKIP-expressing cells treated as above and low RKIP-expressing cells treated as above. All cells were grown for 24 hours in 5% foetal calf serum prior to treatment. **b)** ImageJ analysis of pERK levels (normalised to ERK loading control) from Figures 5.7a and 5.13a, of high RKIP colon cells administered vardenafil citrate or locostatin. **c)** pERK levels (normalised to ERK) of WT RKIP Ls174T cells treated with vardenafil citrate or locostatin, from ImageJ analysis of Figures 5.7a and 5.13a. **d)** pERK levels of low RKIP-expressing colon cells treated with vardenafil citrate and locostatin, normalised to ERK levels by ImageJ analysis of Figures 5.7a and 5.13a.

In the untreated samples, the levels of pERK did not appear to decrease as the levels of RKIP in the cell increased. The low RKIP cells however did display increased pERK levels compared to WT and high RKIP-expressing cells (Figure 5.13a). This was not consistent with the known function of RKIP as an inhibitor of the ERK pathway [143, 144].

Treatment of cells with an increasing concentration of vardenafil citrate resulted in a drop in the levels of pERK. This effect was more apparent in the WT RKIP-expressing cells

than in the high RKIP or the low RKIP-expressing cells (Figure 5.13). This contradicted the TPA- and EGF-stimulated results (Figures 5.11 and 5.12) that suggested that vardenafil citrate may be a weak inhibitor of RKIP function akin to locostatin [178]. Interestingly, growing cells treated with sildenafil citrate also displayed this contradictory result (Figure 5.10), with the pERK levels in the WT and high RKIP cells decreasing sharply at 100 μM sildenafil and vardenafil. Vardenafil citrate-treated growing cells exhibited a trend in pERK levels similar to that observed for sildenafil citrate-treated growing cells. It is likely that these results, which contradict those obtained for TPA- and EGF-stimulated cells, were due to the strong induction of various growth and proliferative cascades that may disrupted the subtle responses of the MAPK pathway to the vardenafil citrate treatment. We suggest this may be the case since the effect of locostatin [178] treatment on growing cells did not concur with its known functions, nor with the data obtained for locostatin-treated Ls174 cells that had been stimulated with EGF or TPA.

However the high RKIP cells displayed a more dramatic decrease in pERK levels than low or WT RKIP cell lines (Figure 5.11). Thus it is possible that there was an element of RKIP-dependent interaction with vardenafil citrate, such that vardenafil citrate was potentiating the actions of RKIP within growing cells. This is contradictory to the results obtained for the EGF and TPA-stimulated cells (Figures 5.9 and 5.10). This result may have been a consequence of vardenafil citrate interacting with other targets within the cell; these interactions could in turn have an effect on ERK signalling *via* pathway cross-talk.

In summary the data presented above suggests that vardenafil citrate may act in a manner similar to the known RKIP inhibitor locostatin, and the PDE5 inhibitor sildenafil citrate, but that the use of FBS over-shadowed the effects of drug treatment.

5.4.4 Vardenafil citrate treatment discussion

Vardenafil citrate treatment and TPA-stimulation of Ls174T cells resulted in a potentially RKIP-dependent effect on pERK levels within the cells. High and WT RKIP-expressing cell lines exhibited a gradual increase in pERK levels when more than 3 μM vardenafil citrate was used to treat the Ls174T cells, with the higher RKIP cells being more sensitive to drug treatment. The low RKIP cells displayed a varied response in pERK expression

thus, as in the case of sildenafil citrate-treated cells; it is possible that vardenafil citrate was displaying RKIP-independent effects on pERK in the low RKIP cell line.
The levels of pERK in vardenafil citrate-treated cells were similar to the levels of pERK observed in the locostatin-treated cells after TPA-stimulation. It is possible that vardenafil citrate was acting as a weaker, potentially indirect inhibitor of RKIP, like the RKIP inhibitor locostatin [178].

After vardenafil citrate treatment and EGF stimulation, there was no marked change in levels of pERK regardless of the drug concentration used to treat the cells. The greatest change occurred in the WT RKIP cells where increasing concentrations of vardenafil citrate resulted in decreased levels of pERK in the cell. It is possible that EGF was masking the effects of the drug treatment; this also appeared to be the case for locostatin and sildenafil citrate-treated cells after EGF stimulation. However, the effect observed after vardenafil citrate treatment did appear to mimic the effect of the RKIP inhibitor locostatin [178].

In the growing cells, pERK levels displayed a response contradictory to the results observed after vardenafil citrate treatment when both EGF and TPA were used as stimulants. Both locostatin and sildenafil citrate also exhibited this contradictory result in pERK levels in growing cells. It is possible that vardenafil citrate was displaying off-target effects, *e.g.* RKIP-independent effects during the growth of the cells such as PDE5 inhibition [372] which may have result in downstream effects on ERK signalling.

Finally, vardenafil citrate also appeared to follow the trend of the RKIP inhibitor locostatin more potently than sildenafil citrate. Vardenafil citrate and sildenafil citrate have different potencies in terms of PDE5 inhibition due to structural differences in the double ring of the molecules; this may also confer increased potency in terms of an interaction with RKIP. Further characterisation of RKIP-vardenafil citrate binding, and the replication of this data would be required before strong conclusions can be made on the effect of vardenafil citrate on RKIP inhibition of the ERK pathway.

5.5 General Discussion of RKIP and PDE5 inhibitors

In general, the levels of the pERK proteins in the untreated Ls174T cells were consistent with the known actions of RKIP. Low levels of RKIP displayed higher levels of pERK than the WT RKIP cell line and the WT cells had higher levels of these proteins that the high RKIP-expressing cell line. This was due to RKIP-dependent inhibition of MEK phosphorylation by Raf-1 kinase, which subsequently prevented the activation/phosphorylation of ERK by pMEK [143, 144]. The Ls174T cell line was therefore a good system upon which to investigate the effects of locostatin and PDE5 inhibitors on RKIP inhibition of the ERK pathway.

Locostatin was employed as a positive control in these experiments because it is known to bind and inhibit RKIP [178]. Locostatin treatment resulted in increased levels of pERK that were more prominent in the high RKIP-expressing cells. Unfortunately, the mechanism by which locostatin modulates RKIP function is not certain [179]. Initial evidence on the functions of locostatin focused on its role as an inhibitor of cell migration. The intracellular target for this purpose was RKIP [178]. It is possible that locostatin disrupts the RKIP and Raf-1 interaction; this would support our observation that locostatin treatment resulted in elevated pERK levels, especially in the high RKIP-expressing cells. Locostatin was therefore a suitable positive control to compare the effects of sildenafil citrate and vardenafil citrate on RKIP-dependent inhibition of the ERK pathway.

In terms of the results we present here, vardenafil citrate and sildenafil citrate appear to exert the same effect as locostatin on pERK. It is suggestive that these drugs could also act as RKIP inhibitors, albeit more weakly and not as direct inhibitors. This data supported our hypothesis that the effects of sildenafil citrate and vardenafil citrate could modulate the functions of RKIP. Further experimentation would be required to confirm this, particularly protein-drug binding studies looking for an interaction between RKIP-sildenafil citrate and RKIP-vardenafil citrate.

The effects of both sildenafil citrate and vardenafil citrate, in our studies on MAPK activity, were best demonstrated in the TPA-stimulated cells. In these cells, sildenafil citrate and vardenafil citrate treatment resulted in the inhibition of RKIP function in a manner similar to that of the RKIP inhibitor locostatin.

EGF-stimulation of Ls174T cells resulted in levels of pERK that were very similar in the untreated samples of all three of the RKIP expressing cell lines. It is likely that EGF-based induction of the MAPK pathway was overcoming the inhibitory effects of RKIP on this pathway. This is because EGF is a strong activator of the Ras-Raf-MEK-ERK pathway *via* its receptor tyrosine kinase EGFR [376]. EGF stimulation also activates B-Raf, which is a more potent MEK activator than Raf-1 and A-Raf [121] and is not regulated by RKIP [379]. The increased levels of B-Raf would lead to a strong pERK signal in EGF-stimulated cells. This would concur with the hypothesis that EGF induction of ERK was overshadowing the subtle effect of the PDE5 drugs on the levels of pERK protein. Indeed the effects of locostatin were not observed until concentrations of 30 μM and above. Despite the potential masking of the drug response by EGF, some of the results indicated that sildenafil citrate and vardenafil citrate were acting as weak and indirect inhibitors of RKIP function, akin to locostatin.

Earlier we mentioned that it was possible that vardenafil citrate was acting as a more potent effector of RKIP function than sildenafil citrate. In the TPA-stimulated high RKIP cell lines, the levels of pERK were higher when cells were treated with vardenafil citrate than when the were treated with sildenafil citrate. This may be due to the structural differences between the two drugs. These differences may make vardenafil citrate a more potent inhibitor of PDE5 which would in turn translate to a more apparent effect on RKIP function [372, 373]. Further experimental analyses would be required to determine whether this is the case.

The phosphorylation of MEK after treatment with the three drugs – locostatin, sildenafil citrate and vardenafil citrate – was also monitored under the same conditions as those of pERK. This data can be found in the Appendix of this thesis. However, pMEK levels displayed little or no change following treatment with all three drugs, and for all of the experimental conditions employed.

It is possible that this lack of change in pMEK could be the attributed to the relatively long induction time. It is possible that incubation with the drugs for 90 minutes may be too long for the presence of the short-lived pMEK protein. The only known function of pMEK is to activate ERK thus is it is reasonable that pMEK is rapidly de-phosphorylated once it has phosphorylated ERK [105, 123, 380, 381]. It is known that the phosphorylation of MEK and its subsequent phosphorylation of ERK is a very dynamic and temporal interaction,

especially since MEK de-phosphorylation after ERK phosphorylation is important for control and maintenance within MAPK signalling [105, 123, 380, 381].
It is possible that the optimisation of the experimental conditions (*e.g.* the length of incubation) would allow the detection of the active pMEK protein. Optimisation of experimental conditions would have been performed had time allowed.

An increase in the levels of pERK as a result of sildenafil citrate treatment has been demonstrated in cardiovascular studies; this occurred *via* a cGMP-dependent protein kinase (PKG) effect [382]. An increase in cGMP due to the activity of PDE5 inhibitors, such as sildenafil citrate, would increase the activation of PKG. This would result in increased phosphorylation of ERK as was demonstated by Das *et al.* [383]. It is not yet known whether this occurs in Ls174T colorectal carcinoma cells, and why RKIP would enhance such interactions is highly speculative at present.

NO signalling has been implicated in the formation of neoplasias in a paradoxical manner and is excellently reviewed in the following references [186, 188]. Depending on the concentration of NO and the cell type, NO can either increase cell proliferation thereby promoting a cancerous phenotype or NO can induce apoptosis in tumours and act as a tumour suppressor. As discussed in the Introduction for this chapter, NO signals through cGMP (Figure 5.2) and recent studies have shown that NO donors can also induce the expression of RKIP [189, 190].
The expression of RKIP by NO can lead to RKIP-dependent inhibition of the NF-κB pathway [189]. How this would affect the ERK pathway is currently unknown. Phosphorylation of RKIP at serine 153 by PKC switches RKIP function from Raf-1 inhibition to GRK2 inhibition [149, 150]. How RKIP interacts with both the ERK and NF-κB pathways under normal conditions and the dynamics of this inhibition is at present unknown. Could RKIP-induction by NO donors lead to an equilibrium change from a balanced ERK and NF-κB inhibition to one favouring NF-κB inhibition and relief of Raf-1 inhibition? Perhaps the increase in pERK observed after sildenafil citrate and vardenafil citrate treatment was the result of such a switch. The mechanism by which RKIP is induced by NO donors is unknown but if cGMP signalling is involved this may explain the data observed when Ls174T cells were treated with sildenafil citrate and vardenafil citrate. Attempts made to characterise the effect of PDE5 inhibitor treatment on NF-κB targets of RKIP such as IκBα were unsuccessful. If time had permitted, further analysis of NF-κB pathway components would have been performed under the conditions described here.

It is unclear whether RKIP interacts with sildenafil citrate and vardenafil citrate. If this interaction occurs, the specificity of this interaction is also unknown. Preliminary results shown in this report suggest that vardenafil citrate and sildenafil citrate may be acting as weak, potentially indirect, inhibitors of RKIP function. Furthermore, the interaction of sildenafil citrate and vardenafil citrate with RKIP may also be affected by other molecules within the cell; these molecules would vary depending on the stimulus used to treat the cells. It is likely that under optimised experimental conditions, a more distinct effect of vardenafil citrate and/or sildenafil citrate on RKIP function will be observed. Future experiments should include the optimisation of the experimental conditions. These experiments would have been performed had time permitted.

It has been reported that there is a significant amount of cross-talk between the cAMP pathway and the ERK pathway. This has been extensively reviewed in the following references [262, 384, 385]. The presence of PDE inhibitors such as sildenafil citrate and vardenafil citrate could theoretically result in an increase in the levels of cAMP in the cells. This could have a knock-on effect resulting in the activation of B-Raf and then of ERK.

At least 30 different isoforms of PDEs have been discovered that are involved in the hydrolysis of cAMP [367, 368]. In particular, there are a number of PDE4 isoforms that can affect cAMP and ERK cross-talk [386]. Elevated cAMP has been shown to inhibit ERK phosphorylation. The increased cAMP results in the activation of cAMP-dependent protein kinase (PKA), and PKA is thought to phosphorylate Raf-1 on serine 43 and serine 233 [262, 386]. This phosphorylation of Raf-1 by PKA prevents Ras activation of Raf-1 and results in the cessation of ERK signalling [262, 386].

To make matters more complicated, cAMP has been observed to increase ERK signalling in certain cell lines. cAMP has been shown to activate Ras and B-Raf leading to increased ERK signalling [385]. B-Raf is a more potent MEK activator than the other two Raf isoforms [121], and is not subject to regulation by RKIP [379], thus any effects on Raf-1 may be masked by the increased activation of B-Raf.

In fact, in this study the lack of discernable drug-dependent effects were attributed to EGF-dependent activation of B-Raf.

However the likelihood of such an interaction is small as sildenafil citrate and vardenafil citrate are highly selective for PDE5 [372]; and PDE5 is highly selective for cGMP as opposed to cAMP [367, 368].

Attempts were made to down-regulate PDE5 within the Ls174T cells to determine whether the data observed were due to PDE5-mediated interactions. We performed PDE5 siRNA transfections but we were unable to confirm suppression of PDE5 by the PDE5 siRNA.
The potential interaction of RKIP with PDE5 inhibitors such as sildenafil citrate and vardenafil citrate is very exciting. PDE5 inhibitors are important pharmaceutical agents and their long-term effects have yet to be characterised. The modulation of signalling pathways, albeit through indirect mechanisms, could have important pharmacological consequences and these need to be investigated.

5.5.1 Characterisation of the RKIP-PDE5 inhibitor interaction using Protein Crystallography and Mass Spectrometry

Collaboration with the Crystallography Department at the Beatson Institute for Cancer Research attempted to co-crystallise RKIP with sildenafil citrate. Crystallisation of RKIP protein was successfully perfomed as described by Banfield *et al.* [140]. Unfortunately, we were unable to co-crystallise RKIP bound to sildenafil citrate despite numerous modifications to the protocol.

RKIP-drug binding studies were also perfomed using Fourier-Transform Ion Cyclotron Resonance Mass Spectrometry (FT-ICR MS) by Dr Logan MacKay of SIRCAMS. Multiple sildenafil citrate and vardenafil citrate molecules were identified bound to RKIP but the levels of protein bound suggested that the interaction was a result of non-specific RKIP-drug interaction. In addition, the binding of drug molecules enhanced protein unfolding which suggested there may be some inhibitory effect, albeit very weak, of the drugs on RKIP. This would concur with the biochemical data shown above that suggested that sildenafil citrate and vardenafil citrate treatment resulted in weak inhibition of RKIP function. Attempts to characterise RKIP-locostatin interactions in the FT-ICR MS were unsuccessful. Unfortunately neither RKIP-locostatin binding, nor locostatin itself, could be detected. Due to the lack of positive controls in the FT-ICR MS experiments, the binding of sildenafil citrate and vardenafil citrate to RKIP needs to be further elucidated before any conclusions can be drawn. In summary, all of the initial results described herein are promising and warrant further investigation.

5.6 Chapter Discussion

A study reported by the Heck laboratory demonstrated an interaction between RKIP and the PDE5 inhibitor PF-3717842 [374]. In this study, analysis of the potential interaction of the PDE5 inhibitors sildenafil citrate and vardenafil citrate with RKIP was performed, by studying the effects of these compounds on the well characterised RKIP-dependent inhibition of the ERK pathway.

The RKIP inihibitor locostatin was used as a positive control in these experiments since it has been shown to bind and inhibit RKIP [178]. This interaction is poorly understood; studies by Zhu *et al.* [178] and Shemon *et al.* [179] disagree on whether locostatin prevents RKIP-Raf-1 interaction. Shemon *et al.* [179] have shown a locostatin-related compound bound to the ligand-binding pocket of RKIP, thus they suggest that locostatin may act in a similar manner. EGF was used as the stimulant in the Shemon study [179]. In our study, the use of EGF as a stimulus appeared to mask the subtle effects of RKIP and locostatin. It is possible that the EGF-directed activation of B-Raf resulted in a lack of detectable locostatin effect, especially since B-Raf is a more potent activator of MEK and is not be subject to regulation by RKIP [121, 379]. On the other hand, locostatin effects on RKIP may be cell-type specific and this requires further investigation.

Our investigations into the effect of locostatin on RKIP function in Ls174T colon carcinoma cells, has increased our understanding of the actions of this molecule on RKIP function. Locostatin-based inhibition of RKIP is dependent on the stimulus used to treat the cells. Whether the locostatin-directed inhibition of RKIP is direct or indirect has to be determined.

Treatment of Ls174T cells with sildenafil citrate and vardenafil citrate resulted in an inhibitory effect on RKIP in Ls174T cells. The levels of ERK activity increased as the concentrations of the drugs used to treat the cells increased. Whether this was due to the direct interaction of the drugs with RKIP, *via* an indirect mechanism or through downstream effects would need to be examined. If these PDE5 inhibitors do alter ERK signalling, it could have important pharmacological consequences. These drugs have shown promise in the treatment of various cardiovascular conditions such as pulmonary hypertension and chronic heart failure [382, 387-389]. However, the long-term effects of

these drugs are not well characterised. Understanding the mechanisms of action for PDE5 inhibitors would also allow us to safely apply them for the treatment of cardiovascular diseases. Furthermore, if RKIP and/or the ERK pathway could be targeted therapeutically for conditions such as cardiovascular disease, this would open a new avenue of research and potentially yield new and exciting drug targets.

On a different note, the involvement of the NO signalling pathway in the treatment of cancer is controversial [188] and NO donors have been shown to sensitise cells to chemotherapy after the induction of RKIP [189]. It is possible that PDE5 inhibitors that could potentiate NO signalling could also interact with RKIP and/or the ERK pathway, thus they may have implications for cancer therapy. Sulindac sulfone (Exisulind) which is from a new class of anti-cancer compounds and is also a non-steroidal anti-inflammatory agent, has been shown to work through the inhibition of cGMP dependent PDE and activation of PKG [390]. It is possible that sildenafil citrate and vardenafil citrate may have other applications that could be utilised in the treatment of cancer, either in combination with other drugs or alone. Since RKIP has a known role in cancer tumour suppression and chemo-sensitivity [153, 154, 159, 166, 168-173, 180-185, 189, 245, 290, 295], a possible interaction between RKIP and these two compounds would be highly exciting.

In summary, this study has shown that there may be a possible interaction between RKIP and the PDE5 inhibitors sildenafil citrate and vardenafil citrate. Further characterisation of this potential interaction would be required before strong assertions can be made; however there appears to be an overlap between the pathways regulated by RKIP and the signalling cascades modulated by sildenafil citrate and vardenafil citrate. This may have important implications in the understanding and treatment of cancer since all of the effects described herein have been implicated in neoplasias and cancer treatment.
It is clear that RKIP may have many different types of interacting partners within the cell. In addition, RKIP appears to affect the response of various pharmaceutical agents. Understanding and elucidating the functions of this unassuming protein at the crossroads of proliferative, apoptotic and tumour suppressive pathways is of great importance. The RKIP protein shows a high degree of promiscuity and flexibility within the cell and it interacts with a range of diverse proteins. There is no doubt that RKIP will have a substantial role to play in the development of future therapeutic targets.

CHAPTER 6:
FINAL DISCUSSION

6.1 Final Discussion

The aims of this PhD were:

1. To establish the role of RKIP in chemo-sensitivity in a colon carcinoma cell line and to subsequently unravel the mechanisms behind RKIP-dependent modulation of the cellular response to chemotherapy.
2. To examine the potential interaction of RKIP with PDE5 inhibitors and to determine whether PDE5 inhibitors could affect RKIP function in the cell.

The discoveries of this PhD are:

1. That RKIP modulates the apoptotic response of colon carcinoma cells to different chemotherapeutics in different ways. Furthermore, differential modulation of chemotherapy-induced apoptosis by RKIP involves members of the NF-κB pathway, TRAF adaptor proteins, the TRAIL receptor DR5, the anti-apoptotic molecule Bcl-xl and the transcriptional regulator YY1.
2. That PDE inhibitors may exert an inhibitory effect on RKIP function within the ERK signalling pathway. However the direct interaction between RKIP and the PDE5 inhibitors studied in this thesis, sildenafil citrate and vardenafil citrate, has yet to be confirmed.

Initially RKIP was not thought to be particularly important in cellular processes. Research has elucidated roles for RKIP in the modulation of ERK and NF-κB pathways. This suggested that changes in the RKIP protein would have a serious impact on cell growth and proliferation. However, RKIP KO mice did not display significant physical defects, nor were they predisposed to cancer. Studies in human carcinoma cells demonstrated that RKIP levels had few effects on primary tumourigenesis. The real interest in RKIP was sparked when it was discovered that the protein was involved in metastases suppression and that it could modulate chemo-sensitivity. A decade after its discovery as an endogenous inhibitor of ERK signalling, this unassuming protein is now an exciting and attractive avenue of research.

In this study we show that RKIP can radically alter the response of colon carcinoma cells to chemotherapy-induced apoptosis. This modulation and manipulation of apoptosis may involve proteins that are essential for proliferation and are supposedly anti-death. Thus it appears that nothing in the field of cancer and signalling is what it seems. Moreover, RKIP

may interact with another class of drugs; the PDE5 inhibitors. The functional implications of this interaction are as yet, unknown. However this may provide a new source of pharmaceutical manipulation for the treatment of disease.

The flexibility of the molecules within the cells is staggering. We are starting to understand and unravel how these individual components interact. Indeed, research on the functions of the RKIP protein, and its interactors within the cell, continue to reveal new information; and can often require a rethink in terms of the role a protein and/or a signalling pathway has to play within the cell. RKIP is a member of a protein family that has no known homology to any other protein family. This allows some latitude when hypothesising about the functions of this protein. There is, however, no doubt that this enigmatic protein has much more to reveal regarding its role within the cell.

The fundamental processes that govern cell decisions, and ultimately cellular fate, are entwined in a complicated and potentially tenuous manner. Signalling it seems, is a double-edged sword.

Human cells can survive tremendous onslaughts ranging from biochemical, to chemical to mechanical and so it is almost surprising that cancer is not more common. This statement in itself highlights how fascinating and flexible the components of the cell are. It is wonder of nature that the components within cells can change their functions and manipulate cellular fate to keep the cells, and in turn humans, alive and healthy. A small experimental change, such as the choice of one drug over another, results in the activation or inhibition of completely different sets of pathways, or even a well-characterised pathway in an unusual manner.

Such then, is the complexity of cancer, that understanding what happens in normal tissues under normal circumstances is still a conundrum. Every cancer cell and every tumour is a unique entity and while their origins may be similar, their evolution is different. Just as we are all unique from a combination of nature and nurture, yet our DNA blueprint and genes that make us humans spring from the same source.

A unified cure for cancer or a single magic bullet is therefore impossible. This is neither pessimistic nor disheartening; in actual fact this is realistic and at the same time promising. By targeting the disease as a whole, opportunities for manipulation and the targeting of potential weaknesses within certain tumours is lost. Targeted therapies aim to exploit the Achilles' heel of cancer cells; each tumour may have similar weaknesses but overall they will have their own individual weaknesses as a result of their separate evolutions. The most

likely therapy would be a combination of classic agents to target the tumour as a whole, followed by targeted therapy to eradicate the potentially resistant cells. This would be unique to the individual being treated and not a panacea.

Taking the protein that this thesis has focused on as an example; what does the modulation of cytotoxic responses by RKIP mean in the real world, to real patients, and in real treatments?
Firstly, it gives support to the targeted therapy revolution in anti-cancer treatments. Altering just one protein can mean the difference between life and death in the treatment of this disease, depending on the cancer type and the individual. Secondly, it gives more weight behind the need for personalised medicine. Individual patients may have different RKIP levels thus will require individual treatment regimes. The diagnosis and treatment of patients according to their genetic and/or proteomic profile will push forward cancer care. Finally, the emergence of resistant strains and metastatic cancers have been two of the biggest problems in cancer treatment and are amongst the most difficult to treat. Manipulation of RKIP levels may provide a new means of counteracting certain insidious forms of cancer, especially as RKIP has been shown to be down-regulated in metastatic cancer.

On another note, in this thesis we demonstrate the potential interaction of RKIP and the PDE5 inhibitors, sildenafil citrate and vardenafil citrate. Other compounds utilising the NO signalling pathway, cGMP and PKG have shown anti-cancer properties. The question we ask is - could the interaction between the PDE5 inhibitors and RKIP (or the ERK pathway) indicate that these compounds could have a role as potential anti-cancer drugs? Further investigation is required; however preliminary data suggests that this is an exciting avenue for future research which could yield new drug targets or new indications for existing drugs.

Every age in history has its afflictions; from the biblical fear of leprosy; the plagues of medieval times; typhoid and cholera during the Industrial Revolution; even the Romantic Era was spoiled by the rise of syphilis. In the future, will cancer be classed as the affliction of our age; could cancer be the by-product of our improved lifestyles? As we evolve and advance, we eradicate old problems but we encounter the new. In addition, our bodies are exposed to different environmental stimuli and we are living longer; giving rise to new and more evolved adversities.

Fortunately with the human thirst for life and with continuing advancements in science, we will strive to live life to the fullest. Surely Lord Byron says it best with " 'tis very certain the desire of life prolongs it."

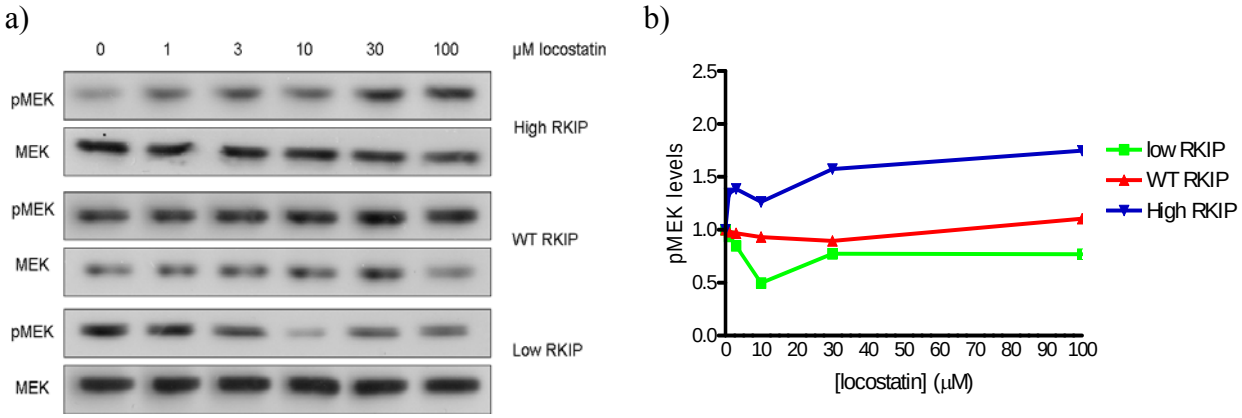
Appendix

Appendix 1

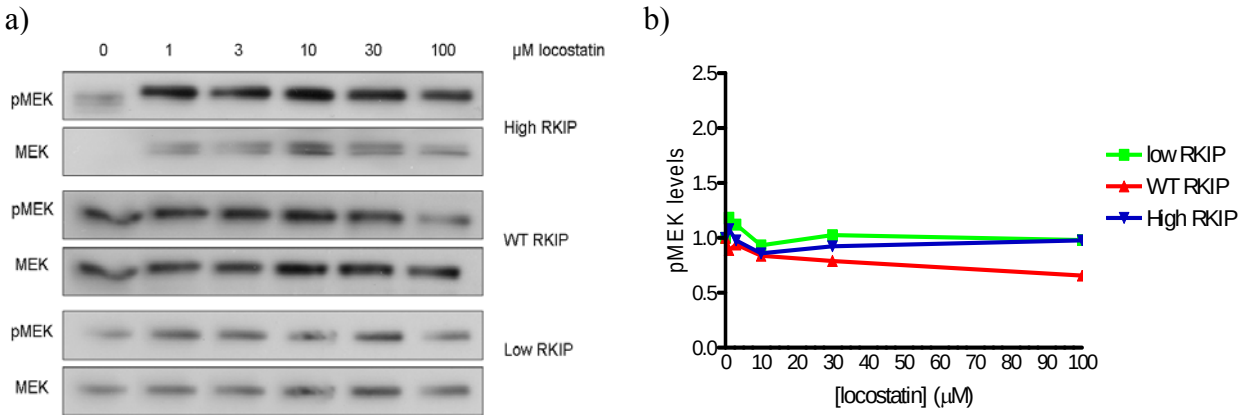
Creation of RKIP-expressing Ls174T colon carcinoma cell lines was performed as stated in the following references [193-195].
1. Low RKIP-expressing cell lines expressing shRNA against RKIP were generated by cloning the annealed oligonucleotides;
5'-GATCCCCGATTCAGGGAAGCTCTACATTCAAGAGATGTA-GAGCTTCCCTGAATCTTTTTA-3' and 5'-AGCTTAAAAAGATTCAGGGAAGC-TCTACATCTCTTGAATGTAGAGCTTCCCTGAATCGGG-3' into pSuperior.retro-Neo (OligoEngine) according to the manufacturer's instructions (human RKIP target sequences are underlined above); the procedure is described in [195].
2. WT RKIP-expressing cells were generated as described in [194].
3. High RKIP-expressing cells expressing FLAG-RKIP were created by subcloning full-length human RKIP with a 5'-single Flag tag into pcDNA5/FRT [194].

Appendix 2

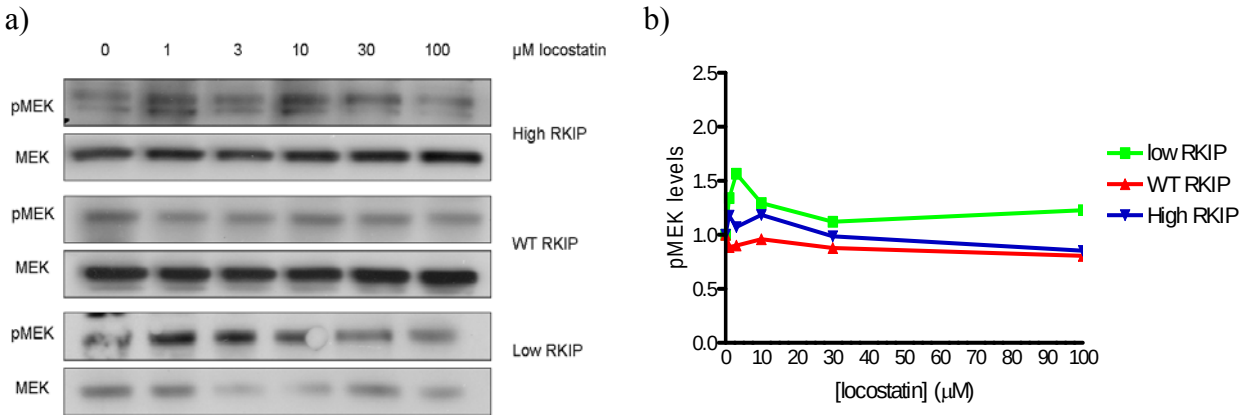
The effect of locostatin, sildenafil citrate and vardenafil citrate in combination with TPA stimulation, EGF stimulation or under growth conditions on pERK levels in Ls174T colon carcinoma cells with low, WT and high levels of RKIP were analysed and discussed in Chapter 5. The same experiments were performed as described in Chapter 5, but with phosphorylated MEK (pMEK) and MEK antibodies in place of pERK and ERK.



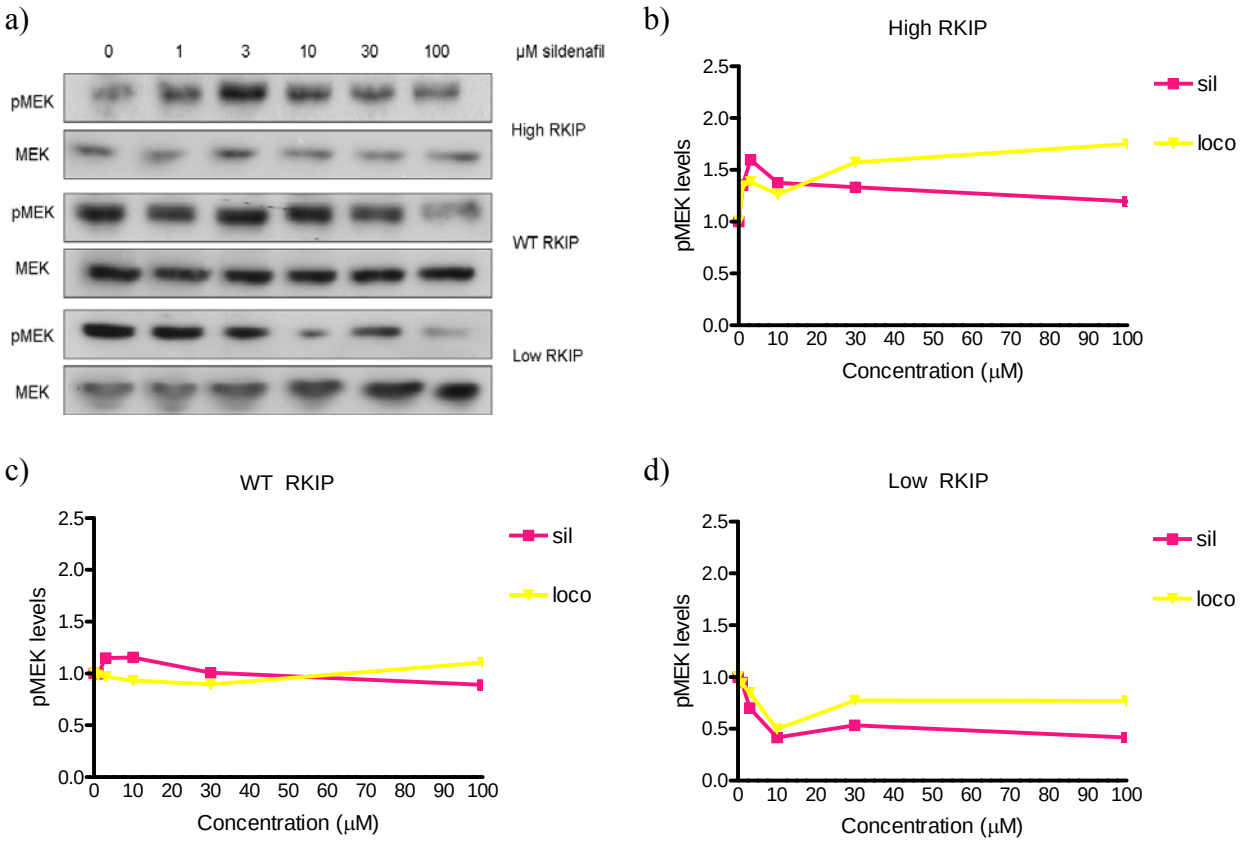
Appendix Figure 1: The effect of locostatin and TPA stimulation on pMEK levels in RKIP-expressing colon carcinoma cells. a) Western blot of pMEK and MEK levels in low, WT and high RKIP cells after treatment with locostatin (0 – 100 µM) and TPA-stimulation. **b)** ImageJ analysis of Appendix Figure 1a showing the effect of locostatin and TPA stimulation on pMEK levels in Ls174T colon carcinoma cells. (pMEK normalised to MEK levels) n=1.



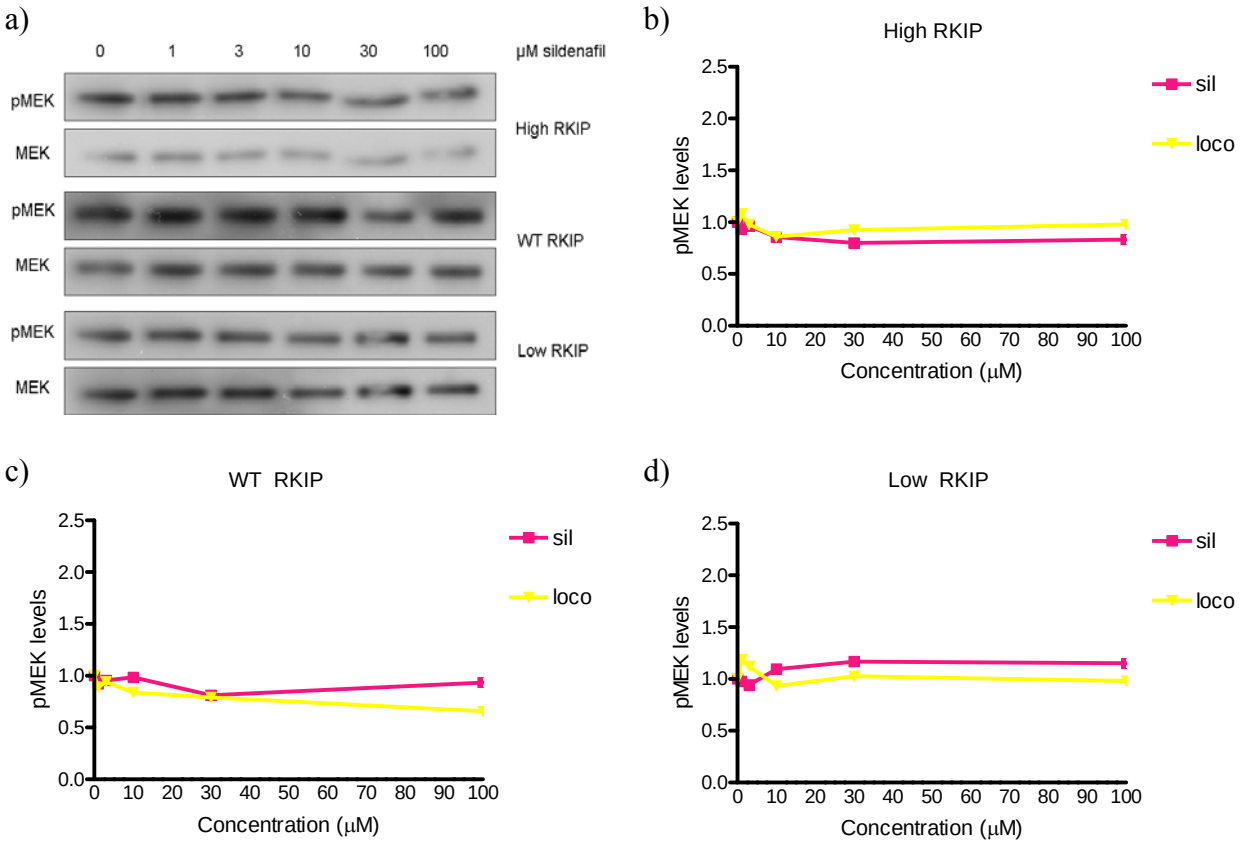
Appendix Figure 2: pMEK levels after EGF-stimulation and locostatin treatment in low, WT and high RKIP Ls174T cells. a) pMEK and MEK western blot analysis after locostatin treatment (0 – 100 µM) and EGF stimulation in RKIP-expressing Ls174T cells. **b)** ImageJ analysis of Appendix Figure 2a showing the effect of EGF stimulation and locostatin treatment on pMEK levels on low, WT and high RKIP colon carcinoma cells (n=1). pMEK levels were normalised to MEK loading control.



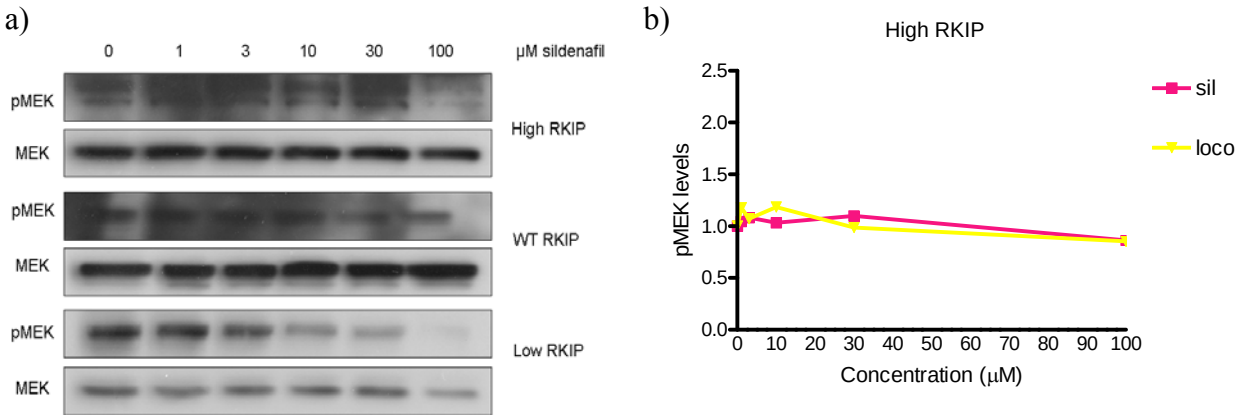
Appendix Figure 3: The effect of locostatin on pMEK levels in growing Ls174T cells with low, WT and high RKIP expression. a) Western blot of pMEK and MEK levels after locostatin treatment (0 – 100 µM) in growing Ls174T colon carcinoma cells expressing low, WT and high levels of RKIP. **b)** ImageJ analysis of Appendix Figure 3a displaying pMEK levels after locostatin treatment in growing cells (n=1). pMEK levels normalised to MEK levels.

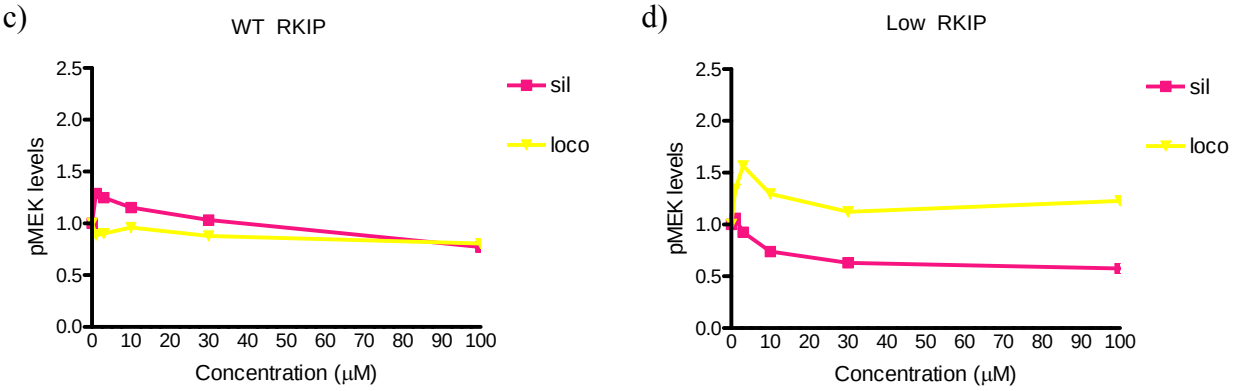


Appendix Figure 4: Comparison of the effect of sildenafil citrate and locostatin on pMEK levels in TPA-stimulated Ls174T cells with low, WT and high RKIP expression. a) pMEK and MEK levels by western blot analysis after sildenafil citrate treatment (0 – 100 μM) and TPA stimuation in low, WT and high RKIP-expressing colon carcinoma cells. **b)** ImageJ analysis of Appendix Figure 4a showing the effect of sildenafil citrate treatment compared to locostatin treatment (Appendix Figure 1b) on pMEK levels in high RKIP cells (n=1). **c)** ImageJ analysis of sildenafil citrate versus locostatin treatment on pMEK levels in WT RKIP cells (n=1). **d)** ImageJ analysis of the effect of sildenafil citrate administration on pMEK levels in low RKIP-expressing cells compared to locostatin treatment (n=1). All pMEK levels were normalised to MEK levels.

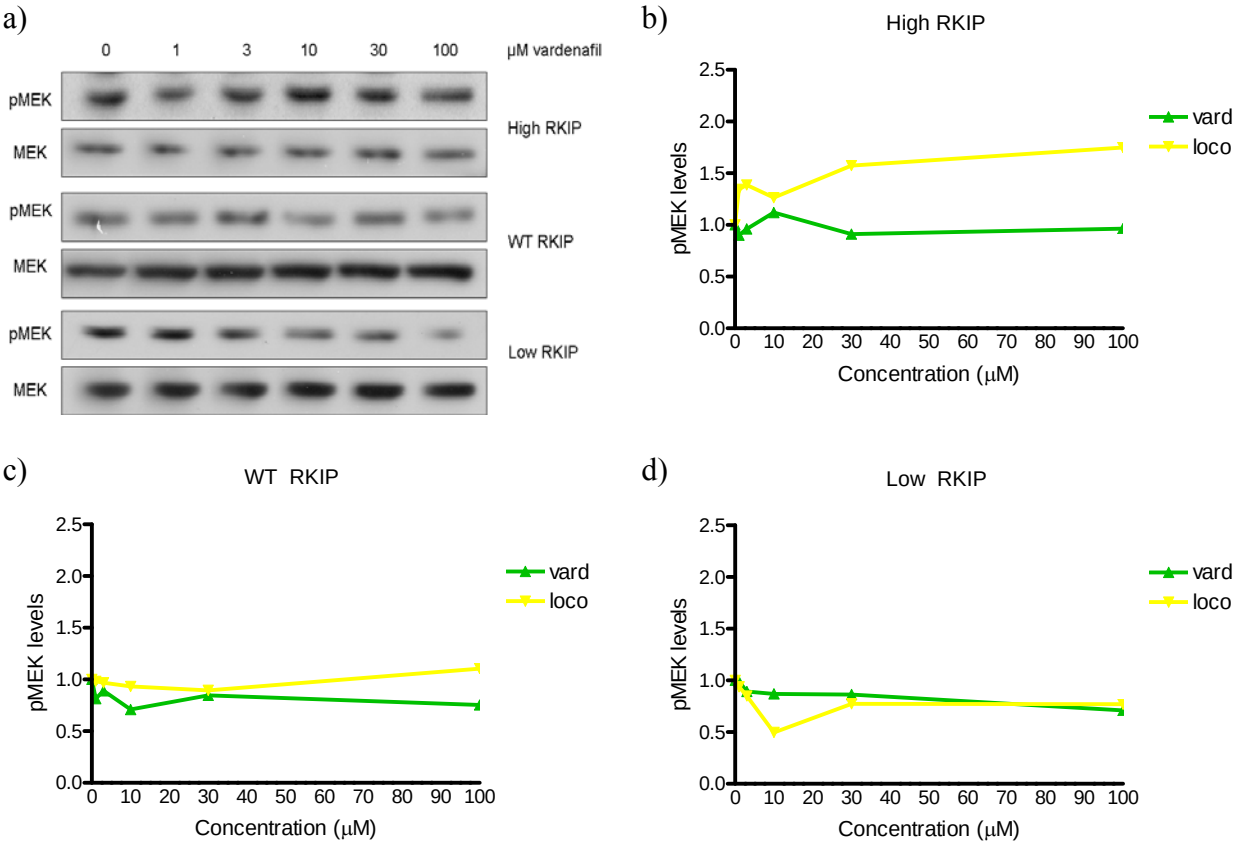


Appendix Figure 5: pMEK levels after sildenafil citrate treatment and EGF stimulation in low, WT and high RKIP colon carcinoma cells compared to locostatin-treated cells. a) pMEK and MEK western blot analysis after sildenafil citrate treatment (0 – 100 µM) and EGF stimulation in Ls174T cells with low, WT and high RKIP levels. **b)** ImageJ analysis of Appendix Figure 5a displaying the effect on pMEK levels in high RKIP-expressing cells of sildenafil citrate treatment compared to locostatin (Appendix Figure 2b) (n=1). **c)** ImageJ analysis showing the effect of sildenafil citrate on pMEK levels in WT RKIP cells compared to locostatin (n=1). **d)** ImageJ analysis of Appendix Figure 5a showing the effect of sildenafil citrate on the pMEK levels of low RKIP cells compared to locostatin treatment (n=1). pMEK levels in all ImageJ analyses were normalised to MEK loading controls.

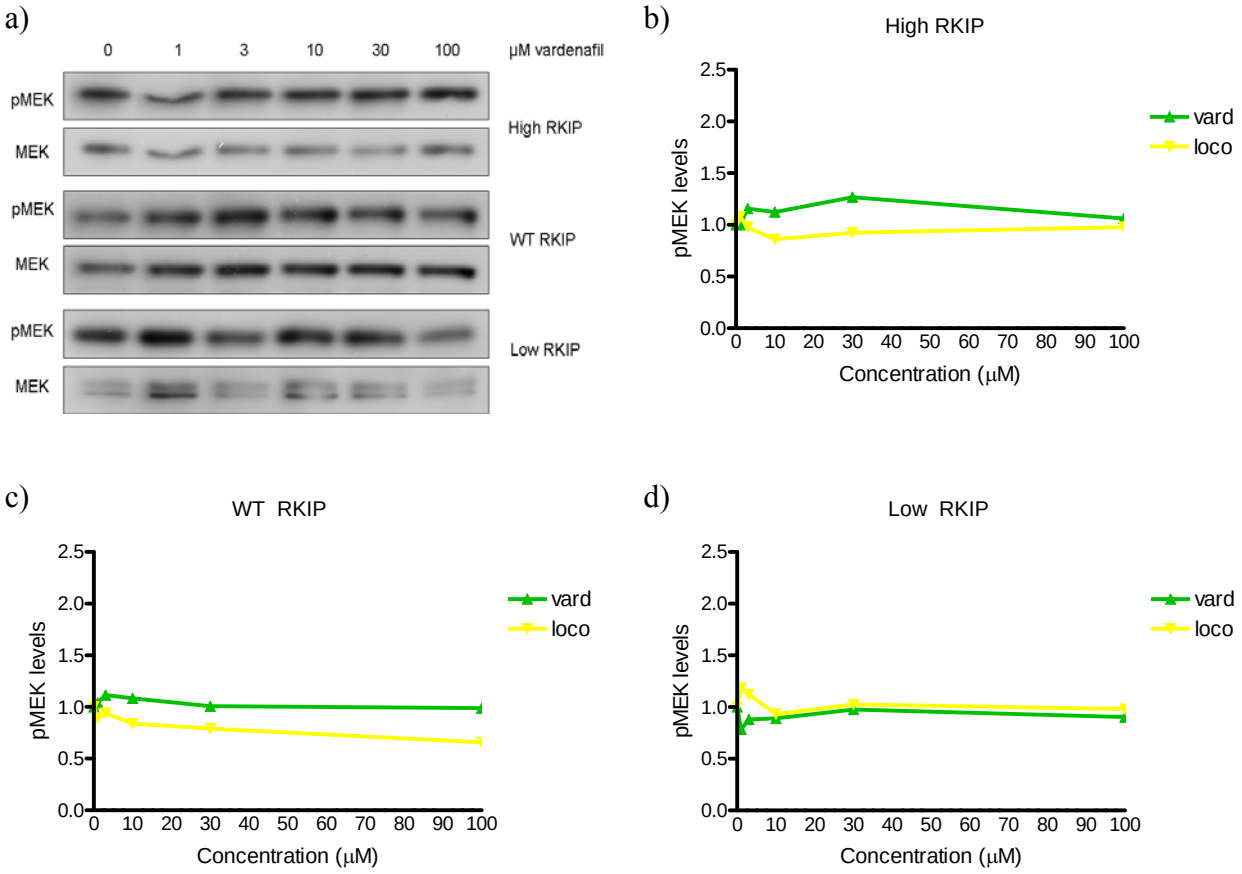




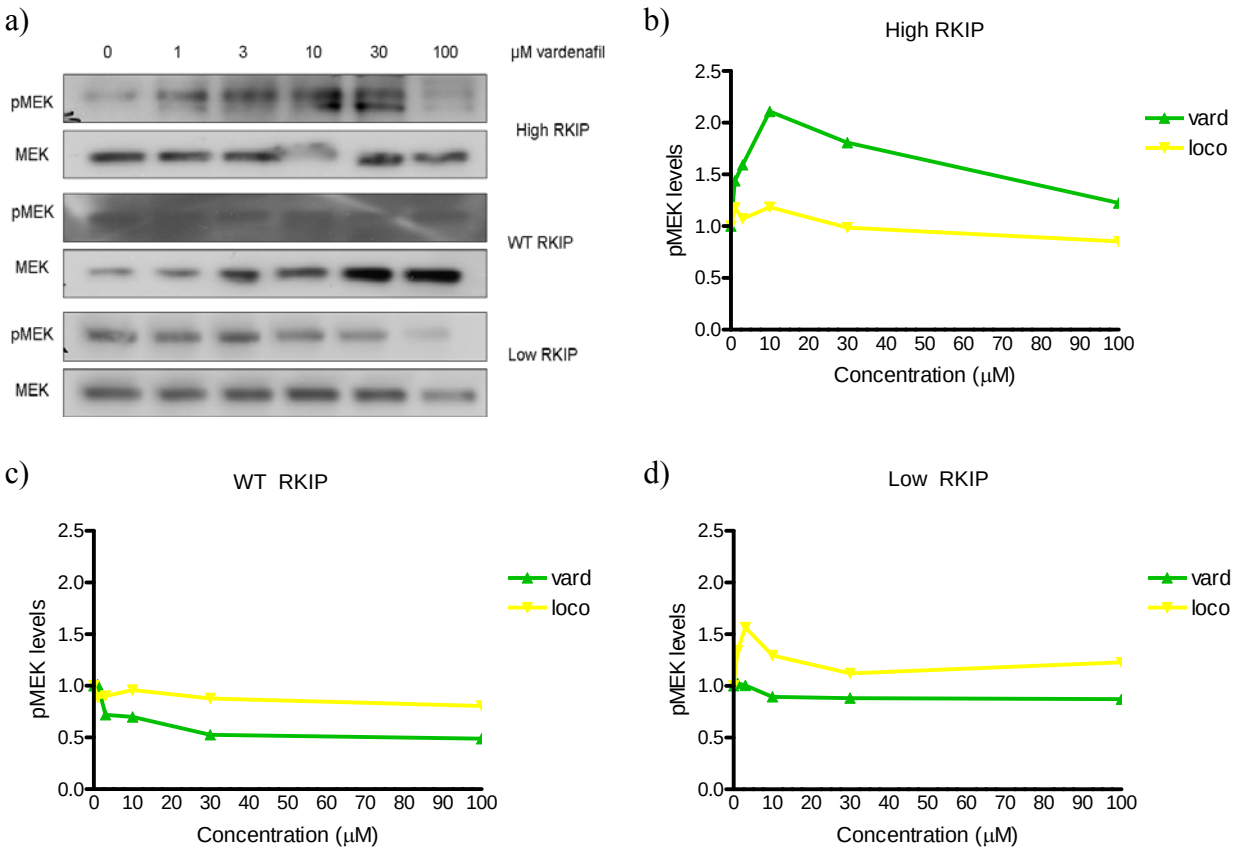
Appendix Figure 6: The effect of sildenafil citrate under growth conditions on pMEK levels in comparsison to locostatin treatment in Ls174T cells. a) pMEK and MEK western blot analysis of low, WT and high RKIP-expressing cells after sildenafil citrate treatment (0 – 100 μM) in growing cells. **b)** ImageJ analysis of Appendix Figure 6a showing the effect of sildenafil citrate treatment on pMEK levels in high RKIP cells (n=1) compared to locostatin-administered cells (Appendix Figure 3b). **c)** ImageJ analysis of pMEK levels in WT RKIP cells after sildenafil citrate treatment compared to locostatin-treated cells (n=1). **d)** ImageJ analysis of pMEK levels in low RKIP cells after sildenafil citrate administration compared to locostatin-treated cells (n=1). All pMEK levels were normalised to MEK loading controls.



Appendix Figure 7: pMEK levels after TPA stimulation and vardenafil citrate administration in RKIP-expressing cells compared to locostatin-treated cells. a) pMEK and MEK western blot analysis of Ls174T cells treated with vardenafil citrate (0 – 100 μM) and TPA stimulation. **b)** ImageJ analysis of Appendix Figure 7a showing the effect of vardenafil citrate on pMEK levels in high RKIP cells compared to locostatin-treated cells (Appendix Figure 1b) (n=1). **c)** ImageJ analysis of pMEK levels in WT RKIP cells after treatment with vardenafil citrate in comparison to locostatin administered cells (n=1). **d)** ImageJ analysis of pMEK levels in low RKIP cells after sildenafil citrate treatment compared to locostatin administered cells (n=1). All pMEK levels were normalised to MEK loading controls.



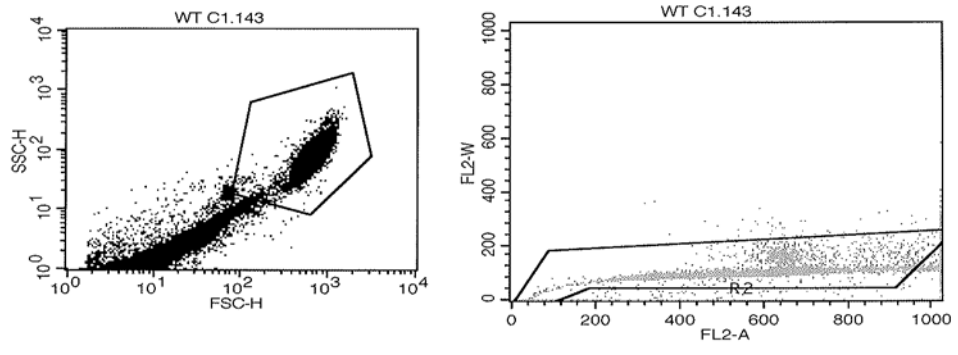
Appendix Figure 8: The effect of vardenafil citrate treatment and EGF stimulation on pMEK levels in comparison to locostatin administration in low, WT and high RKIP colon cancer cells. a) Western blot analysis of pMEK and MEK levels in low, WT and high RKIP-expressing colon carcinoma cells after vardenafil citrate treatment (0 – 100 μM) and EGF stimulation. **b)** ImageJ analysis of Appendix Figure 8a showing the effect of vardenafil citrate treatment on pMEK levels in high RKIP cells (n=1) compared to locostatin-treated cells (Appendix Figure 2b). **c)** ImageJ analysis of pMEK levels after vardenafil citrate treatment in WT RKIP cells compared to locostatin-treated cells (n=1). **d)** ImageJ analysis displaying the effect of vardenafil citrate versus locostatin treatment on pMEK levels in low RKIP-expressing cells (n=1). All pMEK levels were normalised to MEK loading controls.

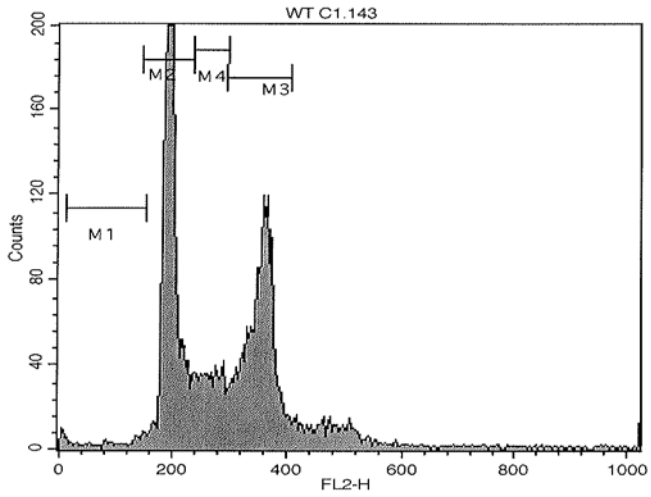


Appendix Figure 9: pMEK levels after treatment with vardenafil citrate in growing Ls174T cells compared to locostatin treatment. a) pMEK and MEK levels by western blot analysis after vardenafil citrate treatment (0 – 100 µM) in low, WT and high RKIP-expressing growing colon carcinoma cells. **b)** ImageJ analysis of Appendix Figure 9a showing pMEK levels in high RKIP cells after treatment with vardenafil citrate compared to locostatin-treated cells (Appendix Figure 3b) (n=1). **c)** ImageJ analysis of pMEK levels after vardenafil citrate treatment in comparison to locostatin-treated cells in WT RKIP cells (n=1). **d)** ImageJ analysis displaying the effect of vardenafil citrate in comparison to locostatin on the pMEK levels of low RKIP-expressing cells (n=1). All pMEK levels were normalised to MEK loading controls.

Appendix 3

An example of the raw FAS data is shown below:





Histogram Statistics

File: WT C1.143
Sample ID:
Tube: Untitled
Acquisition Date: 04-Jul-08
Gated Events: 15000
X Parameter: FL2-H (Linear)

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube List
Gate: G3
Total Events: 359872

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	0, 1023	15000	100.00	4.17	281.42	261.81	38.33	254.00	194
M1	11, 153	193	1.29	0.05	95.82	76.46	51.19	111.00	149
M2	146, 237	6829	45.53	1.90	197.56	197.10	6.88	195.00	194
M3	296, 409	5149	34.33	1.43	353.09	352.23	6.93	357.00	361
M4	237, 300	1840	12.27	0.51	267.80	267.18	6.79	267.50	290

Appendix Figure 10: Snapshot of raw FACS data. Example of a screen snapshot during FACS analysis, taken from a WT RKIP untreated sample.

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