'The Effects of Nonsteroidal Anti-inflammatory Drugs on Nuclear Factor-kappaB and Beta-catenin Signaling in Colorectal Cancer Cells'

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PhD

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Declaration

I declare that:

(A) This thesis has been composed by myself,

(B) the work is my own, except where otherwise stated, and

(C) the work has not been submitted for any other degree or professional qualification except as specified

Carolyn Loveridge

October 2006

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List of Abbreviations

3x κB ConA-Luc	3enhancer-CON-A
5-FU	5-fluorouracil
8-oxo-dG	8-oxo-7,8-dihydroxy-2′-deoxyguanosine
A^{260}	Absorbency at 260 nm
A^{280}	Absorbency at 280 nm
A^{595}	Absorbency at 595 nm
Amp	Ampicillin
AMPK	5'-AMP-activated protein kinase
AOM	Azoxymethane
AP-1	Activator protein-1
APC	Adenomatous polyposis coli
aPKC	Atypical protein kinase C
APS	Ammonium persulfate
ATCC	American type culture collection
ATF	Activating transcription factor
ATM	*Ataxia telangiectasia* mutated
ATP	Adenosine triphosphate
ATR	ATM- and Rad3-related
BAFF	B-cell activating factor
β-catenin	Beta-catenin
BH3	Bcl-2 homology region 3
BMI	Body mass index

BMK1	Big MAPK 1
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CARD	Caspase-activation recruitment domain
CBP	CREB binding protein
CDK	Cyclin-dependent kinase
CDKI	Cyclin-dependent kinase inhibitor
c-FLIP	Caspase-8/FADD-like-IL-1β-converting enzyme inhibitory protein
cGMP	Cyclic guanosine 3′,5′-monophosphate
CHIP	Chromatin immunoprecipitation
CHK	CSK homologous kinase
Chk1	Checkpoint kinase 1
$C_2H_3KO_2$	Potassium acetate
c-IAP	Cellular inhibitor of apoptosis
CKI	Caesin kinase I
CKII	Casein kinase II
ConA-Luc	ConA-Luciferase
COX	Cyclooxygenase
CPT11	Irinotecan
CREB	cAMP response element-binding
Csk	c-Src kinase
C-termunis	Carboxyl terminus
CTNNB1	Gene encoding β-catenin

DAPI	4′,6′-diamido-2-phenylindole
DD	Death domain
dH_2O	Distilled water
DISC	Death-induced signaling complex
Dkk	Dickkopf
DMH	1,2-dimethylhydrazine
DMSO	Dimethylsulfoxide
DNA-PK	DNA-dependent protein kinase
DR	Death receptor
Dsh	Dishevelled
EBV	Epstein-Barr virus
ECACC	European collection of cell cultures
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetate
ELISA	Enzyme-linked immunosorbent assay
EPIC	European prospective investigation into cancer
ERK	Extracellular signal-regulated kinase
EtOH	Ethanol
FADD	Fas associated death domain
FAK	Focal adhesion kinase
FAP	Familial adenomatous polyposis
FasL	Fas ligand
FCS	Foetal calf serum
FITC	Fluorescin isothiocyanate

GFP	Green fluorescent protein
GFP-RelA	pEGFP-RelA
GFP-RelA(Δ27-30)	GFP-tagged RelA(Δ27-30)
GFP-RelA(WT)	GFP-tagged RelA(WT)
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GSK-3β	Glycogen synthase kinase-3-beta
GST	Glutathione *S*-transferase
HAT	Histone acetyl transferase
HCl	Hydrochloric acid
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
HEPES	2 hydroxyethyl)piperazine-N'-2-ethanesulfonic acid
HGF	Hepatocyte growth factor
HIF-1α	Hypoxia-inducible factor-1α
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HMT	Histone methyl transferase
HNPCC	Hereditary non-polyposis colorectal cancer
H_2O_2	Hydrogen peroxide
hr	Hour
HRT	Hormone replacement therapy
HTLV	Human T-cell leukaemia virus
IAP	Inhibitor of apoptosis
ICAM-1	Intracellular adhesion molecule-1

Id2	Dominant negative helix-loop regulator
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor-1
IκB	Inhibitor of κB
IKK	IκB kinase
IL-1	Interleukin-1
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-8	Interleukin-8
iNOS	Inducible nitric oxide synthase
IP	Immunoprecipitation
ITF-2	Immunoglobulin transcription factor-2
JDP	Jun dimerisation partner
JNK	c-Jun N-terminal kinase
Kan	Kanamycin
KCl	Potassium chloride
KH_2PO_4	Potassium phosphate monobasic
KOH	Potassium hydroxide
L-agar	L-broth containing 0.15% agar
L-agar-Amp	L-agar containing Amp (100 μg/ml)
L-agar-Kan	L-agar containing Kan (50 μg/ml)
L-broth	Luria Bertani broth
LEF	Lymphoid enhancer factor
LPS	Lipopolysaccharide

LRP	Lipoprotein receptor-related protein
LTβ	Lymphotoxin β
LTR	Long terminal repeat
MALDI-ToF	Matrix-assisted laser desorption/ionization time-of-flight
MAPK	Mitogen activated protein kinase
MAPKK	Mitogen activated protein kinase kinase or MAP2K
MAPKKK	Mitogen activated protein kinase kinase kinase or MAP3K or MEKK
MEF	Mouse embryonic fibroblast
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
MHC	Major histocompatability complex
Min	Multiple intestinal neoplasia (*APCMin/+*)
min	Minute(s)
MLL	Mixed lineage leukaemia
MMP	Matrix metalloproteinase
MMR	Mismatch repair
MOPS	4-morpholinepropanesulfonic acid
MSI	Microsatellite instability
MSK1	Mitogen- and stress-activated protein kinase-1
MTHFR	Methylene-tetrahydrofolate reductase
mTOR	Mammalian target of rapamycin
NaCl	Sodium chloride
NaF	Sodium fluoride

Na_2HPO_4	Sodium phosphate dibasic (anhydrous)
NaOH	Sodium hydroxide
Na_3VO_4	Sodium orthovanadate
NEMO	NF-κB essential modifier (IKKγ)
NES	Nuclear export signal
NFBP	NF-κB binding protein
NF-κB	Nuclear factor-kappa B
NGF	Nerve growth factor
NIK	NF-κB inducing kinase
NIS	Nuclear import signal
Nkd	Naked cuticle
NLS	Nuclear localisation signal
NoLS	Nucleolar localisation signal
NRF	NF-κB repressing factor
NSAID	Nonsteroidal anti-inflammatory drug
N-terminus	Amino terminus
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	PBS + 0.1% TWEEN® 20
PBSTB	PBS + 5% BSA
PBSTM	PBST + 5% dried milk
PCAF	p300/CBP-associated factor
pCMVβ	pCMV-β-galactosidase
pCMV-Luc	pCMV-Luciferase

PEST	Proline-glutamic acid-serine-threonine
PG	Prostaglandin
PI 3-kinase	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKAc	PKA catalytic subunit
PKC	Protein kinase C
PKG	Protein kinase G
PKQR	Proline-lysine-glutamine-arginine
PMA-PHA	Phorbol 12-myristate 13-acetate plus phytohemagglutinin
PML	Promyelotic leukaemia
POD	PML oncogenic domain
PP1	Protein phosphatase 1
PPAR	Peroxisome proliferator-activated receptor
pRb	Retinoblastoma protein
PSQR	Proline-serine-glutamine-arginine
PTP	Protein tyrosine phosphatase
rDNA	Ribosomal DNA
RelA(Δ27-30)	RelA deleted for amino acids 27-30 (the NoLS)
RelA(WT)	Wild type RelA
RHD	Rel homology domain
RNase A	Ribonuclease A
RSK1	Ribosomal subunit kinase-1
RT	Room temperature
RXR-α	Retinoid-X-receptor-α

SAMP	Serine-Alanine-Methionine-Proline
SAPK	Stress activated protein kinase
SDS	Sodium dodecyl sulphate
sec	Second(s)
Ser	Serine
SFK	Src family kinase
SOCS-1	Suppressor of cytokine signaling-1
SRC-1	Steroid receptor coactivator-1
SSAT	Spermidine/spermine N^1-acetyltransferase
SVV	Survivin
SW480-pBpuro	SW480 cells stably transfected with pBpuro vector
SW480-SrcKD	SW480 cells stably transfected with kinase dead c-Src construct
TAD	Transcriptional activation domain
TAK	TGF-β-activated kinase
TCF	T cell factor
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF	Transforming growth factor
TK	Thymidine kinase
TNFα	Tumour necrosis factor-alpha
TNFR	Tumour necrosis factor receptor
TPA	12-O-tetradecanoylphorbol 13-acetate
TRAF	TNFR-associated factor
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand

TRE	TPA-responsive element
Tris	Tris(hydroxymethyl)methylamine
TSA	Trichostatin A
T/V	Trypsin/Versene
TxRd	Texas red
Tyr	Tyrosine
Ubc	Ubiquitin conjugating enzyme
Ubl	Ubiquitin ligase
UV	Ultraviolet
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
Wnt	Wingless
WT	Wild type
XIAP	X-chromosome-linked inhibitor of apoptosis

Abstract

There is substantial evidence that the use of aspirin and related nonsteroidal anti-inflammatory drugs (NSAIDs) is associated with colorectal cancer prevention. Although some data indicates a role for inhibition of cyclooxygenase (COX) enzymes, the underlying mechanisms of the anti-tumorigenic effects of NSAIDs are complex and poorly understood. It has previously been shown by the host laboratory that aspirin activates the nuclear factor-kappa B (NF-κB) pathway via phosphorylation and degradation of the NF-κB inhibitor protein, IκBα. (Stark *et al.*, 2001). More recent work has established that nucleolar translocation of the RelA component of NF-κB in response to aspirin is causally involved with repression of basal NF-κB transcriptional activity and apoptosis (Stark and Dunlop, 2005).

The first research strand in this thesis was to determine whether non-aspirin NSAIDs also induce apoptosis of colorectal cancer cells via modulation of the NF-κB pathway and to elucidate the upstream mechanisms involved. Experimental evidence presented here shows that sulindac, sulindac sulfone and indomethacin cause NF-κB pathway activation. It was found that non-aspirin NSAIDs induce nucleolar translocation of the NF-κB component, RelA, repression of NF-κB driven transcriptional activity and apoptosis. Furthermore, in a similar manner to aspirin, nucleolar translocation of RelA was demonstrated to be absolutely required for the apoptotic effects of sulindac, sulindac sulfone and indomethacin. However, in contrast to aspirin, activation of the NF-κB pathway by the non-aspirin NSAIDs was independent of IκBα degradation. By means of phospho-specific antibodies, chemical inhibition and genetic intervention, the tyrosine kinase c-Src was found to

be activated in response to NSAIDs and this activation was causally involved in the apoptotic mechanism of sulindac, sulindac sulfone and indomethacin.

The host laboratory was the first to demonstrate nucleolar sequestration of RelA and so the second research strand in this thesis was to further understand the mechanisms involved. Beta catenin (β-catenin) has previously been shown to interact with RelA and modulate nuclear NF-κB activity (Deng *et al.*, 2002). Therefore, the role of the β-catenin pathway in NSAID-effects on the NF-κB pathway and apoptosis was investigated. Aspirin, sulindac, sulindac sulfone and indomethacin all induced activation of the β-catenin pathway and nucleolar sequestration of β-catenin. Blocking aspirin-induced nucleolar translocation of RelA blocked nucleolar localisation of β-catenin, suggesting a possible interaction between the NF-κB and β-catenin pathways.

It is increasingly apparent that post-translational modifications of RelA, particularly acetylation and ubiquitination, are implicated in nuclear regulation of NF-κB. The final strand of research in this thesis was therefore to determine whether these modifications were involved in aspirin effects on NF-κB activity and apoptosis. By means of immunoprecipitation and Western blot analysis, aspirin was found to cause an increase in ubiquitinated RelA. Furthermore, the proteasome inhibitor, MG132, mimicked the effects of aspirin in terms of nucleolar translocation of RelA and β-catenin, repression of NF-κB activity and increased ubiquitinated RelA. Moreover, both aspirin and MG132 mediated a reduction in proteasome levels and caused proteasomes to localise to the nucleolus, providing support for the notion that RelA and/or β-catenin are subject to ubiquitin-mediated proteolysis in the nucleolus. Additionally, the finding that there is a decrease in acetylated RelA after aspirin

treatment provides further evidence that acetylation is important in the response to aspirin.

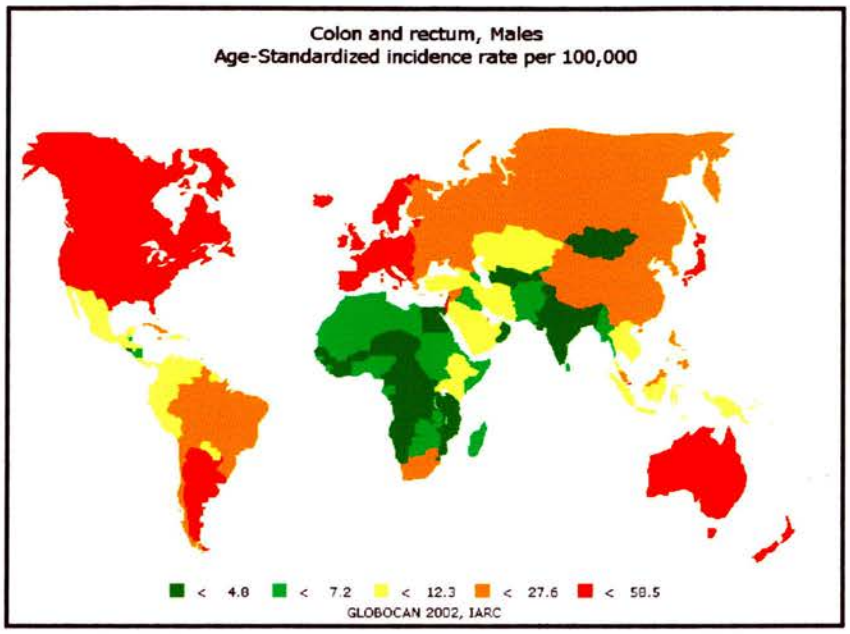
Collectively, the data in this thesis establishes a novel mechanism whereby NSAIDs induce apoptosis of colorectal cancer cells. Activation of the NF-κB pathway is critical in this process, although the upstream mechanism of activation differs between NSAIDs. Cross-talk between the NF-κB and β-catenin pathways could represent an important regulatory mechanism in response to NSAIDs and the nucleolus might be a key site for such cross-regulation. Moreover, post-translational modifications, including ubiquitination and acetylation, seem to play a significant role in sequestration to the nucleolus of key NF-κB and β-catenin pathway components. Elucidating the mechanisms whereby NSAIDs induce apoptosis of colorectal cancer cells is of vital importance for the rational design of new therapeutic agents.

Chapter 1 – Introduction

Colorectal cancer is a substantial public health problem worldwide, particularly among developed populations. The disease has a complex epidemiology, with both genetic and environmental contribution. There is considerable morbidity associated with colorectal cancer and its treatment, which has led to the development of prevention strategies to combat the disease. Nonsteroidal anti-inflammatory drugs (NSAIDs) are known to reduce incidence and mortality from colorectal cancer and so show promise as chemopreventative agents. However, there is a considerable amount of work still needed to determine the optimal means of preventing the disease. While the focus of this thesis is on the effects of NSAIDs on nuclear factor-kappa B (NF-κB) signaling as a key mechanism of the anti-tumour effects of NSAIDs against colorectal cancer, it is important to consider this in the context of colorectal cancer as a whole. Hence, this introduction will discuss the incidence and mortality of colorectal cancer (1.1); treatment and survival rates (1.2); modifiable genetic and environmental risk factors for prevention (1.3); chemoprevention (1.4); mechanism of NSAID action (1.5); and the need for novel therapeutic agents (1.6).

1.1 Incidence and Mortality of Colorectal Cancer

Colorectal cancer is the third most common malignant neoplasm worldwide (http://www.cancer.gov) and Figure 1.1 demonstrates the global distribution of the



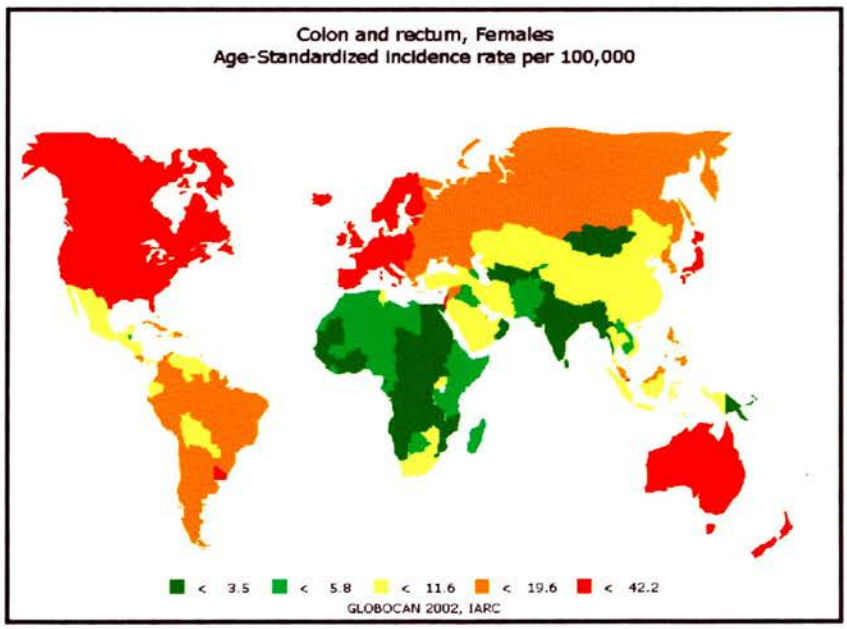


Figure 1.1 – Global Incidence of Colorectal Cancer in Males and Females

[Figures obtained from: http://www-dep.iarc.fr/]

disease in males and females. It is noteworthy that the areas with the highest incidence are similar in males and females, namely United States, Western Europe and Australia. Indeed, colorectal cancer is the second most common fatal malignancy in both sexes combined after lung cancer in the Western population, accounting for over 10% of all cancer related deaths. The annual incidence in the United States is approximately 184,300 (affecting 72,600 males and 75,700 females) with 56,600 deaths (Lynch and de la Chapelle, 2003). In Europe in 2004, colorectal cancer was estimated to account for 13.2% incident cases of cancer diagnosed and approximately 203,700 deaths (Boyle and Ferlay, 2005). In the UK in particular, there were approximately 34,500 new cases of colorectal cancer in 2001 (affecting a higher proportion of males, 18,500, than females, 16,039) with 16,107 deaths from colorectal cancer in 2003 (http://www.cancerresearchUK.org). Figure 1.2 illustrates the mortality rates of bowel cancer in selected countries by sex. It is of significance that not only does colorectal cancer seem to affect a higher proportion of males than females, as mentioned for Europe and the UK, but there are higher mortality rates in general among males than females for bowel cancer. It is also noteworthy that the mortality rate for colorectal cancer is correlated with age – the rate increases exponentially from approximately age 40-45 in both males and females, exemplified by recent Scottish data for 2004 which is documented on the ISD Scottish Health Statistics website (http://www.isdscotland.org) and illustrated in Figure 1.3.

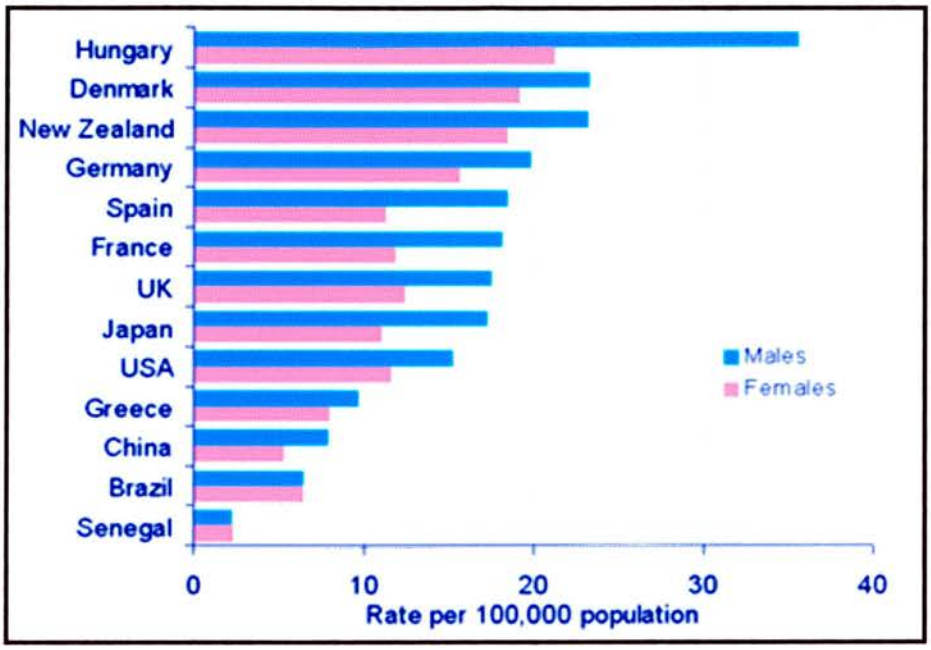


Figure 1.2 – Age Standardised (World) Mortality Rates of Bowel Cancer in Selected Countries, by Sex, 2002 Estimates.

[Figure obtained from: http://www.cancerresearchuk.org]

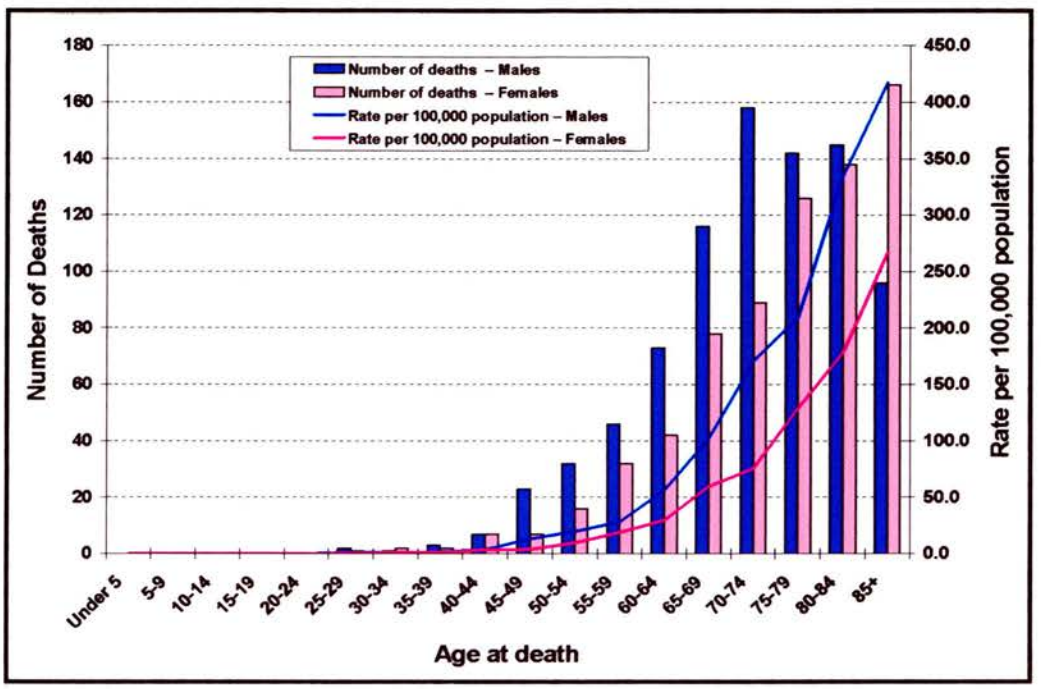


Figure 1.3 – Number of Deaths and Age-standardised Mortality Rates for Colorectal Cancer in Males and Females in Scotland, 2004.

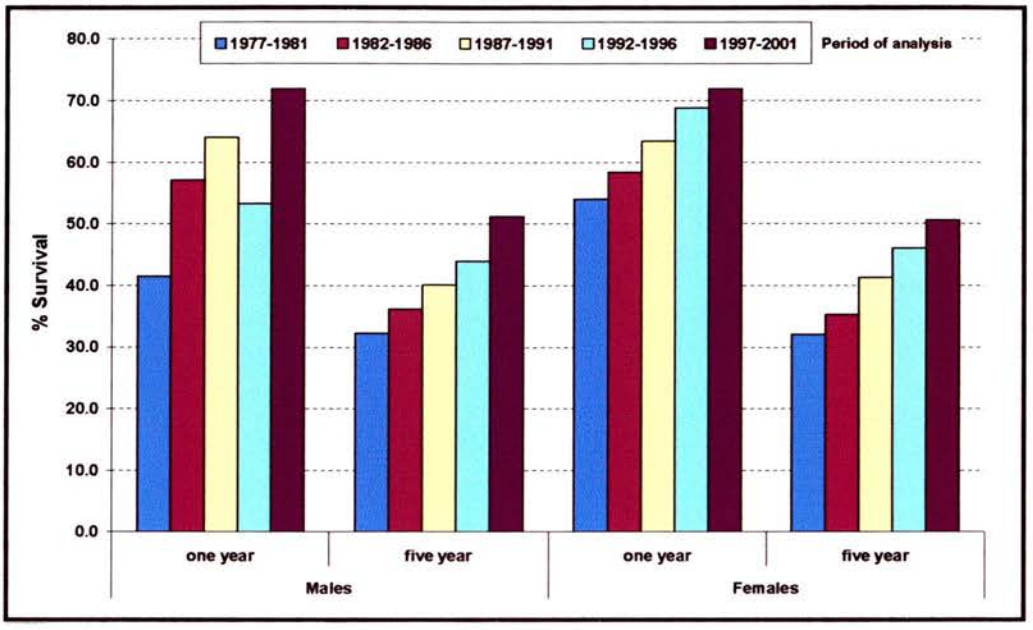
[Data obtained from: http://www.isdscotland.org]

1.2 Treatment and Survival Rates of Colorectal Cancer

There has been significant improvement in the survival rate of colon cancer patients over the past few decades as a result of earlier detection and better treatment of patients, with a current five year survival rate of approximately 51% here in Scotland, based on recent data presented on the ISD Scottish Health Statistics website (http://www.isdscotland.org). In particular, Figure 1.4A highlights the improvement in one year and five year survival rates from colorectal cancer in Scotland for both males and females during the period 1971-2001. Predictably, survival from colorectal cancer is dependent on age at diagnosis, with poorer survival rates observed among patients diagnosed later in life and Figure 1.4B illustrates this in the case of Scotland. Survival can be as high as 70% after curative surgery (Dunlop, 1997). However, this depends on early diagnosis and treatment prior to the development of metastasis.

Approximately 19% of colorectal cancer patients have metastatic disease at initial presentation (Goldberg, 2005). 5-fluorouracil (5-FU) has been basis of conventional chemotherapy treatment for patients with metastatic colorectal cancer over the past 40 years (Köhne *et al.*, 2004) and works by inhibiting thymidylate synthase, which is important in pyrimidine synthesis (Napier and Ledermann, 2000). However, this drug has a significant degree of toxicity, with side effects including nausea/vomiting, diarrhoea and hand-foot syndrome (Xiong and Ajani, 2004). Moreover, resistance to the drug can occur (Banerjee *et al.*, 2002). More recent chemotherapeutic agents that have been developed and used to treat colorectal cancer

A



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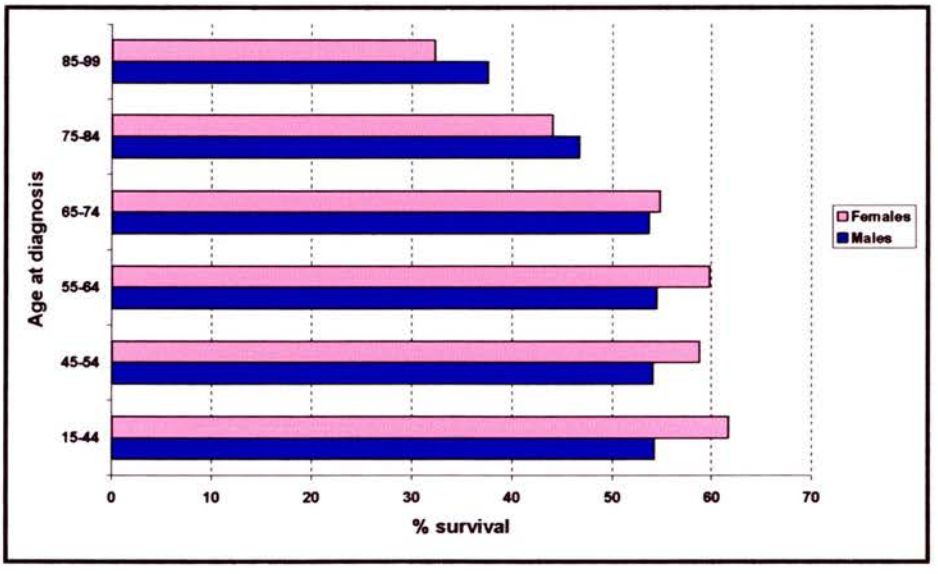

Figure 1.4 – Colorectal Cancer Survival Statistics for Scotland. **(A)** One and five year relative survival rates (%) for colorectal cancer in males and females in Scotland diagnosed during the period from 1971-2001. **(B)** Relative survival (%) rates of colorectal cancer in males and females in Scotland by age at diagnosis, 2004.

[Data obtained from http://www.isdscotland.org]

include: capacitabine (thymidylate synthase inhibitor) and oxaliplatin (diaminocyclohexane platinum, a DNA cross-linker) (Napier and Ledermann, 2000), both of which are now in routine clinical practice. Other second line drugs, for example CPT11 (irinotecan, a DNA topoisomerase I inhibitor), are available but like 5-FU, these agents also have a significant degree of toxicity. Adjuvant chemotherapy and radiotherapy can help reduce the chance of recurrence of disease and significantly improve overall survival in patients with early stages of colorectal cancer undergoing surgery (Chau and Cunningham, 2002). However, for patients with advanced disease, median survival is around six to 12 months, with an overall 5-year survival rate of only 10%. Conventional chemotherapy can perhaps improve this by about three to six months (Goldberg, 2005; Simmonds, 2000).

1.3 Modifiable Risk Factors for Prevention of Colorectal Cancer

The lifetime risk of colorectal cancer in the general population is approximately 5% in females and 6.1% in males, based on recent Scottish data documented on the ISD website (http://www.isdscotland.org). Given that conventional chemotherapy offers only marginal survival benefits and that early detection and prompt surgery in the early stages of colon cancer offer the only hope of long-term cure, there has been a great deal of focus on developing primary, secondary and tertiary prevention strategies to combat the disease. Primary prevention is concerned with etiological factors, such as lifestyle and dietary

modification, to eliminate potential mutagens (Krishnan *et al.*, 1997). Secondary prevention attempts to reduce incidence by identifying and treating pre-malignant conditions in subjects at risk (Rigas and Williams, 2002). In tertiary prevention or chemoprevention, naturally occurring or pharmacological agents are used to prevent the initiation of carcinogenesis in healthy individuals and to prevent recurrence, invasion and metastasis in people diagnosed with symptomatic disease (Vainio and Miller, 2003).

1.3.1 Genetic Susceptibility

Prevention in the general population is a worthy long-term goal but patients identified to be at high risk would gain particular benefits. Studies carried out on cohorts of twins from Sweden, Denmark and Finland suggest that approximately 35% of colorectal cancer incidence can be attributed to a genetic component (Lichtenstein *et al.*, 2000). Moreover, approximately 20% colorectal cancers occur in individuals with a positive family history (Rowley, 2004). It follows that first-degree relatives of a person who has had colorectal cancer are predictably at increased risk of developing the disease themselves, especially if the relative had the cancer at a young age (Hopper, 2005). If there is a clear family history of the disease – those who have two or more first- or second-degree relatives (or both) with colorectal cancer – then the risk is even higher (Lynch and de la Chapelle, 2003). Therefore, there is undoubtedly a genetic contribution to this disease.

Familial colorectal cancers can broadly be divided into two groups: those characterised by the presence of multiple benign colorectal polyps (polyposis) and those characterised by the absence of polyposis (Kinzler and Vogelstein, 1998a). The two major, highly penetrant, autosomal dominant gene disorders that predispose individuals to colon cancer, namely familial adenomatous polyposis and hereditary non-polyposis colorectal cancer, fit into these two categories respectively (Rowley, 2005). Taken together, these syndromes probably account for less than 5% of all cases of colorectal cancer (de la Chapelle, 2004).

1.3.1.1 Familial Adenomatous Polyposis

Familial adenomatous polyposis (FAP) is an autosomal dominant inherited disease that is characterised by the presence of hundreds to thousands of benign adenomatous polyps throughout the colorectum that usually appear at an average age of 16 years (de la Chapelle, 2004; Rowley, 2005). Colorectal cancer subsequently presents at approximately 39 years of age in these patients (Rowley, 2005). FAP occurs in 1:7000 to 1:14,000 live births (Rowley, 2004; de la Chapelle, 2004) and is responsible for less than 1% of all colorectal cancers (Fearnhead *et al.*, 2002). However, the risk of cancer is so high that identification of gene carriers is of vital importance (Dunlop, 1997). The adenomatous polyps observed in FAP patients resemble sporadic polyps that develop in the general population, but the large number of polyps present in FAP patients essentially guarantees that some will

progress to malignancy, although an individual polyp is no more likely to become cancerous than a sporadic polyp (Kinzler and Vogelstein, 1998a).

The genetic basis of FAP lies in a germ line mutation of the adenomatous polyposis coli (*APC*) gene (Fearnhead *et al.*, 2002). Linkage analysis of families with FAP led to the mapping of the *APC* gene to chromosome 5q21 (Bodmer *et al.*, 1987). The *APC* gene was then cloned, identified and characterised in 1991 (Groden *et al.*, 1991; Nishisho *et al.*, 1991). *APC* encodes a multi-functional protein that is involved in several key cellular processes, such as cell adhesion and migration (via interaction with E-cadherin), signal transduction (via interaction with beta-catenin (β-catenin)), microtubule assembly and chromosome segregation [reviewed in (Senda *et al.*, 2005; Bienz and Hamada, 2004; Fodde *et al.*, 2001b; Fearnhead *et al.*, 2001; Sieber *et al.*, 2000; Polakis, 1997)].

As can be seen in Figure 1.5, the *APC* gene consists of 8535 bases that encode a 2843 amino acid multi-domain protein. The functional domains of APC include heptad repeats, which occur within the oligomerisation domain (amino acids 6-57) at the amino-terminus (N-terminus) of the APC protein and mediate APC homodimer formation. Amino acids 453-767, termed the armadillo region, are known to control cell adhesion and motility via modulation of the actin cytoskeleton. Two motifs in APC mediate binding and downregulation of β-catenin respectively: a 15 amino acid repeat (occurring three times between residues 1020-1169) and a 20 amino acid repeat (occurring seven times between residues 1262-2033). Moreover, the 20 amino acid repeats contain a consensus site for glycogen synthase kinase-3-beta (GSK-3β) phosphorylation. Three SAMP (Serine-Alanine-Methionine-Proline)

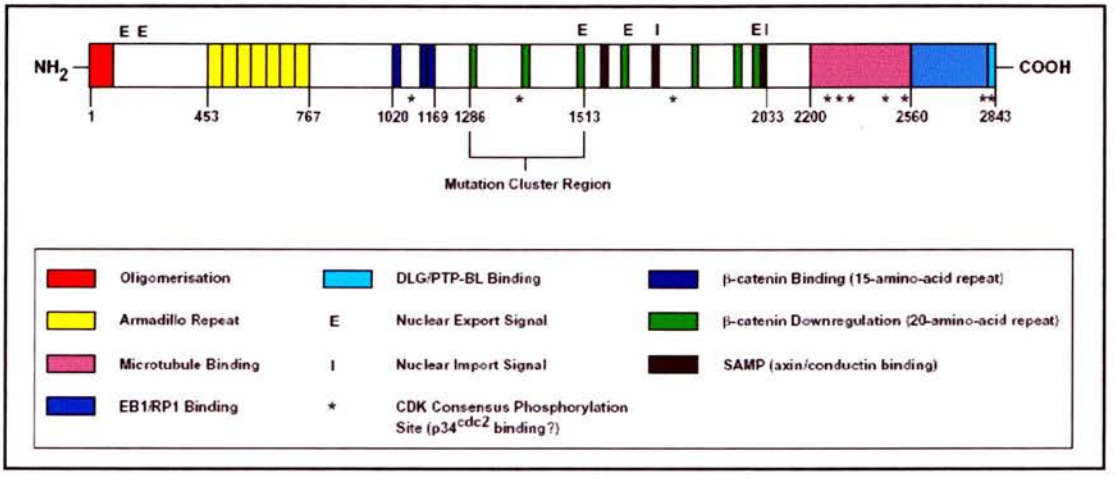

Figure 1.5 – Domain Structure of the APC Gene. Protein domains of APC include: the heptad repeats that mediate APC oligomerisation; the armadillo repeat, which binds to Asef; the 15 and 20 amino acid repeats that are involved in binding and regulating β-catenin and which are interspersed with SAMP amino acid motifs that mediate axin binding; several nuclear localisation and export sequences; a microtubule-binding domain; and sites for binding EB1/RP1. APC also contains several consensus sites for phosphorylation by $p34^{cdc2}$, five nuclear export signals (E) and two nuclear import signals (I). Most somatic mutations of APC occur in the mutation cluster region and these usually lead to truncated proteins being produced.

[Figure adapted from (Fodde *et al.*, 2001b)]

repeats, which are located between the third and fourth, fourth and fifth and after the seventh 20-amino acid repeats, allow interaction of APC with axin/conductin. The 'basic' domain of APC lies within the carboxyl-terminus (C-terminus) between amino acids 2200 and 2400 and has been demonstrated to act as a binding site for microtubules (Munemitsu *et al.*, 1994) and other proteins, including: EB1 (Su *et al.*, 1995), a protein that associates with mitotic spindles, microtubules and centrosomes; HDLG (Matsumine *et al.*, 1996), the human homologue of the *Drosophila* discs large tumour suppressor protein; and the protein tyrosine phosphatase, PTP-BL (Erdmann *et al.*, 2000). Finally, APC contains five nuclear export signals (NES) and two nuclear import signals (NIS).

The involvement of APC in FAP comes as no surprise given that it is such a multi-functional protein and that each of the roles of APC outlined above could potentially be linked with cancer. It was proposed that the single gene, *APC*, essentially acts as the 'gatekeeper' of colonic epithelial cell proliferation and that inactivation of this gatekeeper is required for net cellular proliferation (Kinzler and Vogelstein, 1996). Gatekeepers essentially function to maintain a constant cell number in renewing populations, ensuring that cells respond appropriately to situations requiring cell growth. FAP was therefore postulated to result from an increased rate of tumour initiation due to abrogation of the gatekeeper function of APC.

The vast majority (95%) of *APC* germ line mutations are nonsense or frameshift mutations that result in a truncated protein product with abnormal function (Fearnhead *et al.*, 2002). Colorectal tumours from FAP patients carry additional somatic *APC* mutations or loss of heterozygosity at this locus in addition to the

original germ line mutation (Powell *et al.*, 1992; Miyoshi *et al.*, 1992). These observations are in accordance with Knudson's two-hit hypothesis which states that both copies of *APC* must be inactivated during tumorigenesis. *APC* has therefore been defined as a tumour suppressor gene. It is noteworthy that most sporadic colorectal cancers also carry two inactivating *APC* mutations (Fearnhead *et al.*, 2001).

Another gene found to give rise to the multiple adenoma phenotype is the base excision repair gene, *MutYH* (Al-Tassan *et al.*, 2002). Base excision repair essentially acts to prevent mutagenesis induced by 8-oxo-7,8-dihydroxy-2′-deoxyguanosine (8-oxo-dG) (Sieber *et al.*, 2003). Specifically, MutYH removes adenines mispaired with 8-oxo-dG or guanine and a defect in this repair mechanism was suspected from the observation that tumours from these patients had an excess of somatic mutations comprising G:C→A:T transversions in the *APC* gene, which is typical of changes caused by oxidative damage to DNA (Lipton and Tomlinson, 2004). It is noteworthy that the genetic defect responsible for the polyposis phenotype acts as a recessive trait in these families (Strate and Syngal, 2005) and is very rare.

1.3.1.2 Hereditary Non-polyposis Colorectal Cancer

Hereditary non-polyposis colorectal cancer (HNPCC), also called Lynch syndrome, is the most common form of hereditary colorectal cancer, accounting for approximately 3 to 5% of all colorectal cancer cases (Rowley, 2004). HNPCC is

characterised by the onset of colorectal cancer at an average age of 44 years and frequent occurrence of other cancers such as endometrial, stomach, ovarian, small bowel, hepatobiliary epithelium, uroepithelial epithelium and brain (de la Chapelle, 2004; Rowley, 2005). Patients with HNPCC have a 70-80% risk of developing colorectal cancer (Rowley, 2005) and so the identification of carriers is clearly of importance.

HNPCC is caused by mutations in one of the DNA mismatch repair (MMR) genes, most commonly *hMLH1*, *hMSH2*, *hMSH6*, *PMS1* or *PMS2*, and the condition is inherited as an autosomal dominant (Rowley, 2004). Identification of HNPCC kindreds has been facilitated by Amsterdam Criteria refined in 1999 (Lynch and de la Chapelle, 2003). Individuals at risk in HNPCC kindreds are heterozygous for MMR genes and so loss of MMR function requires both the germ line mutation and a somatic mutation (Fearnhead *et al.*, 2002). The MMR genes normally maintain fidelity of DNA replication by correcting nucleotide mispairs and small insertions or deletions generated by misincorporation or slippage of DNA polymerase during transcription (Rowley, 2004). Dysfunction of these genes essentially causes widespread genome instability, which can manifest as different numbers of simple repeated sequences, such as microsatellites (Fodde *et al.*, 2001a; Hopper, 2005). This phenomenon is known as microsatellite instability (MSI) and disruption of microsatellites is observed in the vast majority (>90%) of HNPCC patients and is also seen in approximately 15% sporadic colorectal cancers (Fearnhead *et al.*, 2002). Unlike FAP, HNPCC patients lack a marked increase in the number of precursor adenomas but tumours that do arise in these patients are genetically unstable and rapidly progress to cancer (Kinzler and Vogelstein, 1998a). Thus, in contrast to FAP,

which mainly affects tumour initiation, HNPCC primarily affects tumour progression.

1.3.1.3 The Benefits of Screening for High Risk Individuals

The identification of a causative germ line mutation for colorectal cancer in a patient's family by genetic testing would aid prevention because the option of chemotherapy or as a last resort, prophylactic surgery, would be available before presentation of metastatic disease (Kinzler and Vogelstein, 1996). Other current screening methods for colorectal cancer include faecal occult blood test, rigid and flexible sigmoidoscopy, colonoscopy and double contrast barium enema (Ahmed, 2003; Vainio and Miller, 2003; Hawk and Levin, 2005). Ultimately, early identification of individuals and/or families at high risk should lead to a reduction in the morbidity and mortality from colorectal cancer because a program of cancer surveillance and treatment can be tailored to personal risk.

Knowledge of the molecular genetic basis of colorectal cancer is vital not only to increase our understanding of the disease but to help identify targets that may be important in treatment and/or prevention. Therefore, in addition to screening for known mutations in genes implicated in hereditary colorectal cancer, the identification of low-penetrance susceptibility alleles and modifier genes responsible for sporadic forms of the disease is also imperative (de la Chapelle, 2004). Prevention may be difficult on a population basis, but these studies would help to identify individuals at risk for targeted prevention.

1.3.2 Non-genetic and Environmental Susceptibility

Numerous genetic, experimental and epidemiological studies carried out to date suggest that colorectal cancer is largely an environmental disease. The majority of colorectal cancers are therefore described as being 'sporadic' as they have no apparent underlying genetic predisposition. Sporadic colorectal cancer is associated with a variety of risk factors, including: age, diet and lifestyle, and these are outlined below.

1.3.2.1 Age

Age is the single most important risk factor for colorectal cancer – the risk of developing colorectal cancer rises exponentially with age, commencing at 40 years, rising to 50 years, and doubling with each decade, where it peaks at 70 years (Heavey *et al.*, 2004). Indeed, approximately 50% of the Western population develops a colorectal tumour by the age of 70 and in about 1 in 10 of these individuals, progression to malignancy ensues (Kinzler and Vogelstein, 1998b; Kinzler and Vogelstein, 1998a).

1.3.2.2 Diet

Epidemiological studies strongly suggest that the prevalence of colon cancer can be influenced by diet. However, due to the complexity of human diets, it has been difficult to determine which dietary components are responsible for this modulation. Most research has therefore concentrated on the main groups of food that are consumed, namely: fats, sugars and complex carbohydrates, proteins, and fruit and vegetables.

1.3.2.2.1 Dietary Fat

There is substantial evidence that diets high in fat, especially saturated animal fats, are associated with an increased risk of colorectal cancer [reviewed in (Martínez, 2005; Corrêa Lima and Gomes-da-Silva, 2005)]. Colon cancer rates are high in populations with high total fat intake but are lower in populations consuming less fat. In high-incidence Western countries, fat comprises about 40-50% of total caloric intake whereas in low-risk countries, fat only accounts for about 10% of dietary calories (http://www.cancer.gov). Furthermore, there is an increased risk of colorectal cancer among populations migrating from low to high risk areas (Key *et al.*, 2002). Laboratory animal studies have shown that increasing the fat composition of the diet is associated with an increase in incidence of colon tumours (Corrêa Lima and Gomes-da-Silva, 2005). The reason for the increased incidence of colon cancer as a result of high fat intake, particularly saturated fats, could be due to increased bile

acid and cholesterol in the colonic lumen (Giovannucci and Willett, 1994; Mason, 2002). Colonic bacteria can convert these compounds to tumour-promoting secondary bile acids and other cytotoxic metabolites that could potentially induce cell proliferation and hence act as promoters of cancer of the colon (Gill and Rowland, 2002). The discovery of the *MOM1* gene in the multiple intestinal neoplasia (*Min*) (*APCMin/+*) mouse model of colorectal cancer further supports the idea that lipids are among the critical dietary components contributing to colorectal cancer risk. The *MOM1* gene encodes secreted phospholipase A2, an enzyme involved in fat metabolism. This gene is a major modifier locus affecting intestinal neoplasia in the *Min* mouse (Kinzler and Vogelstein, 1996). However, underscoring the complexity of genetic susceptibility to colon cancer in humans, PLA2 has not been associated with colon cancer risk (Tomlinson *et al.*, 1996; Papanikolaou *et al.*, 2000; Fijneman, 2005). It is noteworthy that in contrast to saturated animal fats, there is some evidence to suggest that consumption of polyunsaturated fats, in particular marine fish oil and olive oil, has a protective role in colon carcinogenesis (Corrêa Lima and Gomes-da-Silva, 2005). However, despite there being substantial evidence that dietary fat intake may be correlated with incidence of colorectal cancer, the results have not always achieved statistical significance and some studies have found no such association.

1.3.2.2.2 Red Meat

There is reasonably consistent evidence from epidemiological data indicating that that eating red meat (lamb or beef) and processed meat (sausages, salami, ham, bacon) is positively related to risk of colorectal cancer (Martínez, 2005; Ahmed, 2004; Heavey *et al.*, 2004; Lewin *et al.*, 2006). Furthermore, this association tends to be independent of the fat content of meat (Giovannucci, 2003). There are several mechanisms to explain the association between meat consumption and colorectal cancer [reviewed in (Cross and Sinha, 2004)]. Processing of meat may increase the presence of nitrates and nitrites in the diet (Key *et al.*, 2002). Although these chemicals do not cause cancers directly, they can be converted to nitrosamines in the body, which may help cause digestive system cancers. There has also been some suggestion that the excess iron intake, which is more readily absorbed in the haem form found in red meat, may also be associated with increased risk of colorectal cancer (Kushi and Giovannucci, 2002). Haem iron could influence the risk of cancer via the generation of hydroxyl radicals in the colonic lumen (Corrêa Lima and Gomes-da-Silva, 2005). It has been suggested that cooking methods of meat and fish may also play a role, with the hypothesis that consumption of 'well done' meat increases the risk of colorectal cancer compared with rare or medium rare intake (Lewin *et al.*, 2006; Giovannucci, 2002). This is most likely due to higher levels of mutagenic heterocyclic aromatic amines in well done meat (Lewin *et al.*, 2006; Cross and Sinha, 2004). It is noteworthy that while red meat consumption is positively associated with risk for colorectal cancer, non-red meat sources of animal protein,

including fish and poultry, have typically been found to have little or even a slight preventative effect against colorectal cancer (Giovannucci, 2002).

1.3.2.2.3 Dietary Fibre

Another important dietary factor that has been suggested to influence the incidence of colorectal cancer is dietary fibre, although the evidence is conflicting. A very recent pooled analysis of 13 prospective cohort studies revealed that dietary fibre intake was inversely associated with risk of colorectal cancer in age-adjusted analyses, but after accounting for other dietary risk factors, high dietary fibre intake was not found to be associated with a reduced risk of colorectal cancer (Park *et al.*, 2005). These data are in contrast to the majority of animal and epidemiological studies which show a protective effect of dietary fibre on colon carcinogenesis [reviewed in (Ahmed, 2004; Martínez, 2005)]. Fibre is a very complex form of carbohydrate that is either insoluble, typified by wheat bran and cellulose, or soluble, for example legumes (Ahmed, 2004). Inclusion of fibre in the diet could influence carcinogenesis in the large bowel by a number of mechanisms. Fibre generally increases the bulk of stools, thereby decreasing the concentration of lumenal carcinogens and tumour promoters, and reduces colonic transit time, thus limiting the time of exposure of the colonic epithelium to carcinogens (Lao and Brenner, 2004). Fibre can also bind bile acids and hence can lower stool pH, which in turn can prevent the conversion of primary to harmful secondary bile acids (Gatof and Ahnen, 2002). Fibre may also act as a substrate for bacteria in the gut for fermentation,

resulting in an increase in concentration of short chain fatty acids such as butyrate (Campos *et al.*, 2005). Butyrate has been shown to be anti-carcinogenic and can induce apoptosis in colon adenoma and colon cell lines (Key *et al.*, 2002). Furthermore, butyrate is an important fuel for regeneration of the colonic epithelium and may also have protective effects by lowering the pH in the colonic lumen (Heavey *et al.*, 2004). It is not clear whether the possible protective effects of fibre described above against bowel cancer are due to fibre itself or to other protective effects of fruit, vegetables and cereals that contain fibre.

1.3.2.2.4 Fruits and Vegetables

Colorectal cancer is less prevalent in Mediterranean countries where diet is characterised by high consumption of foods of plant origin and low intake of red meat, compared to countries that have a diet rich in meat, high in saturated fat and poor in fibre (Corrêa Lima and Gomes-da-Silva, 2005). It is noteworthy, however, that vegetarians in developed countries have not shown low death rates from colorectal cancer, suggesting that vegetable and meat consumption may not be key factors contributing to high rates of colorectal cancer in these countries (Key *et al.*, 2002). Many epidemiologic studies have examined the role of fruit and vegetable intake in the incidence of colorectal cancer although these studies have provided mixed results. Three large, prospective cohort studies did not observe a protective effect of fruit and vegetables against colorectal cancer [reviewed in (Mason, 2002; Lao and Brenner, 2004)]. In contrast, recent preliminary findings from the European

Prospective Investigation into Cancer (EPIC) study suggest a moderate protective effect for vegetables against colorectal cancer and the results of an Adelaide case-control study have shown that a high intake of certain vegetables, such as onions, legumes, cabbage, green leafy vegetables and raw fruit, is associated with a decreased risk of colorectal cancer (Steinmetz and Potter, 1993). The protective effects of fruits and vegetables have been attributed to various components of these foods. In addition to being rich in fibre, fruits and vegetables contain anticarcinogenic and antioxidant micronutrients such as beta carotene, folic acid, ascorbic acid, phenols and flavonoids (Gatof and Ahnen, 2002). Dark leafy green cruciferous vegetables, such as broccoli, have also been found to contain glucosinolates, which can induce glutathione *S*-transferase (GST) activity and have been shown to exhibit chemopreventive activity by upregulating certain hepatic and gut mucosal detoxification enzymes (Heavey *et al.*, 2004; Martínez, 2005). Furthermore, in one case-control study (Lin *et al.*, 1998), the authors established that subjects with the highest intake of broccoli (an average of 3.7 servings per week) had an odds ratio of 0.47 for colorectal adenomas, when compared with people who reportedly never ate broccoli.

1.3.2.2.5 Vitamins and Minerals

An abundant array of micronutrients, including: vitamins A, C, D, E, folic acid and B6; minerals, for example calcium and selenium; and phytochemicals, such as carotenoids and flavonoids, have been shown to have a protective effect against

colorectal cancer. Table 1.1 summarises the common dietary sources and possible mechanisms for the beneficial effects of some of these compounds.

1.3.2.3 Lifestyle and Colorectal Cancer

In addition to dietary factors, certain lifestyle factors, including: physical activity, energy balance/obesity, alcohol consumption and smoking, are known to influence the risk of colorectal cancer.

1.3.2.3.1 Physical Activity

A substantial body of epidemiological evidence supports an association of a sedentary lifestyle with an increased risk of colorectal cancer (Martínez, 2005). In fact, there is a strong inverse relationship between the levels of physical activity and colon cancer incidence (Giovannucci, 2003; Lao and Brenner, 2004). At the molecular level, dysregulation of the 5'-AMP-activated protein kinase (AMPK), which is a key kinase involved in regulating cellular lipid metabolism and protein metabolism in response to exercise, has been associated with a predisposition to certain cancers, including colon cancer (Luo *et al.*, 2005b). This kinase has therefore been identified as a potential target for anti-cancer agents. In particular, inhibitors of a downstream effector of AMPK, namely mTOR (mammalian target of rapamycin),

Dietary Constituent	*Possible Beneficial Effects*	*Plant Foods*	*References*
Carotenoids	Potentially anti-tumorigenic as well as having anti-oxidant properties	Green-yellow vegetables, citrus fruits, spinach, broccoli, tomato	(Raju and Cruz-Correa, 2006; Lao and Brenner, 2004; Gatof and Ahnen, 2002; Martínez, 2005)
Folate	Sustains appropriate balance of DNA synthesis and biological methylation Enhances DNA repair Guards against DNA damage that can cause cancer Stabilises certain tumour suppressor genes thus preventing further increases in proliferation	Broccoli, spinach, asparagus, orange, dried beans.	(Heavey *et al.*, 2004; Martínez, 2005; Mason, 2002; Giovannucci, 2002; Ströhle *et al.*, 2005; Kim, 2005)
Flavonoids (flavones and isoflavones)	Potent inhibitors of reactive oxygen species Enhance rate of apoptosis by activating caspase enzymes Suppression of cell division and proliferation via activation of enzymes such as telomerase.	Soy, citrus fruits, broccoli, tomato, cabbage, olive oil	(Hoensch and Kirch, 2005; Ahmed, 2004; Corrêa Lima and Gomes-da-Silva, 2005)
Vitamins (A,C,E)	Antioxidants that may alleviate oxidative stress by reducing the toxic effect of reactive oxygen species in cancer causation Reduce nitrosamines/amides Enhance immune response Decrease epithelial cell proliferation	Citrus fruits, most vegetables	(Gatof and Ahnen, 2002; Ahmed, 2004; Lao and Brenner, 2004; Corrêa Lima and Gomes-da-Silva, 2005)
Vitamin D	Required for calcium absorption Reduces colonic cell proliferation	Exposure to sunlight, milk and dairy products, oily fish	(Harris and Go, 2004; Giovannucci and Willett, 1994; Gatof and Ahnen, 2002; Ahmed, 2004; Lao and Brenner, 2004)
Calcium	Binds bile acids, preventing abnormal cell growth	Milk and dairy products, salmon and sardines, broccoli, soy	(Mason, 2002; Giovannucci, 2003; Ahmed, 2004; Heavey *et al.*, 2004)
Selenium	Allows maximal level of expression of enzymes with antioxidant function and inhibits tumorigenesis Modulates the metabolism of some carcinogens Increases the immune response Induces apoptosis	Cereal, meat, fish	(Gatof and Ahnen, 2002; Mason, 2002; Lao and Brenner, 2004; Ahmed, 2004; Martínez, 2005)
Pyridoxine (Vitamin B6)	Reduces colonic cell proliferation Antioxidant activity, suppressing oxidative stress Antiangiogenic	Poultry, fish, legumes, potatoes, bananas, whole grains	(Komatsu *et al.*, 2003; Ahmed, 2004)

Table 1.1 – Summary of Protective Effects of Micronutrients for Colorectal Cancer.

[Table adapted from (Corrêa Lima and Gomes-da-Silva, 2005)]

have been characterised and are in clinical development as anti-cancer agents (Thomas, 2006). In general, an approximately 30% to 50% reduction in colorectal cancer incidence has been documented among individuals with the highest levels of physical activity compared to those with low levels of activity (Giovannucci, 2003; Ahmed, 2004). The biological mechanisms which have been proposed for the observation that increased physical activity protects against colorectal cancer include: maintenance of a healthy body weight and facilitation of the transit time of colonic contents, thus decreasing the time of exposure of the colonic epithelium to potentially harmful agents (Gatof and Ahnen, 2002). At the molecular level, exercise could positively influence metabolic pathways, such as the AMPK pathway, and hence could protect against cancer, perhaps by countering or correcting insulin resistance associated with obesity or by reducing triglyceride levels (Luo *et al.*, 2005b). Moreover, physical activity could have favourable effects on the immune system, such as a reduction in concentration of prostaglandins in the rectal mucosa (Martínez, 2005).

1.3.2.3.2 Obesity

A high calorie intake combined with a sedentary lifestyle results in a positive energy balance and inevitably leads to weight gain, by virtue of increased adipose tissue, and clinical obesity. Many retrospective and prospective studies have demonstrated that a high body mass index (BMI), the result of being obese, is associated with an increased risk of colorectal cancer [reviewed in (Gunter and

Leitzmann, 2006; Ahmed, 2004; Martínez, 2005)]. A BMI of above 30 is associated with about a twofold increased risk of colorectal cancer (Gatof and Ahnen, 2002). Furthermore, there is evidence that a tendency for visceral or central adiposity, expressed as the waist-to-hip ratio, is associated with a higher risk of developing colorectal cancer, independent of the BMI (Giovannucci, 2003). Several mechanisms have been proposed for the association of obesity with an increased risk of developing colorectal cancer. Specifically, insulin resistance and resulting hyperinsulinemia have been proposed as the major mechanisms (Giovannucci, 2002). A high concentration of circulating insulin may have a mitogenic action on colonocytes, affecting cellular proliferation and apoptosis via activation of signal transduction pathways such as protein kinase C (PKC) and mitogen activated protein kinase (MAPK) pathways (Campos *et al.*, 2005; Gunter and Leitzmann, 2006). Higher circulating levels of insulin may additionally increase the levels and availability of insulin-like growth factor-1 (IGF-1), which can also promote cellular proliferation (Giovannucci, 2003; Campos *et al.*, 2005). The insulin hypothesis is further supported by the observations that type 2 diabetes mellitus is associated with a higher risk of colorectal cancer (Giovannucci, 2003; Martínez, 2005).

1.3.2.3.3 Alcohol

There is evidence from case-control and prospective studies of an association between high alcohol intake and elevated risk of colorectal cancer, although there are conflicting reports in the literature (Heavey *et al.*, 2004; Ahmed, 2004; Martínez,

2005). The mechanism whereby alcohol increases the risk for colorectal cancer is unknown but folate metabolism has been implicated (Giovannucci, 2002). Acetaldehyde, a breakdown metabolite from alcohol, may inactivate N^5-methyltetrahydrofolate, the form of folate required for methionine synthesis, or may inhibit the methionine synthetase complex or folate-related enzyme, MTHFR (methylene-tetrahydrofolate reductase) (Giovannucci and Willett, 1994; Giovannucci, 2003; Martínez, 2005). As mentioned previously, folate is implicated in key cellular processes such as DNA synthesis and repair. It follows, therefore, that reduced folate levels as a result of excessive alcohol intake could enhance the process of carcinogenesis by interrupting these critical cellular processes. Indeed, there is a 2-5 fold elevation in colorectal cancer risk among individuals with high intakes of alcohol and low intakes of folate (Giovannucci, 2003). In addition to its effects on folate metabolism, alcohol has been shown to stimulate cell proliferation in the rectum (Corrêa Lima and Gomes-da-Silva, 2005) and can contribute to the MSI phenotype (Heavey *et al.*, 2004).

1.3.2.3.4 Smoking

A higher risk of adenomatous polyps has been consistently observed among smokers in numerous studies (Giovannucci, 2002), although the evidence for a positive association between smoking and colorectal cancer has been conflicting (Ahmed, 2004; Martínez, 2005). An induction period of 30-40 years between smoking and risk of colorectal cancer has been proposed to account for the

discrepancy for the results of adenomas compared to colorectal cancer in relation to smoking (Gatof and Ahnen, 2002). More recently published studies have found an elevated colorectal cancer risk, approximately 2-4 times the average risk, for tobacco smokers (Giovannucci, 2002; Ahmed, 2004). Cigarette smoking likely increases the risk of colorectal cancer due to carcinogenic action of polycyclic aromatic hydrocarbons, nitrosamines and aromatic amines that are found in burned tobacco (Ahmed, 2004). Polycyclic aromatic hydrocarbons form harmful DNA adducts in the colonic mucosa (Giovannucci, 2002) and furthermore, the carcinogenic compounds found in tobacco smoke can cause MSI (Heavey *et al.*, 2004).

1.3.2.3.5 Hormone Replacement Therapy

The correlation between hormone replacement therapy (HRT) and colorectal cancer has been widely researched and the majority of case-control and cohort studies to date have demonstrated that women who use HRT postmenopausally have an approximately 20-40% decreased risk of colorectal cancer, independent of other risk factors (La Vecchia *et al.*, 2005; Hawk *et al.*, 2004; Raju and Cruz-Correa, 2006). For example, a prospective cohort study of 59,002 postmenopausal participants from the Nurses' Health Study found that current users of postmenopausal hormones had a protective effect against the development of colorectal cancer (relative risk 0.65), an association that was attenuated in past users but disappeared by 5 years after cessation of therapy (Grodstein *et al.*, 1998). It is thought that the protective effects of HRT could reflect a direct benefit of estrogens.

Estrogens have been postulated to prevent colorectal cancer by decreasing the production of secondary bile acids and IGF-1, by exerting a direct effect on the colorectal epithelium, or by a combination of both of these mechanisms (Jänne and Mayer, 2000; Ahmed, 2004). However, a recent study (Anderson *et al.*, 2004) documented no protective effect of estrogen-only HRT compared with placebo, suggesting that progestin may be more important or that estrogen-independent mechanisms exist for the protective effects of HRT.

1.3.2.4 Other Risk Factors

In addition to the dietary and lifestyle risks described above, the presence of benign growths, called polyps or adenomas, can significantly increase the risk of developing colorectal cancer (Winawer *et al.*, 1996). Polyps or adenomas are not uncommon in the general population. Indeed, approximately 5% of people have polyps by age 50 and this increases to 50% for people over 70 years old (Kinzler and Vogelstein, 1996). It is important to note, however, that only a small fraction of polyps do develop into cancer and this process takes years.

People with other bowel diseases, such as ulcerative colitis, Crohn's disease and inflammatory bowel disease are at greater risk of developing colorectal cancer (Lichtenstein, 2002). These diseases cause continuous inflammation of the bowel such that the lining of the bowel becomes damaged. This damage is constantly repaired but the resultant increase in cell turnover augments the risk that a cancerous

cell might develop. In terms of prevention, it would therefore be important to closely monitor patients with such bowel diseases for the development of colorectal cancer.

1.3.2.5 Dietary and Lifestyle Goals for Prevention of Colorectal Cancer

General preventative strategies to help combat colorectal cancer have been devised and proposed, based on the knowledge that dietary and lifestyle factors contribute significantly to the development and progression of colorectal cancer, and these are summarised in Table 1.2. Briefly, the consumption of saturated animal fat should be kept to a minimum and only one portion of red meat should be consumed per week. Five servings of fruit and vegetables should be eaten daily and the amount of fibre in the diet should be around 30-35 g per day. Some authors suggest that supplements of vitamins and minerals, in particular vitamins A, C, E and B6, calcium and selenium, would be advisable although sufficient quantities of these micronutrients can be obtained through the diet from eating fruits and vegetables. In general, energy in the form of calories taken in from the diet should not exceed energy expenditure so as to maintain a healthy body weight and prevent obesity. The partaking of regular exercise is also important to help maintain a healthy body weight, in addition to reducing colonic transit time. Finally, less than two drinks of alcohol should be consumed a day and no cigarettes or tobacco smoked to help reduce the risk of colorectal cancer.

Food or Nutrient	*Protective or Risk for Colorectal Cancer*	*Proposed Mechanism*	*Common Dietary Source*	*Dietary or Supplement Goal*
Vegetables and Fruits	Protective	Suppress formation of carcinogens, induce multiplicity of solubilising and activating enzymes, suppress DNA and protein synthesis		≥ 5 servings per day
Fibre	Protective	Increases stool bulk, binds with bile acids and other potential carcinogens, reduces faecal pH and anaerobic flora, ferments to short chain fatty acids	Whole grains, legumes, wheat bran, nuts, barley, beans	30-35 g per day
Vitamins (A, C, D, E, folic acid and B6) and Minerals (calcium, selenium, carotenoids, flavonoids)	Protective	Have antioxidant activity, reducing excess oxidative damage to DNA, sustain appropriate balance of DNA synthesis and biological methylation, enhance DNA repair, reduce bile- and fatty-acid-induced colonocyte cytotoxicity, sustain immune function, decrease epithelial cell proliferation	Fruits and vegetables, dairy products, oily fish	1200 mg calcium per day 400-1000 µg folate per day 200 µg selenium per day
Animal Fat (except fish), Red Meat, Processed Meat	Risk	Increase excretion of bile acids, DNA damage	Beef, lamb, pork	1 serving only of red meat per week
Calories	Risk	Promotes obesity, hyperinsulinemia, growth factors	Fat	To maintain BMI ≤ 25
Alcohol	Risk	Impairs one-carbon (e.g. folate/methionine) metabolism, carcinogenicity of acetaldehyde, phenol and amines	Beer, wine, distilled beverages	< 2 drinks per day
Smoking	Risk	Polycyclic aromatic hydrocarbons, nitrosamines and aromatic amines that are found in burned tobacco	Cigarettes, cigars	Do not smoke or chew tobacco

Table 1.2 – Summary of Dietary and Lifestyle Factors for Colorectal Cancer Prevention.

[Table adapted from (Mason, 2002)]

1.4 Chemoprevention of Colon Cancer

Although dietary and lifestyle intervention can have a considerable impact on reducing the risk of developing colorectal cancer, it is very difficult to devise and implement such prevention strategies on a large scale due to lack of compliance. Chemoprevention therefore represents an attractive, alternative approach to reducing the mortality from colorectal cancer and essentially involves the long-term use of chemical agents that can delay, prevent or reverse the development of adenomas and interfere with the multi-step progression from adenoma to carcinoma (Benamouzig, 2005; Arber and Levin, 2005). The challenge for chemoprevention is to develop safe and effective drugs for colorectal cancer which are easy to administer and of low cost (Thun *et al.* 2002). One class of compounds that have shown considerable promise in the chemoprevention of colorectal cancer are NSAIDs.

1.4.1 NSAIDs Prevent Colorectal Cancer

In recent years, there is substantial evidence from animal models, epidemiological reports, clinical observations, and *in vitro* studies that the use of aspirin and related NSAIDs is associated with colon cancer prevention [reviewed in (Arber and Levin, 2005; Thun *et al.*, 2002; Haanen, 2001; Shiff and Rigas, 1997; Smalley and DuBois, 1997)].

1.4.1.1 Evidence of Protective Effects of NSAIDs from Epidemiological Studies and Clinical Trials

Epidemiological studies with different design, location and population groups have consistently shown that persons who regularly take aspirin or related non-aspirin NSAIDs, have a 40-50% lower risk of developing colorectal neoplasias [reviewed in (Benamouzig *et al.*, 2005; Brown and DuBois, 2005; Imperiale, 2003; Burke *et al.*, 2003; Giovannucci, 1999; Shiff and Rigas, 1997)]. An inverse association between aspirin use and the risk for colorectal cancer has been suggested from studies of patients with rheumatoid arthritis. In both a Swedish cohort study (Gridley *et al.*, 1993) and Finish cohort study (Kauppi *et al.*, 1996), which were conducted on 11,863 and 9,469 patients hospitalised with rheumatoid arthritis respectively using record linkage analysis, a reduction of approximately 40% in risk for colon carcinoma was reported. Similarly, the results of the population-based Melbourne colorectal cancer study (Kune *et al.*, 1988), which included 715 colorectal cancer cases and 727 age/sex-matched controls, found an inverse association between aspirin use and risk for colorectal cancer. A prospective study, including 662,424 men and women from the Cancer Prevention Study II of the American Cancer Society, subsequently reported that regular aspirin use at low doses (≥16 times per month) reduces the risk of fatal colon cancer by approximately 40% in both men and women (Thun *et al.*, 1991). Data from the Nurses' Health Study, comprising a cohort of 121,701 U.S. female registered nurses aged 30-55 years, suggested that regular aspirin use substantially reduces the risk of colorectal cancer, although the protective effects may not be apparent until after a decade of aspirin

consumption (Giovannucci *et al.*, 1995). A more recent population-based cohort study with secondary case-control analysis of 943,903 persons aged 40-79 years, traced from the General Practice Research Database in the UK, reported a 40% reduction in the risk of developing colon cancer among long-term users of aspirin at doses of 300 mg daily (García-Rodríguez and Huerta-Alvarez, 2001).

In addition to aspirin, there is also compelling evidence from epidemiological studies that non-aspirin NSAIDs prevent colorectal cancer. An initial report that sulindac therapy could induce polyp regression in FAP patients (Waddell and Loughry, 1983) was followed by numerous other non-randomised studies documenting similar findings. One study (Rigau *et al.*, 1991) found a marked reduction in the number and size of polyps in 7 patients with different forms of adenomatous polyposis after treatment with sulindac for 6 months, an effect that was reversed after withdrawal of the drug. Similarly, another study (Mäkelä and Laitinen, 1994) demonstrated that sulindac therapy mediated the regression and/or disappearance of rectal polyps in two FAP patients and that discontinuation of treatment resulted in regression. The results of a prospective, controlled, non-randomised study of 38 FAP patients demonstrated that there was a significant reduction of adenomas in response to sulindac treatment after three years of therapy (Winde *et al.*, 1997). In an open prospective study of 17 patients with FAP, it was found that daily treatment with 300 mg sulindac for four months, followed by a washout phase of 6 months, led to a significant reduction in the number and size of adenomas (Guldenschuh *et al.*, 2001). The metabolite of sulindac, sulindac sulfone (exisulind), has also been shown to have a protective effect against colorectal cancer. One study (Stoner *et al.*, 1999) demonstrated a 25% reduction in rectal adenomas in

73 FAP patients treated with exisulind. A subsequent non-randomised Phase I trial of exisulind in 18 FAP patients established that there was a trend towards increased apoptosis in polyps at the maximum tolerated dose (600 mg/day) (van Stolk *et al.*, 2000). The only epidemiological data concerning the protective benefits of indomethacin and risk of colorectal cancer comes from a population-based cohort study with secondary case-control analysis using the UK General Practice Research Database (García Rodríguez and Huerta-Alvarez, 2000). In that study, the relative risk of either colorectal adenoma or colorectal cancer among regular users of indomethacin was documented as being 0.4 (95% confidence intervals 0.2 to 1.0) when compared to NSAID non-users.

It is important to note that all epidemiological studies, by their nature, are observational and therefore provide little information as to the most effective dose and duration of drug use. The most definitive evidence for the anti-tumour effects of NSAIDs comes from randomised controlled trials. Until recently, only one randomised trial of aspirin with respect to reduced risk of colorectal cancer had been conducted, the Physicians' Health Study (Stürmer *et al.*, 1998). The authors reported no significant protective effect either during the 5 year study period or up to 12 years after the study began. However, the results of three new randomised trials suggest that aspirin reduces the risk of recurrent adenomas among persons with a history of colorectal cancer or adenomas. In one study (Sandler *et al.*, 2003), the authors found that daily use of aspirin (325 mg/day) is associated with a significant reduction in the incidence of colorectal adenomas in patients with previous colorectal cancer. In a separate study (Baron *et al.*, 2003), it was demonstrated that low-dose aspirin (81 mg/day), but not a higher dose (325 mg/day), has a moderate chemopreventative

effect on adenomas in the large bowel in patients with a recent history of adenomas. Similarly, another study (Benamouzig *et al.*, 2003) reported preliminary findings from a 4 year trial that daily soluble aspirin intake (160 or 300 mg/day) is associated with a reduction in the risk for recurrent adenomas, as identified by colonoscopy 1 year after starting treatment.

Although the results of a recent randomised control trial of sulindac in 41 FAP patients found that sulindac was ineffective for the primary treatment of FAP and that the long-term use of sulindac resulted in resistance to the medication (Giardiello *et al.*, 2002), three previous randomised control trials have demonstrated a protective benefit of sulindac therapy in FAP patients. A randomised, placebo-controlled, double-blind cross over study was first conducted in 10 FAP patients with rectal polyps, who had previously been treated by colectomy and ileorectal anastomosis, and observed complete (6 patients) or almost complete (3 patients) regression of polyps in patients treated with sulindac (300 mg/day) for two 4-month periods separated by a one month wash-out phase (Labayle *et al.*, 1991). These observations were confirmed by a subsequent randomised control trial in 24 FAP patients who had previously undergone prophylactic colectomy and had advanced duodenal polyposis (Nugent *et al.*, 1993). In that study, six months of sulindac therapy was found to be associated with a trend towards duodenal polyp regression (P=0.12). Similarly, the results of another randomised, double-blind, placebo controlled study of 22 FAP patients, of which 18 had not undergone colectomy, found that patients who received sulindac (150 mg twice a day) for nine months had a statistically significant decrease in the mean number of polyps and mean diameter of polyps, as compared with those given placebo (Giardiello *et al.*, 1993). A very

recent randomised, double-blind, placebo controlled, dose-response study of exisulind in 281 patients with sporadic adenomatous polyps established that treatment with exisulind (400 mg daily) for 12 months caused significant reduction in median polyp size and inhibited disease progression as compared to placebo treatment (Arber *et al.*, 2006). However, in that study, treatment with exisulind was associated with a degree of toxicity. There have been no clinical trials for indomethacin specifically with regards prevention for colorectal cancer, but a randomised, placebo-controlled trial of indomethacin (50 mg twice daily) or prednisolone (10 mg twice daily) in patients with disseminated solid malignancy (colorectal cancer 22% cases, liver/pancreatic cancer 33% cases) demonstrated that indomethacin prolonged mean survival from 250±28 days to 510±28 days ($P<0.05$) compared with placebo treatment (Lundholm *et al.*, 1994).

Collectively, these data from epidemiological observations and clinical trials clearly demonstrate that NSAIDs, in particular aspirin, sulindac, sulindac sulfone and indomethacin, prevent colorectal cancer.

1.4.1.2 Evidence of Protective Effects of NSAIDs from Animal Studies

In addition to epidemiological studies and clinical trials in humans, the anti-neoplastic properties of NSAIDs are clearly apparent in animal (mainly rodent) models of colon cancer. One of the first studies discovered that rats co-administered DMH (1,2-dimethylhydrazine), a colon carcinogen, and indomethacin developed

fewer colon tumours compared with rats just given the carcinogen alone (Pollard and Luckert, 1981). Similar studies in rats treated with azoxymethane (AOM), a carcinogen that causes development of colorectal cancer, have demonstrated that NSAIDs, including aspirin (Reddy *et al.*, 1993), sulindac sulfone (Haanen, 2001) and nimesulide (Fukutake *et al.*, 1998), can suppress AOM-induced colon carcinogenesis. Investigators in these and related studies concluded that NSAIDs inhibit the initiation and development of carcinogen-induced tumours (Shiff and Rigas, 1997). However, it is important to note that in these animal studies, the anticancer effect of NSAIDs was shown to be reversible as tumour occurrence increased when the agent was discontinued (Baron and Sandler, 2000).

More recently, studies have been conducted using mouse models of hereditary colorectal cancer, the most common being the *Min* mouse model. The *Min* (*APCMin/+*) mouse is a neoplasia model in which an autosomal, dominant heterozygous mutation of the mouse *APC* gene leads to spontaneous intestinal neoplasia, resembling FAP in humans (Giovannucci, 1999). However, unlike humans, who show colonic adenomas, the predominant location of tumours in the mouse is in the small intestine. In this mouse model, the administration of the NSAIDs, sulindac (Chiu *et al.*, 1997), aspirin (Barnes and Lee, 1998) and piroxicam (Jacoby *et al.*, 1996), has been shown to reduce the number and size of tumours in both the small and large intestine. Another animal model used to study colorectal cancer is the nude mouse xenograft model, where cultured colorectal cancer cells are xenografted onto the flanks of nude (athymic) mice (Gupta and DuBois, 2001). Using this model, the NSAIDs meloxicam (Goldman *et al.*, 1998) and celecoxib (Williams *et al.*, 2000) have been shown to suppress colorectal cancer cell growth.

Interestingly, very recent studies from the host laboratory have established that, using both the *Min* mouse and nude mouse xenograft models of colorectal cancer, aspirin induces apoptosis in intestinal tumours. Moreover, aspirin-induced apoptosis was associated with activation of the NF-κB pathway, suggesting a possible mechanism for the protective effects of NSAIDs *in vivo* (Reid *et al.*, 2004). The NF-κB pathway as a target for the chemopreventative effects of NSAIDs will be discussed in detail later. It should be noted that although these animal models of colon cancer are useful, the direct implications for human colorectal cancer are unclear [reviewed in (Corpet and Pierre, 2005)].

1.4.1.3 Evidence of Protective Effects of NSAIDs from *in vitro* Studies

I*n vitro*, NSAIDs have been shown to alter cellular functions that are crucial for the development of colorectal tumours, which will be discussed in more detail, including: cell proliferation, cell division, cell cycle phase distribution, induction of cell cycle quiescence and apoptosis [reviewed in (Ricchi *et al.*, 2003; Hull *et al.*, 2003; Thun *et al.*, 2002; Haanen, 2001; Shiff and Rigas, 1997)]. In addition to these effects, NSAIDs modulate several other cellular processes that may be implicated in their protective effects against colorectal cancer. A few studies have indicated that NSAIDs can up-regulate the expression of major histocompatibility complex (MHC) (Rigas *et al.*, 1994) and human leukocyte antigen (HLA) class II molecules (Arvind *et al.*, 1995). This could ultimately restore the ability of the immune system to

eliminate transformed cells expressing tumour-related antigens. For a solid tumour to grow larger than approximately 2 mm in diameter, it must stimulate the formation of new capiliary blood vessels, a process known as angiogenesis (Thun *et al.*, 2002). There is a growing body of evidence that NSAIDs can inhibit angiogenesis [reviewed in (Brown and DuBois, 2005; Thun *et al.*, 2002)]. Both aspirin and the cyclooxygenase (COX)-2-selective inhibitor, NS-398, have been shown to suppress the migration and release of angiogenic growth factors by colorectal cancer cells that are co-cultured with vascular endothelial cells (Tsujii *et al.*, 1998). Moreover, the authors found that aspirin, but not NS-398, suppressed tube formation of vascular endothelial cells. Furthermore, it has been demonstrated that indomethacin and NS-398 inhibit *in vitro* angiogenesis in rat aortic cells and human dermal microvascular endothelial cells (Jones *et al.*, 1999). Finally, NSAIDs can modulate the activation of carcinogens, production of mutagens or formation of harmful peroxyl radicals via their COX-inhibitory activity (Shiff and Rigas, 1997). The role of COX in the mechanism of action of NSAID will be the subject of further discussion later.

1.4.1.3.1 NSAIDs Inhibit Cell Proliferation

Inhibiting colonocyte proliferation has been postulated as a potential mechanism by which NSAIDs retard the growth of colorectal neoplasias and there is evidence to support this. In particular, the NSAIDs aspirin (Ricchi *et al.*, 1997; Qiao *et al.*, 1998a; Smith *et al.*, 2000), sulindac (Shiff *et al.*, 1995; Shiff *et al.*, 1996; Goldberg *et al.*, 1996), sulindac sulfone (Piazza *et al.*, 1995; Richter *et al.*, 2001),

indomethacin (Shiff *et al.*, 1996; Smith *et al.*, 2000; Kralj *et al.*, 2001), naproxen (Shiff *et al.*, 1996), piroxicam (Hanif *et al.*, 1996) and the COX-2-selective inhibitors, NS-398 (Smith *et al.*, 2000) and SC215 (Richter *et al.*, 2001), have been shown to inhibit proliferation of colorectal cancer cells. The major mechanisms whereby NSAIDs inhibit cell proliferation are **1**. inhibition of cell division, through alteration of cell cycle phase distribution or induction of cell cycle quiescence and **2**. promotion of apoptosis. These pathways will now be discussed further.

1.4.1.3.2 NSAID Effects on the Cell Cycle

Progression of a cell through the various phases of the cell cycle is promoted by a number of cyclin-dependent kinases (CDKs) which, when complexed with specific regulatory proteins called cyclins, drive the cell forward through the cell cycle (Schwartz and Shah, 2005). For example, a principal target of CDKs at the G_1/S transition of the cell cycle is the retinoblastoma protein (pRb). pRb is present in a hypophosphorylated state in quiescent or G_0 cells (Cobrinik, 2005). Hypophosphorylated pRb forms an inhibitory complex with the E2F family of transcription factors by directly binding to the transactivation domain of E2F, thus blocking the ability of E2F to activate expression of genes that encode products necessary for S-phase progression (Giacinti and Giordano, 2006). Phosphorylation of pRb by cyclin/CDK complexes (CDK4/6-Cyclin D and CDK2/Cyclin E) leads to the release of E2Fs, which in turn activate the transcription of genes required for cell cycle progression (Schwartz and Shah, 2005). Progression through the cell cycle

mediated by cyclin/CDK complexes is monitored at several positions, known as cell cycle checkpoints (Collins and Garrett, 2005). The activity of the CDK/cyclin complex is further negatively regulated by a number of CDK inhibitors (CDKIs), including p21 (waf1/cip1) and p27 (kip1) (Vermeulen *et al.*, 2003a).

There is a body of evidence that NSAIDs alter the expression levels of cell cycle proteins and induce cell cycle arrest. Sulindac sulfide has been shown to decrease the expression of cyclins B1 and E, and increase the expression of cyclins D1, D2 and D3, particularly in the G_1 phase of the cell cycle, in HT-29 colon cancer cells (Qiao *et al.*, 1998b). It has also been observed that aspirin and indomethacin reduce the levels of two CDKs, namely $p34^{cdc2}$ and $p33^{cdk2}$ (Shiff *et al.*, 1996). Moreover, in parallel with this effect, these agents were found to increase the proportion of cells in the G_0/G_1 phase but reduce the proportion of cells in the S phase and G_2/M phases of the cell cycle. Sulindac and sulindac sulfide have been reported to induce cell cycle arrest at the G_0/G_1 phase (Shiff *et al.*, 1995). Furthermore, this effect was associated with reduced expression and reduced catalytic activity of CDKs. NSAIDs have also been shown to up-regulate CDKIs, such as $p27^{kip1}$ and $p21^{waf1/cip1}$ (Ricchi *et al.*, 2003). One study (Marra *et al.*, 2000) demonstrated that sodium salicylate increases the expression of $p27^{kip1}$ and $p21^{waf1/cip1}$ in vascular smooth muscle cells. Another study (Yang *et al.*, 2001b) reported that $p21^{waf1/cip1}$ is induced by sulindac in SW620 colorectal cancer cells. Furthermore, inactivation of *p21* in $APC^{+/-}$ mice completely eliminated the ability of sulindac to inhibit APC-initiated tumour formation, thus demonstrating that p21 is critical for tumour inhibition by this drug (Yang *et al.*, 2001b). Similarly, it has been shown that the COX-2 selective inhibitor celecoxib induces G_0/G_1 cell cycle arrest,

an effect that was attributed to increased expression of CDKIs $p21^{waf1/cip1}$ and $p27^{kip1}$ (Grösch *et al.*, 2001). More recently, it has been reported that G_0/G_1 cell cycle arrest induced by celecoxib in colorectal cancer cells is correlated with a decrease in the expression levels of cyclin A and cyclin B1 and an increase in the expression of the CDKIs $p21^{waf1/cip1}$ and $p27^{kip1}$ (Maier *et al.*, 2004). It has also been demonstrated that sulindac sulfide-induced cell cycle arrest in MEFs is mediated by pRb and $p21^{waf1/cip1}$ (Jung *et al.*, 2005). Furthermore, MEFs deficient in p21 or pRb were more susceptible to sulindac sulfide-mediated cell death, leading the authors to propose that this agent may selectively target cells with cell cycle checkpoint deficits. In another recent study (Xu and Zhang, 2005) it was reported that indomethacin downregulates the expression of CDK2 and CDK4 and upregulates the expression of $p21^{waf1/cip1}$ in SW480 cells transfected with wild-type p53, but not parental SW480 cells, suggesting that indomethacin induces cell cycle arrest at the G_2 phase and inhibits cell proliferation in a p53- $p21^{waf1/cip1}$ dependent manner.

1.4.1.3.3 NSAIDs Induce Apoptosis

Apoptosis, or programmed cell death, is needed to maintain homeostasis in continuously replicating tissues such as the intestine and partial suppression of apoptosis is known to occur early in colorectal tumorigenesis (Thun *et al.*, 2002). There is substantial evidence that both traditional NSAIDs, including aspirin (Qiao *et al.*, 1998a; Stark *et al.*, 2001; Stark and Dunlop, 2005), sulindac (Haanen, 2001; Jung *et al.*, 2005), sulindac sulfide (Piazza *et al.*, 1995; Shiff *et al.*, 1995; Sinicrope and

Penington, 2005), sulindac sulfone (Piazza *et al.*, 1995; Babbar *et al.*, 2003), indomethacin (Kralj *et al.*, 2001; Smith *et al.*, 2000), piroxicam (Hanif *et al.*, 1996; Shiff *et al.*, 1996) and naproxen (Shiff *et al.*, 1996), and COX-2 selective NSAIDs, such as celecoxib (Grösch *et al.*, 2001; Maier *et al.*, 2004) and NS-398 (Smith *et al.*, 2000; Smartt *et al.*, 2003) induce apoptosis of colorectal cancer cells.

After treatment with NSAIDs, for example sulindac (Piazza *et al.*, 1995), sulindac sulfone (Shiff *et al.*, 1995; Babbar *et al.*, 2003), aspirin (Qiao *et al.*, 1998a; Smith *et al.*, 2000; Stark *et al.*, 2001) and indomethacin (Smith *et al.*, 2000; Kralj *et al.*, 2001), colon cancer cells undergo several morphological changes that are associated with apoptotic activation, including cell shrinkage, development of membrane blebs characterised by externalisation of phosphatidyl serine, nuclear condensation, fragmentation of genomic DNA and formation of apoptotic bodies. Moreover, these effects can be inhibited by agents that block gene expression, such as cyclohexamide, suggesting that cell death induced by NSAID treatment is genuine programmed cell death and not necrotic cell death caused by general toxic effects of the drugs (Chan *et al.*, 1998; Chan, 2002).

Two pathways to apoptosis downstream of the 'death signal' have been recognised, the transmembrane 'extrinsic' pathway and the mitochondrial 'intrinsic' pathway (Hersey and Zhang, 2003). Both pathways depend on activation of cysteine proteases called caspases. These enzymes are synthesised as proenzymes that become activated by adaptor proteins. Once activated, so-called initiator caspases can activate effector caspases, which act on a wide range of substrates to cause apoptosis (Gross *et al.*, 1999). The extrinsic pathway is initiated by ligation of a trans-membrane death receptor, which contains an intracellular globular protein

interaction domain called a death domain (DD) and is typified by members of the tumour necrosis factor receptor (TNFR) family such as Fas (Thorburn, 2004). Upon ligand binding to death receptors, oligomerisation of activated receptors leads to recruitment of the adaptor protein, called fas associated death domain (FADD) via their mutual DDs (Budd, 2001). FADD then recruits caspase-8 via interaction with their N-terminal death effector domains (Boatright and Salvesen, 2003) and it is within this death-induced signaling complex (DISC) that the initiator caspase-8 is activated. Activated caspase-8 can then in turn activate effector caspases, such as caspase-3, -6 and -7, causing the cell to undergo apoptosis via degradation and digestion of various cellular components (Thorburn, 2004).

In contrast, the intrinsic pathway is dependent on the release of pro-apoptotic molecules from mitochondria (Hersey and Zhang, 2003). Following the death trigger, mitochondria may become slightly permeabilised by disruption of the outer membrane, leading to the release of cytochrome *c*, which can complex with Apaf-1 and caspase-9, forming the 'apoptosome' (Budd, 2001). Apaf-1 recruits caspase-9 via its N-terminal caspase-activation recruitment domain (CARD) (Boatright and Salvesen, 2003). Procaspase-9 becomes activated by undergoing a conformational change and thereby can activate effector caspases, in particular caspase-3, -6 and -7, resulting in apoptosis (Hersey and Zhang, 2003). Another factor released from mitochondria that can mediate apoptosis is Smac, which inactivates the inhibitor of apoptosis (IAP) and also results in activation of downstream effector caspases (Budd, 2001).

It has been demonstrated that NSAIDs, including sulindac sulfide (Rice *et al.*, 2001; Richter *et al.*, 2001), sulindac sulfone (Rice *et al.*, 2001; Richter *et al.*, 2001),

aspirin (Gao *et al.*, 2004), indomethacin (Gao *et al.*, 2004) and celecoxib (Grösch *et al.*, 2001; Maier *et al.*, 2004) can activate caspases, in particular caspase-3, caspase-7 and caspase-9, enhance cleavage of PARP, a 112 kDa protein that is specifically cleaved by activated caspase-3 and caspase-6, and stimulate cytochrome *c* release, all of which are key markers of the intrinsic pathway to apoptosis. NSAIDs have also been shown to activate the extrinsic pathway to apoptosis. Indomethacin and sulindac sulfide induce apoptosis in human leukemic Jurkat cells by a mechanism that requires the FADD-mediated activation of caspase-8 (Han *et al.*, 2001). Similarly, apoptosis induced by sulindac sulfide and the COX-2-selective inhibitor, SC-236, is coupled with upregulation of death receptor (DR) 5, caspase-8 activation and Bid cleavage in HCT116 colorectal cancer cells (He *et al.*, 2002). Increased levels of the membrane death receptors, DR4 and DR5, and activation of caspase-8 in response to sulindac sulfide in SW480 colorectal cancer cells have been reported (Sinicrope and Penington, 2005), indicating stimulation of the extrinsic apoptotic pathway. Moreover, it was discovered that sulindac sulfide-mediated stimulation of the extrinsic pathway occurred alongside activation of the intrinsic pathway in response to sulindac sulfide.

The role of Bcl-2 family members in apoptosis is well established and extensively reviewed (Gross *et al.*, 1999; Borner, 2003). Bcl-2 family proteins are central regulators of apoptosis because they integrate diverse survival and death signals that are generated both outside and inside the cell. The family is subdivided into two classes: anti-apoptotic members, such as Bcl-2 and Bcl-X_L (the Bcl-2-like survival factors), which protect cells from apoptosis; and pro-apoptotic members, including Bax and Bak (the Bax-like death factors) and the large group of BH3 (Bcl-

2 homology region 3)-only death proteins, which trigger or sensitize for apoptosis (Borner, 2003). The relative levels of the various members of the Bcl-2 family regulate survival and apoptosis in cells (Ricchi *et al.*, 2003).

A role for the pro-apoptotic gene *Bax* in NSAID-induced colorectal cancer cell death has been proposed (Zhang *et al.*, 2000). In contrast to parental cells, *Bax*$^{-/-}$ HCT116 colorectal cancer cells were discovered to be resistant to apoptosis induced by sulindac or indomethacin. Moreover, in several parental colorectal cancer cell lines, both sulindac and indomethacin inhibited the expression of the anti-apoptotic protein Bcl-X_L, resulting in an altered ratio of Bax to Bcl-X_L and subsequent mitochondria-mediated cell death. Similarly, aspirin and indomethacin were shown to induce apoptosis through upregulation of the pro-apoptotic proteins Bax and Bak and activation of caspase-3 (Zhou *et al.*, 2001). In contrast to these studies, the COX-2 selective inhibitors, SC-58125 and NS-398 have been reported to sensitize colon and prostrate cancer cells, respectively, to apoptosis by downregulating the anti-apoptotic protein Bcl-2 (Liu *et al.*, 1998). Interestingly, in a recent study (Sinicrope and Penington, 2005), co-administration of sulindac sulfide with HA14, a small molecule Bcl-2 inhibitor, or tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), was discovered to enhance sulindac sulfide-induced apoptosis in colorectal cancer cells.

Collectively, *in vitro* studies have demonstrated that NSAIDs can inhibit various cellular processes which are implicated in tumour development and progression, including the recognition of transformed cells by the immune system, angiogenesis and activation of carcinogens. Furthermore, NSAIDs can inhibit cell proliferation by altering the expression levels of cell cycle proteins or induction of

cell cycle arrest. Crucially and most importantly, NSAIDs can induce apoptosis – NSAIDs have been shown to modulate both the extrinsic and intrinsic pathways to apoptosis, via up-regulation of death receptors and activation of caspases, and can regulate the expression of Bcl-2 family member proteins, such as Bax. Moreover, the induction of apoptosis is now recognised as being the predominant anti-tumour activity of these agents.

The compelling evidence from epidemiological studies, clinical trials, *in vivo* studies and *in vitro* studies strongly implicate NSAIDs as important chemopreventative agents against colorectal cancer. It is therefore vital to understand the mechanisms and pathways whereby NSAIDs exert their protective effects for the development of novel therapeutic agents.

1.4.2 The Genetic Model for Colorectal Tumorigenesis

It is important to consider the tumorigenic process itself when trying to establish and understand the mechanisms for chemopreventative agents such as NSAIDs. It is now widely accepted that multiple mutations over time are necessary for the development of malignancy (Kinzler and Vogelstein, 1996). This multi-step process is well illustrated by colorectal cancers, which typically develop over decades and require at least four sequential genetic changes to occur in order to ensure colorectal cancer evolution (Fodde, 2002). A genetic pathway (Figure 1.6) that parallels the histological progression of colorectal cancer from adenoma to

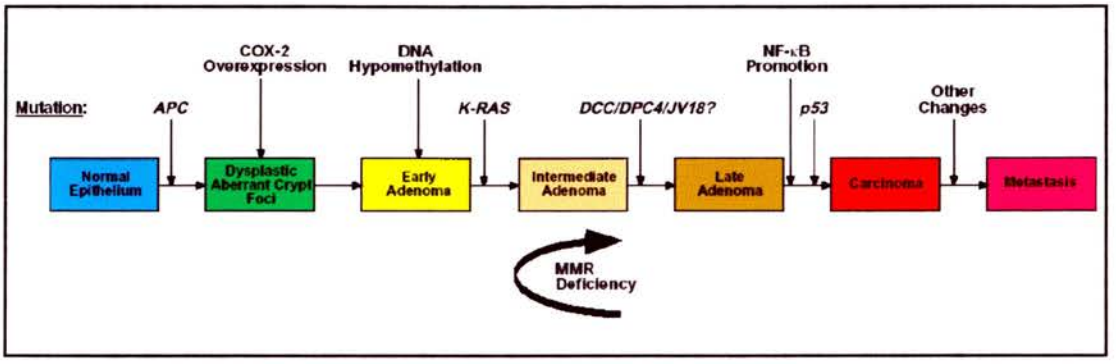

Figure 1.6 – Genetic Changes Associated with Colorectal Tumorigenesis. Tumorigenesis proceeds through a series of genetic alterations involving oncogenes e.g. *K-RAS*, that require only one genetic event for their activation, and tumour suppressor genes e.g. *DCC*, *DPC4* and *JV18*, that require two genetic events (one in each allele) for their inactivation. *APC* mutations initiate the neoplastic process and tumour progression results from mutations in the other genes indicated. The three stages of adenomas represent tumours of increasing size, dysplasia, and villous content. DNA mismatch repair (MMR) deficiency speeds up this process. Tumours continue to progress once carcinomas have formed, and the accumulated loss of suppressor genes on additional chromosomes correlates with the ability of the carcinomas to metastasise and cause death.

[Figure adapted from (Kinzler and Vogelstein, 1996)]

carcinoma has been suggested (Fearon and Vogelstein, 1990) and is now widely accepted. Briefly, the process of tumorigenesis has three stages: initiation, promotion and progression. Mutation and/or loss of *APC* is the earliest genetic alteration in the genesis of colorectal tumours and seems to be required to initiate the adenomatous process, resulting in the clonal growth of a single cell (Fodde *et al.*, 2001b). A small polyp subsequently develops and eventually, one of these cells overgrows its sister cells, promoting the growth of a larger tumour. When a cell has acquired enough mutations and undergone clonal expansions, it acquires the ability to invade and metastasise and progression to malignancy ensues (Kinzler and Vogelstein, 1998a; Hawk and Levin, 2005).

NSAIDs have been shown to modulate several key molecules that are implicated in the progression of colorectal cancer and the specific mechanisms and pathways regulated by NSAIDs will be the subject of the remainder of this discussion.

1.5 Mechanism of NSAID Action

The mechanisms which have been proposed for the anti-tumour effects of NSAIDs are summarised in Figure 1.7. In particular, there is substantial evidence that inhibition of the COX enzymes is implicated in the preventative effects of NSAIDs. However, there is also increasing support for the existence of COX-independent pathways and suggested mechanisms include modulation of peroxisome

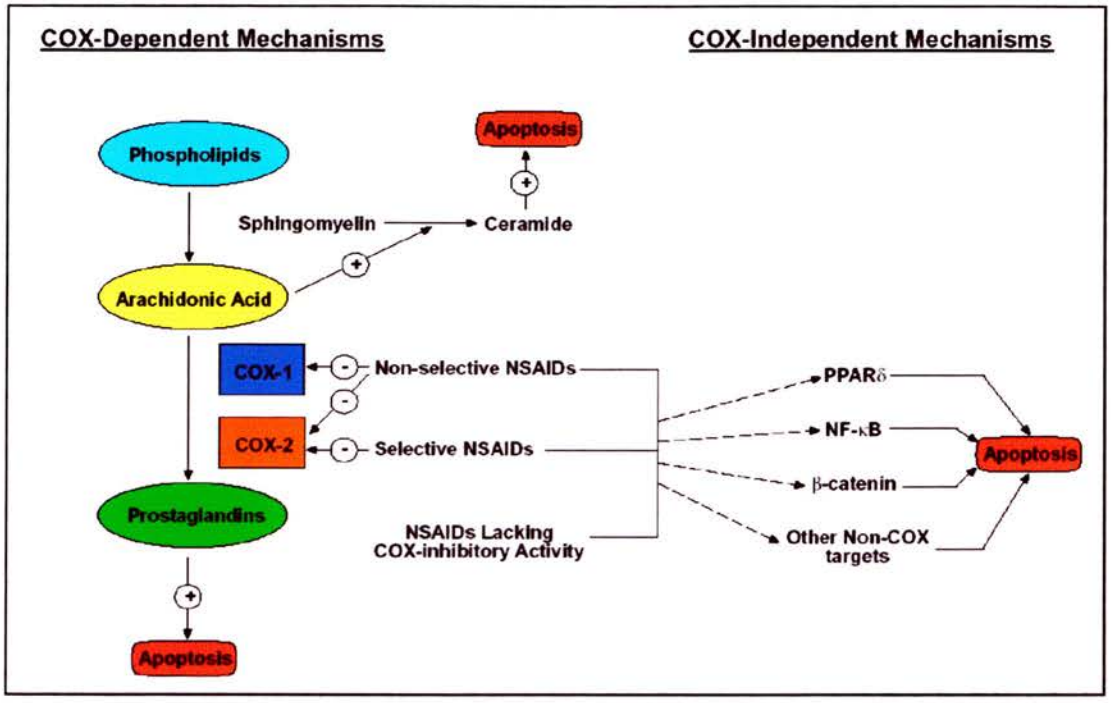

Figure 1.7 – Mechanisms of Action of NSAIDs. Non-selective NSAIDs, for example aspirin, sulindac and indomethacin, can inhibit both cyclooxygenase (COX)-1 and COX-2, whereas COX-2 selective NSAIDs, such as celecoxib and rofecoxib, inhibit only COX-2. These agents induce apoptosis by both COX-dependent and COX-independent mechanisms. NSAIDs that lack COX-inhibitory activity, for example sulindac sulfone, only induce apoptosis via COX-independent mechanisms. The inhibition of COX leads to apoptosis primarily by altering prostaglandin production. In addition, the inhibition of COX leads to an increase in arachadonic acid, which in turn stimulates the conversion of sphingomyelin to ceramide, a mediator of apoptosis. NSAIDs also exert their effects and induce apoptosis by COX-independent mechanisms such as modulation of PPARδ, NF-κB, β-catenin or other non-COX targets. Plus signs indicate stimulation or activation, and minus signs indicate inhibition. Dotted line denotes modulation.

[Figure adapted from (Jänne and Mayer, 2000)]

proliferator-activated receptors (PPARs), NF-κB and β-catenin signaling. It is important to emphasize that the mechanisms for the protective benefits of NSAIDs against cancer are complex and have yet to be fully elucidated. The work of this thesis is focussed on COX-independent pathways, but here I will discuss both COX-dependent and COX-independent effects of NSAIDs.

1.5.1 COX-dependent Effects

COX is the rate-limiting enzyme that catalyses the production of prostaglandins (PGs) from arachidonic acid (Figure 1.8). It occurs as two isozymes: COX-1, a constitutive isoform associated with beneficial housekeeping roles, and COX-2, an inducible isoform associated with inflammation, mitogenesis and/or specialised signal transduction mechanisms (Brown and DuBois, 2005). PGs play critical roles in numerous biological processes, including maintenance of physiological organ function, regulation of immune function, kidney development, reproductive biology and gastrointestinal integrity (Gupta and DuBois, 2001; Backlund *et al.*, 2005). It is noteworthy that since COX-1 is the only COX isoform expressed in platelets and gastric mucosa in healthy individuals, the toxicity of NSAIDs in the gastrointestinal mucosa, leading to ulceration and bleeding, is the result of inhibition of this enzyme (Patrignani, 2000).

It is widely accepted that the anti-inflammatory and analgesic properties of NSAIDs arise mainly from inhibition of the enzymatic activity of COX [reviewed in

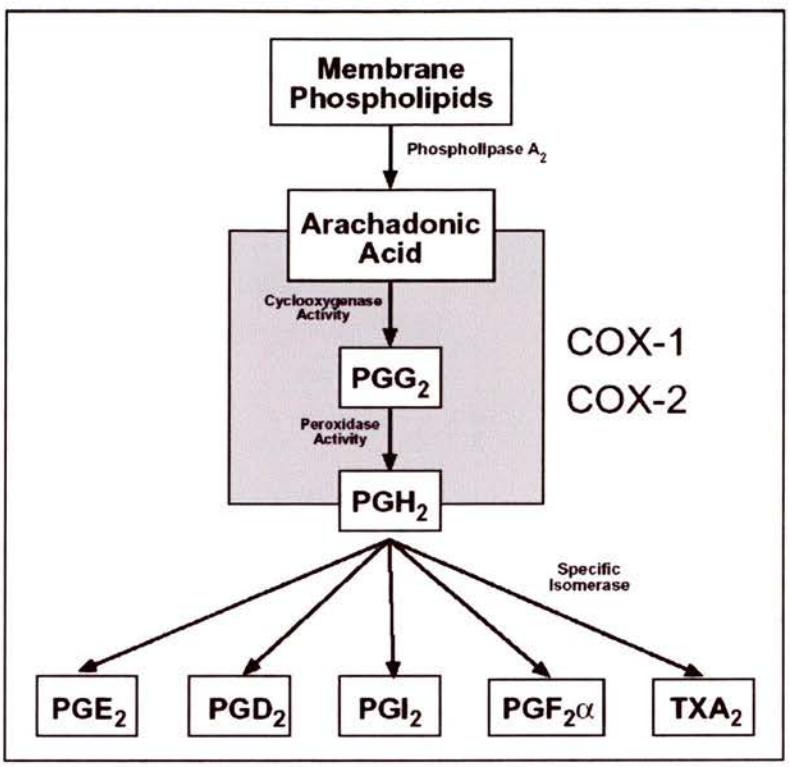

Figure 1.8 – Arachadonic Acid Cascade. Arachadonic acid is released from membrane phospholipids by phospholipase A_2 and is subsequently converted by either cyclooxygenase (COX)-1 or COX-2 to prostaglandin (PG) G_2 and then to PGH_2 by peroxidase catalytic activity. Finally, the production of PGs (PGE_2, PGD_2, PGI_2, $PGF_2\alpha$ and thromboxane A_2) from PGH_2, the precursor of all PGs, is catalysed by a specific isomerase.

[Figure adapted from (Brown and DuBois, 2005)]

(Backlund *et al.*, 2005; Gupta and DuBois, 2001; Prescott and Fitzpatrick, 2000; Patrignani, 2000; Williams *et al.*, 1999b; Shiff and Rigas, 1999)]. As a result, it has generally been assumed that the anti-neoplastic effects of NSAIDs are also dependent on inhibition of COX activity.

There are several lines of evidence that inhibition of COX-2 and PG production contributes to the prevention of colorectal cancer by NSAIDs [reviewed in (Brown and DuBois, 2005; Backlund *et al.*, 2005; Chan, 2002; Patrignani, 2000)]. **1.** COX-2 mRNA and COX-2 protein, but not COX-1, are overexpressed in colorectal polyps and cancers, resulting in increased levels of PGs (Patrignani, 2000). This is important because ecosanoids, including PGs, can stimulate proliferation and reduce apoptosis in colonocytes (Shiff and Rigas, 1999). **2.** PGE_2 levels are elevated in colorectal tumours and adenomas compared to normal adjacent mucosa (Backlund *et al.*, 2005). **3.** COX-2 contributes to colorectal tumorigenesis in *APC* knockout mouse. Both gene disruption and pharmacological inhibition of COX-2 have been shown to reduce the number and size of polyps in $APC^{\Delta 716(+/-)}$ knockout mice (Oshima *et al.*, 1996). **4.** Overexpression of COX-2 in epithelial cell lines results in resistance to apoptosis and changes in cell adhesion properties, both of which are consistent with increased tumorigenic potential (Sakamoto, 1998).

It is also important to consider that the inhibition of COX-2 leads to an increase in arachidonic acid, which in turn stimulates the conversion of sphingomyelin to ceramide, a known mediator of apoptosis (Jänne and Mayer, 2000; Chan *et al.*, 1998).

1.5.2 COX-independent Effects

Despite the compelling evidence for the involvement of COX, in particular COX-2, in the mechanism of NSAID prevention of colorectal cancer, there is a growing body of support for the hypothesis that COX-independent pathways may also be involved in both the anti-inflammatory and anti-tumour activity of NSAIDs [reviewed in (Arber and Levin, 2005; Chan, 2002; Tegeder *et al.*, 2001b; Shiff and Rigas, 1999)]. **1.** The mechanisms that have been suggested to contribute to the anti-tumour activity of NSAIDs, such as inhibition of cell cycle progression, induction of apoptosis and inhibition of angiogenesis, have been observed only at high concentration of the respective NSAIDs, which are 100-1000 fold higher than the amount required to inhibit prostaglandin production (Grösch *et al.*, 2001; Kralj *et al.*, 2001; Smith *et al.*, 2000; Chiu *et al.*, 1997; Qiao *et al.*, 1998a; Ricchi *et al.*, 1997). **2.** Colorectal cancer cell lines (Hanif *et al.*, 1996) or mouse embryo fibroblasts (Zhang *et al.*, 1999) that do not express either COX isoform i.e. *COX-1*$^{-/-}$, *COX-2*$^{-/-}$ and *COX-1*$^{-/-}$/COX-2$^{-/-}$, show similar sensitivity to high dose NSAID treatment. **3.** Complete lack of COX-2 enzyme in mice null for COX-2 did not entirely prevent the development of intestinal tumours when crossed onto *APC*$^{\Delta 716(+/-)}$ knockout mice (Oshima *et al.*, 1996) **4.** NSAIDs are effective in colon cancer cell lines that do not produce PGs, even with exogenous stimulation (Hanif *et al.*, 1996). **5.** Metabolites of NSAIDs that have no COX-inhibitory activity e.g. sulindac sulfone or salicylic acid, have similar growth-inhibitory and chemopreventive activities *in vitro* and *in vivo* (Williams *et al.*, 1999a; Piazza *et al.*, 1997). **6.** NSAIDs modulate signal transduction pathways that are independent of COX [reviewed in (Ricchi *et al.*, 2003; Tegeder *et*

al., 2001b)]. Taken together, these data strongly suggest that other pathways in addition to COX are important for the anti-tumour effects of NSAIDs.

1.5.3 Non-COX Pathways Modulated by NSAIDs

To date, non-COX pathways that have been reported to be modulated by NSAIDs include: PPARs, DNA repair, p38 and activator protein-1 (AP-1) signaling. These pathways will each be discussed in turn before considering in more detail the two major non-COX pathways known to be regulated by NSAIDs, namely the NF-κB and β-catenin pathways.

1.5.3.1 PPARs

PPARs function as members of the nuclear hormone superfamily, which includes the steroid hormone, thyroid hormone and retinoid nuclear hormone receptors (He *et al.*, 1999). These receptors are ligand-activated transcription factors, which directly regulate the transcription of target genes (Wang *et al.*, 2006). Three distinct PPAR isoforms: PPARα, δ and γ, which exhibit distinct tissue distribution, have been isolated and characterised (Gupta and DuBois, 2001; Wang *et al.*, 2006). PPARγ is highly expressed in adipose tissue and functions as a master regulator of adipocyte differentiation (Lehmann *et al.*, 1997). PPARα is predominantly expressed in hepatocytes, cardiomyocytes, proximal tubule cells of the kidney and enterocytes

(Wang *et al.*, 2006), and is required for the pleiotropic hepatic response to peroxisome proliferators. PPARδ is more widely and abundantly expressed than the other isoforms although little is known about its physiological function. In addition to adipose tissue, PPARγ is highly expressed in the normal large intestine and has been implicated in breast, colon and prostrate cancer (Tegeder *et al.*, 2001b). Significantly, activation of PPARs in colon cancer cells reduces their growth and induces differentiation *in vitro* (Shiff and Rigas, 1999). PPARs could therefore be a target of NSAIDs in the colon.

It has been shown that indomethacin, and several other NSAIDs, can bind to and induce the transcriptional activity of PPAR isoforms α and γ (Lehmann *et al.*, 1997). Similarly, another report (Jaradat *et al.*, 2001) documented that a panel of NSAIDs comprising ibuprofen, naproxen and indomethacin can activate PPAR α and γ isoforms in a concentration-dependent manner and with varying efficacy. PPARδ has been identified as a direct target of sulindac (He *et al.*, 1999). In that study, PPARδ was initially discovered to be a major target of APC through analysis of global gene expression profiles in human colorectal cancer cells. PPARδ expression was repressed by APC and this repression was mediated by β-catenin/TCF-4-responsive elements in the PPARδ promoter. Sulindac could mimic the effects of APC by downregulating the transcriptional activity of PPARδ and overexpression of PPARδ could rescue the ability of high doses of sulindac to induce apoptosis (He *et al.*, 1999). Another study (Babbar *et al.*, 2003) established that apoptosis induced by sulindac sulfone is mediated, at least in part, by PPAR-dependent transcriptional activation of the spermidine/spermine N^1-acetyltransferase (SSAT) gene. Interestingly, sulindac sulfide can act as a PPARγ agonist and PPARδ antagonist in a

human prostrate epithelial cell line (Jarvis *et al.*, 2005). Moreover, PPARγ was required for both growth inhibition and $p21^{waf1/cip1}$ overexpression in these cells. In addition, overexpression of PPARδ partially rescued these cells from growth inhibition and sulindac sulfide-mediated $p21^{waf1/cip1}$-upregulation. Genetic ablation of PPARδ in colorectal cancer cells does not alter their sensitivity to sulindac-induced apoptosis, suggesting that there are other targets of sulindac in addition to PPARδ (Park *et al.*, 2001). Similarly, a recent study (Hawcroft *et al.*, 2003) demonstrated that indomethacin could bind and activate PPARγ but that this was not necessary for the antiproliferative effect associated with indomethacin *in vitro*, indicating that there are other targets of this agent in addition to PPARγ.

1.5.3.2 DNA Repair

As mentioned above, defective DNA MMR is implicated in colorectal cancer and is usually characterised by MSI. There are several reports that MMR genes could be a target of NSAIDs. One report (Rüschoff *et al.*, 1998) suggested that the MSI phenotype in colorectal cancer cells deficient for a subset of the human MMR genes (*hMLH1*, *hMSH2* and *hMSH6*) is markedly reduced during exposure to aspirin or sulindac. In that study, cell lines deficient in MMR genes, but not with a *hPMS2* mutation, were found to die selectively by apoptosis in response to NSAID treatment, an effect that was reversible, time- and concentration-dependent and appeared to be independent of COX function. More recently, in a study comparing MMR-deficient (HCT116) and MMR-proficient ($HCT116^{+ chr3}$ and SW480) cells, it

was shown that treatment with aspirin results in an increase in MMR protein expression in the MMR-proficient cell lines, but not the MMR-deficient cell line (Goel *et al.*, 2003). Moreover, the authors reported that aspirin induced apoptosis in all the cell lines studied and caused G_0/G_1 cell cycle arrest in HCT116 cells. Although the DNA MMR-proficient cells were perhaps more resistant to the apoptotic effects of aspirin when compared to the MMR-deficient cells, the observations of that study suggest that aspirin could in fact be used as a chemopreventive agent irrespective of MSI status. In keeping with these data, recent studies from the host laboratory have shown that aspirin-induced apoptosis is an effect that is common to colorectal cancer cell lines, but not non-colorectal cancer cell lines examined, irrespective of the mutation status of DNA MMR genes (Din *et al.*, 2004). Specifically, aspirin treatment was found to induce apoptosis in both HCT-116 and $HCT116^{+\ chr3}$ colorectal cancer cells, indicating that the effect of aspirin is independent of MMR status (Din *et al.*, 2005).

1.5.3.3 p38

p38 is a MAPK, which act as serine/threonine protein kinases and are members of discrete signaling cascades (Ono and Han, 2000). The MAPK family can be divided into at least three distinct subgroups, namely extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinase (JNK)/stress activated protein kinases (SAPKs), and p38 (Engelberg, 2004). Moreover, ERK5/big MAPK 1 (BMK1) has recently been described as another subfamily of MAPKs (Kasler *et al.*, 2000).

MAPKs are activated by dual specificity kinases, called MAPK kinases (MAPKKs or MAP2Ks) (Saklatvala, 2004). MAPKKs are in turn activated by a family of serine-threonine kinases called MAPKK kinases (MAPKKKs, MAP3Ks or MEKKs). These signaling cascades essentially act as focal points for diverse extracellular stimuli and function to regulate key cellular processes, including: cell proliferation, oncogenesis, differentiation, inflammation and stress responses (Huang *et al.*, 2004). It is noteworthy that four mammalian isoforms of p38 have been identified (p38α, p38β, p38δ and p38γ) (Lee *et al.*, 2000) and that p38 has both oncogenic and tumour suppressing activities, depending on the cellular context [reviewed in (Engelberg, 2004; Bulavin and Fornace, Jr., 2004; Rennefahrt *et al.*, 2005)].

There is a growing body of evidence that NSAIDs can modulate p38 signaling. Sodium salicylate has been shown to activate p38 (Schwenger *et al.*, 1998). Moreover, activation of p38 by sodium salicylate led to inhibition of TNF-induced IκBα phosphorylation and degradation. More recent data indicates that sodium salicylate activates p38 via phosphorylation at Thr 180/Tyr 182 and partially activates ERK1/2 by phosphorylation at Thr 202/Tyr 204 in HCT116 colorectal cancer cells (Lee *et al.*, 2003). Furthermore, the authors reported that a specific p38 inhibitor (SB203580), but not other inhibitors, significantly prevented salicylate-induced apoptosis, strongly implicating p38 in the mechanism of apoptosis caused by this agent. p38 is also activated by indomethacin in colorectal cancer cells (Kim *et al.*, 2002) and this activation may play a partial but significant role in indomethacin-induced apoptosis. Both sulindac sulfide and the selective COX-2 inhibitor, NS398 activate p38 and $ERK^{p44/42}$ in a dose- and time-dependent manner in HCA-7 and HCT-15 colorectal cancer cells (Sun and Sinicrope, 2005). In that study, activation of

p38 and ERK$^{p44/42}$ was accompanied by an increase in COX-2 expression, an effect which could be suppressed by selective inhibitors of these MAPKs. Furthermore, sulindac sulfide-induced apoptosis was significantly enhanced by using MAPK inhibitors or reducing constitutive COX-2 expression via antisense oligonucleotides, leading the authors to conclude that MAPK activation mediates COX-2 induction by sulindac sulfide and that selective inhibitors of MAPKs potentiate apoptosis induction by this NSAID. It is of interest to note that recent work from the host laboratory has shown that aspirin rapidly activates the p38 MAPK pathway in SW480 colorectal cancer cells (Thoms *et al.*, 2004).

1.5.3.4 AP-1

The AP-1 transcription factor complex is produced by homo- or hetero-dimeric combinations of basic region-leucine zipper proteins that belong to members of the Jun (c-Jun, JunB and JunD) and Fos (c-Fos, FosB, Fra-1 and Fra-2) family, Jun dimerisation partners (JDP1 and JDP2) and the closely related activating transcription factor (ATF2, LRF1/ATF3 and B-ATF) subfamilies (Kundu and Surh, 2004; Chun and Surh, 2004). AP-1 is activated in response to a variety of stimuli, including ultraviolet (UV) radiation, growth factors, tumour necrosis factor-alpha (TNFα) and interleukin-1 (IL-1) (Tegeder *et al.*, 2001b). Activation of AP-1 is mediated predominantly by MAPK cascades, particularly ERK, JNK and p38 (Kundu and Surh, 2004). After dimerisation, the AP-1 complex binds to TPA (12-O-tetradecanoylphorbol 13-acetate)-responsive elements (TREs; TGAC/CTCA) in the

promoter and enhancer regions of target genes (Chun and Surh, 2004; Milde-Langosch, 2005). It is noteworthy that targets regulated by AP-1 include genes involved in the immune and inflammatory response, proliferation, invasion, metastasis, differentiation and survival (Milde-Langosch, 2005).

There is a body of evidence suggesting that AP-1 could be a target of NSAIDs. Aspirin and Sodium salicylate have been shown to inhibit epidermal growth factor and UV-induced AP-1 activation in mouse epidermal cells (Dong *et al.*, 1997). Similarly, another study (Tegeder *et al.*, 2001a) observed that R-flurbiprofen can inhibit lipopolysaccharide (LPS)-induced AP-1 activation in mouse macrophages. Aspirin and sodium salicylate have been demonstrated to reduce Epstein-Barr-mediated upregulation of matrix metalloproteinase (MMP)-9, thus reducing tumour invasion, via simultaneous inhibition of NF-κB and AP-1 (Murono *et al.*, 2000). However, in contrast to these studies, a very recent paper (Niederberger *et al.*, 2006) established that the COX-2-selective inhibitors, etoricoxib and lumiracoxib had no effect on LPS-induced activation of the AP-1 subunits, c-jun and c-fos.

1.5.3.5 The NF-κB Signaling Pathway

There is compelling evidence that the NF-κB pathway is a very good potential target for NSAIDs in the colon and this notion is strongly supported by previous work from the host laboratory which identified the NF-κB pathway as a critical target for the apoptotic effects of aspirin (Stark *et al.*, 2001; Stark and

Dunlop, 2005). The major focus of research in the host laboratory, and consequently this thesis, has therefore been to investigate the mechanism by which aspirin mediates the apoptosis of colorectal cancer cells and to investigate whether this effect is specific to aspirin or is a general effect common to other NSAIDs.

NF-κB is an inducible and ubiquitously expressed transcription factor that regulates genes involved in cell survival, cell adhesion, inflammation, differentiation and cell growth (Bharti and Aggarwal, 2002). Active NF-κB complexes are comprised of homodimers or heterodimers of various combinations of the Rel family of polypeptides. Mammals express five Rel proteins that belong in two classes (Greten and Karin, 2004) and these are illustrated in Figure 1.9A. The first class are synthesised as mature proteins and includes: RelA (p65), RelB and c-Rel. The second class consists of the precursor proteins, NF-κB1 (p105/p50) and NF-κB2 (p100/p52), which undergo proteolysis to yield their mature products, p50 and p52 respectively (Perkins, 2000). Each member of this family contains a conserved amino-terminal region of approximately 300 amino acids called the Rel homology domain (RHD), within which lies DNA-binding and dimerisation domains, a nuclear localisation signal (NLS) and a site for binding of the NF-κB inhibitors, the IκBs (inhibitor of κBs). RelA, RelB and c-Rel additionally have a carboxy-terminal transactivation domain (TAD) (Sun and Andersson, 2002; Chen and Ghosh, 1999; Hayden and Ghosh, 2004). In resting cells, NF-κB dimers are generally sequestered in the cytoplasm by interaction with a member of the IκB family, which are illustrated in Figure 1.9B and includes IκBα, IκBβ, IκBε, IκBγ, Bcl-3, p100 and p105 (Ravi and Bedi, 2004). IκB family members are characterised by the presence of

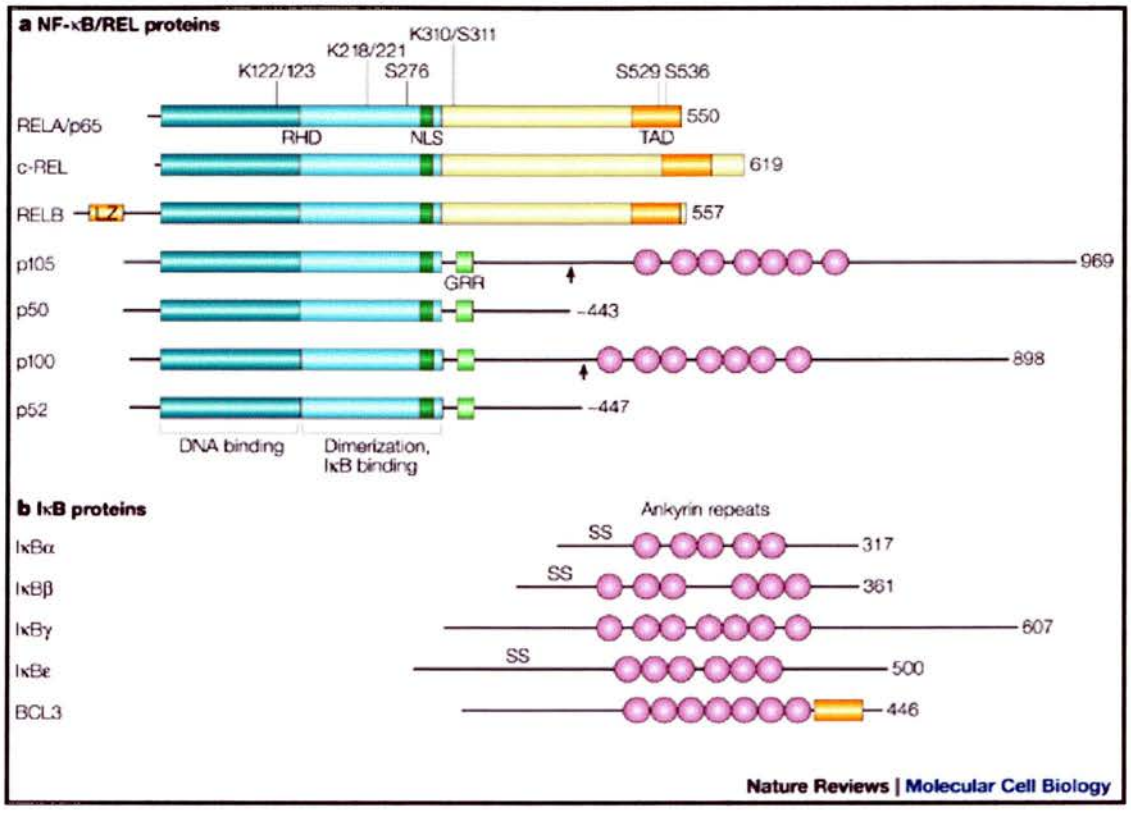

Figure 1.9 – The Domain Structure of the Family of Mammalian NF-κB/Rel and IκB Proteins. **(A)** The mammalian NF-κB/Rel family members are characterised by the presence of a Rel homology domain (RHD) at their amino terminus. This region is responsible for both DNA binding and dimerisation with IκBs. Only RelA, c-Rel and RelB contain carboxy-terminal transcriptional activation domains (TADs). The p105 and p100 precursor proteins contain glycine rich regions (GRRs), which are important for the processing of these transcripts to p50 and p52 respectively, and ankyrin repeats (denoted by pink circles). RelB is the only family member that has a leucine zipper (LZ) at its amino terminus and this motif is required for transactivation by RelB. The location of key phosphorylation (serines (S) 276, 311, 529 and 536) and acetylation (lysines (K) 122, 123, 218, 221 and 310) sites on RelA are also illustrated. **(B)** The mammalian IκB protein family members are characterised by the presence of an ankyrin repeat domain, which mediates interaction with Rel proteins. Phosphorylation of two key serine residues (SS) at the amino-terminal region, serines 32 and 36 in IκBα, triggers polyubiquitination and proteasome-mediated degradation. It is noteworthy that Bcl-3 has a TAD at its carboxyl terminus and so complexes formed between either p50 or p52 and Bcl-3 have transcriptional activation properties.

[Figure from (Chen and Greene, 2004)]

multiple ankyrin repeats, which are protein-protein interaction domains that interact with NF-κB via the RHD (Ghosh and Karin, 2002). Interaction of IκB with NF-κB blocks the NLS of NF-κB, thus sequestering the protein in the cytoplasm (Thanos and Maniatis, 1995; Stancovski and Baltimore, 1997).

1.5.3.5.1 Mechanism of Activation of NF-κB

NF-κB can be activated by a variety of signals including: cytokines, such as IL-1 and TNFα, tumour promoters, bacterial or viral infection and cellular stress (Courtois, 2005). The 'classical' pathway to activation of NF-κB is outlined in Figure 1.10. The key event in this pathway is phosphorylation of two critical serine (Ser) residues of IκBα, Ser32 and Ser36 (Viatour *et al.*, 2005). The kinase responsible for catalysing serine phosphorylation of IκBα is the multi-protein complex, IκB kinase (IKK). IKK has a molecular mass of approximately 700-900 kDa and consists of three subunits: IKK-α (IKK1) and IKK-β (IKK2) (the catalytic subunits), IKK-γ (the regulatory subunit also known as NF-κB essential modifier (NEMO)), and other proteins yet to be identified (Hayden and Ghosh, 2004; Karin *et al.*, 2004). IKK-α and IKK-β are related members of a family of intracellular signal transduction kinases that comprise an amino-terminal kinase domain and a carboxy-terminal region with two protein interaction motifs, a leucine zipper and a helix-loop-helix motif (Karin, 1999a and 1999b). Activation of IKK by proinflammatory and innate stimuli is dependent on phosphorylation of IKK-α or IKK-β at two conserved

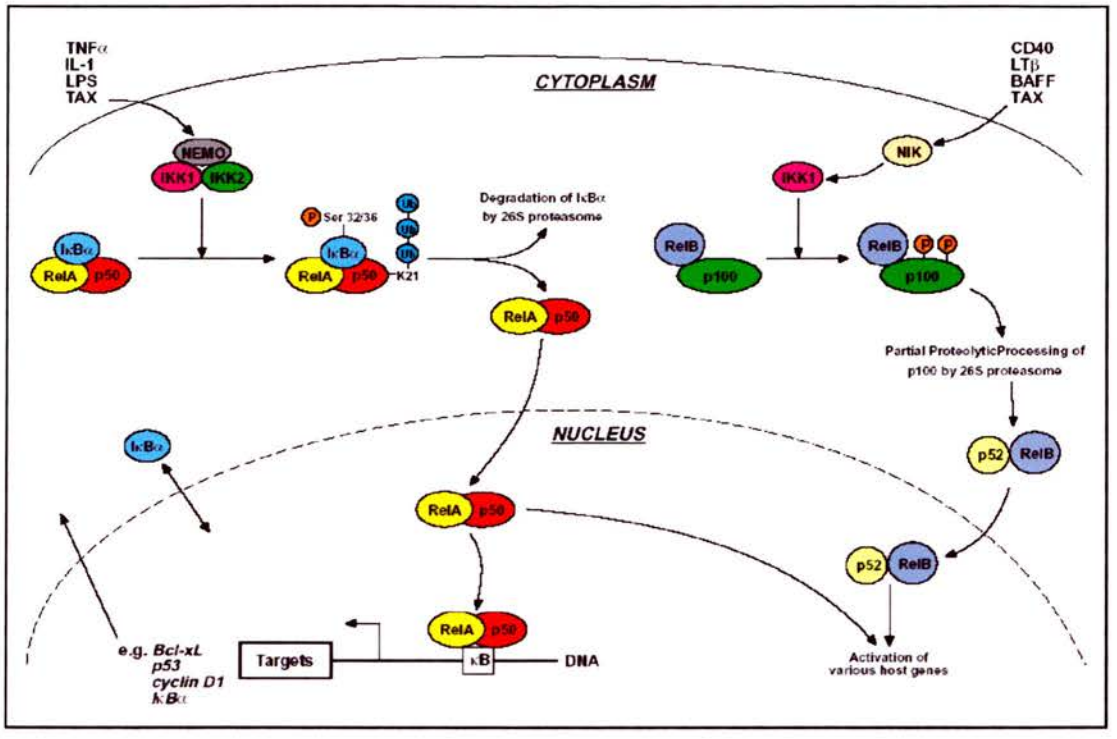

Figure 1.10 – Activation of the NF-κB Pathway. In the 'classical' pathway to activation of NF-κB, heterodimers of p50 and RelA polypeptides (the prototypical NF-κB complex), are sequestered in the cytoplasm by their interaction with the inhibitor protein, IκBα. Following stimulation by an external signal, such as tumour necrosis factor-α (TNFα), interleukin-1 (IL-1), lipopolysacchardie (LPS) or the human T-cell leukaemia virus (HTLV-1) TAX protein, IκBα is phosphorylated (P) by the IKK complex, comprising IKK1, IKK2 and NEMO, at the two critical serine residues, Ser32 and Ser36. Phosphorylated IκBα is then ubiquitinated (Ub) on lysine 21 (K21) and subsequently degraded by the 26S proteasome. Degradation of IκBα unmasks the nuclear localisation signal on RelA, allowing NF-κB to translocate from the cytoplasm to the nucleus, where it binds to κB sites within the promoter region of target genes e.g. *Bcl-xL*. A parallel non-canonical pathway for stimulus-coupled activation of specific Rel proteins involves the inducible proteolytic processing of p100 (NF-κB2). Different members of the TNF-receptor superfamily, including CD40 and B-cell activating factor (BAFF), selectively activate NIK and IKK1, leading to the phosphorylation of p100 and subsequently, its ubiquitination and partial proteolytic processing by the 26S proteasome to yield p52. This pathway generally generates p52-RelB heterodimers, in contrast to the p50-RelA dimmers produced by the canonical pathway. Once produced, p52-RelB dimers can translocate to the nucleus and activate transcription of various host genes.

[Figure adapted from (Chen and Greene, 2004)]

serine residues within their activation loop and ubiquitination and phosphorylation of IKKγ (Ghosh and Karin, 2002; Perkins and Gilmore, 2006). It is probable that IKK-α and IKK-β are phosphorylated and activated by one or more upstream kinases, although there is some evidence to suggest that *trans*-autophosphorylation caused by induced proximity and/or conformational change could be a mechanism of IKK activation (Hayden and Ghosh, 2004). Upstream kinases that have been proposed to act as IKK kinases include members of the MAP3K family, for example NF-κB inducing kinase (NIK) (Ling *et al.*, 1998), MEKK3 (Yang *et al.*, 2001a) and Tpl2 (Lin *et al.*, 1999). Other candidate IKK kinases include atypical PKC (aPKC) (Lallena *et al.*, 1999) TGFβ-activated kinase (TAK)-1 (Wang *et al.*, 2001; Chen, 2005) and Act-1 (Mauro *et al.*, 2003).

It is noteworthy that IKKβ is both necessary and sufficient for phosphorylation of IκBα on Ser32 and 36 (Hayden and Ghosh, 2004). Upon phosphorylation at Ser32 and 36, IκBα becomes a substrate for the Skp1/Cul1/F-box protein-β-TrCP ubiquitin ligase complex and ubiquitinated IκBα is subsequently degraded by the proteasome (Perkins and Gilmore, 2006). NF-κB complexes, freed from their association with IκBα, translocate from the cytoplasm to the nucleus due to unmasking of the NLS (Greten and Karin, 2004). Once inside the nucleus, NF-κB binds to specific sequences in the promoter/enhancer regions of target genes (see below) and regulates their transcription (Karin *et al.*, 2002; Pahl, 1999).

1.5.3.5.2 The Noncanonical NF-κB Pathway

In addition to the classical pathway mentioned above, a 'noncanonical' or alternative pathway of activating NF-κB (illustrated in Figure 1.10), which is triggered by cytokines (such as lymphotoxin β (LTβ), B-cell activating factor (BAFF) and CD40 ligand) or by viruses (including human T-cell leukaemia virus (HTLV) and the Epstein-Barr virus (EBV)), and that only affects the NF-κB dimer between RelB and NF-κB2, has been described (Ravi and Bedi, 2004; Campbell and Perkins, 2006). Briefly, in response to these stimuli, the IKK-α subunit is selectively activated by the upstream kinase, NIK (Greten and Karin, 2004). Together, IKKα and NIK induce the phosphorylation-dependent proteolytic removal of the IκB-like C-terminal domain of NF-κB2. The proteolytic processing of NF-κB2 to p52 in turn results in activation and translocation to the nucleus of NF-κB complexes containing this subunit, which generally consist of RelB-p52 heterodimers, (Karin *et al.*, 2002). Activation of this dimer is known to be important for lymphoid organ development and adaptive immune responses (Delhalle *et al.*, 2004).

1.5.3.5.3 Activation of NF-κB by Atypical Agents

Most stimuli activate NF-κB via IKK-mediated IκBα phosphorylation by the mechanisms outlined thus far. However, further complexity is introduced due to activation by other, atypical inducers including ultraviolet (UV-C and UV-B) light; ionizing radiation; hypoxia; phorbol esters; and chemotherapeutic agents, such as

etoposide (a DNA-damage inducing agent), and anthracyclines (for example daunorubicin and doxorubicin), where both IKK-dependent and independent mechanisms of activation have been proposed (Perkins, 2004b; Campbell and Perkins, 2004 and 2006; Perkins and Gilmore, 2006). Several reports have indicated that UV-C-, camptothecin-, doxorubicin- and etoposide-induced NF-κB DNA-binding is IKK-dependent (Huang *et al.*, 2002; Bottero *et al.*, 2001; Campbell and Perkins, 2004), but exhibits distinct functional differences to other IKK-dependent activation pathways. For instance, NF-κB induction in response to genotoxic stimuli, such as UV-C, has been demonstrated to have a requirement for the zinc-finger domain of IKKγ that is not seen with TNFα (Huang *et al.*, 2002; Perkins and Gilmore, 2006). In contrast to these studies demonstrating IKK-dependent effects of atypical agents, another paper reported that IκBα degradation in response to UV-C treatment occurs via a p38 MAPK-dependent mechanism involving phosphorylation of IκBα at a cluster of serine residues contained in its C terminus, which are specifically targeted by casein kinase II (CKII) (Kato, Jr. *et al.*, 2003). Doxorubicin, a chemotherapeutic agent, has also been shown to induce proteasome-mediated IκBα degradation in mouse embryonic fibroblasts (MEFs) devoid of *IKK-α* and *IKK-β* (Tergaonkar *et al.*, 2003). It is noteworthy that the kinetics of activation of NF-κB by atypical agents is much slower than typical activators, such as TNFα or IL-1 (Campbell and Perkins, 2004 and 2006). Additionally, roles are emerging for specific kinases in the mechanism to activation of the NF-κB pathway by atypical stimuli. These include DNA-PK (DNA-dependent protein kinase) and ATM (*Ataxia telangiectasia* mutated) kinase (Schmitz *et al.*, 2004).

1.5.3.5.4 Tyrosine Phosphorylation of IκBα

In addition to the well established serine (Ser32 and Ser36) phosphorylation mechanism, an alternative pathway for activating NF-κB, which involves phosphorylation of IκB on tyrosine (Tyr42), has been described. Tyrosine phosphorylation of NF-κB has been reported in response to various stimuli, including pervanadate (a protein tyrosine phosphatase inhibitor) (Imbert *et al.*, 1996; Mukhopadhyay *et al.*, 2000), hypoxia/Reoxygenation (Imbert *et al.*, 1996; Fan *et al.*, 2003), oxidative stress (Schoonbroodt *et al.*, 2000), crystalline silica (Kang *et al.*, 2000; Kang *et al.*, 2006), nerve growth factor (NGF) (Bui *et al.*, 2001) and even TNFα, a classical inducer of NF-κB (Abu-Amer *et al.*, 1998). Furthermore, protein-tyrosine phosphatase inhibitors have been shown to block TNFα dependent activation of NF-κB (Singh and Aggarwal, 1995). The tyrosine kinase responsible for this alternative pathway is not known but candidates that have been proposed include: $p56^{lck}$ (Imbert *et al.*, 1996; Livolsi *et al.*, 2001; Mahabeleshwar and Kundu, 2003); ZAP-70 (Livolsi *et al.*, 2001); c-Abl (Kawai *et al.*, 2002); Syk (Takada *et al.*, 2003) and c-Src (Abu-Amer *et al.*, 1998; Fan *et al.*, 2003; Kang *et al.*, 2006). It is important to note that activation of NF-κB mediated by tyrosine phosphorylation of IκBα can occur in the presence or absence of IκBα degradation. Hydrogen peroxide-induced phosphorylation of IκBα was shown to be followed by its degradation, a process that required the C-terminal PEST (proline-glutamic acid-serine-threonine) domain of IκBα (Schoonbroodt *et al.*, 2000). Similarly, pervanadate was found to activate NF-κB through tyrosine phosphorylation and degradation of IκBα (Mukhopadhyay *et al.*, 2000). In contrast to these studies, it has been reported that

tyrosine phosphorylation of IκBα in response to pervanadate treatment or hypoxia/re-oxygenation in Jurkat T cells results in nuclear translocation and activation of NF-κB without degradation of IκBα (Imbert *et al.*, 1996). Similarly, it was demonstrated that pervanadate treatment induces tyrosine phosphorylation of IκBα, an event that was found to protect IκBα from inducible degradation (Singh *et al.*, 1996). More recently, it was established that NGF-induced activation of NF-κB can occur without significant degradation of IκBs (Bui *et al.*, 2001).

1.5.3.5.5 Activation of NF-κB by NSAIDs

Only a few studies, which will be discussed in more detail later, have demonstrated activation of NF-κB by NSAIDs. Significantly, previous work from the host laboratory demonstrated that aspirin activates NF-κB through the classical pathway, involving serine phosphorylation and degradation of IκBα (Stark *et al.*, 2001). In particular, aspirin was found to mediate a reduction in cytoplasmic IκBα levels and this effect could be blocked by pre-treatment of cells with the proteasome inhibitor, MG132 and by mutation of IκBα at the critical Ser32/36 phosphoacceptor sites. In keeping with these data, a recent study (Cho *et al.*, 2005) demonstrated degradation of IκBα in response to diclofenac. The COX-2-selective inhibitor, NS-398 has also been reported to cause degradation of IκBα (Smartt *et al.*, 2003). However, it is important to note that the precise upstream mechanisms to activation of NF-κB by NSAIDs are unknown. Studies are ongoing in the host laboratory to identify these pathways in response to aspirin and other NSAIDs.

1.5.3.5.6 Regulation of NF-κB Activity by Post-translational Modifications

Although the primary level of regulation of NF-κB activity lies in the cytoplasmic release from the IκB inhibitor protein, there is accumulating evidence that NF-κB is also subject to an IκB-independent level of regulation [reviewed in (Schmitz *et al.*, 2001 and 2004; Vermeulen *et al.*, 2002; Chen and Greene, 2004; Viatour *et al.*, 2005)]. Moreover, it is conceivable that such mechanisms could be very important for modulation of NF-κB signaling by NSAIDs.

1.5.3.5.7 Enhanced NF-κB Transcriptional Activity by RelA Phosphorylation

Phosphorylation of NF-κB itself can affect several functions of NF-κB, including DNA binding and transactivation potential, that is the ability to recruit the transcriptional apparatus and stimulate target gene expression (Vermeulen *et al.*, 2002). The RelA subunit of NF-κB is a principal target for phosphorylation by various kinases (Chen and Greene, 2004) and a summary of the inducible phosphorylation sites within RelA and the implicated protein kinases is illustrated in Figure 1.11. These phosphorylation events are found in both the C-terminal transactivation domains and the RHD (Vermeulen *et al.*, 2002), are stimuli-specific and probably cell-type-specific, and occur in the cytoplasm or the nucleus (Viatour *et al.*, 2005; Perkins and Gilmore, 2006).

Phosphorylation of RelA at serine 276, which is situated in the RHD, by the catalytic subunit of protein kinase A (PKAc) in response to treatment with LPS has been shown to increases the transcriptional activity of RelA by increasing the interaction between RelA and the transcriptional co-activator, cAMP response element-binding (CREB)-binding protein (CBP)/p300 in the nucleus (Zhong *et al.*, 1998). Serine 276 of RelA can also be phosphorylated by the mitogen- and stress-activated protein kinase-1 (MSK1) in the nucleus in response to TNFα, resulting in enhanced NF-κB transcriptional activity (Vermeulen *et al.*, 2003b).

TNFα induces phosphorylation of RelA at serine 311, which is also located in the RHD, through the action of yet another kinase, PKCζ (Duran *et al.*, 2003). In that study, phosphorylation of RelA at serine 311 was found to promote the interaction between RelA and CBP and the recruitment of CBP and RNA polymerase II to the interleukin-6 (IL-6) promoter. Furthermore, PKCζ-deficient fibroblasts exhibit normal activation of IKK and nuclear translocation of RelA but have reduced NF-κB DNA-binding activity *in vitro* (Schmitz *et al.*, 2004).

RelA is also subject to phosphorylation at two sites within the C-terminal TAD. After treatment with IL-1 or TNF-α, serine 529 is phosphorylated by CKII (Bird *et al.*, 1997; Wang and Baldwin, Jr., 1998; Wang *et al.*, 2000). This phosphorylation event requires IκBα degradation and is prevented by RelA binding to IκBα in unstimulated cells. Upon stimulation with TNF-α, activation of IKK results in phosphorylation of RelA at serine 536 (Sakurai *et al.*, 1999). The ribosomal subunit kinase-1 (RSK1), a downstream effector of p53 which activates NF-κB in response to DNA damage, has also recently been shown to mediate phosphorylation

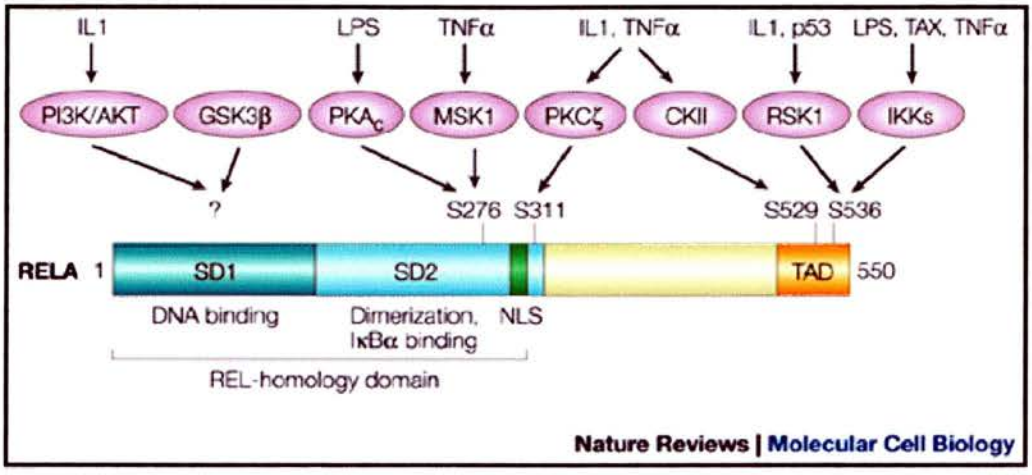

Figure 1.11 – Summary of Inducible Phosphorylation Sites Within RelA and Implicated Protein Kinases in Response to Distinct Stimuli. The four major phosphorylation sites in RelA include serines (S) 276 and 311, which are located in the dimerisation and IκBα binding sub-domain (SD) at either side of the nuclear localisation signal (NLS) within the Rel homology domain; and serines 529 and 536, which are located in the transcriptional activation domain (TAD). Phosphorylation of S276 is mediated by the catalytic subunit of protein kinase A (PKA_C) in response to LPS, or by mitogen- and stress-activated protein kinase-1 (MSK1) in response to tumour necrosis factor-α (TNFα). Activation of protein kinase C-ζ (PKCζ) by either interleukin-1 (IL1) or TNFα triggers phosphorylation of S311. TNFα and IL1 also stimulate casein kinase II (CKII), leading to phosphorylation of serine 529. Targeting of S536 for phosphorylation occurs by either activation of IKKs in response to LPS, TNFα or the viral protein TAX; or stimulation of ribosomal-subunit kinase-1 (RSK1), which is itself activated by IL1 or p53.

[Figure from (Chen and Greene, 2004)]

of serine 536 of RelA in an IKK-independent manner in response to treatment with drugs such as doxorubicin or etoposide (Bohuslav *et al.*, 2004). It is noteworthy that phosphorylation of serines 529 and 536 of RelA is functionally relevant as NF-κB-dependent transcription is greatly impaired by mutating these residues (Ravi and Bedi, 2004).

RelA is also subject to regulation by GSK-3β and phosphatidylinositol 3-kinase (PI 3-kinase), which can phosphorylate the RelA C-terminus *in vitro*, although the exact phosphorylation sites remain to be determined (Schwabe and Brenner, 2002; Chen and Greene, 2004; Schmitz *et al.*, 2004). Interestingly, phosphorylation of RelA at threonine 505 has been implicated in $p14^{ARF}$-mediated repression of NF-κB transactivation (Rocha *et al.*, 2003). A more recent study from the same group identified the checkpoint kinases, ATR (ATM- and Rad3-related) and Chk1 (checkpoint kinase 1), as the kinases responsible for this phosphorylation event (Rocha *et al.*, 2005).

It is noteworthy that no studies to date have demonstrated phosphorylation of RelA in response to NSAIDs. However, the discovery that RelA can be phosphorylated by GSK-3β, a component of the Wnt signaling pathway, is of particular interest given that the wingless (Wnt)/β-catenin pathway is also a strong target of NSAIDs and this will be discussed later.

1.5.3.5.8 Regulation of RelA by Acetylation

In addition to phosphorylation, the transactivation function of RelA has also been shown to be regulated by reversible acetylation of RelA itself or histones at target promoters [reviewed in (Quivy and Van Lint, 2004; Schmitz *et al.*, 2004)]. It is now recognised that various post-translational modifications, including phosphorylation, acetylation, ubiquitination, methylation and sumoylation, occur on the amino-terminal tail, in addition to residues located at exposed sites within the globular domain, of histones. Such modifications represent information contained in chromatin, not resulting from mutation or changes in the actual DNA sequence, which defines a heritable pattern of gene expression and is often referred to as 'epigenetic' information (Mellor, 2006).

Post-translational modifications on histones can serve to create or stabilise binding sites for regulatory proteins, for instance transcription factors, or may even disrupt or mask chromatin binding sites. Furthermore, the role of a particular modification may be influenced by the degree and stability of the modification (Santos-Rosa and Caldas, 2005; Grewal and Rice, 2004). Distinct histone modifications, on one or more tails, in effect act sequentially or in combination to form a 'histone code' which is read by proteins containing specific interaction domains, for example, the bromodomain functions as an acetyl-lysine binding domain and is found in most histone acetyl transferases (HATs); the chromodomain recognises and binds methylated lysines, can bind DNA and is found in histone methyl transferases (HMTs) (de la Cruz *et al.*, 2005; Santos-Rosa, 2005). It is noteworthy that bromodomains and chromodomains are also present in some ATP-

dependent chromatin remodelling enzymes, factors which are involved in causing conformational changes by ATP-dependent movement of nucleosomes (de la Cruz *et al.*, 2005). Proteins containing functional domains, such as the bromodomain and chromodomain, which recognise histone modifications on DNA are therefore the effectors that mediate downstream responses, such as transcriptional activation/repression.

It is well established that NF-κB transcriptional activity is dependent upon interaction with multiple cellular co-activators, such as CBP/p300, p300/CBP-associated factor (PCAF) or steroid receptor co-activator-1 (SRC-1), which possess HAT activity (Gerritsen *et al.*, 1997; Perkins *et al.*, 1997; Sheppard *et al.*, 1999; Zhong *et al.*, 1998; Quivy and Van Lint, 2004). The association of NF-κB with CBP/p300 has been shown to be dependent upon phosphorylation of RelA at serine 276 by protein kinase A (PKA) and is essential for transcriptional activity of NF-κB (Zhong *et al.*, 1998). Furthermore, a very recent study (Chen *et al.*, 2005) established that phosphorylation of RelA at serines 276 and 536 increased the assembly of RelA with p300, which in turn enhanced acetylation of RelA on lysine 310. It has also been established that the inactivation of CBP, or PCAF, or SRC-1 by nuclear antibody microinjection prevents NF-κB-dependent transactivation (Sheppard *et al.*, 1999). Furthermore, the exogenous expression of CBP, or PCAF or SRC-1, in a background where RelA is overexpressed, enhances RelA-dependent transcriptional activation, suggesting a positive regulatory function for each of these three proteins on NF-κB driven transcription (Gerritsen *et al.*, 1997; Sheppard *et al.*, 1999). Activation of the IL-6 promoter activity by RelA and CBP/p300 appears to be highly dependent on the HAT activity of CBP/p300 (Vanden Berghe *et al.*, 1999).

Moreover, although NF-κB DNA binding is rapid at constitutively active promoters, hypoacetylated promoters require additional events leading to their hyperacetylation prior to NF-κB recruitment and activation of transcription (Campbell and Perkins, 2006).

In addition to effects on NF-κB-driven transcriptional activity, stimulus-induced acetylation of RelA has been shown to control the duration of the NF-κB transcriptional response (Chen *et al.*, 2001). A summary of the key inducible acetylation sites within RelA and the resultant effects on its nuclear activity is shown in Figure 1.12. In one study (Kiernan *et al.*, 2003), lysines 122 and 123 were identified as key residues for acetylation of RelA by both p300 and PCAF. Moreover, acetylation of RelA on those residues was discovered to reduce the ability of RelA to bind target κB sites on DNA and facilitated the removal of RelA from DNA, thus increasing its export from the nucleus. In another study (Chen *et al.*, 2002), it was established that the HATs, p300 and CBP, principally target lysines 218, 221 and 310 of RelA for modification. By using hypoacetylated RelA mutants containing lysine to arginine substitutions at these sites or wild-type RelA co-expressed in the presence of a dominantly interfering mutant of p300, the authors found that acetylation at lysine 221 in RelA enhances DNA binding and disrupts assembly with IκBα. Moreover, it was shown that acetylation of RelA on lysine 218, in combination with acetylation on lysine 221, is likely important for RelA assembly with IκBα. In contrast, acetylation of lysine 310 was discovered to be required for the full transcriptional activity of RelA, in the absence of effects on DNA binding or IκBα assembly (Chen *et al.*, 2002).

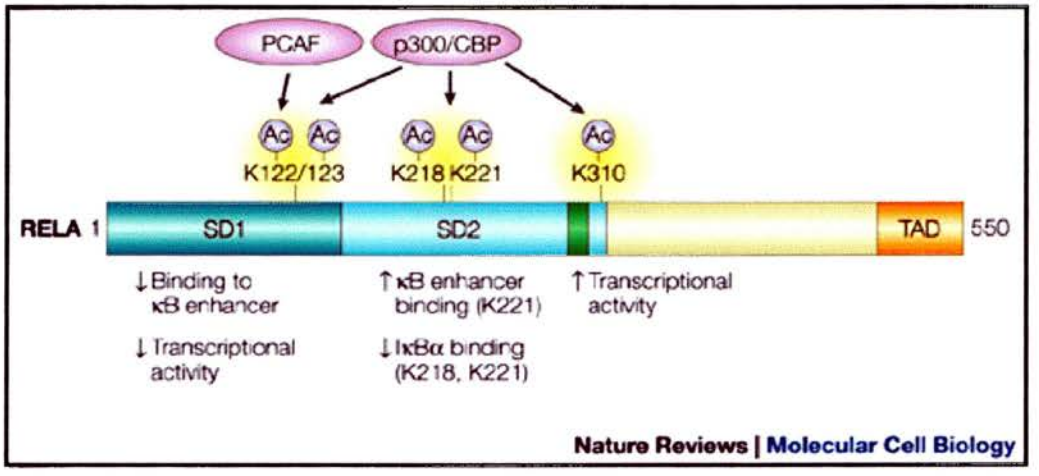

Figure 1.12 – Summary of Inducible Acetylation Sites Within RelA and Resultant Effects on its Nuclear Activity. Acetylation (Ac) of lysines (K) 122 and 123 by p300/CREB-binding protein (CBP)-associated factor (PCAF) and p300/CBP respectively decreases transcriptional activity of RelA by reducing the binding of RelA to the κB enhancer. Conversely, acetylation of K221 by p300/CBP slightly increases transcriptional activity of RelA and increases DNA-binding affinity of RelA for the κB enhancer. Acetylation of K221, together with acetylation of K218, also prevents the association of RelA with IκBα. In contrast to the other residues mentioned, acetylation of K310 does not control DNA binding or IκB assembly, but is instead required for the transactivation function of RelA. SD denotes sub-domain; TAD denotes transactivation domain; green box denotes nuclear localisation signal.

[Figure from (Chen and Greene, 2004)]

In addition to co-activators, NF-κB has also been shown to recruit, either directly or indirectly, co-repressor complexes, which posess histone deacetylase (HDAC) activity (Quivy and Van Lint, 2004). Deacetylation is thought to play a role in the termination of transcriptional activity by enhancing binding affinity of NF-κB to IκBα (Schmitz *et al.*, 2004). It is now widely accepted that the acetylation status of RelA is subject to regulation by deacetylases. One study demonstrated that RelA can interact with the HDAC co-repressor proteins, HDAC1 and HDAC2 (Ashburner *et al.*, 2001). Acetylated RelA has also been shown to be deacetylated through a specific interaction with HDAC3 (Chen *et al.*, 2001; Kiernan *et al.*, 2003). Moreover, deacetylation of lysine 310 by the HDACs, SIRT1 or HDAC3, inhibits the transcriptional activity of RelA and can augment cellular apoptosis in response to TNFα (Chen *et al.*, 2002; Yeung *et al.*, 2004). Interestingly, a recent study established that interaction of RelA with HDACs 1, 2 and 3 in response to UV-C and daunorubicin resulted in repressed expression of NF-κB-regulated genes (Campbell *et al.*, 2004). It is important to note that a role for acetylation in the regulation of NF-κB activity is further supported by studies showing that deacetylase inhibitors, such as trichostatin A (TSA) or sodium butyrate, can enhance NF-κB-dependent gene expression in the presence of TNF-α (Ashburner *et al.*, 2001; Chen *et al.*, 2001; Inan *et al.*, 2000b).

Similar to phosphorylation of RelA, no studies to date have demonstrated acetylation of RelA in response to NSAIDs. However, modulation of RelA activity by acetylation is of particular interest to the host group as recent unpublished data generated in the host laboratory has indicated that acetylation of RelA is implicated

in the apoptotic response to aspirin and this will be discussed more fully in Chapter 6.

1.5.3.5.9 Regulation of RelA by Ubiquitination

Two studies to date have suggested a role for ubiquitination in the regulation of RelA activity. Cytokine-induced phosphorylation of RelA at threonine 254 has been reported to stabilise the interaction between the peptidyl-prolyl isomerase, Pin1 and RelA (Ryo *et al.*, 2003). Binding of Pin1 to RelA was found to inhibit the interaction of RelA with IκBα, enhance RelA nuclear localisation and increase RelA stability via decreased proteasomal degradation of ubiquitinated RelA. Moreover, ubiquitin-mediated proteolysis of RelA in turn was facilitated by the E3 ubiquitin ligase, suppressor of cytokine signaling-1 (SOCS-1). In another study (Saccani *et al.*, 2004), degradation of promoter-bound RelA was shown to be essential for the termination of the NF-κB pathway in response to TNFα. The potential role for ubiquitination of RelA in the response to aspirin will be discussed in Chapter 6.

1.5.3.5.10 Regulation of RelA by Other Signaling Pathways

There are several signaling pathways known to modulate the transcriptional activity of NF-κB which are not involved in nuclear translocation of NF-κB. Neither p38 nor JNK directly phosphorylate NF-κB but inhibition of these pathways, using

specific inhibitors or dominant negative mutants, strongly reduces NF-κB-dependent transcription (Vanden Berghe *et al.*, 1998; Schmitz *et al.*, 2001). Nuclear translocation and DNA binding of NF-κB are also unaffected by inhibiting these pathways, suggesting that p38 and JNK regulate the transactivation function of NF-κB. However, the precise mechanisms for this are still not clear. It is of interest that recent unpublished work from the host laboratory has shown that activation of the p38 MAPK pathway is implicated in the NF-κB response to aspirin (Thoms *et al.*, 2004). In particular, specific inhibition of p38 signaling by using chemical inhibitors or siRNA knock-down attenuates the NF-κB response to aspirin and aspirin-induced apoptosis in SW480 colorectal cancer cells.

The PI 3-kinase signaling pathway has also been implicated in the transcriptional activation of NF-κB, but the evidence for this is quite controversial. Several studies support a role for PI 3-kinase and Akt (a downstream kinase of PI 3-kinase) in regulation of RelA transcriptional activity in response to IL-1 (Schmitz *et al.*, 2001; Vermeulen *et al.*, 2002; Ghosh and Karin, 2002; Viatour *et al.*, 2005). However, the PI 3-kinase inhibitors wortmannin and LY 294002 have no effect on inducible NF-κB nuclear translocation, although the latter can prevent IL-1-induced RelA phosphorylation (Béraud *et al.*, 1999; Sizemore *et al.*, 1999). Neither Akt nor PI 3-kinase directly phosphorylate RelA (Schmitz *et al.*, 2004) but it has been proposed that Akt targets the transactivating function of NF-κB by activating p38 and IKKβ (Viatour *et al.*, 2005).

1.5.3.5.11 Target Genes of NF-κB

The active NF-κB transcription factor regulates a large number of target genes, which can be split into four broad functional categories: immunoregulatory and inflammatory genes; genes involved in apoptosis; genes that regulate cell proliferation; and genes that encode negative feedback regulators of NF-κB itself (Karin *et al.*, 2002; Campbell and Perkins, 2006) (for examples, see Table 1.3).

As mentioned earlier, NF-κB plays a crucial role in the inflammatory response and has been implicated in a variety of inflammatory diseases such as rheumatoid arthritis, asthma and Crohn's disease (Pande and Ramos, 2005; Perkins, 2000). It is noteworthy that NF-κB controls the expression of the cytokines, IL-1 and TNFα, which are essential mediators of chronic inflammation (Delhalle *et al.*, 2004). As both IL-1 and TNFα can also activate NF-κB, an interdependence of persistent NF-κB activation and sustained levels of IL-1 and TNF-α is implicated (Makarov, 2000; Sun and Andersson, 2002). This essentially allows for the rapid induction of a response such that NF-κB activation could spread from cell to cell, within a tissue and beyond. It is important to note that, in addition to mediating the immune response, the wide variety of NF-κB inducers and target genes have implicated NF-κB more generally as a 'central regulator of stress responses' (Pahl, 1999).

NF-κB is subject to negative feedback regulation as it controls the transcription of its inhibitor, IκB, and the p105 (NF-κB1) and p100 (NF-κB2) precursors (Karin *et al.*, 2002). Indeed, the critical step for termination and down-regulation of NF-κB activity involves binding of newly synthesised IκBα to NF-κB

Gene Category	*Examples*	
	Gene Product	*Function*
Immunoregulatory/Inflammatory	IFN-γ IL-1α and -1β TNF-α and –β CCR5 ICAM-1 VCAM-1 COX-2	Interferon Interleukin-1α and -1β Tumour Necrosis Factor α and β Chemokine receptor Intracellular adhesion molecule-1 Vascular cell adhesion molecule Cyclooxygenase, prostaglandin endoperoxide synthase
Apoptosis	Bcl-xL cIAPs Bfl1/A1 CCD95 (Fas) Fas-Ligand	Pro-survival Bcl-2 homologue Inhibitors of apoptosis Pro-survival Bcl-1 homologue Pro-apoptotic receptor Inducer of apoptosis
Cell Proliferation	Cyclin D1 c-Myc p53	Cell Cycle Regulation Proto-oncogene Tumour suppressor
Negative Feedback Regulators	IκBα NF-κB1 NF-κB2 A20	Inhibitor of NF-κB NF-κB p105 precursor NF-κB p100 precursor TNF-inducible zinc finger

Table 1.3 – **Examples of Target Genes of NF-κB and Their Functions.**

in the nucleus (Rothwarf and Karin, 1999; Sun and Andersson, 2002). These NF-κB:IκBα complexes can then be transported from the nucleus to the cytoplasm by means of the nuclear export signal present on IκBα (Rothwarf and Karin, 1999; Sun and Andersson, 2002).

The fact that NF-κB targets include genes that are implicated in critical cellular processes, in particular regulation of apoptosis and cell proliferation, makes it a very attractive target pathway for chemopreventative agents such as NSAIDs. Therefore, the role of NF-κB in regulation of apoptosis and cell proliferation will now be discussed.

1.5.3.5.12 Regulation of Apoptosis and Cell Proliferation by NF-κB

It is well established that NF-κB has a key role in regulation of apoptosis, having both pro-apoptotic and anti-apoptotic functions depending on the cell type and stimulus used (Bours *et al.*, 2000; Kucharczak *et al.*, 2003; Perkins, 2004a; Luo *et al.*, 2005a; Campbell and Perkins, 2006). A major insight into this functional aspect of NF-κB came from the observation that the *RelA* knockout mouse is embryonic lethal, due to extensive liver apoptosis and that cells from these mice have an enhanced sensitivity to TNF-induced cell death (Sun and Andersson, 2002).

NF-κB exerts its pro-survival function by inducing the expression of anti-apoptotic genes, whose products include: c-FLIP (caspase-8/FADD-like-interleukin-1β (IL-1β)-converting enzyme inhibitory protein), Bcl-X_L, A1/Bfl-1, cellular inhibitor of apoptosis (c-IAP), X-chromosome-linked inhibitor of apoptosis (XIAP),

TRAF (TNFR-associated factor) 1 and TRAF2 (Luo *et al.*, 2005a; Delhalle *et al.*, 2004; Ravi and Bedi, 2004). c-FLIP is a proteolytically inactive analogue of caspase-8 and thus prevents death receptor-induced activation of the initiator pro-caspase-8 and subsequent apoptosis (Kreuz *et al.*, 2001). Members of the IAP family can inhibit the activation of various effector caspases by direct interaction, and therefore prevent death receptor-induced apoptosis (Deveraux and Reed, 1999). The anti-apoptotic Bcl-2 family members, including Bcl-X_L, prevent apoptosis by inhibiting cytochrome *c* release and depolarisation of mitochondria, thus avoiding apoptosome formation and the apoptotic cascade (Luo *et al.*, 2005a). In addition to promoting expression of anti-apoptotic genes, NF-κB activation can also lead to repression of pro-apoptotic genes, such as *bax* (Bentires-Alj *et al.*, 2001). NF-κB has also been demonstrated to interfere with the transcriptional activity and pro-apoptotic function of p53 (Webster and Perkins, 1999). The mechanism for the transcriptional antagonism between NF-κB and p53 involved competition for a limiting pool of their shared transcriptional co-activators, p300 and CBP. There is also accumulating evidence that suppression of JNK, a regulator of programmed cell death, contributes to the anti-apoptotic function of NF-κB (Kucharczak *et al.*, 2003; Ravi and Bedi, 2004; Luo *et al.*, 2005a).

In contrast to the pro-survival role mentioned above, there is a growing body of evidence that activation of NF-κB may promote cell death under certain circumstances (Barkett and Gilmore, 1999; Sun and Andersson, 2002; Campbell and Perkins, 2004 and 2006). NF-κB can activate expression of some pro-apoptotic genes, including TNF receptor superfamily members, DR4 and DR5 (Ravi *et al.*, 2001), DR6 (Kasof *et al.*, 2001), Fas (Kimura *et al.*, 2003), and Fas ligand (FasL)

(Wiener *et al.*, 2004). Under certain conditions, NF-κB may also function to repress expression anti-apoptotic genes. NF-κB activation has been shown to be required for doxorubicin-induced apoptosis (Ashikawa *et al.*, 2004). Another report documented that UV-C and daunorubicin inhibit TNF-α-induced NF-κB transcriptional activity by a mechanism involving enhanced association of RelA with HDACs and repression of anti-apoptotic genes (Campbell *et al.*, 2004). Similarly, repression of NF-κB transcriptional activity has been shown to be causally involved in aspirin-induced apoptosis of colorectal cancer cells (Stark and Dunlop, 2005). However, the authors established that sequestration of RelA in the nucleolus was a central component of the mechanism. The observations of that paper will be discussed more fully later in this introduction and in Chapter 3.

In addition to its role as a regulator of apoptosis, NF-κB can control cellular proliferation. NF-κB influences cellular proliferation by directly stimulating the transcription of genes required for cell cycle progression, for example G1 cyclins (*cyclin D1* and *cyclin E*), c-*myc*, and c-*myb* (Kucharczak *et al.*, 2003). Furthermore, NF-κB can activate the expression of cytokines, such as IL-2, granulocyte-macrophage colony-stimulating factor (GM-CSF) and CD40 ligand, which are growth factors that stimulate the proliferation of lymphoid and myeloid cells (Karin *et al.*, 2002).

1.5.3.5.13 Role of NF-κB in Tumorigenesis

Given that NF-κB is involved in the regulation of critical cellular processes, such as apoptosis and proliferation, it comes as no surprise that there is a strong link between aberrant regulation of NF-κB and cancer [reviewed in (Karin *et al.*, 2002; Bharti and Aggarwal, 2002; Gilmore, 2003; Greten and Karin, 2004; Ravi and Bedi, 2004)]. Furthermore, NF-κB has been implicated in the tumorigenic process and this provides further support for the notion that it is a very good target pathway for chemopreventative agents such as NSAIDs.

NF-κB is known to be activated by a variety of carcinogens and tumour promoters, for example UV radiation and phorbol esters (Bharti and Aggarwal, 2002). Certain viral oncoproteins, including EBV latent membrane protein-1 and HTLV-1 Tax, stimulate cellular pathways which activate NF-κB (Ravi and Bedi, 2004). Moreover, the transforming ability of some viral and cellular oncoproteins, for example Ras and Bcr-Abl, requires the activation of NF-κB (Greten and Karin, 2004).

An important aspect of tumour growth is angiogenesis, a process that requires both migratory and invasive capabilities of vascular epithelial cells (Karin *et al.*, 2002). Invasion and angiogenesis are in turn critical events for tumour metastasis (Bharti and Aggarwal, 2002). There is substantial evidence demonstrating a role for NF-κB in angiogenesis. Cells with elevated NF-κB activity deregulate production of chemokines (chemotactic factors that induce cell migration), such as interleukin-8 (IL-8), thereby leading to increased migratory activity and promotion of angiogenesis (Karin *et al.*, 2002). Various genes known to be involved in tumour cell invasion and

angiogenesis are regulated by NF-κB (Bharti and Aggarwal, 2002). NF-κB promotes the expression of cell adhesion molecules [VCAM-1 (vascular cell adhesion molecule-1), ICAM-1 (intracellular adhesion molecule-1), E-selectin] and MMP-2 and -9, which are proteolytic enzymes that are involved with degradation of extracellular matrix proteins, hence promoting tumour invasion of the surrounding tissue (Ravi and Bedi, 2004). NF-κB also enhances expression of iNOS (inducible nitric oxide synthase), a protein required for vasodilation, VEGF (vascular endothelial growth factor) and COX-2, which in turn can induce the expression of several angiogenic factors (Bharti and Aggarwal, 2002).

Constitutive nuclear NF-κB activity is observed in numerous tumour cell types, including leukaemia, lymphoma, myeloma, breast and colon (Bharti and Aggarwal, 2002; Kucharczak *et al.*, 2003). Interestingly, most tumours that have constitutive NF-κB activity show an increased resistance to chemotherapeutic drugs (Delhalle *et al.*, 2004). Furthermore, many chemotherapeutic agents induce NF-κB activity, thereby increasing drug resistance in tumour cells (Greten and Karin, 2004). The use of chemopreventative agents, such as NSAIDs or resveratrol, to inhibit NF-κB in combination with chemotherapeutic agents was therefore proposed (Bharti and Aggarwal, 2002). It has been shown that inhibition of NF-κB activity not only leads to enhanced apoptosis but also to synergy with radiation or chemotherapy in several tumour cell lines and xenograft models of cancer (Greten and Karin, 2004). The design and development of drugs that inhibit activation of the NF-κB pathway, in particular IKK inhibitors, has therefore been of considerable importance for the treatment of cancer (Greten and Karin, 2004). However, it is important to re-

emphasize that in response to certain agents, for example aspirin, activation of NF-κB as opposed to inhibition may be therapeutically relevant.

There is a body of evidence that inappropriate NF-κB signaling is implicated in colorectal cancer in particular. The observation that primary colon tumours exhibit constitutive activation of NF-κB (Gilmore, 2003) is supported by data from *in vitro* studies which found that colon cancer cell lines exhibit abnormally high NF-κB activity and low IκBα levels (Dejardin *et al.*, 1999). Significantly, several growth regulatory genes that are known to be involved in colorectal cancer progression e.g. *p53*, *COX-2*, *c-myc*, are regulated by NF-κB (Karin *et al.*, 2002; Ravi and Bedi, 2004).

Collectively, these data provide compelling evidence that the NF-κB pathway is a strong potential target for the anti-tumour effects of NSAIDs in the colon. In spite of this knowledge, however, there have only been a few studies investigating the effects of NSAIDs on the NF-κB pathway and these will now be discussed.

1.5.3.5.14 The Effects of NSAIDs on the NF-κB Pathway

The first study to investigate the effects of NSAIDs on NF-κB (Kopp and Ghosh, 1994) demonstrated that sodium salicylate inhibits activation of the NF-κB pathway by two different inducers [LPS and PMA-PHA (phorbol 12-myristate 13-acetate plus phytohemagglutinin)]. Furthermore, in that study, sodium salicylate and aspirin also inhibited stimulus-induced (PMA-PHA) activation of NF-κB-dependent transcription as measured from two different reporter constructs [immunoglobulin

(Ig)-κ enhancer (containing 2 κB sites)-luciferase and the human immunodeficiency virus (HIV)-1 long terminal repeat (LTR) repeat (containing two inducible NF-κB sites)-luciferase] in transfected cells. In a similar study (Yin *et al.*, 1998), it was found that sodium salicylate and aspirin, but not indomethacin, inhibit stimulus-induced (TNFα, NIK, TAX or MEKK1) activation of NF-κB-dependent transcription from the HIV-1 LTR-luciferase reporter in transfected cells. Moreover, the mechanism for the effects of sodium salicylate and aspirin was established to be, at least in part, the result of binding of these agents to IKKβ to reduce ATP binding. Another study by the same group established that sulindac, sulindac sulfone and sulindac sulfide, but not indomethacin or ibuprofen, reduced TNFα-mediated and NIK-mediated activation of NF-κB-driven transcription from the HIV-1 LTR-luciferase reporter in transfected cells (Yamamoto *et al.*, 1999). Furthermore, the authors established that sulindac, but not ibuprofen or indomethacin, inhibited stimulus-induced (TNFα or NIK) nuclear translocation of NF-κB and like aspirin and sodium salicylate, sulindac specifically inhibited IKK-β activity. A very recent study (Niederberger *et al.*, 2006) established that the COX-2-selective inhibitors, etoricoxib and lumiracoxib, can inhibit both LPS stimulated DNA-binding activity of NF-κB and nuclear accumulation of RelA in response to LPS.

In contrast to previous reports, the host laboratory demonstrated that aspirin mediates its anti-tumour effect by activating the NF-κB pathway even in the absence of TNF or other stimulating agents (Stark *et al.*, 2001). It was found that aspirin induces a reduction in cytoplasmic IκBα that is both time- and concentration-dependent. Furthermore, the degradation of IκB mediated the nuclear translocation of p50/RelA NF-κB complexes, thus confirming stimulation of the NF-κB pathway.

This response to aspirin occurred before detectable cell death by apoptosis, suggesting a causal relationship. Inhibiting nuclear translocation of NF-κB using super-repressor IκBα, a dominant negative form of IκBα that is resistant to phosphorylation on the two critical serine residues (Ser 32 and 36), effectively blocked aspirin-induced cell death confirming that aspirin-induced apoptosis is due, at least in part, to nuclear translocation of NF-κB (Stark *et al.*, 2001). It is also noteworthy that the observed effects of aspirin on the NF-κB pathway are cell-type specific to colorectal cancer cell lines (Din *et al.*, 2004) and independent of both p53 status and, as mentioned above, DNA MMR gene proficiency (Din *et al.*, 2005). Several other papers have reported activation of the NF-κB pathway in response to NSAIDs. A recent study (Cho *et al.*, 2005) demonstrated that the NSAID diclofenac mediates degradation of cytoplasmic IκBα and nuclear accumulation of RelA. The selective COX-2 inhibitor, celecoxib, has been found to increase nuclear translocation of RelA, NF-κB DNA binding activity and NF-κB-dependent gene transcription, strongly suggesting that this agent has COX-independent actions (Niederberger *et al.*, 2001). Another COX-2-selective inhibitor, NS-398, was reported to cause degradation of IκBα and a delayed increase in NF-κB DNA-binding, but not NF-κB-dependent transcriptional activity, in colon cancer cells in the absence of additional NF-κB activators (Smartt *et al.*, 2003).

Overall, the results from these studies indicate that activation of the NF-κB pathway is common to NSAIDs and independent of their COX inhibitory nature. It is also very likely that there is a further level of complexity in the upstream mechanism of activation of the NF-κB pathway, depending on the cell type and NSAID used.

1.5.3.5.15 The Nucleolus and Regulation of Transcription, Cell Growth and Apoptosis

Recent data from the host laboratory has shown that aspirin not only induces translocation of NF-κB components to the nucleus, but also leads to nucleolar sequestration of the RelA component of NF-κB (Stark and Dunlop, 2005). Compartmentalisation of RelA in the nucleolus was found to be stimulus specific. The classical NF-κB activators, TNF and TRAIL, caused RelA to be concentrated in the nucleoplasm with no detectable nucleolar staining. However, serum withdrawal and UV-C radiation (pro-apoptotic stimuli) resulted in sequestration of RelA in the nucleolus. Using aspirin as a model system, nucleolar accumulation of RelA was found to require de-novo protein synthesis from the observations that both cyclohexamide and actinomycin D prevented RelA localisation in the nucleolus. Furthermore, nucleolar sequestration of RelA was independent of the Crm1p nuclear export pathway and required translocation from the cytoplasm to the nucleus. Using a dominant negative RelA construct, nucleolar accumulation of RelA was demonstrated to be causally involved in aspirin-induced repression of NF-κB transcriptional activity and apoptosis (Stark and Dunlop, 2005).

The nucleolus from most higher eukaryotes is assembled around clusters of tandemly repeated ribosomal genes (ribosomal DNA (rDNA) genes) and primarily functions as a factory for ribosome assembly (Olson *et al.*, 2000). The three major morphological components of the nucleolus are: the fibrillar centre, the dense fibrillar component, and the granular component (Dundr and Misteli, 2001). In addition to being a site dedicated to ribosome biogenesis, non-conventional roles for

the nucleolus are emerging, which include processing and/or nuclear export of certain mRNAs, sequestration of regulatory molecules, modification of small RNAs, ribonucleoprotein particle assembly, and control of ageing [reviewed in (Pederson, 1998a; Pederson, 1998b; Olson *et al.*, 2002)]. Many nonribosomal proteins, such as survivin-deltaEx3 (Song and Wu, 2005), c-Myc (Arabi *et al.*, 2003), promyelotic leukaemia gene product (PML)-containing nuclear body associated proteins (Mattsson *et al.*, 2001), growth factors (Antoine *et al.*, 1997; Galcheva-Gargova *et al.*, 1998; Pederson, 1998b), virus proteins (Stauber and Pavlakis, 1998; Wurm *et al.*, 2001) and regulators of apoptosis, for example p53, DEDD, MDM2 and p14ARF (Stegh *et al.*, 1998; Zhang and Xiong, 1999; Lohrum *et al.*, 2000), have been demonstrated to localise to the nucleolus under certain conditions. It has therefore been proposed that the nucleolus may have a key role in cell-cycle regulation and apoptosis (Carmo-Fonseca *et al.*, 2000; Horký *et al.*, 2002). Indeed, one of the most notable features of the cell cycle is the disassembly of the nucleolus during mitosis and its subsequent reassembly as the daughter cells proceed into interphase (Dimario, 2004).

The nucleolus has been identified as a key site for regulation of c-Myc, an oncoprotein that regulates the transcription of genes that are associated with cell growth, proliferation and apoptosis (Oster *et al.*, 2002). c-Myc and proteasomes have been observed to accumulate at the nucleoli of cells containing elevated c-Myc protein levels (achieved by overexpression or proteasome inhibition) (Arabi *et al.*, 2003), leading the authors to propose that c-Myc is subject to ubiquitin-mediated proteolysis in the nucleolus. A more recent study (Datta *et al.*, 2004) demonstrated that c-*myc*-induced progression through the cell cycle is inhibited by the

sequestration of the protein in the nucleolus. Furthermore, interaction between c-Myc and the Fbw7 ubiquitin ligase within the nucleolus is important for regulation of c-Myc's growth promoting function (Welcker *et al.*, 2004). c-Myc has also been shown to associate with ribosomal DNA and activate RNA polymerase I transcription, indicating that regulation of ribosome biogenesis by c-Myc could be important for its effects on cell growth (Arabi *et al.*, 2005).

The nucleolus has also been implicated in the regulation of the tumour suppressor protein, p53 [reviewed in (Olson, 2004; Dundr and Misteli, 2001; Zimber *et al.*, 2004)]. p53 activity is primarily controlled by its interaction with the MDM2 oncoprotein, which regulates p53 degradation in the cytoplasm through its targeting to the ubiquitination pathway. MDM2 has been shown to undergo nucleocytoplasmic shuttling and its activity is regulated by ARF, which blocks the export of MDM2 to the cytoplasm and sequesters MDM2 in the nucleolus. Furthermore, ARF inhibits the E3 ubiquitin-ligase activity associated with MDM2. As a consequence, p53 is not degraded and its level in the nucleus rises. Increased levels of ARF essentially induce cell-cycle arrest by inhibiting the MDM2-dependent degradation of p53. Specifically, ARF has been shown to differentially regulate the E2F-1/DP-1 complex (Datta *et al.*, 2002), a complex known to be involved in G_1/S cell cycle progression.

Interestingly, ARF has recently been shown to regulate the transactivation function of RelA and was found to inhibit both NF-κB-driven transcription and anti-apoptotic activity, independent of MDM2 and p53 (Rocha *et al.*, 2003). Moreover, ARF induces ATR- and Chk1-mediated phosphorylation of RelA on threonine 505, a site required for ARF-dependent repression of RelA transcriptional activity (Rocha *et al.*, 2005; Rocha and Perkins, 2005). The nucleolar protein, NF-κB binding protein

(NFBP), has been identified in a yeast two-hybrid screen for novel NF-κB interacting proteins (Sweet *et al.*, 2003). Similarly, it was discovered that the nucleolar protein, nucleophosmin/B23, is an NF-κB-interacting partner (Dhar *et al.*, 2004). It is also noteworthy that nucleolar shuttling of the NF-κB regulator, NIK, has recently been shown to affect the function of that kinase (Birbach *et al.*, 2004). Another recent paper reported that nucleolar localisation of NRF (NF-κB repressing factor) correlates inversely with its mobility.

Collectively, these data indicate that nucleolar sequestration of key transcription factor components, for example RelA, might be very important in the apoptotic response to NSAIDs, in particular aspirin. It follows, therefore, that the nucleolus could be a good candidate target site for chemopreventative agents in general.

1.5.3.6 β-catenin Signaling

In addition to COX, NF-κB and the other pathways mentioned so far, the cellular factor β-catenin has been identified as a major target of NSAIDs. β-catenin is a 92 kDa protein that has two major roles in cells. As a component of adherens junctions, β-catenin was first implicated in cell-cell adhesion (Bienz, 2005). β-catenin, together with α-catenin and γ-catenin, binds to the intracellular domain of E-cadherin, a transmembrane protein involved in homotypic cell-cell contacts, thus linking cadherin adhesion receptors to α-catenin, which in turn links to the actin cytoskeleton (Morin, 1999; Harris and Peifer, 2005). This function of β-catenin is

based on the stable, membrane-associated subcellular pool of β-catenin (Bienz, 2005). In addition to its role in cell adhesion, β-catenin was found to have signaling functions when it was discovered to be the mammalian homologue of *armadillo*, a segment polarity gene involved in the Wnt signaling pathway in *Drosophila* (Morin, 1999). In this role, β-catenin functions as a transcriptional co-activator by forming a complex with the lymphoid enhancer factor (LEF) and T cell factor (TCF) classes of transcription factors to activate transcription of Wnt target genes (Lustig and Behrens, 2003; Harris and Peifer, 2005). This signaling function of β-catenin is conferred by a soluble cytoplasmic pool that is highly unstable in the absence of a Wnt signal (Bienz, 2005).

1.5.3.6.1 The Wnt Signaling Pathway

An overview of the canonical Wnt pathway is shown in Figure 1.13 [reviewed in (Behrens and Lustig, 2004; Lustig and Behrens, 2003; Giles *et al.*, 2003; Polakis, 2000; Dale, 1998)]. Briefly, Wnts are a large family of secreted glycoproteins, with at least 19 known human members, that have key roles in development (Giles *et al.*, 2003). Wnt signaling is initiated following Wnt ligand binding to seven transmembrane receptors called frizzleds, with low-density lipoprotein receptor-related protein (LRP) family receptors 5 and 6 acting as essential co-receptors of Wnt ligands (Lustig and Behrens, 2003). Secreted factors, for example WIF, FrzB and Dickkopf (Dkk) can modulate the interaction of Wnts with frizzled receptors by acting as direct or indirect antagonists (Giles *et al.*, 2003).

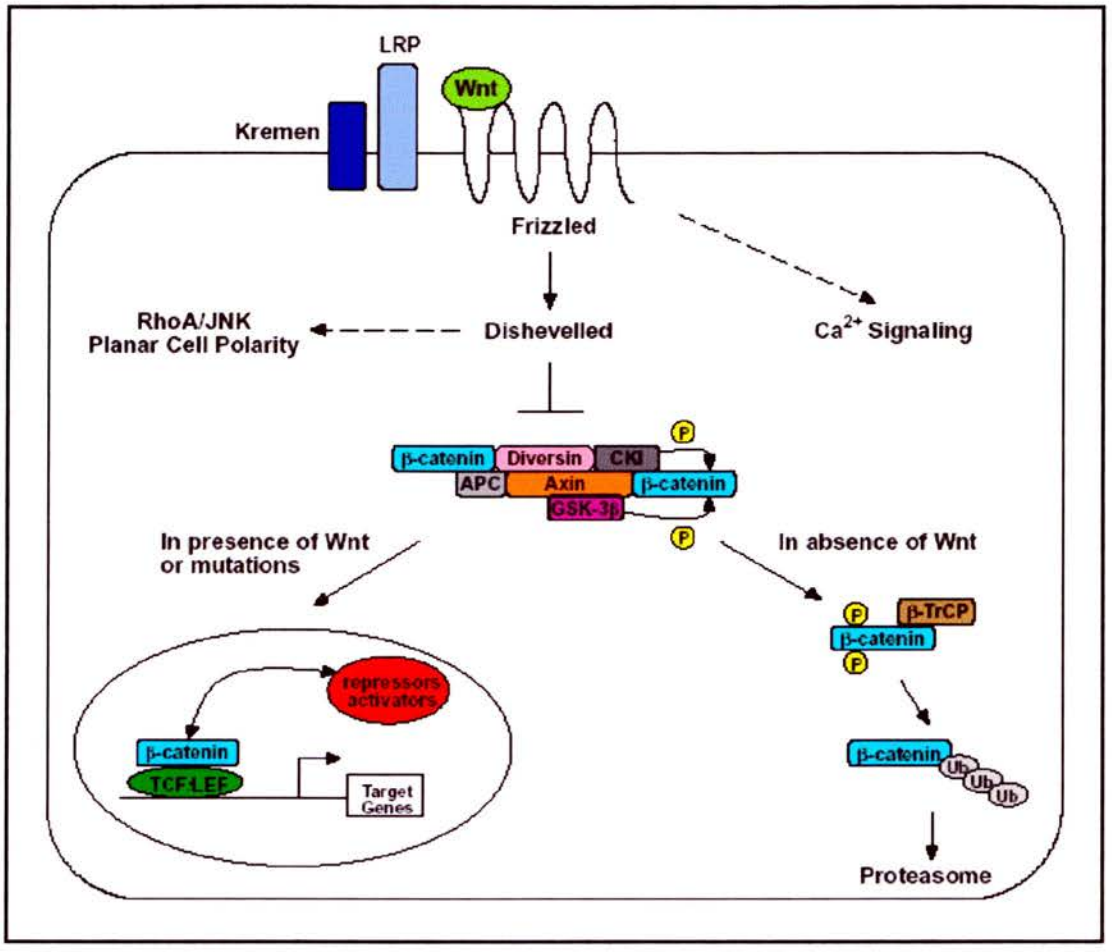

Figure 1.13 – Overview of The Wnt Signaling Pathway. Binding of Wnt ligand to frizzled receptors activates dishevelled, which in turn blocks the function of the multi-protein complex comprising: APC, the scaffold proteins axin or conductin, GSK-3β, diversin and CKI. In the absence of Wnts, the multi-protein complex phosphorylates (P) β-catenin via CKI and GSK-3β. Phosphorylated β-catenin then becomes multi-ubiquitinated (Ub) by β-TrCP and is subsequently targeted for degradation by the proteasome. In the presence of Wnt or after mutations of *APC*, the phosphorylation and degradation of β-catenin is blocked, which allows the association of β-catenin with TCF transcription factors. TCF/β-catenin complexes bind to specific promoters on DNA and hence activate transcription of Wnt target genes, together with the appropriate transcriptional repressors or activators. The canonical Wnt signaling pathway also branches to the planar cell polarity and Ca^{2+} signaling pathways (indicated by dashed lines).

[Figure adapted from (Lustig and Behrens, 2003)]

In the absence of Wnt stimulation, cytoplasmic β-catenin levels are normally regulated by a multi-protein destruction complex which targets β-catenin for degradation in proteasomes (Behrens and Lustig, 2004). This multi-protein destruction complex comprises axin (or its homologue conductin), GSK-3β, APC, casein kinase I (CKI) and diversin (Lustig and Behrens, 2003). Axin/conductin, together with APC, form a structural scaffold in this complex that facilitates the efficient phosphorylation of β-catenin (Giles *et al.*, 2003). Phosphorylation of β-catenin occurs via a two-step mechanism at four amino terminal residues (Behrens and Lustig, 2004). First, Ser45 is phosphorylated by CKI, creating a primary binding site on β-catenin for GSK-3β, which subsequently phosphorylates Ser33, Ser37 and Thr41. Phosphorylation of β-catenin targets it for binding to β-TrCP, an F box protein and E3 ubiquitin ligase, thus triggering ubiquitination of β-catenin and its subsequent degradation in proteasomes (Giles *et al.*, 2003).

In the presence of Wnt stimulation, binding of Wnt to the frizzled receptor leads to phosphorylation and activation of dishevelled (Dsh), which is subsequently recruited to the cell membrane (Bienz and Clevers, 2000). Activated Dsh can recruit axin and the destruction complex to the plasma membrane, leading to degradation of axin (Moon *et al.*, 2004). Furthermore, activated Dsh can also inhibit GSK-3β activity (Ben-Ze'ev and Geiger, 1998). Collectively, these events stabilise β-catenin levels by reducing phosphorylation and degradation of β-catenin by the destruction complex. Stabilised (hypophosphorylated) β-catenin is then free to translocate to the nucleus, where it associates with TCF/LEF transcription factors, leading to activation of transcription of Wnt target genes (Giles *et al.*, 2003). Accumulation of β-catenin is therefore critical for activation of the Wnt transcriptional response (Taipale and

Beachy, 2001). The interaction between stabilised β-catenin and TCFs can be modulated by several direct and indirect mechanisms. In the absence of Wnt signaling, TCF's can associate with transcriptional repressors, such as groucho, to block the expression of Wnt target genes (Lustig and Behrens, 2003). Furthermore, the transcriptional activity of TCF/β-catenin complexes can be enhanced by transcriptional activators, including p300/CBP (Hecht *et al.*, 2000). Several nuclear effector proteins, namely ICAT and Chibby, can bind to β-catenin and in doing so, disrupt the interaction of β-catenin with TCF (Behrens and Lustig, 2004).

Having outlined the Wnt/β-catenin pathway, it is clear that there are strong parallels with the NF-κB pathway. These similarities and the potential for cross-talk between the NF-κB and Wnt/β-catenin pathways will be discussed more fully later but in keeping with this notion, it is noteworthy that the NF-κB and Wnt signaling pathway regulate a similar panel of target genes. Therefore, in order to highlight similarities with the NF-κB pathway and to emphasize the relevance for the anti-tumour effects of NSAIDs, Wnt target genes that are implicated in cancer development will be the major focus of the following discussion of target genes of β-catenin.

1.5.3.6.2 Target Genes of β-catenin

Several Wnt targets (for examples see Table 1.4) [reviewed in (Doucas *et al.*, 2005; Lustig and Behrens, 2003; Behrens and Lustig, 2004)] that have been

Gene Category	Examples	
	Gene Product	Function
Cell Cycle/Apoptosis	c-Myc	Proto-oncogene
	Cyclin D1	Activator of cyclin-dependent kinases
	c-Jun	Proto-oncogene
	Fra-1	Proto-oncogene
Growth Factors	VEGF	Vascular endothelial growth factor
	c-Met	Receptor for the epithelial growth, motility and survival factor, hepatocyte growth factor (HGF)/scatter factor
	WISP-1	Wnt-1-induced secreted protein 1
	BMP-4	Bone morphogenetic protein-4
Tumour Progression	MMP7	Matrix metalloproteinase 7
	Nr-CAM	Cell adhesion molecule
	CD44	Cell surface molecule
Transcription Factors	ITF-2	Immunoglobulin transcription factor-2
	Id2	Dominant negative helix-loop regulator
	AF17	Transcriptional regulator and fusion partner of MLL in certain acute lymphoblastic leukaemia and myeloid leukaemia
Negative Feedback Regulators	Conductin	β-catenin degradation
	Nkd	Naked cuticle (interferes with *dsh*)
	TCF-1	T cell factor-1 transcription factor
	β-TrCP	E3 Ubiquitin Ligase
Other	COX-2	Cyclooxygenase
	PPARδ	Peroxisome proliferator-activated receptor δ

Table 1.4 – Examples of Target Genes of β-catenin and Their Functions.

identified include genes which have roles in cell cycle progression and apoptosis. The promoters of both c-*myc*, a proto-oncogene, and *cyclin D1*, an activator of cyclin dependent kinases, contain TCF binding sites and are controlled by TCF/β-catenin complexes (He *et al.*, 1998; Tetsu and McCormick, 1999). c-Myc regulates the transcription machinery and its expression has been shown to be inversely related to apoptosis in colorectal cancer cells (Greco *et al.*, 2001). Furthermore, upregulation of c-Myc leads to repression of the CDKI, $p21^{waf1/cip1}$, and thus stimulates G_1/S progression through the cell cycle (van de Wetering *et al.*, 2002). Cyclin D1 regulates the G_1/S phase of the cell cycle by direct activation of G_1 CDKs (Behrens and Lustig, 2004) and so TCF/β-catenin complexes can also promote cell cycle progression through upregulation of cyclin D1 (Lustig and Behrens, 2003). Interestingly, adenomas from FAP patients have elevated cyclin D1 levels (D'Orazio *et al.*, 2002) and sporadic colorectal tumours showing nuclear accumulation of β-catenin exhibit cyclin D1 overexpression (Oda *et al.*, 1999). Expression of *survivin*, an anti-apoptotic gene known to be upregulated in colon tumours, has been shown to be downregulated by APC and analysis of the promoter revealed TCF-4-binding sites, thus implicating it as a Wnt target (Zhang *et al.*, 2001a).

Wnt targets also include several growth factors and their receptors, which could affect cell proliferation and hence influence tumour progression. The levels of VEGF, a pro-angiogenic protein, have been shown to be elevated in colorectal cancer and moreover, expression of VEGF is upregulated by Wnt signaling through TCF-binding sites in its promoter, which could lead to stimulation of angiogenesis (Zhang *et al.*, 2001b). The tyrosine kinase c-Met is the receptor for the epithelial growth, motility and survival factor, hepatocyte growth factor (HGF)/scatter factor. c-Met

has been shown to be upregulated in the polyps of FAP patients, an effect that can be abrogated by dominant-negative TCF in colorectal tumour cells (Boon *et al.*, 2002). Amplification of c-Met occurs in approximately 10% primary colon tumours and this may promote cell motility and invasion (Lustig and Behrens, 2003).

Wnt targets that are specifically implicated in tumour progression include members of the MMP family. B-catenin/TCF complexes can activate the expression of MMP-7 (Crawford *et al.*, 1999), a protein that is frequently upregulated in colorectal cancer (Oving and Clevers, 2002). Deficiency of the MMP matrilysin in *Min* mice results in a decrease in tumour number and size (Crawford *et al.*, 1999). The cell adhesion molecules, CD44 (a protein implicated in metastasis formation through interaction with proteoglycans) and Nr-CAM (a member of the Ig superfamily of adhesion receptors) have been implicated as Wnt targets, and could therefore affect the motility of tumour cells and their capacity to metastasise (Behrens and Lustig, 2004). CD44 is strongly expressed in aberrant crypt foci (Wielenga *et al.*, 1999) and Nr-CAM has been demonstrated to be overexpressed by β- or γ-catenin in colorectal cancer cell lines (Conacci-Sorrell *et al.*, 2002).

Other Wnt targets include transcription factors, including ITF-2 (immunoglobulin transcription factor-2), Id2 (dominant negative helix-loop regulator) and AF17 (transcriptional regulator and fusion partner of MLL (mixed lineage leukaemia) in certain acute lymphoblastic leukaemia and myeloid leukaemia) (Lustig and Behrens, 2003), thus allowing for potential cross-talk between different pathways.

Like NF-κB and other signal transduction cascades, the Wnt pathway incorporates mechanisms for negative feedback control. The scaffold component of

the destruction complex, conductin/axin2, is a direct target of Wnt signaling (Jho *et al.*, 2002) and conductin/axin2 is overexpressed in early colorectal adenomas (Lustig *et al.*, 2002). Negative control of the pathway can also be achieved via naked cuticle (Nkd), which interferes with dishevelled; dominant negative forms of TCF-1; or expression of the ubiquitin ligase β-TrCP, which is activated by Wnt signaling at the post-transcriptional level (Spiegelman *et al.*, 2000).

It is of particular interest that *COX-2* and *PPARδ*, which were described above as being well defined targets of NSAIDs, are targets of Wnt signaling (Giles *et al.*, 2003). PPARδ is upregulated early in tumorigenesis by binding of TCF/β-catenin complexes to TCF-responsive elements (He *et al.*, 1999). Another study (Howe *et al.*, 1999) demonstrated that COX-2 is upregulated in mouse mammary cell lines by induction of stabilised β-catenin through Wnt-1. COX-2 catalyses the production of eicosanoids, including PGs, from arachidonic acid and eicosanoids in turn can promote cell viability through binding and activation of PPARδs (Lustig and Behrens, 2003). Hence a plausible hypothesis is that NSAIDs block PPARδ function at two levels: indirectly by blocking eicosanoid synthesis through COX-2 and directly by stimulating interaction of PPARδ with specific promoters.

1.5.3.6.3 Alterations of Wnt Signaling in Colorectal Cancer

In support of the notion that β-catenin is a potential target for NSAIDs in the colon, there is a body of evidence that defects in the Wnt signaling pathway have a central role in the development and progression of colorectal cancer. In addition to

being a component of the Wnt pathway, *APC* is a key tumour suppressor gene in colorectal cancer. It is mutated in the germ line in FAP, a hereditary syndrome with a penetrance of 100% (Jo and Chung, 2005). Moreover, *APC* is somatically mutated in approximately 80% of sporadic colorectal cancers and its inactivation is generally one of the earliest events in colon tumorigenesis (Gregorieff and Clevers, 2005; Polakis, 1999). The majority of sporadic mutations in *APC* are nonsense or frameshift mutations that lead to a truncated APC protein (Fearnhead *et al.*, 2002). Furthermore, approximately 60-80% of these mutations occur in the 700 bp 'mutation cluster region', which corresponds to the β-catenin/axin binding domain (Sieber *et al.*, 2000; Lustig and Behrens, 2003). Mutational inactivation of *APC* leads indirectly to reduced phosphorylation and subsequent degradation of β-catenin. This causes an accumulation of stabilised β-catenin and activation of target genes (Moon *et al.*, 2004). Loss of *APC* function is therefore equivalent to a constitutive, positive Wnt signal (Taipale and Beachy, 2001). In support of this, β-catenin is overexpressed in colon cancer cells with mutant *APC* (Munemitsu *et al.*, 1995). Moreover, by reintroducing wild-type *APC* into those cells, β-catenin levels could be reduced, strongly suggesting that the major tumour suppressive function of APC lies in its ability to regulate free β-catenin levels. Similarly, constitutive transcriptional activation of a β-catenin-Tcf complex, as measured by transcription of a *Tcf* reporter gene, has been observed in $APC^{-/-}$ colorectal cancer cells (Korinek *et al.*, 1997). Furthermore, reintroduction of wild-type *APC* into those cells abrogated the transcriptional transactivation.

While mutations in APC have the effect of mimicking constitutive Wnt signaling through an indirect effect on β-catenin, activating mutations in the gene

encoding β-catenin (*CTNNB1*) have an equivalent functional effect. Activating *CTNNB1* mutations have been identified in approximately 50% colorectal cancers that express wild-type APC, although, since the majority of colon cancers have APC defects, this represents less than 10% of all cases of colorectal cancer (Morin, 1999; Lustig and Behrens, 2003). *APC* and *CTNNB1* mutations are mutually exclusive in colorectal cancer, consistent with the view that mutation of either gene has the same effect on β-catenin stability and TCF activation (Giles *et al.*, 2003). Mutations in *CTNNB1* which are functionally relevant to cancer are activating and occur in the amino-terminal regulatory region, in or around exon 3, affecting one or more of the four putative phosphorylation sites for GSK-3β (Polakis, 2000; Behrens and Lustig, 2004). These mutations render β-catenin refactory to destruction by the axin complex and hence increase the stability of β-catenin (Bienz and Clevers, 2000). It is noteworthy that mutations of *CTNNB1* have mainly been detected in colorectal tumours exhibiting the MSI mutator phenotype, characterised by defective DNA MMR due to somatic mutation or epigenetic inactivation of genes encoding DNA MMR proteins or, in the case of HNPCC, germ line DNA MMR gene mutations (Kitaeva *et al.*, 1997; Sparks *et al.*, 1998). Moreover, both hereditary and sporadic forms of MSI colorectal cancers have been shown to have a relatively high frequency of *CTNNB1* mutations and a low incidence of *APC* mutations (Mirabelli-Primdahl *et al.*, 1999). Similar to mutation of *APC* in the *Min* mouse, transgenic mouse models with a conditional knock-in mutation of one of the β-catenin alleles to an oncogenic form (lacking GSK-3β target sites) produces adenomatous polyps in the intestine and microadenomas in the colon (Harada *et al.*, 1999). Hence, there is substantial evidence that any mutation leading to stabilised nuclear β-catenin is sufficient for

neoplastic transformation in colonic mucosa and is one of the earliest events in tumorigenesis in the mammalian intestinal tract [reviewed in (Gregorieff and Clevers, 2005; Bienz and Clevers, 2000)].

1.5.3.6.4 The Effects of NSAIDs on the β-catenin Pathway

Several studies have investigated the effects of NSAIDs on β-catenin. One study (Hawcroft *et al.*, 2002) found that indomethacin induces a dose-dependent decrease in β-catenin protein levels and that this down-regulation of β-catenin was associated with indomethacin-induced G_1 cell cycle arrest. Another study (Dihlmann *et al.*, 2001) reported that aspirin and indomethacin inhibit transcription of a β-catenin/TCF-responsive reporter gene in a dose-dependent manner. However, this effect did not involve changes in levels of endogenous β-catenin. Similarly, repressed expression of β-catenin-dependent genes in response to diclofenac has been observed in the absence of any effect on cytoplasmic β-catenin levels (Cho *et al.*, 2005). It appears that the observed reduction of β-catenin/T-TCF signaling by aspirin and indomethacin is due to increased stabilisation of phosphorylated β-catenin (Dihlmann *et al.*, 2003). In contrast, one paper documented that sulindac sulfone caused a reduction in the high levels of accumulated β-catenin in SW480 colorectal cancer cells (mutated in *APC*) and suggested that induction of apoptosis by sulindac sulfone was the result of activation of protein kinase G (PKG) in response to sustained increases in cyclic guanosine 3′,5′-monophosphate (cGMP) levels (Thompson *et al.*, 2000). More recently, the same group have demonstrated that exisulind-induced β-

catenin degradation precedes induction of apoptosis and that the down-regulation of inappropriate β-catenin-activated genes such as *cyclin D1* accounts in part for the pro-apoptotic effects of exisulind in colorectal cancer cells (Li *et al.*, 2002). A dose-dependent down-regulation of β-catenin has been observed in response to indomethacin (Veeramachaneni *et al.*, 2003). Similarly, sulindac metabolites can induce degradation of β-catenin (Rice *et al.*, 2003). A recent study of a panel of NSAIDs (Gardner *et al.*, 2004) established that some, but not all of the NSAIDs, mediated a reduction in catenin-related transcription. Another study has proposed that the anti-proliferative effects of indomethacin may contribute to enhanced cell adhesion via translocation of β-catenin from the nucleus to the cell membrane (Kapitanović *et al.*, 2006). Interestingly, a recent study demonstrated that repression of β-catenin signaling by a panel of 19 different NSAIDs requires both PPARγ and its co-receptor, retinoid-X-receptor-α (RXR-α) and is independent of COX-inhibitory activity (Lu *et al.*, 2005). Furthermore, β-catenin was found to interact with RXR-α and PPARγ by immunoprecipitation.

The findings of *in vitro* studies are supported by several *in vivo* studies examining the effects of NSAIDs on β-catenin. One study (McEntee *et al.*, 1999) reported that sulindac caused a ≥50% decrease in β-catenin levels and diminished Bcl-2 levels in small intestinal tumours from *Min* mice harvested between 2 and 4 days of treatment when compared to untreated controls. Moreover, colonic tumours had elevated Bcl-2 expression compared to their small intestinal counterparts and the levels of Bcl-2 and β-catenin were unaffected by sulindac treatment for 2 or 4 days. This strongly suggested that reduction of Bcl-2 was associated with regression of small intestinal tumours and provides a possible explanation for the observed

differences in sensitivity of colonic and small intestinal tumours to sulindac-induced regression. A recent study (Roy *et al.*, 2005) demonstrated that the NSAID, nabumetone, can reduce β-catenin levels in non-neoplastic intestinal mucosa of the *Min* mouse but had no effect on β-catenin levels in the uninvolved colonic mucosa of AOM-treated rats. However, in both the *Min* mice and AOM-treated rats, treatment with nabumetone led to an increase in GSK-3β levels. Nabumetone supplementation reduced the level of nuclear β-catenin in AOM-induced tumours and nuclear cyclin D1 in both the non-neoplastic mucosa and adenomatous tissue in AOM-treated rats. E-cadherin was also found to be significantly increased in the uninvolved mucosa of both the *Min* mouse and AOM-treated rat in animals supplemented with nabumetone. Taken together, the results from that study implicate β-catenin signaling in the mechanism of chemoprevention by nabumetone. Finally, it has been established that sulindac can target nuclear accumulation of β-catenin and β-catenin/TCF-mediated transcription in adenomas of patients with FAP, in addition to colorectal cancer cell lines (Boon *et al.*, 2004).

Collectively, the data from these *in vitro* and *in vivo* studies provide compelling evidence to implicate the involvement of β-catenin in the mechanism by which NSAIDs mediate their anti-tumour effects.

1.5.3.7 Interaction of NF-κB and β-catenin

As mentioned above, parallels exist between the Wnt/β-catenin and NF-κB pathways and several lines of evidence now suggest that cross-talk occurs between

the two pathways. Firstly, the kinases, GSK-3β and IKK, are common to both signaling pathways. In addition to phosphorylation of β-catenin through Wnt signaling, GSK-3β has been shown to be required for cell survival and NF-κB activation (Hoeflich *et al.*, 2000). In that study, it was established that GSK-3β-deficient embryos could be rescued by inhibition of TNF, a known potent activator of NF-κB and treatment of wild-type fibroblasts with lithium, an inhibitor of GSK-3β, was demonstrated to sensitize cells to TNFα treatment and inhibit transactivation of NF-κB. These findings led the authors to propose that NF-κB is regulated by GSK-3β at the level of the transcription complex. β-catenin is also subject to regulation by IKK, a component of the NF-κB signaling cascade (Lamberti *et al.*, 2001) since both IKKα and IKKβ interact with and phosphorylate β-catenin *in vitro* and *in vivo*. Secondly, both β-catenin and the NF-κB inhibitor protein, IκBα, interact with the same E3 ubiquitin ligase component, β-TrCP and are thus targeted for destruction via the ubiquitin pathway (Amit and Ben Neriah, 2003). Moreover, expression of β-TrCP is itself activated by Wnt signaling, providing the potential for cross-talk between the Wnt and NF-κB pathways (Maniatis, 1999). Finally, as highlighted above, the NF-κB and Wnt/β-catenin pathways regulate the transcription of similar categories of genes involved in cell proliferation, cell cycle/apoptosis and tumour progression. Moreover, many of these genes, for example *cyclinD1* and *c-myc*, contain both NF-κB and TCF/LEF binding sites.

The similarities between the NF-κB and Wnt/β-catenin pathways described above provide strong circumstantial evidence that that they could be mechanistically linked. In support of this suggestion, RelA was found to specifically suppress β-catenin/TCF-dependent transcription, an effect that was independent of the DNA

binding ability of the β-catenin/TCF complex (Masui *et al.*, 2002). β-catenin can also physically interact with both RelA and p50 by coimmunoprecipitation (Deng *et al.*, 2002), resulting in reduced NF-κB DNA binding, reduced transactivation activity and reduced target gene expression. Furthermore, a more recent study by the same group established that APC/GSK-3β, through β-catenin, can cross-regulate the NF-κB pathway (Deng *et al.*, 2004). Interestingly, diclofenac has been shown to attenuate Wnt/β-catenin signaling via activation of NF-κB in colon cancer cells (Cho *et al.*, 2005).

Collectively, these studies demonstrate that cross-regulation of the NF-κB and Wnt/β-catenin pathways does occur and that such interactions may be implicated in the mechanisms underlying the protective effects of NSAIDs.

1.6 The Need for Safer Alternatives to NSAIDs

Despite the compelling evidence for benefit from case/control, cohort, controlled trials and clinical observational studies, the prolonged use of NSAIDs is limited by significant toxicity. Harmful side effects include: gastrointestinal bleeding and perforation; intracranial haemorrhage; renal side effects; hypersensitivity reaction to salicylate and accelerated cartilage destruction in osteoarthritis (Kaza *et al.*, 2002; Rainsford, 1999). The combination of the side effect profile and the fact that NSAIDs provide only partial protection against colorectal cancer, provides the rationale for development of safer and even more effective alternatives.

1.6.1 Rational Design of COX-2 Selective NSAIDs

The major use of NSAIDs is for pain relief and reduction of inflammatory action in musculoskeletal disorders, especially arthritic processes. The rational drug design and development of new specific, preferential COX-2-selective drugs, such as celecoxib, rofecoxib and nimesulide, was on the premise that side effects of traditional NSAIDs such as aspirin, which inhibit both COX-1 and COX-2, could be avoided or at least minimised. Clinical trials of COX-2-selective drugs in preventing or reducing colorectal neoplasia have recently been carried out, although there has been some controversy surrounding them. One study (Steinbach *et al.*, 2000) reported that twice-daily treatment with 400 mg celecoxib for 6 months in patients with FAP resulted in a significant (28%) reduction in the mean number of colorectal polyps and a similar (30.7%) reduction in polyp burden. Another study demonstrated a significant reduction (14.5% overall and 31% in patients with clinically significant disease) in duodenal polyposis in FAP patients after treatment with celecoxib 400 mg twice daily (Phillips *et al.*, 2002). Similarly, once-daily treatment with 25 mg rofecoxib was found to significantly reduce the number and size of rectal polyps in FAP patients (Higuchi *et al.*, 2003). Maximal effects were seen after 9 months treatment, with a 6.8% reduction in polyp number and a 16.2% reduction in polyp size. A separate study (Hallak *et al.*, 2003) established that there was a highly significant reduction in the rate of polyp formation (70-100%) in 10 FAP patients receiving 25 mg rofecoxib once-daily for up to 30 months.

Despite encouraging evidence to suggest selective agents might be effective and perhaps be associated with less side effects, the results of two major trials have

called into question the safety of COX-2-selective drugs. The first trial results to emerge were from the Rofecoxib (Vioxx) study. The authors reported an increased risk of thrombotic events in patients receiving the drug for more than 18 months (Bresalier *et al.*, 2005). These safety concerns over increased cardiovascular risk resulted in a 'black box' warning being issued by the Food and Drug Administration. Subsequently, rofecoxib was removed from the market by Merck on September 30th 2004 (Topol, 2004; Samoha and Arber, 2005). Similar to the findings of the rofecoxib trial, the results from an independent evaluation of a trial of celecoxib also demonstrated cardiovascular side effects after several months of treatment, especially in the high-dose group (Solomon *et al.*, 2005).

1.6.2 The Need for Rational Design of Novel Therapeutic Agents

In light of the above safety concerns over COX-2-selective NSAIDs and given the toxicity of traditional NSAIDs, it is therefore of vital importance to fully understand the mechanism of action of NSAIDs so that safer drugs can be developed. The identification of pathways targeted by NSAIDs, in particular the NF-κB and β-catenin pathways, and the specific mechanisms involved in their regulation is therefore critical for the rational design of novel therapeutic agents.

1.7 Preliminary Results From MSc Mini Project

Modulation of the NF-κB pathway by a panel of non-aspirin NSAIDs, comprising sulindac and indomethacin (non-selective COX inhibitors) and sulindac sulfone (lacks COX-inhibitory effect), was investigated to determine whether the effects were similar to those of aspirin (Loveridge, 2001). Preliminary results from this mini-project were as follows: **1**. Annexin V-FITC analysis of SW480 colon cancer cells showed that sulindac, sulindac sulfone and indomethacin induced apoptosis of SW480 colon cancer cells in a dose-dependent manner, similar to the effect observed for aspirin. **2**. Using immunocytochemistry, it was found that the non-aspirin NSAIDs caused nuclear translocation of the RelA component of NF-κB in a similar fashion to aspirin. **3**. All NSAIDs mediated repression of NF-κB transcriptional activity in colorectal cancer cells as measured by the 3enhancer-CON-A (3x κB ConA-Luc) NF-κB dependent luciferase reporter plasmid. **4**. In contrast to aspirin, Western blot analysis demonstrated that non-aspirin NSAID treatment had no apparent effect on IκBα levels. These preliminary results indicated that apoptosis associated with activation of the NF-κB pathway is common to NSAIDs and independent of their COX-inhibitory nature. However, the differences observed between aspirin and the other NSAIDs also suggested a further level of complexity in modulation of the NF-κB signaling pathway by NSAIDs. Tyrosine phosphorylation of IκBα has previously been shown to cause nuclear translocation of NF-κB without degradation of IκBα (Imbert *et al.*, 1996) (see section 1.5.3.5.5 and Chapter 4), and so this mechanism could possibly explain the differences observed between aspirin and the other NSAIDs.

1.8 Hypothesis of PhD Project

In view of the evidence from the literature, previous work carried out in the host laboratory, and work from my MSc project, the following hypothesis was set up: NSAIDs as a class impart their anti-tumour effect by modulation of the NF-κB pathway and effects on β-catenin signaling regulate this response.

1.9 Research Aims

The central theme of the work presented in this thesis was to understand the mechanisms underlying the antineoplastic properties of NSAIDs so that safer alternatives can be developed. The specific aims were:

1. To establish whether modulation of NF-κB is absolutely required for the effects of non-aspirin NSAIDs on apoptosis.
2. To define the mechanism by which non-aspirin NSAIDs activate the NF-κB pathway.
3. To determine the importance of NSAID effects on Wnt/β-catenin signaling in modulation of the NF-κB response to NSAIDs, in particular nuclear regulation of NF-κB activity.
4. To establish the role of post-translational modifications, specifically ubiquitination and acetylation, in nucleolar sequestration of RelA in response to NSAIDs.

1.10 Experimental Approach

The research presented in this thesis utilises the SW480 colorectal cancer cell line, which was derived from a primary Dukes' stage B colon carcinoma that arose in a 50-year-old male patient (Leibovitz *et al.*, 1976). There are potential limitations in examining a single cell line because effects may be cell line specific and may not be representative of the *in vivo* situation. However, the host laboratory has a wealth of data indicating that SW480 cells do represent the generality of responses to aspirin. Furthermore, the practicality of studying different agents at various time points means that using panels of cell lines is unfeasible. In addition, it has been shown that appropriate phenotypic features are retained in SW480 cells, even with long-term *in vitro* culture (Hewitt *et al.*, 2000). Hence, the investigation of SW480 as a single cell line is backed up by substantial work in other cell lines and systems and is considered supportable and practically deliverable.

Initially, previous results from my MSc project demonstrating that non-aspirin NSAIDs activate the NF-κB pathway were confirmed and the importance of NF-κB activation for non-aspirin NSAID effects on apoptosis was determined by using a dominant negative RelA construct generated by the host laboratory (Chapters 3 and 4). The upstream mechanism of NF-κB activation upon NSAID treatment was investigated by use of chemical and molecular inhibitors. Immunoprecipitation for tyrosine phosphorylated IκBα and kinase assays for candidate kinases were also carried out.

The second research strand focussed specifically on investigating the role of Wnt/β-catenin signaling in modulation of the NF-κB response to NSAIDs and

regulation of nuclear NF-κB activity (Chapter 5). The effect of NSAIDs on cytoplasmic β-catenin levels were assessed by Western blotting. Immunocytochemistry was used to study the subcellular localisation of β-catenin in response to NSAIDs. NSAID effects on β-catenin driven transcription were subsequently investigated by using the TOP/FOP reporter plasmids that contain TCF/LEF binding sites upstream of the firefly luciferase gene. Interaction of the NF-κB and Wnt/β-catenin pathways was then examined by means of immunoprecipitation, to determine whether RelA co-immunoprecipitated with β-catenin after NSAID treatment, and co-localisation studies were subsequently undertaken using immunocytochemistry. Finally, the dominant negative RelA construct was used to determine whether blocking NSAID-induced nucleolar localisation of RelA had any effect on β-catenin.

In the third strand, the role of ubiquitination and acetylation in nucleolar localisation of RelA in response to NSAIDs were investigated by means of immunoprecipitation and subsequent Western blot analysis, to establish the change in these modifications of RelA after NSAID treatment (Chapter 6). The proteasome inhibitor, MG132, was then used to determine whether this agent induced similar effects to those observed for NSAIDs on the NF-κB and Wnt/β-catenin pathways. The effects of NSAIDs and MG132 on localisation and levels of proteasomes were also studied by Western blot analysis and immunocytochemistry respectively.

Chapter 2 – Materials and Methods

This chapter outlines the experimental protocols and materials used throughout the course of this thesis. For all procedures outlined, standard safety and COSHH regulations were adhered to. All chemicals referred to in this chapter were supplied by Sigma, unless otherwise stated. Certain stock solutions [marked with an asterisk (*)] were prepared, sterilised by autoclaving and stored at the appropriate temperature by the Technical Services Department at the MRC HGU. Where the pH of solutions had to be adjusted, this was accomplished by adding concentrated sodium hydroxide (NaOH), potassium hydroxide (KOH) or hydrochloric acid (HCl) as appropriate while monitoring the pH using a microprocessor pH meter (Hanna Instruments).

2.1 Cell Culture and Reagents

2.1.1 Cell Lines, Maintenance and Media

SW480 cells, an adherent cell line derived from a Dukes' stage B colon carcinoma (see section 1.10), were obtained from the American Type Culture Collection/European Collection of Cell Cultures (ATCC/ECACC; number CCL-228). SW480 cells constitutively expressing a kinase dead mutant of c-Src (SW480-SrcKD) and equivalent control cells (SW480-pBpuro) were a kind gift from Prof. M. Frame (Cancer Research UK Beatson Laboratories, Glasgow).

SW480 cells were maintained in L-15 medium (Gibco BRL) supplemented with 10% foetal calf serum (FCS)* and 1% penicillin and streptomycin* at 37°C in a humidified atmosphere containing 5% CO_2. SW480-pBpuro and SW480-SrcKD cells were maintained in RPMI medium (Gibco BRL) supplemented with 10% FCS and 1% penicillin and streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. All cell lines were grown in 25 cm^2 or 75 cm^2 flasks (Greiner Bio-one) until a confluent monolayer was formed.

Cells were passaged or seeded for experimentation at a 1:3 → 1:5 dilution by washing in phosphate buffered saline (PBS)* [137 mM sodium chloride (NaCl), 2.7 mM potassium chloride (KCl), 10 mM sodium phosphate dibasic (anhydrous) (Na_2HPO_4), 2 mM potassium phosphate monobasic (KH_2PO_4), pH 7.4], followed by a 5 minute (min) incubation with Trypsin/Versene (T/V)* (1:1).

To freeze cell lines, 1 ml of appropriate cell culture media was added to detached cells prior to centrifugation at 200 x *g* for 5 minutes at room temperature. The media was then discarded and the cell pellet resuspended in 1 ml freezing media [10% dimethylsulfoxide (DMSO) in FCS] before being transferred to a cryotube. Cells were subsequently incubated on ice for 1 hour (hr) then stored in liquid nitrogen. To retrieve frozen cells, aliquots were rapidly thawed at 37°C, cells collected by centrifugation at 200 x *g* for 5 min, washed in culture medium then seeded into 25 cm^2 tissue culture flasks.

2.1.2 Reagents (NSAIDs, Other Stimuli and Inhibitors)

Stock solutions of Aspirin (Sigma) (0.5 M, pH 7.0), Sulindac (Sigma) (0.1 M, pH 7.0) and Sulindac Sulfone (MP Biomedicals) (0.1 M, alkaline pH) were prepared by solubilising the reagents in distilled water (dH_2O), using 5 M NaOH to adjust the pH as required. Stock solution of indomethacin (Sigma) (0.25 M) was prepared by solubilising the reagent in ethanol (EtOH). All drugs were filter sterilized using a 0.2 micron filter (Schleicher and Schuell Microscience) prior to use in cell culture.

Stock solution of TNFα (R&D Systems, Minneapolis, USA) (100 mg/ml) was prepared in PBS containing 1% bovine serum albumin (BSA) (Sigma). Stock solution of LPS (from *E. coli* 026:B6) (Sigma) (1 mg/ml) was prepared in PBS. Pervanadate was made by adding 40 µl of a 500 mM sodium orthovanadate (Na_3VO_4) stock solution [prepared in dH_20 (pH 10) by boiling yellow solution until it appeared colourless and allowing to cool to room temperature, then readjusting the pH to 10 and repeating the boiling and cooling steps until the solution remained colourless and the pH stabilised at 10] and 5 µl of 30% hydrogen peroxide (H_2O_2) (Sigma) to 455 µl of PBS. This mixture was incubated for 5 min at room temperature prior to the addition of catalase (200 µg/ml) (Sigma) to remove the excess H_2O_2. The pervanadate solution (final concentration 100 µM) was further incubated for 5 min at room temperature before being diluted and immediately applied to cells.

Stock Solutions of PP2 (calbiochem) (2.5 mM), MG132 (calbiochem) (50 mM) and genistein (Sigma) (100 mM) were prepared in DMSO.

2.1.3 Treatment Protocol

Cells were seeded at a density of 1 x 10^5 in 25 cm^2 ml flasks or 0.5 x 10^5 on sterile coverslips (22 mm x 22 mm) (VWR International) in 6 well plates (Greiner Bio-one), grown until 60-80% confluent then treated continuously with the relevant NSAID or stimulus in low serum (0.5% FCS) medium for a further 16 hrs or as specified. Following treatment, cells were washed with PBS and harvested by the method appropriate to the assay (see below).

To study the effects of inhibitors on the response to NSAIDs, cells were seeded and grown as above. Once 60-80% confluent, cells were pre-treated with the required inhibitor in low serum medium for 1 hr prior to continuous treatment with NSAIDs as specified.

2.2 Generation and Amplification of Plasmids

The plasmids used in these studies and details of their construction or manufacturer are summarised in Table 2.1.

Plasmid	*Amount Used for Transfection* (per no. cells)	*Comment*	*Construction/ Manufacturer*
pCMV-β-galactosidase (pCMVβ)	3 µg/5 x 10^6 cells	pCMV-β contains an intron (splice donor/splice acceptor; 2) and polyadenylation signal from SV40, and the full-length *E. coli* β-galactosidase gene with eukaryotic translation initiation signals	Clontech Laboratories, Inc.; product number: 6177-1
3enhancer-CON-A (3x κB ConA-Luc)	6 µg/5 x 10^6 cells	The 3enhancer-CON-A vector contains three synthetic copies of the NF-κB-consensus sequence, driven by an Ig-κ chain promoter	Gift from Prof Ron Hay, University of Dundee; described in (Rodriguez *et al.*, 1996)
ConA-Luciferase (ConA-Luc)	6 µg/5 x 10^6 cells	As for 3x κB ConA-Luc but minus the NF-κB consensus sequences	Gift from Prof Ron Hay, University of Dundee; used in (Hay *et al.*, 2003)
pCMV-Luciferase (pCMV-Luc)	6 µg/5 x 10^6 cells	pCMV-Luc vector contains a single copy of the firefly luciferase gene driven by a CMV promoter	Stratagene; now discontinued
pEGFP-RelA (GFP-RelA)	6 µg/5 x 10^6 cells	RelA cDNA fused in frame to the carboxyl terminus of EGFP downstream of the CMV promoter	Gift from E. Qwarnstrom, University of Sheffield; described in (Carlotti *et al.*, 1999)
GFP-RelA(Δ27-30)	6 µg/5 x 10^6 cells	GFP-RelA with a deletion of amino acids 27 to 30	Described in (Stark and Dunlop, 2005)
pEGFP-C1	6 µg/5 x 10^6 cells	pEGFP-C1 contains a single copy of the GFPmut1 variant driven by an SV40 promoter.	Clontech Laboratories, Inc.; now discontinued
pTOPFlash	6 µg/5 x 10^6 cells	TCF reporter plasmid containing two sets (with the second set in the reverse orientation) of three copies of the TCF binding site (wild type) upstream of a Thymidine Kinase (TK) minimal promoter and Luciferase open reading frame.	Upstate Cell Signaling Solutions; product no: 21-170
pFOPFlash	6 µg/5 x 10^6 cells	TCF reporter plasmid containing two full and one incomplete copy of the Tcf binding site (mutated) followed by three copies in the reverse orientation, upstream of a TK minimal promoter and Luciferase open reading frame	Upstate Cell Signaling Solutions; product no: 21-169
WT IκBα	6 µg/5 x 10^6 cells	WT IκBα (PK-tagged) subcloned under the control of a CMV immediate early promoter into the pCDNA3.1 expression vector, which carries the prokaryotic phage T7 RNA polymerase promoter	Gift from Prof Ron Hay, University of Dundee; described in (Rodriguez *et al.*, 1996)
pCDNA3.1	6 µg/5 x 10^6 cells	Commercially available expression vector	Invitrogen Life Technologies; product number: V790-20

Table 2.1 – Plasmids Used for Transfections.

2.2.1 Transformation of Competent Cells

In order to generate a sufficient quantity of plasmid, vectors were amplified in *E. coli* cells. Each of the plasmids used carried the gene for ampicillin or kanamycin resistance, allowing selection of bacteria that were successfully transformed. A single transformed bacterial clone could then be grown in bulk and the required plasmid subsequently extracted. Initially, the vectors were transformed into *E. coli* that had been made chemically competent for transformation. The bacteria used were Subcloning Efficiency™ DH5α™ Competent Cells (Invitrogen Life Technologies Life Technologies) and the method as per manufacturers instructions. Briefly, Competent *E. coli* cells were thawed on ice prior to transformation by the gentle addition of 10-15 ng of ligated plasmid DNA to approximately 5 x 10^6 bacteria and incubating cells on ice for 30 min. Following heat shock at 42°C for 45 seconds (sec), cells were incubated on ice ice for 2 min and 900 µl SOC medium (Invitrogen Life Technologies) [2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM magnesium chloride ($MgCl_2$), 10 mM magnesium sulphate ($MgSO_4$), 20 mM glucose] added prior to incubation at 37°C for 60 min with vigorous shaking (225 rpm). An aliquot (100 µl) of the transformed bacteria was plated onto L-agar (Luria Bertani Broth (L-broth)* [0.1% tryptone, 0.05% yeast extract, 171 mM NaCl] containing 0.15% agar) plates containing the appropriate antibiotics [L-agar-Amp* (L-agar containing ampicillin (100 µg/ml)) or L-agar-Kan* (L-agar containing kanamycin (50 µg/ml))] and the plates were then incubated at 37°C overnight to allow colony formation.

2.2.2 Growth of Transformed *E. coli*

Following transformations, a single transformed *E. coli* colony was picked from the plate and grown in 2 ml L-broth containing the appropriate antibiotics [ampicillin (100 μg/ml) or kanamycin (50 μg/ml)] overnight at 37°C with vigorous shaking (225 rpm). An aliquot (500 μl) of this culture was used to innoculate 500 mls of L-broth, containing antibiotics as above, which in turn was grown overnight to allow large scale amplification of the plasmid in *E. coli*. Transformed *E. coli* colonies were stored on L-agar plates at 4°C for up to one month or at -70° in L-broth containing 50% glycerol for longer term storage. Strains were re-isolated from glycerol stocks by spreading some of the frozen cells using a sterile loop onto an L-agar plate containing the appropriate antibiotic and then incubating the plate overnight at 37°C.

2.2.3 Plasmid Preparation

Plasmids were harvested from bacterial cells using a purification kit (Qiagen) and reagents provided by the manufacturer according to their instructions. Briefly, cells were collected by centrifugation at 6,000 x *g* for 15 min at 4°C. Cell pellet was then resuspended in 10 ml chilled buffer P1 [10 mM ethylenediaminetetraacetate (EDTA), 50 mM Tris(hydroxymethyl)methylamine (Tris)-HCl (pH 8.0), 100μg/ml ribonucleaese A (RNase A)] and subsequently lysed with 10 ml buffer P2 [200mM NaOH, 1% sodium dodecyl sulphate (SDS)] to allow disruption of the bacterial cell

wall. This alkaline lysate was neutralised with 10 ml chilled buffer P3 [3.0 M potassium acetate ($C_2H_3KO_2$) (pH 5.5)] and then incubated on ice for 20 min. Cell debris was removed by centrifugation at 20,000 x *g* for 30 min. The soluble fraction (including the plasmid DNA) was applied to a pre-equilibrated QIAGEN-tip 500, which contains a specific Anion-Exchange resin. The plasmid DNA adhered to the resin whilst the remaining debris, such as RNA, protein and metabolites, was removed by washing the tip with 60 ml buffer QC [1.0 M NaCl, 50 mM 4-morpholinepropanesulfonic acid (MOPS) (pH 7.0), 15% isopropanol]. The plasmid DNA was detached from the resin by the addition of buffer QF [1.25M NaCl, 50mM Tris-HCl (pH 8.5), 15% isopropanol], precipitated with isopropanol and immediately pelleted by centrifugation at 15,000 x *g* for 30 min at 4°C. The DNA was then washed with 70% EtOH, re-pelleted by centrifugation at 15,000 x *g* for 10 min at 4°C and allowed to air-dry before being dissolved in 300 µl TE buffer* [10mM Tris-HCl (pH 8.0), 1mM EDTA] and stored at -20°C.

Plasmid DNA was quantified by optical densomitery on a UV spectrophotometer (GeneQuant Pro, Biochrom Ltd.). 1 µl DNA was diluted with 99 µl dH_2O and placed in quartz cuvettes. DNA quantities were calculated automatically from the measured absorbency at 260 nm (A^{260}) and 280 nm (A^{280}), taking into account the dilution factor of 100. An A^{260}/ A^{280} ratio of 1.8 was taken as optimum purity of DNA.

It is noteworthy that endotoxin-free plasmid purification kits are now available from Qiagen and these should be used in the future to minimise the possibility of contamination of DNA samples, and hence experiments, with such

toxins. Endotoxins, for example LPS, are known to activate NF-κB and so contamination with these agents could potentially affect the results of experiments.

2.3 Transient Transfection of Adherent Cells

Cells were grown in 25 cm^2 flasks or on sterile coverslips in the wells of 6 well plates until 50-60% confluent prior to transfection. Transient transfection of cells with plamid DNA was carried out using Lipofectin (Invitrogen Life Technologies) as per manufacturers instructions. Briefly, for each transfection, 20 μl lipofectin was added to 200 μl optimem (Gibco BRL) and left for 45 min at room temperature. 200 μl optimem was added to the plasmid DNA (3-6 μg) to be used for transfection prior to the addition of 200 μl of the lipofectin/optimem mix. Samples were then left for 15 min at room temperature before a further 1.6 ml optimem was added to each sample. Cells were washed twice with 2 ml optimem before 2 ml DNA mix was added. Cells were then incubated for 5-6 hrs at 37°C. After this incubation, the DNA mix was discarded and cells were grown for 24hrs in the appropriate low serum medium before being harvested or treated as described in section 2.1.3.

For overexpression of wild type (WT) IκBα, SW480 cells were transiently transfected with WT IκBα or pCDNA3.1 (mock) using lipofectin as described above. After transfection, cells were incubated for 36 hrs in high serum medium (10 % FCS), to allow expression of WT IκBα or pCDNA3.1, prior to treatment as specified.

2.4 Reporter Assays

Cells were transiently transfected with 6 μg of a luciferase reporter vector (either ConA-Luciferase (ConA-Luc), 3x κB ConA-Luc, pCMV-Luciferase (pCMV-Luc), pTOPFlash or pFOPFlash) and 3 μg of the pCMV-β-galactosidase (pCMVβ) reporter vector as described above, prior to treatment as specified. Luciferase and β-galactosidase activity were then measured in cell extracts using a luciferase reporter assay kit (Promega) and a β-galactosidase assay kit (Promega), as per manufacturers instructions. Briefly, transfected cells were washed twice with 2 ml PBS then adherent cells lysed with 500 μl 1 X Reporter Lysis Buffer (made by diluting 5 X Reporter Lysis buffer (Promega) in dH_20) for 15 min at room temperature. Lysate was then scraped, placed into a 1.5 ml eppendorf and vortexed prior to centrifugation at 15,800 x *g* for 1 min. The supernatant was then collected and assayed.

To determine relative light units of β-galactosidase activity, 50 μl reporter cell extract was added to 50 μl 2 X assay buffer (Promega) in a well of a 96 well plate (Sero-Wel, Bibby Sterilin Ltd.). The plate was incubated at 37°C for 30 min (or until a yellow colour appeared) then the reaction stopped by adding 150 μl sodium carbonate (Promega). The β-galactosidase activity was determined by measuring the the absorbance of the samples at 420 nm using an ELISA plate reader (Labsystems, Original Multiskan MS).

Luciferase activity was measured by adding 50 μl luciferase reagent (Promega) to 10 μl reporter cell extract using a Lumat LB 9507 (EG + G Berthold) luminometer. Relative luciferase activity was calculated as unit of luciferase activity

per unit of β-galactosidase activity. Where used, FOPFlash activity was expressed relative to TOPFlash activity.

2.5 Preparation of Protein Extracts

2.5.1 Cytoplasmic and Nuclear Extracts

Treated cells were washed with 2ml PBS prior to harvesting by scraping cells into 5 ml PBS. Cells were subsequently pelleted by centrifugation at 200 x *g* for 5 min and then lysed for five min at 4°C in approximately 3 X cell volume of lysis buffer [50 mM NaCl, 10 mM N-(2 hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES) (pH 8.0), 500 mM sucrose, 1mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, 0.2% triton X-100] containing Complete™ protease inhibitor cocktail (Roche Applied Science) at 1:1250 dilution, 1 mM pepstatin A (Sigma) and 100 mM Pefabloc SC (Roche Applied Science). Phosphatase inhibitors [100 μM sodium fluoride (NaF), 10 mM β-glycerophosphate, 10 mM Na_3VO_4] were also added where appropriate, that is for the analysis of phosphorylated proteins. Nuclei were pelleted by centrifugation at 3,300 x *g* for 20 min at 4°C and the supernatant (cytoplasmic extract) collected and stored at -20°C prior to analysis.

Nuclear extracts were obtained by resuspending and lysing nuclei in approximately 1.5 X nuclear volume of hypotonic buffer [350 mM NaCl, 10 mM HEPES, 25% glycerol, 0.1mM EDTA, 0.5 mM spermidine, 0.15 mM spermine] containing protease inhibitors, pepstatin A and Pefabloc SC as per above for

cytoplasmic extracts, and then incubating on ice for 30 min. Debris was cleared by centrifugation at 15,800 x *g* for 5 min at 4°C and the supernatant (nuclear extract) collected and stored at -20°C prior to analysis.

2.5.2 Whole Cell Extracts

Treated cells were washed twice with 2 ml PBS then adherent cells lysed in approximately 4 X cell volume whole cell lysis buffer [50 mM HEPES (pH 7.4), 150 mM NaCl, 20 mM EDTA, 0.5% NP-40 (Calbiochem)], containing Complete™ protease inhibitor cocktail at 1:1250 dilution, 1 mM pepstatin A, 100 mM Pefabloc SC, 1 mM PMSF and phosphatase inhibitors [100 μM NaF, 10 mM β-glycerophosphate, 10 mM Na_3VO_4] (where appropriate), for 30 min at 4°C. Cell lysate was then scraped off, placed into 1.5 ml eppendorfs and centrifuged at 10,000 x *g* for 15 min at 4°C. The supernatant (whole cell extract) was subsequently collected and stored at -20°C prior to analysis.

2.6 Calculation of Protein Content by Bradford Assay

Bradford Reagent (Bio-RAD) was diluted 1 : 5 with dH_20 and filtered using Whatman filter paper (27.0 cm) prior to use. 1 μl protein sample was added to 200 μl Bradford Reagent in the wells of a 96 well plate. A concentration curve was included in each experiment using BSA standards (0.2 μg/μl, 0.5 μg/μl, 0.8 μg/μl, 1.0 μg/μl,

1.5 μg/μl and 2.0 μg/μl). The standards were loaded in duplicate and samples loaded in triplicate. Following a 10 min incubation at room temperature, the absorbance was measured at 595 nm (A^{595}) using an ELISA (enzyme-linked immunosorbent assay) plate reader. The protein concentration was calculated from the A^{595} automatically by the plate reader, taking into account the standards. The average of the 3 readings for each sample was determined and subsequently used.

2.7 SDS Polyacrylamide Gel Electrophoresis (PAGE)

A tenth of the volume of Sample Buffer [20% glycerol, 2% SDS, 0.25% bromophenol blue, 1 X Stacking Buffer [500 mM Tris, 0.4% SDS, pH 6.8], 5% β-mercaptoethanol] was added to protein samples (10-30 μg) prior to boiling at 95°C for 5 min and then placing on ice. The Protean II mini-gel apparatus (Bio-RAD) and a two tier gel system, comprising a lower resolving gel and upper stacking gel, were used to resolve protein extracts by denaturing SDS PAGE. Resolving gels were 8% polyacrylamide, 8.5% polyacrylamide, or 10 % polyacrylamide. Stacking gels were 4% polyacrylamide. 10μl of Kaleidoscope pre-stained molecular weight standards (Bio-RAD) were loaded in a single well of each gel and run in parallel with protein samples to aid the identification of proteins of interest. Gels were electrophoresed in 1 X Running Buffer [25 mM Tris, 0.2 M glycine, 0.1% SDS] at 160 volts for approximately 1 hr.

2.7.1. Resolving Gel Monomer Solutions

	8%	8.5%	10%
dH_2O	4.02 ml	3.93 ml	3.65 ml
40% Acrylamide/ Ratio: 19:1 – bis acrylamide (Severn Biotech Ltd.)	1.48 ml	1.57 ml	1.85 ml
4 X Resolving Buffer [1.5 M Tris, 0.4% SDS, pH 8.8]	1.9 ml	1.9 ml	1.9 ml

N.B. Immediately before pouring, 112 µl 10% ammonium persulfate (APS) and 5 µl N,N,N',N'-tetramethylethylenediamine (TEMED) were added to the solution.

2.7.2 Stacking Gel Monomer Solution

	4%
dH_2O	1.081 ml
40% Acrylamide/ Ratio: 19:1 – bis acrylamide	0.218 ml
4 X Stacking Buffer	0.444 ml

N.B. Immediately before pouring, 28 µl 10 % APS and 5 µl TEMED were added to the solution.

2.8 Western Blot Analysis

Antibodies used for Western blot analysis and details of the host species they were raised in, dilutions, incubation time and manufacturer are summarised in Table 2.2. Western blot analysis was carried out using standard procedures. Briefly, protein extracts were resolved on SDS PAGE gels as described in section 2.7. Proteins were then transferred to PVDF membrane (Bio-RAD) using a semi-dry blotter (Bio-RAD) with 1 X semi-dry transfer buffer [47 mM Tris, 40 mM glycine, 0.037% SDS, 100 mM methanol] at 10 volts for 30 min as per manufacturers instructions. The membrane was then blocked in PBS containing 0.1% TWEEN® 20 (Sigma) (PBST) plus 5 % dried milk (PBSTM) for one hr at room temperature or overnight at 4°C prior to the addition of primary antibody for 1 hr at room temperature or overnight at 4°C. The membrane was subsequently washed with PBST (3 X 10 min) before being incubated with the appropriate secondary antibody conjugated to horseradish peroxidase for 30 min at room temperature. The membrane was then washed with PBST (3 X 10 min) before detection of specifically bound antibody by chemiluminescence. ECL reagents (Santa Cruz Biotechnology) were mixed 1 : 1, applied to the membrane for 1 minute, excess removed then the membrane covered with cling film and exposed to Hyperfilm (Amersham Biosciences) for 10 sec to 10 min. Films were subsequently developed using an SRX-101A X-ray processor (Konica Minolta, Medical + Graphic Inc.).

Antibody	Host Species and Antibody Type	Dilution	Incubation Time	Manufacturer
Anti-IκBα	Sheep (Polyclonal)	1 : 4000 in PBSTM (PBS + 0.1% TWEEN®20 (Sigma) + 5% dried milk)	1 hr at room temperature (RT)	Gift from Ron Hay
Anti-IκBα	Rabbit (Polyclonal)	1: 1000 in PBSTM	1 hr at RT	Santa Cruz
Anti-Cu/Zn SOD	Sheep (Polyclonal)	1 : 4000 in PBSTM	1 hr at RT	The Binding Site
Anti-β-catenin	Mouse (IgG) (Monoclonal)	1 : 2000 in PBSTM	1 hr at RT	BD Transduction Laboratories
Anti-β-catenin	Goat (Polyclonal)	1 : 1000 in PBSTM	1 hr at RT	Santa Cruz Biotechnology
Anti-Actin	Mouse (IgM) (Monoclonal)	1 : 15,000 in PBSTM	1 hr at RT	Calbiochem
Anti-Phospho-tyrosine	Mouse (IgG) (Monoclonal)	1 : 500 in PBSTM	1hr at RT	Upstate Cell Signaling Solutions
Anti-PK Tag	Mouse (IgG) (Monoclonal	1 : 1000 in PBSTM	1 hr at RT	TCS Cell Works
Anti-RelA	Rabbit (Polyclonal)	1 : 1000 in PBSTM	1 hr at RT	Santa Cruz Biotechnology
Anti-RelA	Mouse (IgG) (Monoclonal)	1 : 1000 in PBSTM	1 hr at RT	Santa Cruz Biotechnology
Anti-Caspase-3	Goat (Polyclonal)	1 : 4000 in PBSTM	1 hr at RT	R & D Systems
Anti-c-Src	Mouse (IgG) (Monoclonal)	1 : 500 in PBSTM	16 hrs at 4 °C	Upstate Cell Signaling Solutions
Anti-Phospho-c-Src (Tyr 139)	Rabbit (Polyclonal)	1 : 200 in PBSTM	16 hrs at 4°C	Upstate Cell Signaling Solutions
Anti-Phospho-c-Src (Tyr 416)	Mouse (IgG) (Monoclonal)	1 : 500 in PBSTM	16 hrs at 4°C	Upstate Cell Signaling Solutions
Anti-Ubiquitin	Rabbit (Polyclonal)	1 : 1000 in PBSTM	1 hr at RT	Dako Cytomation
Anti-Acetylated Lysine	Rabbit (Polyclonal)	1 : 1000 in PBSTB (PBST + 5 % BSA)	16 hrs at 4°C	Cell Signaling Technology
Anti-S5a-18	Mouse (IgG) (Monoclonal)	1 : 1000 in PBSTM	1 hr at RT	Calbiochem
Anti-Mouse IgG (HRP Conjugate)	Donkey (Polyclonal)	1 : 1000 in PBSTM	30 min at RT	Amersham Biosciences
Anti-Mouse IgM (HRP Conjugate)	Goat (Polyclonal)	1 : 1000 in PBSTM	30 min at RT	Calbiochem
Anti-Sheep IgG (HRP Conjugate)	Donkey (Polyclonal)	1: 1000 in PBSTM	30 min at RT	Jackson Immunoresearch
Anti-Rabbit IgG (HRP Conjugate)	Donkey (Polyclonal)	1 : 1000 in PBSTM	30 min at RT	Amersham biosciences
Anti-Goat IgG (HRP Conjugate)	Donkey (Polyclonal)	1 : 1000 in PBSTM	30 min at RT	Santa Cruz Biotechnology

Table 2.2 – Antibodies Used for Western Blot Analysis.

2.8.1 Stripping Membranes

Membranes were stripped in a fume hood with 50 ml strip buffer [62.4 mM Tris, 2 % SDS, pH 6.7], which was pre-warmed in a glass dish to 60°C using a heat block. One or two membranes were placed into the buffer and 400 µl β-mercaptoethanol added and gently mixed prior to heating at 60°C for a further 30-45 min. The strip solution was then discarded and membranes washed with PBST (3 X 10 min) before being re-blocked in PBSTM for 1 hr or overnight at 4°C. Membranes were then re-probed with primary antibody as above.

2.9 Annexin V-Fluorescin Isothiocyanate (FITC) and AnnexinV–Biotin Apoptosis Assays

SW480, SW480-pBpuro and SW480-SrcKD cells were treated as specified. Staining for cell surface phosphatidyleserine was used as a marker for apoptosis and was carried out using an AnnexinV-FITC (non-transfected cells) or Annexin V-Biotin (transfected cells) apoptosis detection kit (Calbiochem), as per manufacturers instructions. Briefly, for both protocols, the media from a flask of treated adherent cells was transferred to a 15 ml conical tube and placed on ice. Adherent cells were then washed with PBS prior to incubation with T/V at 37°C until cells appeared to be detached. Cells were then released from flasks with firm tapping and subsequently resuspended in the media that was initially removed. The number of cells/ml was determined by haemocytometric counts then cells were resuspended at a

concentration of approximately 1 x 10^6 cells/ml. 0.5 ml cell suspension was then transferred to a 1.5 ml eppendorf along with 10 µl media binding reagent (Calbiochem) and 1.25 µl Annexin V-FITC or Annexin V-Biotin (Calbiochem). Cells were incubated for 15 min at room temperature in the dark, pelleted by centrifugation at 1,000 x *g* for 5 min at room temperature and then resuspended in 0.5 ml cold 1 X binding buffer (made by diluting 5 X binding buffer (Calbiochem) with dH_2O). The percentage of cells undergoing apoptosis in the population was determined using fluorescent microscopy. For non-transfected cells, the total number of cells per field of view was determined using brightfield microscopy then the number of apoptotic cells quantified using the FITC channel. Where the Annexin V-Biotin kit was used, 15 µl of streptavidin-texas red (TxRd) conjugate (Calbiochem) (diluted to 15 µg/ml in 1 X binding buffer) was added prior to microscope analysis. In this case, GFP expressing cells were identified in the FITC channel then the number of these cells undergoing apoptosis quantified using the TxRd channel. For all cases, at least 200 cells from a minimum of 10 independent fields of view were analysed.

2.10 Immunocytochemistry

Antibodies used for immunocytochemistry and details of the host species in which they were raised, dilutions, incubation time and manufacturer are summarised in Table 2.3. SW480, SW480-pBpuro and SW480-SrcKD cells were grown on

Antibody	*Host Species and Antibody Type*	*Dilution*	*Manufacturer*
Anti-Nucleolin (C23)	Mouse IgG (Monoclonal)	1 : 100	Santa Cruz
Anti-Fibrillarin	Mouse (IgG) (Monoclonal)	1 : 1000	Cytoskeleton, Inc.
Anti-β-catenin	Mouse (IgG) (Monoclonal)	1 : 100	BD Transduction Laboratories
Anti-β-catenin	Goat (Polyclonal)	1 : 100	BD Transduction Laboratories
Anti-RelA	Rabbit (Polyclonal)	1 : 100	Santa Cruz Biotechnology
Anti-S5a-18	Mouse (IgG) (Moniclonal)	1 : 100	Calbiochem
Anti-MTS4	Rabbit (Polyclonal)	1: 100	Gift from Dr C. Gordon, MRC Human Genetics Unit, Edinburgh
Anti-mouse IgG (FITC Conjugate)	Donkey (Polyclonal)	1 : 100	Jackson Immunoresearch
Anti-mouse IgG (TxRd Conjugate)	Donkey (Polyclonal)	1 : 100	Jackson Immunoresearch
Anti-rabbit IgG (FITC conjugate)	Donkey (Polyclonal)	1 : 100	Jackson Immunoresearch
Anti-rabbit IgG (TxRd conjugate)	Donkey (Polyclonal)	1 : 100	Jackson Immunoresearch
Anti-goat IgG (TxRd conjugate)	Donkey (Polyclonal)	1 : 100	Jackson Immunoresearch

Table 2.3 – Antibodies Used for Immunocytochemistry.

sterilised coverslips in 6 well plates until 60-80% confluent then treated as specified. After treatment, cells were washed briefly with PBS then fixed in 1 : 1 methanol : acetone at -20°C for 20min. Cells were subsequently washed with PBS (2 X 10 min) prior to incubation with 10% donkey serum (Sigma) for 30 min to block non-specific binding. After blocking, cells were incubated with primary antibody diluted in 10% donkey serum for 1 hr and washed with PBST (2 X 10 min) prior to incubation with secondary antibody diluted in 1.5% donkey serum for 30 min. Cells were washed with PBS (2 X 10 min) then coverslips mounted in Vectastain (Vector Laboratories) containing DAPI (4′,6′-diamido-2-phenylindole) (1 μg/ml) (Sigma) to stain DNA.

Stained cells were analysed using fluorescent microscopy, which was performed with a Zeiss Axioplan microscope, 63X Plan Neofluor objective and Chroma 83000 filter set. Each channel was recorded independently and pseudocolor images superimposed. Images were captured using in house scripts written for IPLab Spectrum 3.6.

2.11 Live Cell Imaging

SW480 cells were grown until 50-60% confluent and then transiently transfected with GFP-tagged expression constructs using lipofectin as described in section 2.3 prior to treatment as specified. Cells were washed briefly with PBS then analysed under 1 ml PBS using an Axiovert 100 inverted microscope (Zeiss). Phase contrast and fluorescent images were captured from random fields of view and these images represent the typical localisation of RelA within the cell population.

2.12 Immunoprecipitation

Antibodies used for immunoprecipitation and details of the host species they were raised in, dilutions, incubation time and manufacturer are summarised in Table 2.4. SW480 cells were grown in 75 cm^2 flasks until 60-80% confluent and then treated as specified. After treatment, cells were washed twice with 5 ml PBS then whole cell extracts prepared as described in section 2.5.2. The protein content of the extracts was then immediately determined by Bradford Assay as described in section 2.6.

500 µg of whole cell extract described above was added to ~ 30 µl ProteinA-sepharose (Amersham) (used for polyclonal antibodies) or Protein A/G-Sepharose (Santa Cruz Biotechnology) (used for monoclonal antibodies) beads, which had been pre-washed with 500 µl immunoprecipitation (IP) buffer [50 mM HEPES (pH 7.4), 150 mM NaCl, 20 mM EDTA, 0.5% NP-40 (Calbiochem)], containing Complete™ protease inhibitor cocktail at 1:1250 dilution, 1 mM pepstatin A, 100 mM Pefabloc SC, 1 mM PMSF and phosphatase inhibitors [100 µM NaF, 10 mM β-glycerophosphate, 10 mM Na_3VO_4] (where appropriate). Samples were rotated at 4°C for 1 hr with beads alone to reduce non-specific binding. Extracts that had been pre-cleared (as above) were added to another 30 µl ProteinA-sepharose or ProteinA/G-spepharose beads (pre-washed with 500 µl IP buffer) then the appropriate antibody [or no antibody (control)] was added to the required dilution factor. Samples were incubated with rotation at 4°C overnight then beads washed 3 X 5 min with 500 µl IP buffer followed by 2 X 5 min washes with 500 µl IP wash

Antibody	*Host Species and Antibody Type*	*Dilution*	*Manufacturer*
Anti-IκBα	Rabbit (Polyclonal)	1 : 100	Santa Cruz
Anti-β-catenin	Mouse (IgG) (Monoclonal)	1 : 100	BD Transduction Laboratories
Anti-β-catenin	Goat (Polyclonal)	1 : 50	Santa Cruz Biotechnology
Anti-RelA	Rabbit (Polyclonal)	1 : 50	Santa Cruz Biotechnology
Anti-RelA	Mouse (IgG) (Monoclonal)	1 : 50	Santa Cruz Biotechnology
Anti-c-Src	Rabbit (Polyclonal)	1 : 25	Upstate Cell Signaling Solutions

Table 2.4 – Antibodies Used for Immunoprecipitation

buffer (IP buffer without NP-40). 30 µl PBS and 3 µl 10 X Sample Buffer (see section 2.7) was added to each sample before boiling at 95°C for 5 min to release proteins from beads. Samples were then centrifuged at 10,000 x *g* for 1 min to remove beads and the supernatants resolved by SDS polyacrylamide gel electrophoresis as described in section 2.7. N.B. a small sample (30 µg) of cell extracts which had not been immunoprecipitated (inputs) were also loaded onto each gel. Western blot analysis was subsequently carried out as outlined in section 2.8 to detect proteins of interest.

2.13 Detection of Tyrosine Phosphorylated IκBα by Mass Spectrometry

Whole cell extracts were prepared from treated cells, then immunoprecipitation using anti-IκBα antibody carried out as described in sections 2.5.2 and 2.12 respectively. Samples were then resolved by SDS PAGE as described in section 2.7. Recombinant IκBα (gift from Ron Hay, University of Dundee) (2.5 µg) was loaded onto each gel alongside samples to aid identification of the band containing immunoprecipitated IκBα. After electrophoresis, the gel was placed in a clean tray and washed with dH_20 (3 X 5 min) with gentle shaking. To stain proteins, the gel was incubated with 20 ml GelCode® Blue Stain Reagent (Pierce), again with gentle shaking. After staining, the gel was de-stained by washing with dH_20 for a 1-2 hr period, changing the water several times. Once de-stained, the relevant bands were

cut out from the gel in a fume hood using a clean scalpel and placed in 1.5 ml eppendorfs prior to freezing at -70°C.

Samples to be examined by mass spetrometry were sent to Dr Alexander Henzing at the University of Edinburgh Chemistry Department, where he carried out the analysis. Briefly, matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectrometry was employed and polypeptides identified by peptide mass fingerprinting and sequence database searching as previously described (Jensen *et al.*, 1997). In-gel digestion of excised polypeptide bands was performed with trypsin as previously documented (Shevchenko *et al.*, 1996), followed by sample preparation using miniaturized sample concentration/desalting techniques as previously reported (Gobom *et al.*, 1999). For the mass spectrometric analysis, a PerSeptive Biosystems Voyager DE™STR MALDI-ToF mass spectrometer (Applied Biosystems) was used. Peptide ion signals were assigned with a mass error less than 50 parts per million. Lists of tryptic peptide masses were used to search protein sequence databases using the ProFound tool (http://www.unb.br/cbsp/paginiciais/profound.htm)

2.14 *In Vitro* Kinase Assays

c-Src kinase was immunoprecipitated from 500 µg whole cell extracts using anti-c-Src rabbit polyclonal antibody (Santa Cruz Biotechnology) as described in section 2.12. After beads were washed with 500 µl IP buffer (3 X 5 min), the beads were washed again with 500 µl kinase assay buffer [20 mM HEPES (pH 7.0), 6 mM

$MgCl_2$, 20 mM Na_3VO_4] (2 X 5 min). *In vitro* Kinase assays were carried out by standard methods. Briefly, where untagged recombinant IκBα (gift from Prof Ron Hay, University of Dundee) was used as a substrate, 5 μg of this substrate was added to the washed beads in the presence of 19 μl kinase reaction mix [18.5 μl kinase assay buffer, 0.5 μl 100 mM adenosine triphosphate (ATP)] to give a final volume of 25 μl. Reactions were then incubated for 30 min at room temperature and subsequently stopped by the addition of 2.5 μl 10 X Sample Buffer (as per section 2.7) and boiling at 95°C for 5 min. Samples were then centrifuged at 1000 x *g* for 1 min to remove the beads and supernatants resolved by SDS PAGE as described in section 2.7 on 10% polyacrylamide gels. Western blot analysis using anti-phospho-tyrosine antibody was carried out as described in section 2.8 to determine the extent of c-Src mediated tyrosine phosphorylation of IκBα.

Where GST-tagged recombinant IκBα (Santa Cruz Biotechnology) and synthetic c-Src substrate (Santa Cruz Biotechnology) were used in kinase assay reactions as substrates, the samples were prepared as outlined above but in screw cap eppendorfs. 5 μg of the appropriate substrate was added to washed beads in the presence of 20 μl kinase reaction mix (19.5 μl kinase assay buffer, 0.25 μl 100 mM ATP) containing 0.25 μl [^{32}P]γATP (~2.5 μCi) (Amersham Biosciences) to give a final volume of 25 μl. Reactions were incubated for 30 min at room temperature then samples centrifuged at 1000 x *g* for 1 min to remove beads and the supernatant removed and placed into a new screw cap eppendorf. Proteins were then precipitated by adding 45 μl cold 10% Trichloroacetic Acid (Riedel de-Haën), vortexing, standing for 5 min and then placing back on ice. 5 μl of the total kinase reaction was spotted independently onto P81 phosphocellulose cation-exchange paper (Whatman)

to allow calculation of the specific activity of ATP in the kinase reaction (in cpm/pmol). 35 µl of each reaction was then spotted independently onto P81 phosphocellulose cation-exchange paper prior to washing three times with 500 µl cold 0.5% phosphoric acid and then once with 500 µl acetone to eliminate low specificity/non-specific binding. Filters were then left to dry prior to placing into Wheaton liquid scintillation vials (Wheaton Scientific) and adding 2 ml Fluoran Safe XE scintillation fluid (BDH Laboratory Supplies). ^{32}P incorporation was then measured by scintillation counting using an LS 6500 Multi-Purpose Scintillation Counter (Beckman Coulter™). Counts per minute obtained in the kinase reaction [minus blank (no substrate control)] were divided by the specific activity to determine the moles of phosphate transferred in each reaction.

Chapter 3 – Effects of NSAIDs on NF-κB Pathway

3.1 Introduction

The NF-κB pathway was identified in the host laboratory as being a central component of the mechanism responsible for aspirin-induced apoptosis in colorectal cancer cells (Stark *et al.*, 2001; Stark and Dunlop, 2005). As discussed in Chapter 1, aspirin inhibits both COX-1 and COX-2 and there is substantial evidence that inhibition of COX-2 in particular is implicated in the anti-neoplastic effects of this agent and other related NSAIDs [reviewed in (Brown and DuBois, 2005; Backlund *et al.*, 2005; Kawai *et al.*, 2002; Patrignani, 2000)]. It is therefore important to determine whether the NF-κB effect observed in response to aspirin is related to COX inhibition. This was addressed here by studying NF-κB signaling in response to NSAIDs that have COX-2 non-selective activity and also an NSAID that lacks COX-inhibitory effects. The panel of NSAIDs used in this research comprised: aspirin, sulindac and indomethacin (non-selective COX-inhibitors); and sulindac sulfone, an oxidative metabolite of sulindac (lacks COX-inhibitory activity). The chemical structures of these agents are shown in Figure 3.1. It is noteworthy that aspirin (acetylsalicylic acid) is a member of the salicylate family whereas indomethacin, sulindac and sulindac sulfone are members of the arylalkanoic acid family and have a greater molecular mass and more complex chemical structure than aspirin.

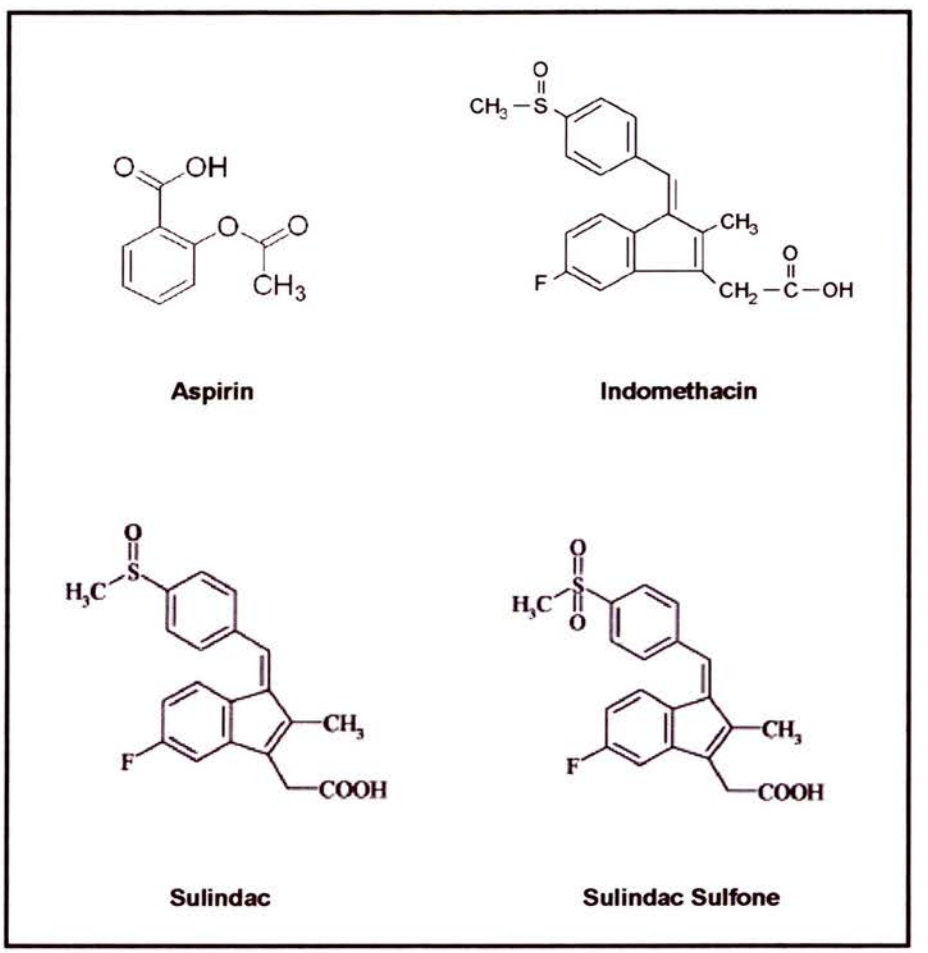

Figure 3.1 – Chemical Structures of NSAIDs Used in Study.

In addition to their COX-inhibitory profiles, it is important to emphasize that the panel of NSAIDs chosen for this study have all been previously shown to have a protective effect against colorectal cancer (see Chapter 1). Significantly, the results of three recent randomised trials have reported that low-dose aspirin can prevent the recurrence of adenomas in patients with previous colorectal cancer (Baron *et al.*, 2003; Sandler *et al.*, 2003; Benamouzig *et al.*, 2003). Three randomised control trials have demonstrated that sulindac reduces the number and size of colorectal adenomas in patients with FAP (Labayle *et al.*, 1991; Nugent *et al.*, 1993; Giardiello *et al.*, 1993). A recent randomised control trial reported that sulindac sulfone caused considerable regression of sporadic adenomatous polyps (Arber *et al.*, 2006). To date, no randomised trials specifically looking at the treatment of colorectal cancer with indomethacin have been carried out. However, a randomised, placebo-controlled trial found that indomethacin prolonged mean survival in patients with disseminated solid malignancy, 22% of which were colorectal cancer cases (Lundholm *et al.*, 1994). Support for the protective effects of aspirin sulindac, sulindac sulfone and indomethacin has also come from animal and *in vitro* studies (see Chapter 1). These NSAIDs have all been shown to reduce the size and number of polyps in chemical-induced models of colorectal cancer (Barnes and Lee, 1998; Chiu *et al.*, 1997; Mahmoud *et al.*, 1998; Reddy *et al.*, 1993; Haanen, 2001; Pollard and Luckert, 1981), and induce cell cycle arrest (Shiff *et al.*, 1995; Shiff *et al.*, 1996; Xu and Zhang, 2005) and apoptosis (Stark *et al.*, 2001; Haanen, 2001; Piazza *et al.*, 1995; Smith *et al.*, 2000) of colorectal cancer cells *in vitro*.

The objective of this research strand was to investigate the effects of non-aspirin NSAIDs on NF-κB signaling in SW480 colon cancer cells. The specific aim

was to establish whether sulindac, sulindac sulfone and indomethacin could induce apoptosis of colorectal cancer cells via modulation of the NF-κB signaling pathway in a similar fashion to aspirin. In addition to answering the question of COX-dependency, it is important to understand the effects of non-aspirin NSAIDs in terms of identifying potential pathways affected by these agents. Such understanding could lead to the design of new therapeutic agents. Furthermore, this work could further extend understanding about the generality of modulation of the NF-κB signaling pathway by NSAIDs as a class in the context of their effects on prevention and/or treatment of colorectal cancer.

3.2 Results

3.2.1 NSAIDs Induce Apoptosis of SW480 Colorectal Cancer Cells

The first objective was to confirm that the non-aspirin NSAIDs induce apoptosis in SW480 colorectal cancer cells. Annexin V-FITC apoptosis assays were employed to detect cells showing externalisation of phosphatidylserine, a marker for apoptosis, in response to treatment with sulindac (0-500 μM), sulindac sulfone (0-500 μM), indomethacin (0-250 μM) and aspirin (0-5 mM) (for comparison). It is noteworthy that the concentrations of NSAIDs used in this study are comparable to those published in other studies (Piazza *et al.*, 1995; Chan *et al.*, 1998; Qiao *et al.*, 1998a; Smith *et al.*, 2000). Figure 3.2A indicates that all of the NSAIDs induce

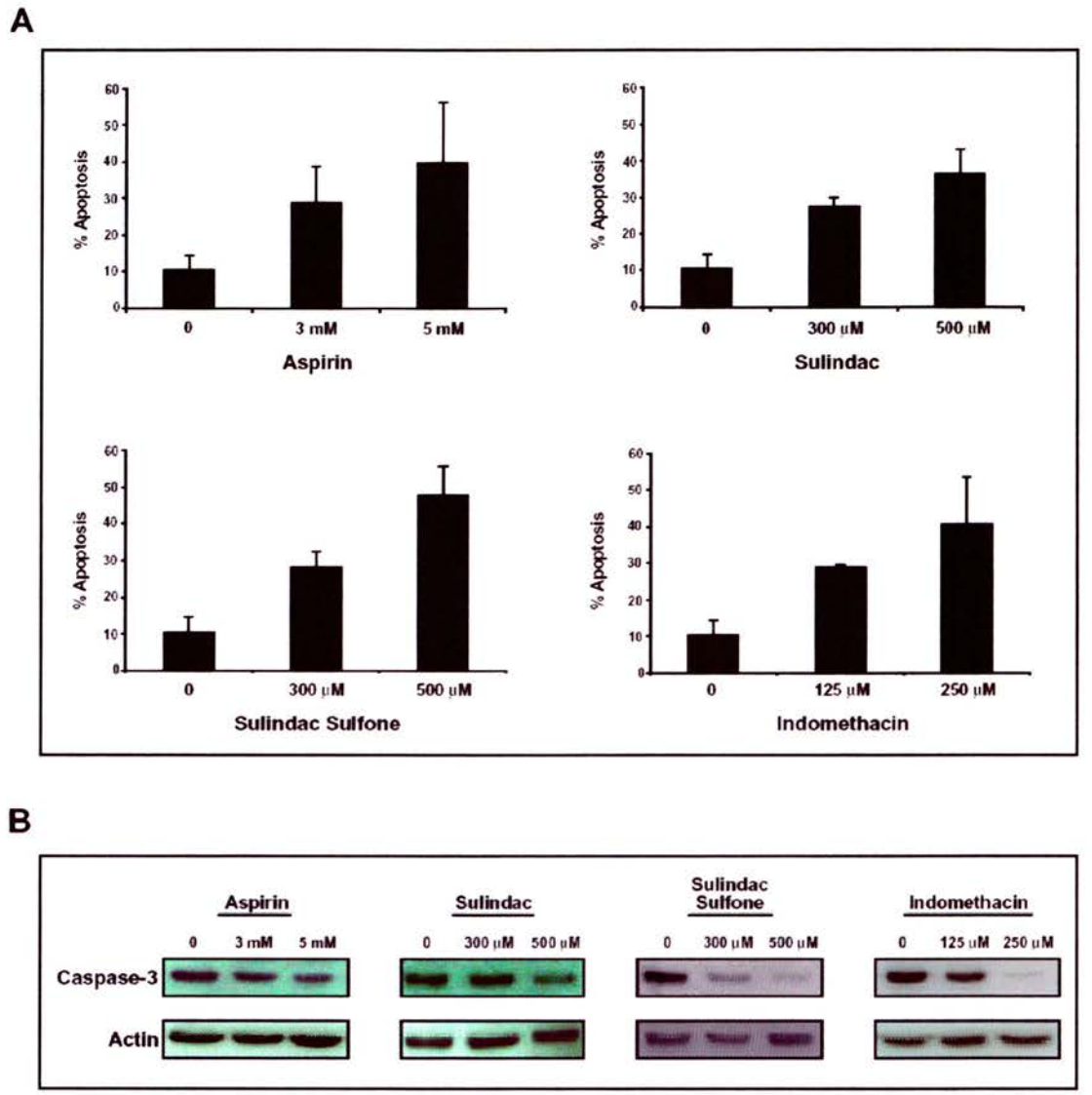

Figure 3.2 – NSAIDs Induce Apoptosis of SW480 Colorectal Cancer Cells. **(A)** SW480 colon cancer cells were treated with Aspirin (0, 3 mM, 5 mM), Sulindac (0, 300 µM, 500 µM), Sulindac Sulfone (0, 300 µM, 500 µM) or Indomethacin (0, 125 µM, 250 µM). The percentage of apoptotic cells was determined using fluorescent microscopy, in a minimum of 200 cells from multiple fields of view, to detect Annexin V-FITC binding to externalised phosphatidyl serine. The values for non-treated cells were pooled together and the results presented are the mean of three independent experiments (+/- standard deviation). **(B)** SW480 cells were treated overnight (16 hrs), in three independent experiments, with Aspirin (0, 3 mM, 5 mM), Sulindac (0, 300 µM, 500 µM), Sulindac Sulfone (0, 300 µM, 500 µM) or Indomethacin (0, 125 µM, 250 µM) then cytoplasmic extracts (15 µg) resolved by SDS PAGE on 10% polyacrylamide gels and anti-Caspase-3 Western blot carried out. Actin was used as a loading control. Representative blots are shown.

apoptosis in a concentration-dependent manner compared to non-treated controls. To further verify NSAID-induced apoptosis in SW480 colorectal cancer cells, Western blot analysis was used to examine cytoplasmic caspase-3 levels, an established marker of apoptosis (Borner, 2003; Thorburn, 2004). Figure 3.2B demonstrates that there is a dose-dependent cleavage of caspase-3 in response to all the NSAIDs, verifying that these drugs do indeed induce apoptosis in SW480 colorectal cancer cells.

3.2.2 NSAIDs Induce Nucleolar Accumulation of RelA

Having confirmed that the panel of NSAIDs induce apoptosis, the next goal was to determine whether this was associated with activation of the NF-κB pathway. Since previous data indicates that aspirin mediates nucleolar translocation of RelA (Stark and Dunlop, 2005), immunocytochemistry was used to examine the subcellular localisation of RelA in SW480 colon cancer cells treated with the panel of NSAIDs. RelA was found to be predominantly cytoplasmic in untreated control cells (Figure 3.3). However, in response to each of the NSAIDs, RelA accumulated in the nucleoplasm and localised to distinct sub-nuclear bodies. These nuclear bodies were observed within areas devoid of DAPI staining and co-localised with nucleolin (C23), a critical structural nucleolar protein. This provides strong evidence that RelA does indeed accumulate in the nucleolus in response to NSAIDs (see Red arrows in Figure 3.3 for good examples of nucleolar RelA). Moreover, these data indicate that

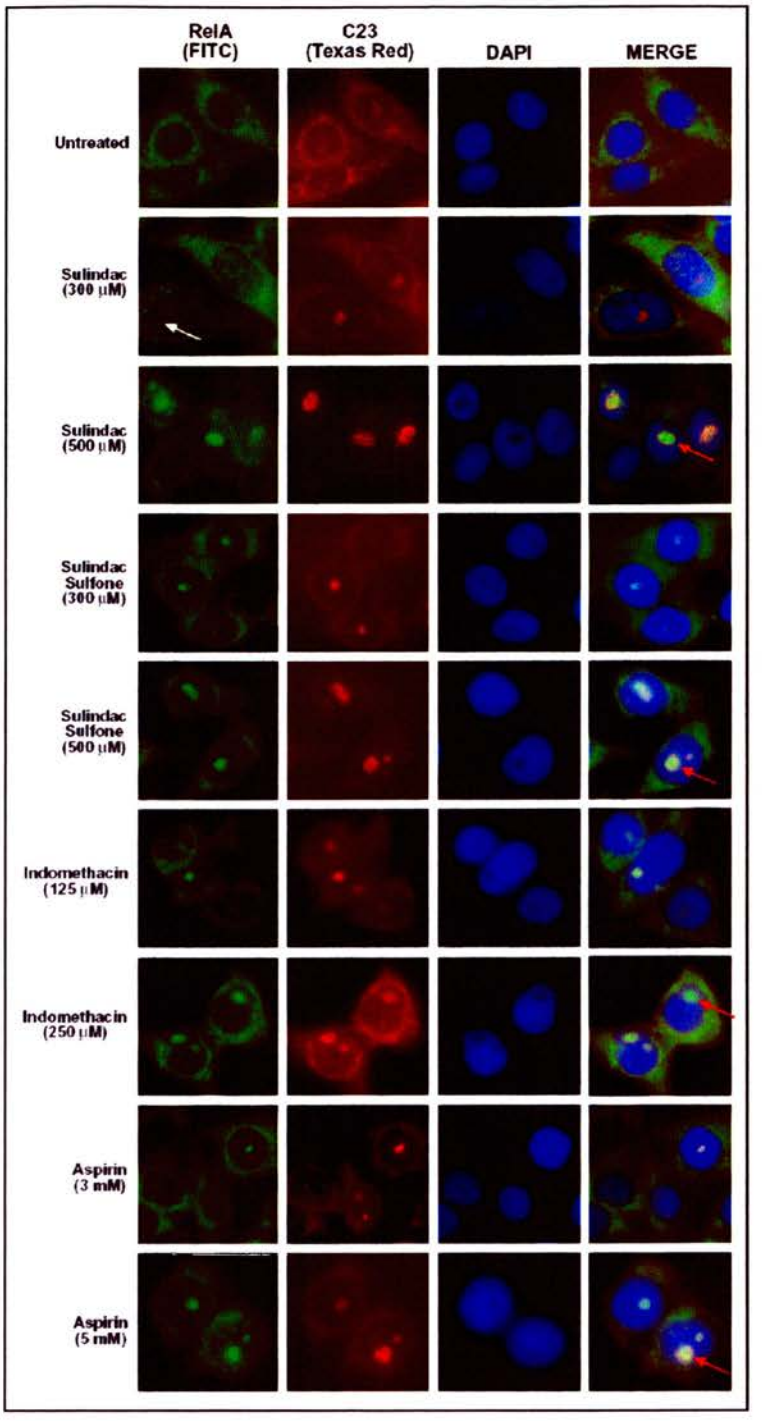

Figure 3.3 – NSAIDs Induce Nucleolar Translocation of RelA. In three independent experiments, SW480 cells were treated for 16 hrs with Sulindac (300 µM, 500 µM), Sulindac Sulfone (300 µM, 500 µM), Indomethacin (125 µM, 250 µM), Aspirin (3 mM, 5 mM) or untreated (control). Representative micrographs (63 x) illustrating localisation of RelA and the nucleolar protein, nucleolin (C23), are shown. DAPI depicts DNA. White arrow indicates nuclear RelA that is excluded from the nucleolus. Red arrows highlight examples of nucleolar RelA.

activation of the NF-κB pathway and nucleolar translocation of RelA is not a restricted response to aspirin, but is induced by multiple NSAIDs. It is noteworthy that the response seems to vary slightly with the different NSAIDs. The lower dose of sulindac (300 μM) induced mainly nuclear RelA (see white arrow in Figure 3.3) whereas a higher concentration (500 μM) was required for nucleolar sequestration of RelA. This is in keeping with previous data showing that in response to aspirin, RelA is nuclear then nucleolar with increasing concentration (Stark and Dunlop, 2005). In contrast, the lower doses of aspirin (3 mM), sulindac sulfone (300 μM), and indomethacin (125 μM) were all sufficient to cause nucleolar sequestration of RelA. It is also of interest that there was more distinct cleavage of caspase-3 with the lower doses of aspirin (3 mM), indomethacin (125 μM) and particularly sulindac sulfone (300 μM), than was observed for sulindac (300 μM).

3.2.3 NSAID-mediated Nucleolar Accumulation of RelA is Associated with Repression of NF-κB Transcriptional Activity

In light of the observation that RelA accumulates in the nucleolus in response to treatment with non-aspirin NSAIDs, the next focus of investigation was to determine whether, as with aspirin, this compartmentalization regulates nuclear NF-κB transcriptional activity. Thus, SW480 colon cancer cells were transiently transfected with the 3x κB ConA-Luc NF-κB-dependent luciferase reporter plasmid, along with the pCMVβ control plasmid, then treated with aspirin, sulindac, sulindac

sulfone and indomethacin as per the previous experiments. All the NSAIDs were found to induce a significant *decrease* in levels of relative NF-κB-driven luciferase activity, compared to untreated controls (Figure 3.4A). This decrease was dependent upon NSAID concentration and ranged from 2.5 fold [Aspirin (3 mM)] to 25 fold [Indomethacin (250 μM)]. It is noteworthy that a greater repression was seen with sulindac sulfone and indomethacin compared to sulindac or aspirin. Significantly, sulindac, sulindac sulfone and indomethacin had a minimal effect on relative luciferase activity when cells were transfected with a pCMV-Luc plasmid, indicating a considerable degree of specificity in the observed decrease in transcription from the 3x κB ConA-Luc reporter construct in response to these agents (Figure 3.4B). It should be pointed out that the pCMV-Luc experiment shown in Figure 3.4B was performed by Dr L. Stark. The specificity of the aspirin effect on repression of NF-κB-driven transcriptional activity has previously been established (Stark and Dunlop, 2005).

3.2.4 Nucleolar Accumulation of RelA is Causally Involved in NSAID-induced Apoptosis

The next goal was to confirm that apoptosis induced by non-aspirin NSAIDs was primarily caused by nucleolar translocation of RelA. Previous studies from the host laboratory identified a motif at the N-terminus of RelA that was responsible for nucleolar localisation of the protein, termed the nucleolar localisation signal (NoLS)

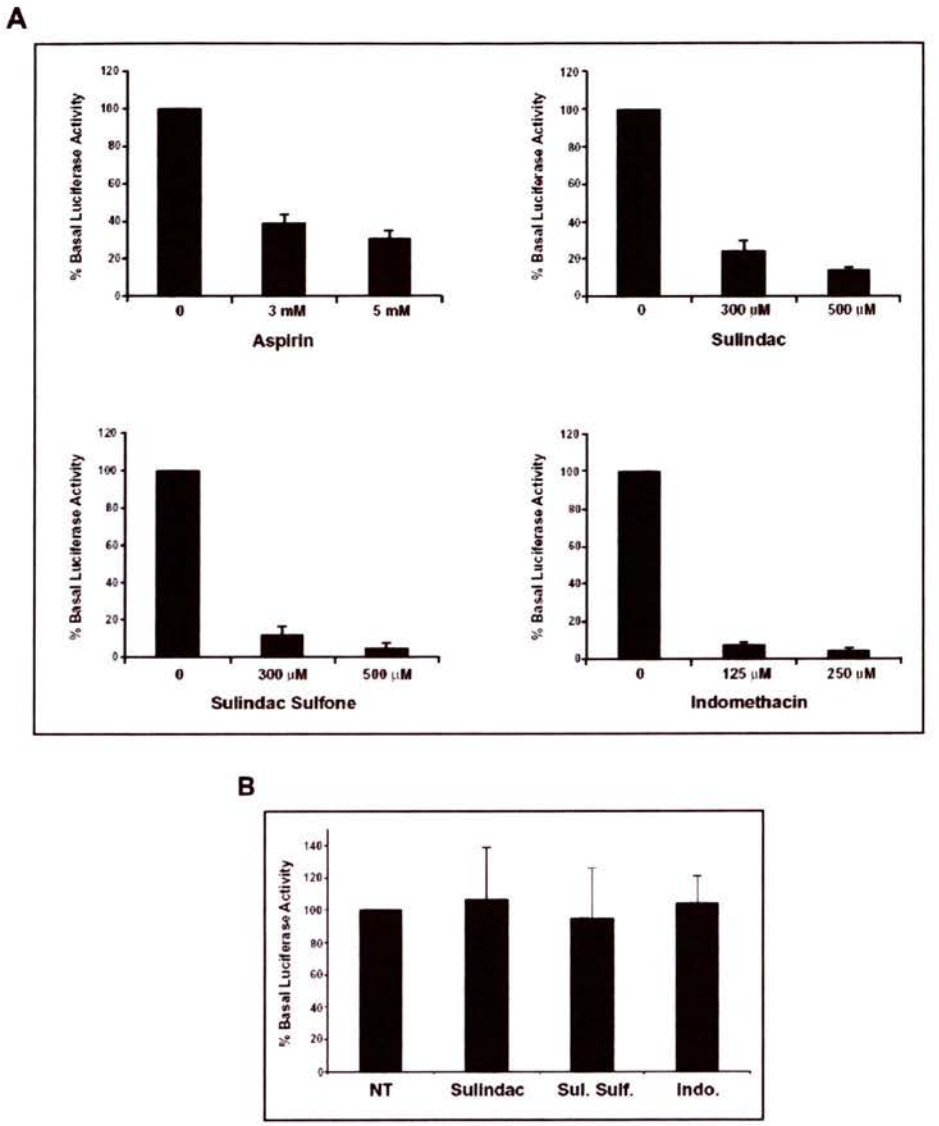

Figure 3.4 – Nucleolar Translocation of RelA is Associated with Repression of NF-κB Driven Transcription in Response to NSAIDs. **(A)** SW480 cells were transiently transfected with 3x κB ConA-Luc and pCMVβ prior to treatment with Aspirin (0, 3 mM, 5 mM), Sulindac (0, 300 µM, 500 µM) Sulindac Sulfone (0, 300 µM, 500 µM) or Indomethacin (0, 125 µM, 250 µM). **(B)** SW480 cells were transiently transfected with pCMV-Luc and pCMVβ then were treated with Sulindac (500 µM) Sulindac Sulfone (Sul. Sulf.) (500 µM) Indomethacin (Indo.) (250 µM) or non-treated (NT) (control). Luciferase assays were carried out to measure NF-κB transcriptional activity and β-galactosidase assays were used to normalise these results for transfection efficiency. % transcriptional activity was calculated relative to non-treated controls. Results presented are the mean of three **(A)** or two **(B)** independent experiments (+/- standard deviation).

N.B. Experiments outlined in **(B)** above were performed by Dr L. Stark

(Stark and Dunlop, 2005). RelA deleted for this motif [RelA(Δ27-30)] acted in a dominant negative manner in that it blocked nucleolar translocation of endogenous protein. Furthermore, expression of RelA(Δ27-30) blocked aspirin-mediated repression of NF-κB transcription and apoptosis. This dominant negative mutant was therefore used to investigate whether inhibiting nucleolar translocation of RelA also blocked apoptosis induced by sulindac, sulindac sulfone and indomethacin. It should be noted that all experiments described in this section were performed by Dr L. Stark.

The first question to be addressed was whether deleting the NoLS abrogated nucleolar translocation of RelA induced by these agents. Using live cell imaging of SW480 cells transfected with GFP-tagged WT RelA [GFP-RelA(WT)] or Δ27-30 RelA [GFP-RelA(Δ27-30)], RelA was shown to be mainly cytoplasmic after transfection of either construct but prior to NSAID exposure (Figure 3.5). However, in response to sulindac, sulindac sulfone and indomethacin, GFP-RelA(WT) showed distinct nuclear and nucleolar localisation of RelA. In contrast, GFP-RelA(Δ27-30) (lacks N-terminal NoLS), did not translocate to the nucleolus and consequently, accumulated in the nucleoplasm. This was not a non-specific effect because NSAID exposure had no effect on the localisation of GFP expressed from a construct lacking RelA sequences (data not shown). These data confirm the critical importance of the N-terminal NoLS of RelA for nucleolar translocation of the protein in response to NSAIDs. Furthermore, the effect was generic across each of the NSAIDs studied.

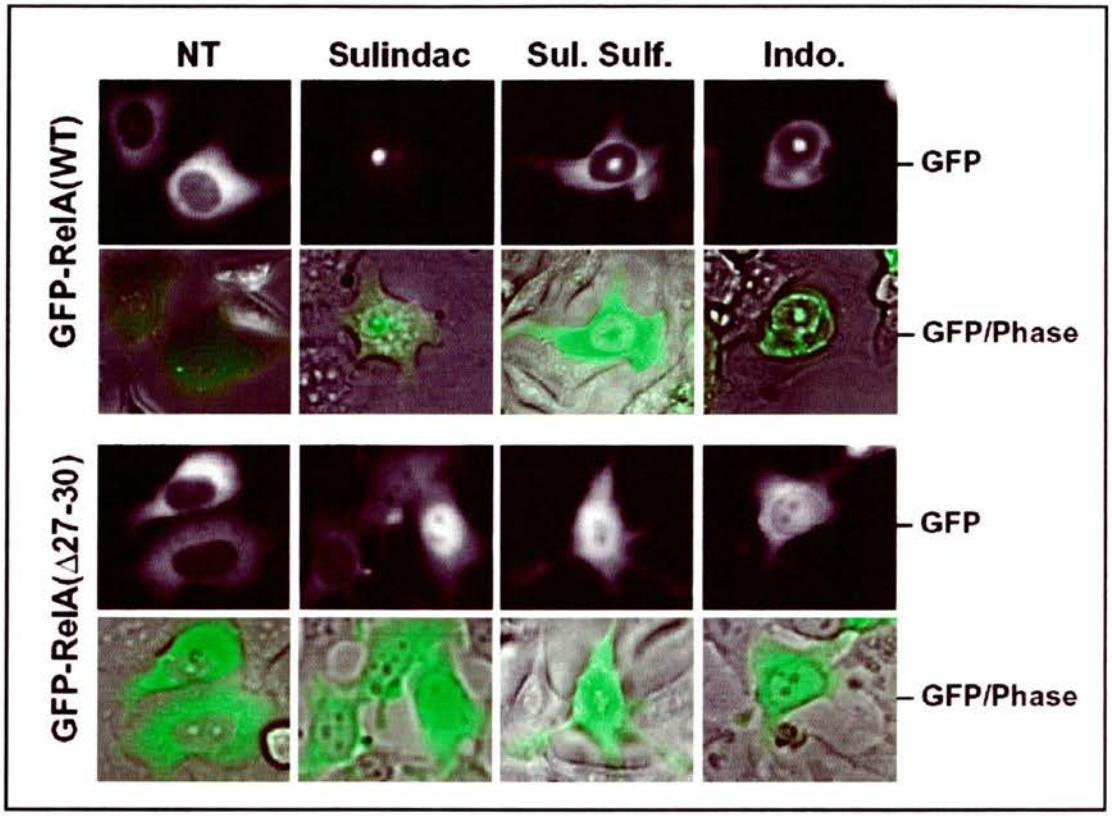

Figure 3.5 – RelA(Δ27-30) Blocks NSAID-induced Nucleolar Localisation of RelA. In three independent experiments, SW480 cells were transiently transfected with GFP-RelA(WT) or GFP-RelA(Δ27-30) and subsequently treated for 16 hrs with Sulindac (300 μM), Sulindac Sulfone (Sul. Sulf.) (300 μM), Indomethacin (Indo.) (125 μM) or non-treated (NT) (control). Cellular distribution of GFP-tagged protein was determined in live/adherent cells using an Axiovert 100 inverted fluorescent microscope (magnification, 40 x). Representative GFP and GFP/Phase images are shown.

N.B. Experiments outlined above were performed by Dr L. Stark

The next question to be addressed was whether the observed effects of NSAIDs on cell viability were also dependent on nucleolar localisation of RelA. To investigate this, the GFP-RelA(Δ27-30) expression construct was utilized to determine if it could block the effects of NSAIDs on cell viability by inhibition of nucleolar sequestration of RelA. Haemocytometric counts showed that sulindac, sulindac sulfone and indomethacin caused a marked reduction in the number of viable SW480 cells in cultures expressing GFP alone or GFP-RelA(WT) (data not shown). Moreover, this effect was blocked in cultures transfected with RelA(Δ27-30) and these cells showed inhibition of nucleolar translocation as described above. Phase-contrast microscopy confirmed that sulindac, sulindac sulfone and indomethacin cause a marked reduction in viable cells in cultures expressing GFP-RelA(WT) (Figure 3.6). Again, this effect on cell number and morphology was blocked in cells expressing RelA(Δ27-30). It is important to note that transfection efficiency was comparable for both vectors – Western blot analysis confirmed that WT and mutant protein were expressed at a similar level and were unchanged by NSAID treatment (data not shown).

Finally, the dependency of RelA on NSAID-induced apoptosis as an explanation for the observed reduction in cell viability was investigated. AnnexinV-Biotin assay, along with a streptavidin-TxRd conjugate, was used to determine cells that were apoptotic in relation to cells which were transfected with WT and mutated RelA. The percentage of cells undergoing apoptosis was then determined in transfected and non-transfected cell populations. As in the cell viability studies described above, apoptosis induced by sulindac, sulindac sulfone and indomethacin

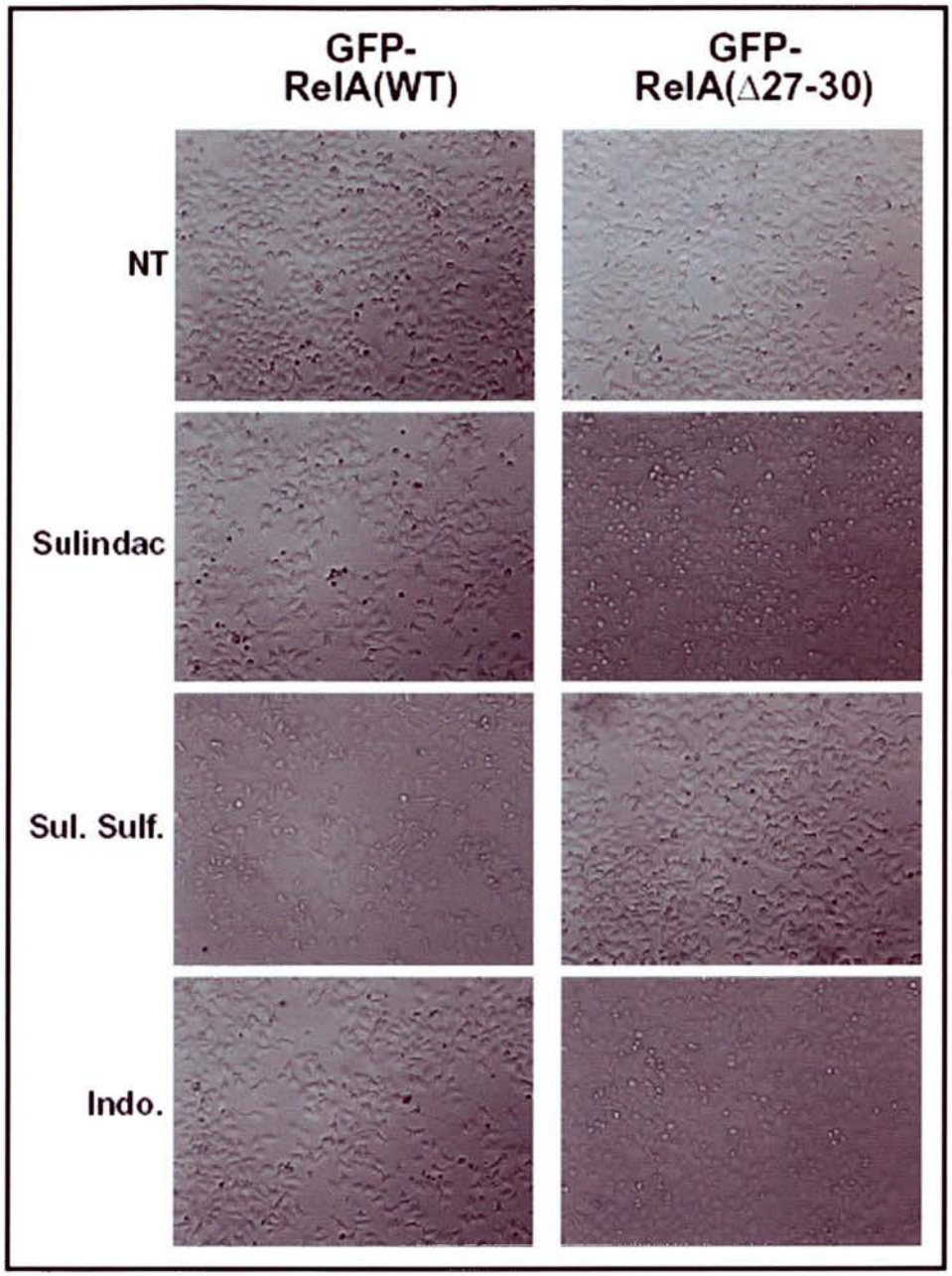

Figure 3.6 – RelA(Δ27-30) Blocks NSAID-induced Reduction in Cell Viability. In three independent experiments, SW480 cells were transiently transfected with GFP-RelA(WT) or GFP-RelA(Δ27-30) and subsequently treated for 16 hrs with Sulindac (300 μM), Sulindac Sulfone (Sul. Sulf.) (300 μM), Indomethacin (Indo.) (125 μM) or non-treated (NT) (control). Effect of deletion of amino acids 27-30 of RelA on cell viability in response to NSAIDs was analysed by phase-contrast microscopy. Representative phase-contrast images (magnification, 20 x), taken from randomly selected fields of view, are shown.

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was inhibited in cells expressing RelA(Δ27-30) (Figure 3.7). In keeping with previously published findings (Stark and Dunlop, 2005), apoptosis was actually enhanced in cells expressing GFP-RelA(WT) compared with cells expressing GFP alone (data not shown). NSAID-induced apoptosis was observed to a similar extent in all non-transfected cells (data not shown). Collectively, these data provide compelling evidence that each of the NSAIDs studied cause signal-specific nucleolar localisation of RelA and that this effect is of pivotal importance for the apoptotic effects of NSAIDs.

3.2.5 Non-aspirin NSAIDs Induce Nuclear Translocation of RelA Without IκBα Degradation

It has previously been shown in the host laboratory that degradation of IκBα in response to aspirin is required for nuclear translocation of NF-κB and nucleolar accumulation of RelA (Stark *et al.*, 2001; Stark and Dunlop, 2005). Having established that nucleolar sequestration of RelA is critical for NSAID-induced apoptosis, the upstream mechanism of activation of the NF-κB pathway in response to non-aspirin NSAIDs was further investigated. Using Western blot analysis, cytoplasmic levels of IκBα were examined in SW480 colon cancer cells after NSAID treatment. Figure 3.8 demonstrates that IκBα is degraded in a dose-dependent manner after aspirin treatment, as previously reported (Stark *et al.*, 2001). However, in marked contrast, there was no change in levels of cytoplasmic IκBα in response to

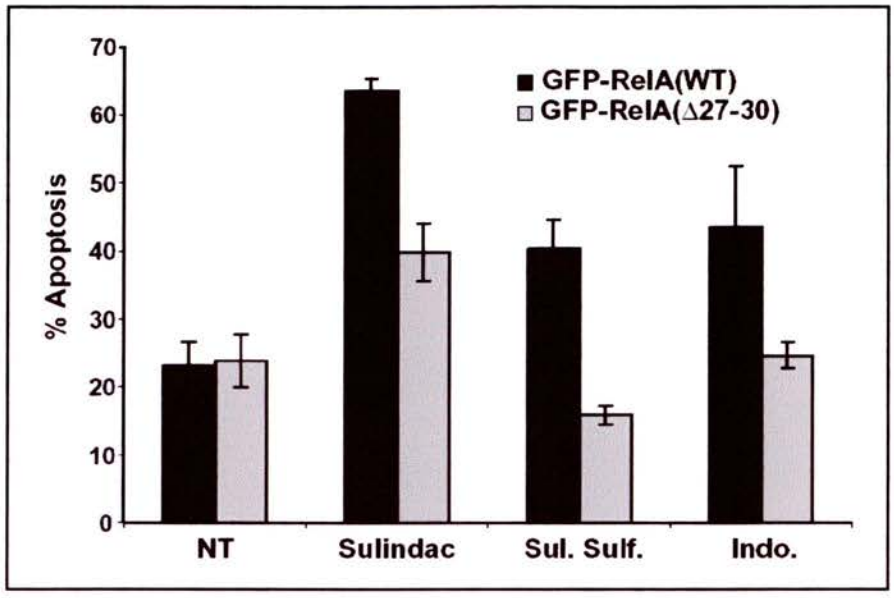

Figure 3.7 – Inhibiting Nucleolar Translocation of RelA Blocks NSAID-induced Apoptosis. SW480 cells were transiently transfected with GFP-RelA(WT) or GFP-RelA(Δ27-30) and subsequently treated for 16 hrs with Sulindac (300 μM), Sulindac Sulfone (Sul. Sulf.) (300 μM), Indomethacin (Indo.) (125 μM) or non-treated (NT) (control). Annexin V-Biotin staining, with a streptavidin-TxRd conjugate, was used to identify apoptotic cells. The percentages of cells expressing GFP-tagged RelA undergoing apoptosis were determined by fluorescence microscopy in a minimum of 250 transfected cells for each sample. The number of apoptotic cells in the non-transfected population was also determined (data not shown). Results presented are the mean of three independent experiments (+/- standard deviation).

N.B. Experiments outlined above were performed by Dr L. Stark

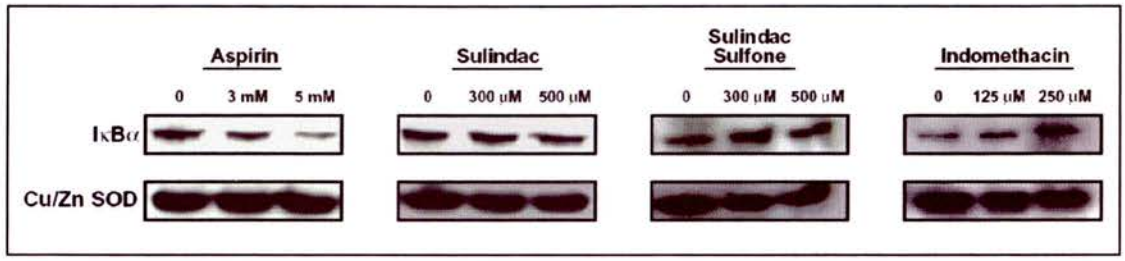

Figure 3.8 – Non-aspirin NSAIDs do not Induce Cytoplasmic IκBα Degradation. SW480 cells were treated overnight (16 hrs), in three independent experiments, with Aspirin (0, 3 mM, 5 mM), Sulindac (0, 300 µM, 500 µM) Sulindac Sulfone (0, 300 µM, 500 µM) or Indomethacin (0, 125 µM, 250 µM) then cytoplasmic extracts (15 µg) resolved by SDS PAGE on 10% polyacrylamide gels and anti-IκBα Western blot carried out. Copper/zinc super oxide dismutase (Cu/Zn SOD) was used as a loading control. Representative blots are shown.

sulindac, sulindac sulfone or indomethacin, even at the highest doses. This is in keeping with previous work in the host laboratory which found no change in cytoplasmic IκBα levels at time points up to 16 hrs of exposure to sulindac. It is important to consider that the predominant isoform of IκB in colonic epithelial cells is IκBβ (Wu *et al.*, 1999; Inan *et al.*, 2000a). However, preliminary data from the host laboratory has shown that IκBβ does not change after sulindac treatment and so it is unlikely that this isoform is targeted in response to NSAIDs.

3.3 Discussion

In previous reports from the host laboratory, the NF-κB pathway was identified as a key target pathway for the anti-tumour effects of aspirin in large bowel epithelial cells. To address the question of whether the NF-κB effect is COX-dependent and given that previous studies from the host laboratory have mainly focused on aspirin, a panel of non-aspirin NSAIDs with varying COX-inhibitory profiles was used in the series of experiments presented in this chapter. The aim was to determine whether these agents also induce apoptosis of colorectal cancer cells via modulation of NF-κB signaling in a similar manner to that has been described for aspirin (Stark *et al.*, 2001).

Sulindac, sulindac sulfone and indomethacin all induced apoptosis (Figure 3.2), nucleolar translocation of RelA (Figure 3.3) and repression of basal NF-κB activity in a similar manner to aspirin (Figure 3.4). Furthermore, using the RelA(Δ27-30) mutant, nucleolar sequestration of RelA was demonstrated to be

causally involved in sulindac, sulindac sulfone and indomethacin-induced apoptosis (Figure 3.7). When reviewing these data, it would have been a good idea to quantitate the immunocytochemistry results showing localisation of RelA to the nucleolus in response to NSAIDs as this would have allowed the use of statistics to verify an effect. The use of statistics to quantitate and authenticate an effect for apoptosis data in response to NSAIDs would also have been informative. It should be noted that the internal control plasmid (pCMVβ) which was used to normalise results for transfection efficiency when looking at the effects of NSAIDs on NF-κB transcriptional activity (Figure 3.4) does itself contain several κB sites. It would therefore be a good idea in the future to look at expression of a few downstream genes to substantiate reporter assay data showing repression of NF-κB activity. Nevertheless, taken together, the findings presented in this chapter strongly support the notion that NSAIDs as a class activate the NF-κB pathway. Moreover, NSAID-induced activation of NF-κB ultimately leads to apoptosis through sequestration of RelA in the nucleolus in association with repression of NF-κB driven transcription. Interestingly, these effects are likely to be COX-independent as sulindac sulfone lacks COX-inhibitory activity.

The observation that RelA accumulates within the nucleolus after NSAID exposure (Figure 3.3) raises questions on the functional consequences of such localisation and on the mechanisms by which it gets there. As discussed in Chapter 1, it is increasingly apparent that sequestration of transcription factors in the nucleolus has a role in cell-cycle regulation and apoptosis. However, the cellular mechanisms for routing of proteins to the nucleolus are not known. There is no structural or functional evidence for the existence of a frontier separating the nucleolus from the

surrounding nucleoplasm and so soluble molecules could, in principle, diffuse between subnuclear compartments (Carmo-Fonseca *et al.*, 2000). However, nucleolar accumulation is generally mediated by the presence of a specific nucleolar targeting sequence (Scheer and Hock, 1999) or through molecular interaction with a nucleolar interaction partner. One example is ARF, which has been shown to sequester MDM2 in the nucleolus (Olson, 2004).

Previous studies from the host laboratory demonstrated that amino acids 27-30 of RelA are responsible for nucleolar localisation of the protein (Stark and Dunlop, 2005). It was also postulated from that study that an additional co-factor is required for nucleolar translocation of RelA. Kinetic studies demonstrated that induced RelA distributes throughout the nucleoplasm in complexes prior to localisation in the nucleolus. However, it accumulates in the nucleoplasm, but not the nucleolus, in response to aspirin exposure or serum withdrawal when protein synthesis is blocked. Potential candidate co-factors include the nucleolar proteins NFBP and nucleophosmin (B23), which have both recently been shown to interact with RelA (Sweet *et al.*, 2003; Dhar *et al.*, 2004). Moreover, nucleophosmin/B23 transcription has been identified as an early gene response to specific external stimuli (Wu and Yung, 2002). The NF-κB regulator, NIK and NRF are also good candidates as nucleolar shuttling has been shown to affect the function and mobility of these proteins respectively (Birbach *et al.*, 2004; Niedick *et al.*, 2004). The nucleolar protein, ARF, is a particularly strong candidate as it has recently been found to regulate the transactivation function of RelA and can inhibit both NF-κB-driven transcription and anti-apoptotic activity (Rocha *et al.*, 2003).

Although there is substantial evidence for a pro-survival function of NF-κB, there is a growing body of evidence supporting a pro-apoptotic or tumour suppressor role for NF-κB. 'Atypical' activators of NF-κB, such as UV-C and daunorubicin, result in DNA binding of NF-κB complexes with much slower kinetics than TNF-α (Perkins, 2004b; Campbell and Perkins, 2006). Furthermore, these atypical stimuli have been shown to repress rather than activate transcription of the RelA-responsive anti-apoptotic target genes, *Bcl-X_L*, *X-IAP* and *A20* (Campbell *et al.*, 2004). In that study, the observed repression of anti-apoptotic genes by UV-C and daunorubicin was demonstrated to be the result of induction of RelA by interaction with HDACs, indicating that repression is an active process. Interestingly, a subsequent study from the same group (Campbell *et al.*, 2006) demonstrated that induction of NF-κB DNA-binding and transcriptional repression in response to topoisomerase inhibitors, including daunorubicin, relates to the capacity of these agents to intercalate with DNA. Furthermore, oxygen free radicals were not required for the NF-κB to repress transcription.

In the work presented here, activation of the NF-κB pathway by the panel of NSAIDs induced nuclear translocation of NF-κB complexes. The functional end result of NSAIDs is equivalent to that published for UV-C and daunorubicin, since both cause transcriptional repression of NF-κB. However, there are important differences in the mechanism by which this is brought about. Whereas UV-C and daunorubicin actively repress RelA by increasing the association of NF-κB with HDACs (Campbell *et al.*, 2004), the NSAIDs used in this study cause sequestration of the RelA component in the nucleolus. Once in the nucleolus, RelA is physically

separated from its target promoters, resulting in a decrease in transcription of anti-apoptotic genes and consequently, apoptosis.

An interesting observation from the data outlined in Figure 3.3 is that nucleoli are very large and pronounced upon treatment with NSAIDs when compared to the untreated control. In particular, the lack of DAPI staining in regions corresponding to nucleoli is very striking in cells treated with sulindac (500 μM) and aspirin (5 mM). It is well established that the appearance of the nucleolus changes during the various phases of the cell cycle. Fully active nucleoli are large, with extensive intermingling of fibrilar centres, dense fibrilar component and granular component. As the cell approaches mitosis, the nucleolus first decreases in size and then disappears as chromosomes condense and RNA synthesis stops, so that there is generally no nucleolus in a metaphase cell. At the end of mitosis when ribosomal RNA synthesis restarts, tiny, compact nucleoli reappear at the chromosomal locations of ribosomal RNA genes (Carmo-Fonseca *et al.*, 2000; Alberts *et al.*, 1983). It is noteworthy that a few papers have demonstrated that certain proteins localise to the nucleolus at particular stages of the cell cycle. In a study of the subcellular distribution of Rad52, a protein involved in DNA repair, a fusion protein comprising GFP and Rad52 was found throughout cells in M phase, localised to the nucleoplasm, but was excluded from nucleoli, in G_1 phase and accumulated in nucleoli in both S phase and quiescent (G_0) cells (Liu *et al.*, 1999). Protein phosphatase 1 (PP1) γ, a ubiquitous serine/threonine phosphatase that regulates many cellular processes, has also been found to have distinct localisation patterns throughout the cell cycle (Trinkle-Mulcahy *et al.*, 2003). Specifically, PP1γ, when expressed as a fluorescent protein fusion, showed a nucleolar accumulation during interphase, localised to kinetochores

on entry to mitosis, dramatically re-localised to chromosome-containing regions at the transition from early to late anaphase and also accumulated at the cleavage furrow and midbody by telophase. In light of the above, it may therefore be very informative in the future to examine cell cycle dynamics and subcellular localisation of RelA.

Another interesting finding from the data presented was that substantial transcriptional repression was observed with the lower concentration of sulindac (300 μM) but there was minimal nucleolar localisation of RelA at this concentration (see Figures 3.3 and 3.4). This suggests the possibility that repression of NF-κB driven transcription by NSAIDs could also involve other mechanisms and so apoptosis may not be due exclusively to transcriptional repression through nucleolar localisation of RelA. Hence, it is interesting to speculate that other, as yet undefined, active mechanisms associated with nucleolar translocation could be implicated in repression of NF-κB driven transcription by NSAIDs.

The observation that all the NSAIDs used in this study activate the NF-κB pathway calls into question several previous studies demonstrating that NSAIDs inhibit cytokine-mediated activation of NF-κB. One report (Kopp and Ghosh, 1994) documented inhibition of cytokine-induced NF-κB-dependent transcription by sodium salicylate and aspirin. It was subsequently demonstrated that the NSAIDs aspirin (Yin *et al.*, 1998) and sulindac (Yamamoto *et al.*, 1999) inhibit cytokine-mediated activation of NF-κB by stereochemical inhibition of IKK and reduction of ATP binding, consequently blocking IKK activity. Another group have also very recently reported inhibition of LPS-induced NF-κB DNA-binding and nucleolar accumulation of RelA by the COX-2-selective inhibitors, etoricoxib and lumiracoxib

(Niederberger *et al.*, 2006). However, it is important to note that in those studies, cells were pre-treated with the appropriate NSAID for only a short period of time before a burst of treatment with powerful NF-κB stimuli. These experimental conditions are not necessarily representative of the *in vivo* situation. Moreover, NSAIDs have been shown to inhibit the growth of colorectal cancer cells without additional cytokine stimulation (Qiao *et al.*, 1998a). Furthermore, several papers support the findings of this study that NSAIDs activate NF-κB in the absence of additional NF-κB stimuli. The NSAID, diclofenac (Cho *et al.*, 2005) and the COX-2-selective inhibitors, celecoxib (Niederberger *et al.*, 2001) and NS-398 (Smartt *et al.*, 2003), have been reported to activate the NF-κB pathway without additional cytokine stimulation. Even so, it would be of interest in the future to establish whether aspirin and the other NSAIDs used in this study inhibit cytokine-mediated activation of the NF-κB pathway in SW480 colon cancer cells.

Having established that activation of the NF-κB pathway was common to the panel of NSAIDs used in this study, it was presumed that the upstream mechanism of activation by the other NSAIDs would be the same as aspirin, involving phosphorylation and degradation of IκBα. However, the discovery that IκBα is not degraded in response to sulindac, sulindac sulfone or indomethacin treatment (Figure 3.8) suggested that there is an alternative upstream mechanism and a further layer of complexity in the pathway. One possible alternative pathway utilised by the non-aspirin NSAIDs will be the focus of investigation and discussion in the next chapter. Nevertheless, it should be noted that it would be important in the future to confirm preliminary data from the host laboratory from time course experiments which indicated that IκBα is not degraded in response to sulindac (500 μM) treatment at any

In summary, the data presented in this chapter indicates that NSAIDs as a class activate the NF-κB pathway and that this is a critical mechanism whereby these agents induce apoptosis. In keeping with the mechanism previously described by the host laboratory for aspirin (Stark and Dunlop, 2005), nucleolar sequestration of RelA, with associated repression of NF-κB-driven transcription, was demonstrated to be vital for induction of apoptosis by sulindac, sulindac sulfone and indomethacin. However, in contrast to aspirin, there was no degradation of IκBα in response to treatment with sulindac, sulindac sulfone or indomethacin. This observation indicates that although NSAIDs have the same end point, that is activation of NF-κB, they have a different upstream mechanism to achieve activation of the pathway. Hence, the focus of the next part of research in this thesis was to look at other mechanisms by which IκBα is induced to dissociate from RelA.