Development of novel therapies for colorectal carcinoma using dietary indoles

Thesis submitted for the degree of

Doctor of Medicine

at the University of Leicester.

By

Alastair Forbes Brookes

Department of Cancer Studies

and Molecular Medicine

2014

Abstract

Development of novel therapies for colorectal carcinoma using dietary indoles

Alastair Forbes Brookes

Colorectal carcinoma is the third commonest cancer in the UK and is the second commonest cause of cancer related death. There is a need for efficacious chemotherapeutic and chemopreventative agents against colorectal carcinogenesis.

Indole-3-carbinol and its acid condensation product, 3,3 diindolylmethane (DIM), exhibit anti-tumourigenic activity in preclinical models. This study investigated their therapeutic potential in colorectal carcinoma.

A panel of four colorectal carcinoma cell lines (HT29, HCT116, SW480 and SW620) was used to compare effects of DIM and I3C on cell viability, induction of apoptosis and cell cycle arrest. Treatment with DIM or I3C resulted in dose-dependent reductions in cell viability. Cell lines were sensitive to 25–75 μM DIM (GI_{50} = 45 μM), but not to 200 μM I3C.

DIM (40 μM) induced apoptosis in all cell lines. This was associated with downregulation of survivin and appeared to be independent of p53. Furthermore it induced G_1 arrest in HCT116, SW480 and SW620 and G_2/M arrest in HT29. DIM induced activatory phosphorylation of Chk2 in HT29 but not in HCT116. DIM downregulated cell cycle proteins: PLK1, cdc25C, cdc2 and cyclins B1, D1 and E.

Since all colon cancer cell lines expressed significant levels of Src, their response to Src inhibition was investigated. Colon cancer cells were modestly sensitive to AZD0530 (AstraZeneca), but combination with indoles reduced viability to a greater extent than single agent treatment. Combining 40 μM DIM with 1 μM AZD0530 was particularly effective, reducing viability by half after 48 hours on average. Decreases were sustained and progressive over extended timepoints.

Attempts were made to establish primary cultures from resected hepatic metastases however issues were identified with cell death, fibroblast overgrowth and slow cell turnover. Potential changes to address these were identified.

These data suggest chemotherapeutic potential of DIM in colon cancer, particularly in combination with Src inhibition.

Acknowledgments and dedication

Contents

List of Figures

List of Tables

List of abbreviations

Apaf1	Apoptosis protease activating factor 1
APC	Anaphase promoting complex
ATM	Ataxia-telangectasia-mutated
ATP	Adensoine tri-phosphate
ATR	Ataxia-telangectasia and rad3 related
AZD	AZD0530
Bax	Bcl2 associated protein
Bcl-2	B Cell Lymphoma-2
Bcl-XL	B Cell lymphoma Extra Large
Bid	Bcl-2 interacting domain
Bim	Bcl-2 interacting mediator of cell death
BSA	Bovine serum albumin
CAK	CDK activating kinse
Cdc	Cell division cycle
CDK	Cyclin Dependent Kinase
CDKI	Cyclin Dependent Kinase Inhibitor
CHK	Checkpoint kinase
DNA	Deoxyribose Nucleic Acid
DISC	Death-inducing signalling complex
DIM	3,3 Diindolylmethane
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
E2F	E2 transcription factor
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FAK	Focal adhesion kinase
FAP	Familial adenomatous polyposis
FDA	Food and Drug Administration
GADD153	Growth arrest and DNA damage induced gene-153
FADD	Fas associated death domain
FCS	Foetal calf serum
FITC	Fluoroscein isothiocyanate

HCEC	Human colonic epithelial cells
HNPCC	Hereditary non-polyposis colorectal cancer
hTERT	Human telomerase reverse transcriptase
I3C	Indole-3-Carbinol
IBMX	Isobutylmethylxanthine
IGF1	Insulin-like growth factor 1
JNK	Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
MEM	Modified Eagle medium
MHRA	Medicines and healthcare products regulatory agency
MMP	Matrix metalloproteinase
MWM	Molecular weight marker (KDa)
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OCT	Optimal cutting temperature embedding medium
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween
PDGF	Platelet derived growth factor
PI	Propidium iodide
PI-3K	Phosphoinositide 3-kinase
PLK	Polo like kinase
polyHEMA	Poly(2-hydroxyethyl methacrylate)
pRb	Product of Retinoblastoma gene
RIP	Receptor interacting protein kinase
RPMI	Roswell Park Memorial Institute medium
SFK	Src family kinases
SH	Src homology domain
TE	Trypsin ethylenediaminetetraacetic
TNFR1	Tumour necrosis factor receptor 1
TRAIL	TNF-related apoptosis-inducing ligand
VEGF	Vascular endothelial growth factor
XIAP	X-linked inhibitor of apoptosis protein

Presentations arising from this project

Brookes A., Moiseeva E., Berry D.P., Manson M. Diindolylmethane demonstrates activity in preclinical models of colorectal carcinoma both as monotherapy and in combination with AZD0530, a Src inhibitor.
4th NCRI Institute Cancer Conference , Birmingham, 2008.

Brookes A.F., Moiseeva E., Neal C.P., Manson M.M., Berry D.P. Diindolylmethane demonstrates activity in pre-clinical models of colorectal carcinoma both as monotherapy and in combination with AZD0530, a Src inhibitor.
12th Annual Scientific Meeting of the Association of Upper Gastrointestinal Surgeons for Great Britain and Ireland, Liverpool, 2008

Chapter 1

Introduction

1.1. Cancer

Cancer is a major cause of mortality and morbidity worldwide. In the United Kingdom 250,000 new cases are diagnosed per annum and cancer is deemed the cause of over a quarter of all deaths, making it the second commonest cause after circulatory diseases, in this country. Overall it is estimated that greater than 1 in 3 people will develop some form of cancer within their lifetime (1,2).

Tumours are classified according to their original cell type and whether they are malignant or benign. Examples include adenomas which are benign tumours of epithelial glandular tissue and adenocarcinomas which are malignant tumours derived from epithelial glandular tissue.

Cancer occurs as a result of DNA damage leading to an imbalance between proliferative and pro-apoptotic signalling factors. This damage may be due to extrinsic environmental factors, referred to as mutagens or carcinogens, or may be intrinsic due errors during cell division. In order for malignant transformation to occur a number of mutations must be accrued by the cancer cell enabling it to acquire the 'hallmarks' for full malignant potential. As described by Hanahan and Weinberg these are (3):

1. Self sufficiency in growth signals
2. Insensitivity to anti-growth signals
3. Ability to invade tissues and to metastasise
4. Limitless replicative potential
5. Sustained angiogenesis
6. Evasion of apoptosis

7. Reprogramming of energy metabolism

8. Ability to evade the immune system

Various mutations can give rise these characteristics and will vary between individual tumours. Over half of cancers are attributable to environmental and lifestyle factors. However in a small proportion of cases the genetic mutations are inherited and in approximately 5% of tumours a strong heritable genetic basis is identifiable (2,4). Examples of the later group include Li-Fraumeni syndrome, resulting from an inherited mutation of the p53 gene, which predisposes to a number of cancers, including breast, ovarian, uterine, pharyngeal and gastric carcinomas, and mutations in the breast cancer 1 and 2 (BRCA1 and BRCA2) genes which predispose to the development of breast, ovarian, pancreatic and prostate carcinomas. The genes that are mutated in carcinogenesis usually fall into one of two broad categories: oncogenes and tumour suppressor genes. Oncogenes, when mutated, upregulate the processes involved in the evolution of malignant change, whereas tumour suppressor genes retard the process of cancerous change, for example they may promote apoptosis (5).

Other factors also influence the incidence of cancer, in particular age, with only 1% of tumours occurring in children and the majority occurring in the elderly population (2). This reflects the fact that cancers usually develop through the progressive build up of mutations, often secondary to prolonged exposure to carcinogens, and then the amassing of tumour bulk through replication of the mutated cells.

1.1.1 Colorectal carcinoma

Colorectal carcinoma is the third most common cancer in the UK after lung and breast carcinomas and is the second most common cause of cancer related death, accounting

for 10% of cancer related mortality. Approximately 35000 new cases of colorectal carcinoma are diagnosed each year (1,2,6).

Colorectal cancer is an adenocarcinoma. The aetiology of colorectal carcinoma has undergone extensive research and recent studies have clearly shown that the development of colorectal cancer is determined by a series of genetic and epigenetic events. The genetic events may be divided into two major types: microsatellite instability and chromosomal instability (7). In the microsatellite instability pathway tumours arise as a result of alterations on tandem repeats of DNA sequences due to mutations in genes encoding DNA mismatch repair enzymes (7). The key pathway remains the chromosomal instability pathway, also called the adenoma-carcinoma sequence, first described by Fearon and Vogelstein (8) and subsequently refined by others (9). In this pathway tumours are proposed to arise within pre-existing adenomas through a series of genetic mutations giving rise to a progressive change in the epithelial cells from normality through dysplasia to invasion. There is evidence of cross talk between the two pathways and the likelihood is that varying combinations of the two occur (7).

The majority of colorectal carcinomas are sporadic. However there are some hereditary conditions accounting for around 5% of cases and overall 20-30% are believed to have a familial basis where no distinct genetic basis for the predisposition has yet been identified (10). The commonest two genetic conditions are hereditary non-polyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP) accounting for approximately 3% and 1% of all colorectal carcinomas respectively (10-12). FAP is transmitted in an autosomal dominant fashion and is characterised by the formation of a large number of colonic polyps. Mutation of a single tumour

suppressor gene, the adenomatous polyposis coli (APC) gene on chromosome 5, has been identified as being responsible for FAP, though over 300 different mutations have been demonstrated. HNPCC is also transmitted in an autosomal dominant fashion. A number of responsible genes have been identified of which 6 (MLH1, MSH2, MSH3, MSH6, PMS1 and PMS2) are routinely assessed in individuals and tumours deemed to be at increased risk (13-15). Unlike FAP, HNPCC, as its name implies is not characterised by extensive polyp formation. Patients with FAP have an almost 100% risk of developing colorectal cancer by the age of 40, whereas patients with HNPCC have an 80% lifetime risk (10,12).

A number of lifestyle factors have been shown to correlate with an increased risk of developing colorectal carcinoma and specifically, epidemiological studies have shown a link between a diet low in vegetables and fruit and an increased risk of colorectal cancer (16,17).

The presentation of a tumour varies according to its position - for example a right colonic lesion may give anaemia due to occult bleeding, weight loss or a palpable mass, whereas left colonic lesions may present with abdominal pain, altered bowel habit, rectal bleeding and, in the case of low rectal tumours, a sensation of incomplete defecation (tenesmus). Forty percent of tumours will present as emergencies with symptoms of obstruction or perforation and these cases are associated with a poorer outcome.

Colorectal carcinoma is usually staged using the modified Dukes' classification:

- A: Tumour confined within muscularis propria
- B: Tumour extends through muscularis propria

- C1: One or more regional lymph nodes involved but apical node free of disease

- C2: One or more regional lymph nodes, including the apical node, involved

- D: Distant metastases

Currently the primary treatment modality is surgical resection of the tumour aiming to remove a clear margin of uninvolved tissue and the regional lymph nodes as far as the origin of the feeding vessel. Adjunctive treatment depends upon both the site of the primary tumour, evidence of spread and the condition of the patient. Prognosis is related to the stage of the disease at presentation ranging from 90% survival for patients with Dukes' A disease to 5% for those with Dukes' D disease (18,19).

After regional lymph nodes, the liver is the commonest site for metastatic disease; at the time of surgery for the primary tumour 20-30% of patients will have evidence of hepatic metastases and a further 40-50% will subsequently develop hepatic metastases. At the present time, without treatment the 1 year survival rate is 31% and this falls to near 1% at 3 years. Surgery offers the only potentially curative treatment modality for these patients however only a relatively small proportion are considered suitable and even then the 5 year survival is 26-49% (20,21).

1.1.2. Diet and Cancer

There is increasing epidemiological evidence that diet influences the risk of developing cancer to the extent that dietary intake has been identified as one of the most readily modifiable determinants of cancer risk with up to 35% of cancers being attributable to dietary factors (22). Studies have demonstrated carcinomas of the

colon, breast, prostate and pancreas are commoner in developed countries of Western Europe and North America than in Africa or Asia (23,24). Further studies of migrant populations have demonstrated that these trends are dependent on environmental factors rather than having a genetic basis (25,26). Specific risk factors have been identified, for example a high alcohol intake is associated with an increased incidence of head and neck, oesophageal and breast cancers (27-29) and a poor fruit and vegetable intake is associated with an increased risk of colon cancer (16,17).

Following on from the epidemiological data, several dietary compounds have been identified which appear to exert a protective effect against the risk of malignancy including reservatrol, a polyphenol present in grapes; vitamin D; curcumin, a polyphenol found in turmeric; and indole-3-carbinol, an indole found in cruciferous vegetables. Several potential pathways for the bioactivity of these compounds have been proposed and investigation of these is ongoing (23,30-32). The potential uses of these bioactive compounds are two fold. Firstly they may have a chemopreventative role and secondly they offer the potential for the development of further treatments for patients with proven malignancies, particularly when one considers that a number of chemotherapeutic agents in current use were originally derived from natural sources, for example the vinca alkaloids.

1.1.3. Cancer chemoprevention

Chemoprevention is defined as the use of agents to inhibit, reverse or retard tumorigenesis (32). Chemopreventative agents can be divided into two subgroups:

1. Blocking agents – these prevent DNA damage by blocking the action or activation of the carcinogen

2. Suppressing agents – these inhibit cell growth and tumour progression once damage has occurred (30).

These actions may take place at various points throughout the progression from a normal cell to an established metastasis (see figure 1.1 below).

There is considerable interest in the use of chemopreventative agents both in healthy populations and in treated patients. The key advantages of chemoprevention are the reduction in the incidence and therefore morbidity of the relevant pathologies and also the lower side effect profile in comparison with the established chemotherapeutic agents.

Blocking agents
- ROS scavenging
- Alter carcinogen metabolism

Blocking agents
- Prevent further DNA damage
Suppressing agents
- Induce apoptosis

Blocking agents
- Prevent further DNA damage
Suppressing agents
- Induce cell cycle arrest
- Induce apoptosis
- Inhibit angiogenesis

Blocking agents
- Prevent further DNA damage
Suppressing agents
- Inhibit angiogenesis
- Inhibit invasion

Normal Cell → Initiated Cell → Preneoplasia → Tumour → Metastasis

Figure 1.1: Potential points of action of chemopreventative agents in carcinogenesis. Reproduced from Manson 2003

Prospective chemopreventative agents should fulfil the following general criteria (33):

- Low costs

- Easily administered – oral ingestion

- Acceptable to patients

- High efficacy at achievable doses

- Minimal or no side effects

- Known mechanism(s) of action

Thorough appraisal of any prospective agents is crucial as the effects can be contrary to expectations/preclinical data. For example contrary to preclinical data large scale trials of vitamin E and β-carotene as chemopreventative agents in prostate and lung cancer respectively actually demonstrated increased malignancy in the treatment arms (34). Furthermore, some agents are proving to be of limited use due to side effects. For example studies of vitamin D have demonstrated that its therapeutic use is restricted by a high incidence of hypercalcaemia (23). The main difficulty of studying chemopreventive agents is testing their effectiveness given the duration of the process of tumorigenesis (35).

1.2. Cell cycle:

A cell reproduces through a progressive series of steps which duplicate the cellular contents prior to the cell dividing into two daughter cells. These daughter cells then enter the same cyclical process. This regulated progression is termed the cell cycle and forms the basis of organism growth and reproduction. As will be explored later in the introduction aberrations within the regulation of this process are integral to the pathology of cancer.
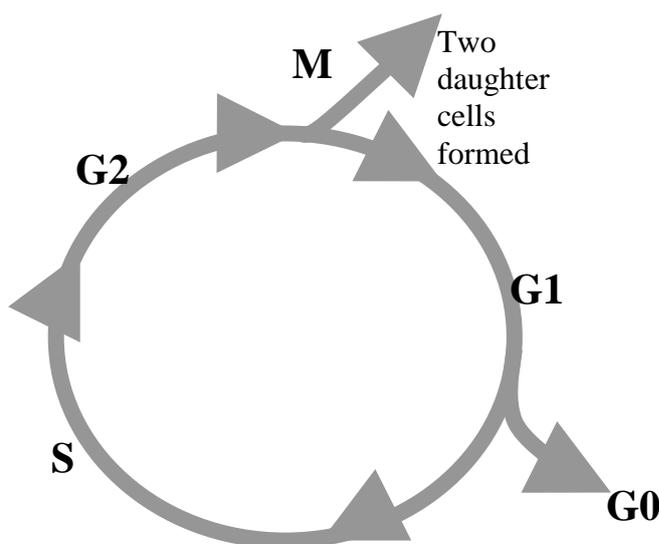
Figure 1.2: Cell cycle overview

24

The mammalian cell cycle may be divided into four distinct phases. The two key phases are the S or synthesis phase, during which replication of the nuclear DNA takes place, and M or mitosis phase, during which the nucleus and subsequently the cell divides to form two identical daughter cells. M phase is further subdivided 5 subphases: prophase, prometaphase, metaphase, anaphase and telophase. The duration of these phases and of the cell cycle in total varies significantly between cell types. Eukaryotic cells in culture typically have a cycle time of 16-24 hours. However, certain liver cells can have a cell cycle duration of greater than one year. M phase typically lasts approximately one hour and S phase twelve hours (36,37). The majority of cells need more time to grow, double their organelles and other cellular contents than for DNA replication and division. In part to accommodate this, two gap phases are inserted into the cell cycle, G1 after the M phase and G2 after the S phase. Thus the sequence of the four stages of the cell cycle is M-G1-S-G2. G1, S and G2 are often referred to collectively as interphase with M phase also described as division. The description above is an over simplification of the purpose of the gap phases as these periods also permit the cell to scrutinise the intracellular and extracellular environment with respect to integrity of the genome, suitability and requirement for cell division, prior to committing to progression through to the next stage of the cell cycle. Several distinct checkpoints have been identified. This is particularly relevant in G1 at what is termed the restriction point. Arrest at these points can lead to delays in the cell cycle; instigation of DNA repair pathways; exit into a quiescent phase, referred to as G0, or diversion into programmed cell death, termed apoptosis (38-42). Cells can remain in quiescence (G0) for extended periods, even years, before resuming progression through the cell cycle. Indeed the majority of non-dividing cells in the adult body will be in G0 (43). Once a cell has progressed through the restriction

point in G1 DNA replication is inevitable, even if all extracellular stimuli and signalling pathways are removed or blocked (43).

As intimated above progression through the cell cycle occurs in response to a variety of stimuli. However, this process is closely regulated by a variety of cellular proteins, key amongst which are cyclin dependent kinases (CDKs). These are a group of serine/threonine protein kinases whose activities rise and fall cyclically leading in turn to changes in phosphorylation of intracellular proteins that initiate or regulate cell cycle events. CDKs, as their name implies, are dependent on regulatory proteins called cyclins. Binding of a cyclin to CDK results in the formation of a cyclin-CDK complex and a conformational change in the catalytic subunit of the CDK. This change partially activates the CDK. However, it may then be further activated through phosphorylation by a separate kinase termed a CDK activating kinase (CAK).

Conversely CDKs can be inhibited through inhibitory phosphorylation at the active site by the Wee1 protein kinase, an effect which is reversed by the phosphatase cdc25. CDK activity may also be inhibited by forming a complex with a CDK inhibitor (CKI) protein. Two distinct families of CKIs have been identified, the INK4 CKIs and the Cip/Kip CKIs. The INK4 family includes p15 (INK4b), p16 (INK4a), p18 (INK4c) and p19 (INK4d) which inhibit cyclin D binding to CDKs 4 and 6 (See below). The Cip/Kip family includes p21 (Waf1, Cip1), p27 (Cip2) and p57 (Kip2) which inhibit a number of processes including binding cyclin B to CDK1, binding of CDKs 4 and 6 by cyclin D (all three) as well as DNA synthesis (p21) (41,43-45).

The final mechanism of regulation of CDK activity is through control of the levels of cyclin in the cell. During the cell cycle CDK levels remain constant, whereas cyclins are dependent on two main factors:

1. Transcriptional regulation determining the rate of synthesis
2. Organised proteolysis at defined stages in the cell cycle where the CDK-cyclin complexes are no longer required.

The latter of these processes occurs via activated enzyme complexes binding multiple copies of ubiquitin protein to the cyclin thus marking it as a target for ubiquitin ligase catalysed destruction in proteasomes. In the human cell there are two main ubiquitin ligases responsible for degradation of cyclins and other cell cycle regulatory proteins. The first is SCF, which acts on G1/S cyclins and some CKIs controlling S-Phase initiation and the second is anaphase promoting complex (APC), which acts on M-cyclins and other mitosis regulators during M-phase.

More than 20 mammalian CDKs have been identified though not all are active during the cell cycle (46,47). Currently there are in excess of

CDK	Cyclin	Associated process/Cell cycle phase of activity
CDK1	Cyclin A1/A2	G2/M phase transition
CDK1	Cyclin B	Mitosis
CDK2	Cyclin E1/2	G1/Sphase transition
CDK2	Cyclin A	G2 + S phases
CDK3	Cyclin C	G0/G1 transition
CDK4	Cyclin D1-3	Mitosis
CDK6	Cyclin D1-3	G1
CDK7	Cyclin H	CAK, all phases
CDK8	Cyclin C	Transcription
CDK9	Cyclin K	Transcription
CDK9	Cyclin T1/2	Transcription
CDK11	Cyclin L	RNA splicing

Table 1.1: Cyclin-CDK complexes and their associated cell process or phase of cell cycle activity. This list is illustrative and research into this field is ongoing. (43,48-54)

20 known cyclins, but as with the CDKs, not all are implicated in cell cycle regulation

27

(47). Of those relevant to the cell cycle there are five classes binding differing CDKs and these are required for different phases of the cell cycle (See table 1) (43). Cyclins may also be classified according to the point at which they act during the cell cycle: (43,48,49,49-54)

1. G1/S cyclins – commit the cell to DNA replication

2. S cyclins – initiate DNA replication.

3. M cyclins – promote mitosis

4. G1 cyclins – promote passage through the restriction point in G1.

The D type cyclins (D1-3) are the first to be produced during the cell cycle. Unlike the other cyclins which are expressed periodically, D cyclins are synthesised as long as there is growth factor stimulation. The D type cyclins bind to CDKs 4 and 6 producing complexes required for entry into G1. The primary function of these complexes is inhibitory phosphorylation of the product of the retinoblastoma tumour suppressor gene (pRb), an inhibitor of cell cycle progression. pRb suppresses the activity of the gene regulatory protein E2F, whose function is upregulation of genes encoding proteins functioning during S-phase or at the G1/S transition including cyclins as well as up regulation of its own gene in a positive feedback loop (44). One of the effects of E2F upregulation is increased synthesis of cyclin E which complexes with CDK 2 during the latter stages of G1. Cyclin E-CDK 2 has a role secondary to the cyclin D-CDK complexes in phosphorylation of pRb. Cyclin E-Cdk2 complex in turn promotes S-phase protein activity through phosphorylation of proteins involved in centrosome duplication and DNA synthesis and repair as well as feeding back into cell cycle regulation through phosphorylation of the Cdk inhibitors p21 and p27 (55).

Cyclin A is also partially regulated via E2F and so accumulates during the G1/S phase transition. During S phase it initially complexes with CDK2 and in the later stages with CDK 1. It has been demonstrated that cyclin A is required for progression through S phase and that, in complex with CDK1, is required for entry into M-phase (43,45). During S phase cyclin A-CDK2 localises to sites of DNA replication indicating potential roles either in DNA synthesis or in prevention of excess DNA replication. Cyclin A has a further role in negative feedback on E2F via the phosphorylation of the E2F heterodimerisation partner, thus inhibiting the DNA binding activity of E2F (45).

Following S phase the second interval or gap phase, G2, enables the cell to synthesise the proteins it requires prior to progression through M phase and mitosis. Transition from G2 to M phase occurs under the control of cyclin B-CDK 1. Cyclin B is synthesised during S phase in response to cell cycle mediated transcription. However, cyclin B mRNA is more stable in G2 than in G1 becoming less stable following DNA damage thus it is also regulated by mRNA stability providing a further check point in cell cycle progression (40). As cyclin B is synthesised throughout S phase cyclin B-CDK1 complex accumulates in the cell but, despite binding with CAK, it is held in an inactive state through phosphorylation at neighbouring threonine sites (Thr14 and Thr15) by Wee1. This inhibition is reversed late in G2 through dephosphorylation by Cdc25 in response to Polo like kinase (Plk). The activated cyclin B-CDK phosphorylates Cdc25, further activating it, and Wee1, inhibiting it, thus forming a positive feedback loop (40,56). Commencement of and progress through mitosis requires the reorganisation of the architecture of the cell, during metaphase, as a result of cyclin B-CDK1 mediated phosphorylation of key subcellular structures. These include; lamin subunits, resulting in nuclear lamina breakdown, the actin binding

protein caldemon, resulting in dissociation from actin microfilaments and therefore cell rounding, and the kinesin motor protein Eg5, leading to the formation of a bipolar spindle. Furthermore it appears that cyclin B-CDK1 also downregulates transcription during mitosis (40). Following these structural rearrangements the duplicated chromatids separate. The chromatids are associated by protein complexes along their length termed cohesins and in order for separation to occur, these are indirectly degraded by the ubiquitin ligase, anaphase promoting complex (APC). APC initiates a protein cascade via proteolysis of the securin protein whose action is inhibition of a protein termed separase. The removal of this inhibition results in separase cleaving the cohesins. APC activation occurs through the transient binding of the co-factor Cdc20. Cdc20 binding can only occur when APC has been phosphorylated on several of its subunits by mitotic kinases including PLK1 and CDK1 (57).

Exit from mitosis and the formation of two daughter cells requires a reversal of the initial cyclin B-CDK mediated steps of mitosis i.e. chromatid segregation, spindle disassembly, decondensation of the chromosomes and formation of the nuclear envelope. This occurs under the control of the ubiquitin ligase APC which degrades the M phase cyclins A and B. This degradation is initiated by the same binding of Cdc20 and APC that triggers the protein cascade leading to chromatid separation. As the cyclin B-CDK complex activates APC-Cdc20, this degradation in turn leads to down regulation of APC and thus enables accumulation of cyclins A and B to recommence during G1 of the following cell cycle (40,56).

1.2.1. Cell cycle checkpoints

As indicated in the initial overview of the cell cycle there are distinct check points within the cell cycle which enable the cell to scrutinise the intracellular environment

and DNA integrity prior to further progression. If the cell and its microenvironment do not fulfil the criteria of the checkpoint, progression through the cell cycle is arrested until it does or if damage is too severe the cell undergoes death by apoptosis. This ensures a degree of protection of the integrity of the genome. The main checkpoints are the G1/S and G2/M transitions though other checkpoints have been identified - for example cells are also able to arrest during S phase, if DNA damage is detected at this stage (40), or M phase, if loss or impairment of functional connections between spindle microtubules and kinetochores are detected (58).

The descriptions in the following sections detail the final intracellular mechanisms of cell cycle control. However, these are in turn mediated by complex intracellular pathways in response to various extracellular stimuli. These extracellular factors may be divided into three broad categories:

1. Mitogens – stimulate cell division.

2. Growth factors – stimulate an increase in cell mass by promoting protein and other macro-molecule synthesis and down regulating their degradation.

3. Survival factors – promote cell survival by suppressing pro-apoptotic pathways.

1.2.1.1. G1 Checkpoint

At the G1 checkpoint DNA damage induces cell cycle arrest which is dependent on p53. DNA damage leads to rapid induction of p53 activity which in turn promotes the transcription of several proteins including p21, Mdm2 and Bax. As previously described p21, a CKI, inhibits CDK1 and thus prevents DNA replication. Mdm2 activation leads to negative feedback on p53 by binding to p53 inhibiting its pro-

31

transcriptional action and promoting proteolytic degradation of p53 by ubiquitination. Increased transcription of Bax leads to diversion of the damaged cell into apoptosis. DNA damage is detected by several protein kinases such as ataxia-telangectasia-mutated (ATM) and ataxia-telangectasia and rad3 related (ATR). These phosphorylate p53 reducing its affinity for Mdm2 binding, resulting in decreased proteolytic degradation and therefore a potentiation of activated p53 in the cell (43). G1 arrest also occurs in response to other extrinsic and intrinsic stimuli including replicative senescence and TGFβ signalling. These lead to activation of the INK4 family of CDKIs and inhibition of dephosphorylation of cdc25. These in turn yield inhibition
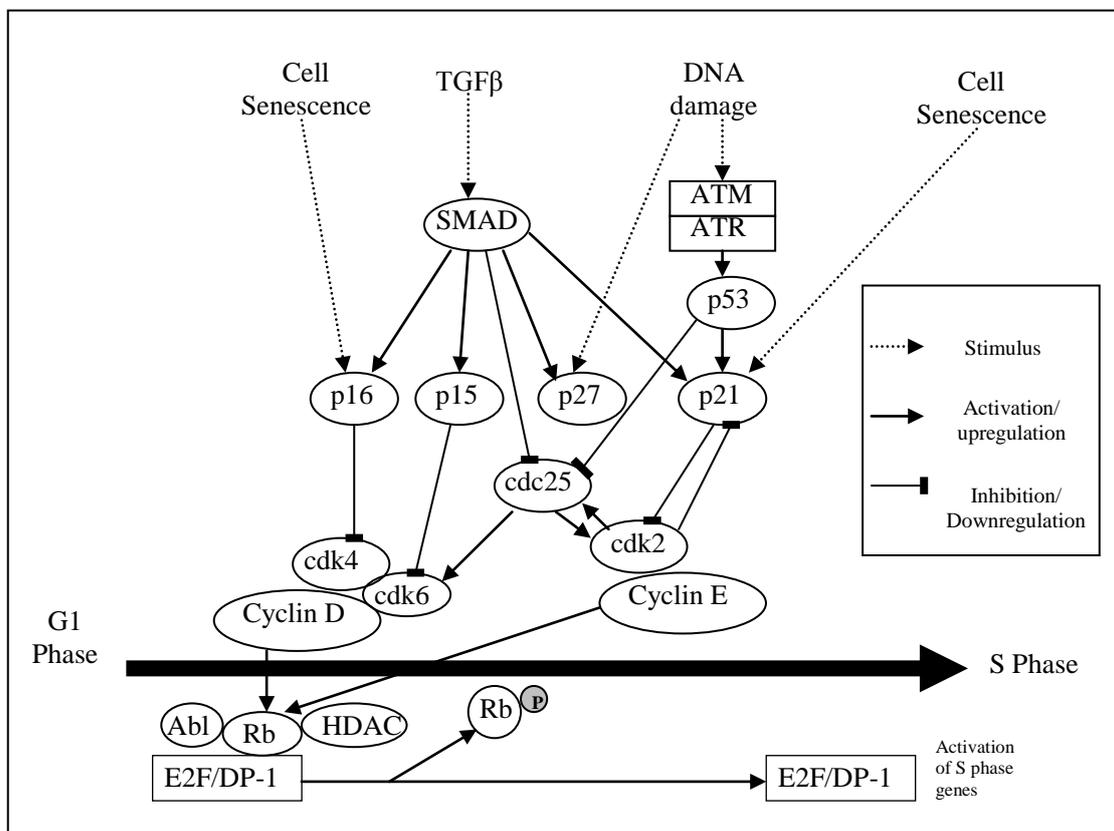
Figure 1.3: Regulation and signalling pathways at the G1 checkpoint.
In G1 the pRb-HDAC repressor complex binds to the E2F-DP1 transcription factors, inhibiting downstream transcription. pRb phosphorylation separates the complex enabling transcription of genes for S phase proteins and therefore cell cycle progression to occur. Multiple stimuli induce G1 arrest including TGFβ, DNA damage and cell senescence. Arrest is mediated by two pathways: 1) induction of members of the INK4 families of cell cycle kinase inhibitors and inhibition of cdc25; 2) induction and dissociation from Mdm2 of p53 and induction of p21. These pathways ultimately inhibit dissociation of the pRb-HDAC complex and thus induce cell cycle arrest. Adapted from www.biocarta.com /pathfiles/h_g1pathway.asp

of CDKs 4/6 and 2 with resultant inhibition of dissociation of phosphorylation of pRb and therefore of dissociation of the pRb-HDAC complex from E2F preventing transcription of genes encoding S phase proteins (43).

1.2.1.2. G2/M or DNA damage checkpoint

At the G2/M checkpoint DNA damage induced cell cycle arrest can occur independent of p53. Progression into mitosis is prevented by inhibition of the cyclin B-CDK1 complex either by inhibitory phosphorylation of the Cdc25 family of phosphatases, by protein kinases Chk1 and Chk2, or by sequestration of the complex
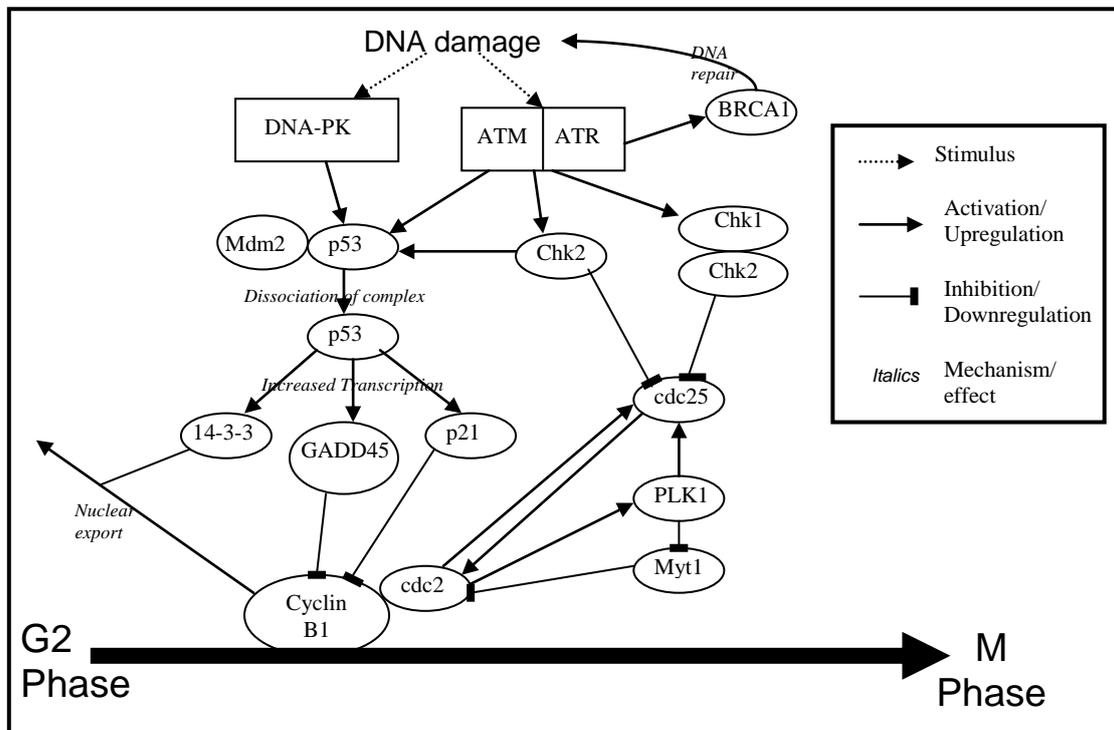
Figure 1.4: Regulation and signalling pathways at the G2/M checkpoint.
The cdc2-cyclin B kinase is central to regulation of the transition from G2 to M phase. During G2 cdc2(cdk1) activation is inhibited by Myt1. Under favourable conditions PLK1 activates cdc25 phosphatase and inhibts Myt1 yielding cdc2 dephosphorylation and activation. DNA damage leads to activation of two pathways yielding cell cycle arrest. Increased dissociation of p53 from Mdm2 results in increased transcription of 14-3-3, GADD45 and p21 with subsequent destabilisation and increased nuclear export of cyclin B1. ATM and ATR mediated activation of CHK1 and CHK2 produces cdc25 inhibition with resultant decreased phosphorylation of cdc2.
Adapted from www.**biocarta**.com/pathfiles/h_g2pathway.asp

outside the nuclear envelope. Chk1 and Chk2 are activated in response to DNA damage by ATM and ATR. p53 also plays a similar role at the G2/M checkpoint as at the G1/S checkpoint in terms of increased transcription of p21, 14-3-3 σ protein and growth arrest and DNA damage inducible protein (Gadd45). 14-3-3 σ binds to cyclin B-CDK1 preventing its transport into the nucleus and Gadd45 mediates dissociation of the complex (40,43). Furthermore as described earlier, cyclin B mRNA is destabilised, inhibiting cyclin B synthesis, in the presence of DNA damage, thus adding a further bar to progress into mitosis.

1.3. Cell death:

Cell death can be defined as an irreversible loss of plasma membrane integrity. A number of types of cell death, distinguished by morphological criteria, have been identified. These are apotoptosis, autophagy, senescence, mitotic catastrophe and necrosis (59-61).

Apoptosis, also termed programmed cell death, is characterised by specific changes in nuclear morphology including chromatin condensation and fragmentation, cell shrinkage, blebbing of plasma membranes and formation of apoptotic bodies prior to the loss of cell membrane integrity. This occurs as part of embryological development, tissue homeostasis and in response to DNA damage (59,62).

Autophagy, is a catabolic process characterised by cell digestion of cell constituents and the formation of double membrane, autophagic vacuoles in the cytoplasm termed autophagosomes. In response to cell stress, targeted cellular components are isolated from the remaining cell constituents in the autophagosomes. These then fuse with a

lysosome resulting in digestion of the contents. The process enables the cell to remove and recycle unnecessary or damaged cellular contents, resulting in either promotion of cell survival or cell death depending on the conditions (59,63).

Cell senescence was initially recognised as the process by which cells terminated at the end of a limited number of replications and as such was also referred to as cell ageing. The process is characterised by shortening of the telomere with each mitotic division ultimately leading to the loss of the telomere and permanent cell cycle arrest and death. More recently it has been demonstrated that cellular senescence can occur in response to oncogenic activation and stressors such as ionising radiation and chemotherapeutic agents. In some of these scenarios the senescent cell cycle arrest appears to be reversible rather than permanent (61,63,64).

Necrosis is predominantly defined negatively as cell death lacking the characteristics of the types described above. This mode of cell death is often associated with unwarranted cell loss in pathological processes and the uncontrolled release of cellular contents from dying cells into the adjacent tissues leading to an inflammatory response (59,65).

There are no established definitions of mitotic catastrophe and there is ongoing debate as to whether it constitutes a mode of cell death or a process leading to other modes of cell death, in particular apoptosis (66). It is frequently defined as cell death occurring following premature or inappropriate entry into or aberrant mitosis (60,66-68). Mitotic catastrophe is thought to be the primary mode of cell death in response to ionising radiation and has been associated with a multitude of mechanisms. It associated with certain characteristic morphological changes including

35

micronucleation, formation of nuclear envelopes around abnormal or non-segregated chromosomes, prior to cell termination (60,67).

1.3.1. Apoptosis

At the outset it was anticipated that of the three types, apoptosis would be of greatest relevance to this project and will therefore be considered in more detail. Apoptosis was first described by Kerr et al in 1972 (69) and constitutes the mechanism by which cells normally die (70). It is a mode of programmed cell death that is critical in both development and tissue homeostasis (62). Apoptosis is characterised by the nuclear and cellular changes listed in section 1.3. The main initiators and effectors of apoptosis are a family of cysteine-aspartate specific proteases termed caspases. At present 14 caspases have been identified and these may be classified according to their function into three classes:

1. Inflammatory caspases (Caspases 1, 4, 5, 11, 12, 13 and 14) – these are integral to the inflammatory response rather than apoptosis.
2. Apoptotic initiator caspases (Caspases 2, 8, 9 and 10)
3. Apoptotic effector caspases (Caspases 3, 6 and 7) – these are activated by upstream initiator caspases and cleave multiple cellular substrates.

They are synthesised as an inactive procaspase, which is cleaved to form the active form of the enzyme following the onset of apoptosis. Apoptosis is triggered via two distinct pathways, namely the intrinsic (Mitochondrial) and extrinsic (Death receptor) pathways.

The extrinsic pathway is stimulated by a wide range of proapoptotic stimuli, such as the binding of death inducing ligands to cell surface receptors. It is mediated by cell
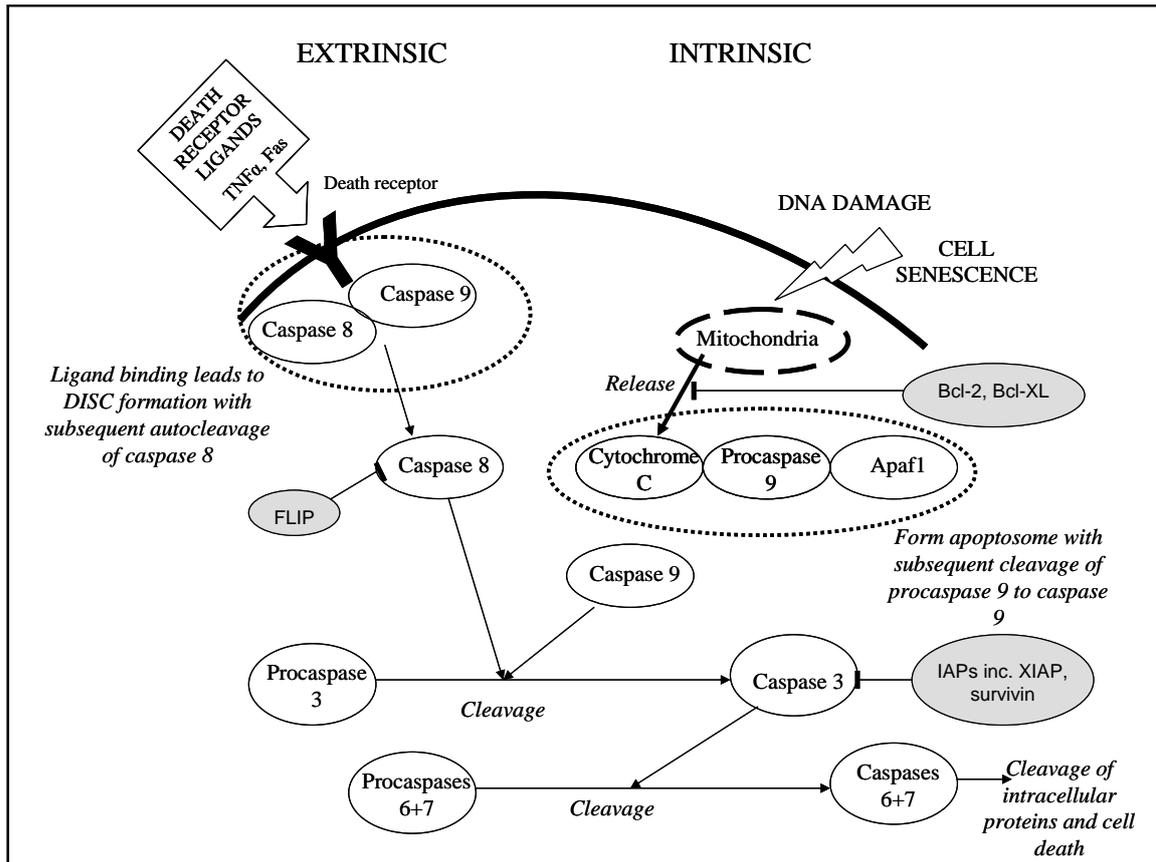
Figure 1.5: Schematic overview of the apoptosis pathways.
Apoptosis is triggered by two distinct pathways. The extrinsic pathway is triggered by binding of ligands to death receptors leading to formation of a death-inducing signalling complex (DISC) and autocleavage of caspase 8. The intrinsic pathway is activated by stimuli including DNA damage and cell senescence. Activation leads to increased mitochondrial membrane permeability and release of cytochrome C which complexes with Apaf1 and procasase 9 to form an apoptosome. Subsequent cleavage of procaspase 9 to the active caspase 9 occurs. The terminal pathway is common to both pathways. Caspases 8 and 9 cleave procaspase 3 to the active caspase 3 which in a cascade cleaves procaspases 6+7 to the active effector caspases 6+7. These cleave numerous intracellular target proteins yielding cell death. These processes may be inhibited at a number of stages including FLIP inhibition of caspase 8, Bcl-2 and Bcl-XL inhibition of cytochrome C release and inhibition of caspase 3 by inhibitory apoptotic proteins (IAPs).
Adapted from http://health.ucsd.edu/news/2003/06_30_Insel.html

surface death receptors such as TNF-related apoptosis-inducing ligand (TRAIL), Fas and tumour necrosis factor receptors. Binding of a death ligand leads to oligomerisation of the receptor, recruitment of the Fas associated death domain (FADD) and of the initiator caspase 8 to form a death-inducing signalling complex

(DISC). Caspase 8 subsequently autoactivates, enabling it to cleave the effector procaspases 3, 6 and 7. These act on a range of downstream targets:

- Structural proteins – this leads to destruction of the cellular architecture.

- DNA repair proteins – cleavage of these prevents unnecessary consumption of intracellular reserves which may be required by the apoptotic process such as ATP.

- Cell cycle regulatory proteins – cleavage of these may have a proaoptotic effect for example loss of pRb leads to an accumulation of procaspases (71).

The final steps of apoptosis are the formation of apoptotic bodies and subsequent phagocytosis of the cell. The latter is induced by the expression of prophagocytic signals on the cell surface for example phosphatidylserine (72).

The intrinsic pathway is triggered by a range of intracellular stimuli, such as DNA damage, leading to the release of cytochrome c, from the mitochondria, which binds to apoptosis protease activating factor 1 (Apaf 1) and procaspase 9. This results in the formation of a DISC like structure, termed an apoptosome, within which procaspase 9 is cleaved and activated. Caspase 9 then activates the effector caspase 3 as a cascade effect. The intrinsic and extrinsic pathways converge upon activation of the effector caspases with the subsequent terminal steps being identical (72).

Apoptosis is influenced by a range of cell signalling pathways and proteins including Myc, MAPK/JNK, Bcl-2, p53, PI-3K/AKT and NF-κB pathways. A number of these interactions are complex and/or controversial, for example JNK has been proposed as having either a pro-apoptotic effect, an anti-apoptotic effect or even no effect on apoptosis, although the predominant data indicate a proapoptotic effect (62,73-77).

Bcl-2 principally suppresses apoptosis, however, at high concentrations this reverses such that it is proapoptotic. Bcl-2 also forms a target of NF- κB with respect to apoptosis in that it upregulates expression of Bcl2. The role of c-Myc has apparently conflicting functions in that it promotes cell growth by suppression of Gadd45, p15, p21 and p27, whereas under or overexpression of c-Myc promotes apoptosis and though the precise mechanism of this remains unclear it does appear to occur in both a p53 dependent and independent manner (62)

Apoptosis usually occurs in a fine balance with cell replication. However, dysregulation of this homeostasis is integral to a number of pathologies including cancer (insufficient apoptosis) and neurodegenerative disorders (excess apoptosis) (78-82). Apoptosis also offers a number of potential targets for therapeutic interventions in these pathologies.

1.3.2.2. Necrosis

Necrosis, is primarily defined by an absence of the characteristic features of the other two cell death mechanisms usually occurring following metabolic failure associated with severe depletion of ATP, classically ischaemia, or mechanical trauma. The damage to or increased permeability of organellar membranes leads to release of proteolytic enzymes from the lysosomes and destruction of intracellular structures. It has previously been considered an accidental, i.e. non-programmed, cell death pathway however recent research indicates that it may occur in a regulated fashion with characteristic changes and can be triggered by death receptors such as TNFR1 (83). The precise mechanisms of necrosis activation and its downstream effectors have yet to be elucidated though some mechanistic data have been reported. TNFR1

signalling usually induces apoptosis via downstream activation of caspase 8 but it has been shown that if caspase 8 is inhibited, necrosis rather than apoptosis ensues. It has been proposed that this is occurs through loss of PARP cleavage by caspases and thus cellular depletion of ATP in turn inhibiting apoptosis, which is heavily ATP dependent. It has also been shown that, in the absence of caspase 8, the protein kinases RIP1 and RIP3, which are implicated in NFκB induction of apoptosis, form a complex critical to the induction of necrosis. It is of note that intracellular depletion of ATP in a cell undergoing apoptosis will result in conversion to necrosis (59,83).

1.4. Src

The proto-oncogene c-Src, herein referred to as Src, was first identified in 1976 as the cellular variant of the transforming oncogene present in avian Rous Sarcoma Virus (RSV), encoding a 60 kDa non-receptor tyrosine kinase (84,85). Subsequently nine structurally and functionally related proteins referred to as Src Family Kinases (SFKs) have been identified (86).

Increased Src activity is implicated in oncogenesis and progression of a number of malignancies though Src dysregulation is more commonly related to non genetic events yielding increased activation or persistence of an activated state rather than underlying mutations (86,87). In particular increased Src activity is associated with a worse prognosis and a tendency towards formation of metastases although data in bladder cancer are more equivocal suggesting a potentially protective role (85,87-91).

In colorectal cancer 12% of patients have Src activating mutations and Src is overexpressed in up to 80% of tumours (92-94). A role for Src in colon carcinogenesis

has been suggested, as activity is increased in adenomas relative to normal mucosa, with greater increases observed in malignant polyps and those with a high malignant potential (95). Additionally, Src overexpression induced colonic tumour formation in transgenic mice (96). Furthermore, overexpression has been shown to correlate with Dukes staging and to be an independent indicator of a poor prognosis (97).

Structurally the SFKs consist of an N terminal Src homology 4 (SH4) domain, the SH3 and SH2 domains, a linker sequence, the tyrosine kinase domain and the C-terminal tail. c-Src activity is determined by inhibitory phosphorylation at tyrosine 530 in humans (527 in avian analogues) which interacts with the SH2 domain enabling the protein to adopt a closed conformation. Upon dephosphorylation at tyrosine 530 a sequence of molecular interactions weaken, resulting in autophosphorylation at tyrosine 419 (416 in avian forms), allowing the molecule to

Figure 1.6: Activation of c-Src. Inhibitory phosphorylation at Tyr530 interacts with SH2 domain and induces a closed inactive closed conformation. Dephosphorylation at Tyr530 leads to destabilisation of molecular interactions resulting in autophosphorylation at Tyr419 and adoption of an open active conformation.

Adapted from Wheeler *et al* The Oncologist 2009;14:667-678

open to a conformation in which the SH2 and SH3 domains are able to interact with receptor tyrosine kinases, G-protein-coupled receptors and focal adhesion kinase (FAK) (figure 1.6) (84).

Src has a key role in many cellular functions that contribute to tumour progression. Src decreases apoptosis and promotes cell survival via Akt and PI3K mediated pathways in addition to promoting MAPK mediated cell proliferation (98-101). Data also indicate a role for Src in angiogenesis, which is critical both for survival of the primary tumour and also for formation of metastases; cells with increased Src activity display a greater increase in vascular endothelial growth factor (VEGF) expression in response to hypoxic stimulation and greater VEGF mediated endothelial barrier disruption which in turn results in increased tumour extravasation and metastasis (102,103). Src also has a crucial function in regulation of cell adhesion and maintenance of the actin cytoskeleton of the cell. Src overexpression leads to decreased cell-cell adhesion and increased cell migration through modification of integrin signalling, upregulation of metallo-matrix proteinases and loss of E-cadherin both functionally through phosphorylation and by increased degradation (104-109).

Src has been implicated in resistance to chemotherapeutic agents and data indicate that Src inhibition may chemosensitise previously resistant cell lines(110). EGFR is commonly overexpressed in colorectal cancer and current chemotherapeutic regimes frequently combine EGFR inhibitors such as cetuximab with cytotoxic agents. However, resistance to EGFR inhibition is proving a limitation to its use. Data indicating that Src may be implicated in acquired resistance to EGFR inhibitors and that Src blockade induces re-sensitisation are of particular interest (84,111,112).

Despite promising preclinical data, clinical trials of Src inhibitors as monotherapies have shown moderate benefits at best, though, as might be anticipated, combination therapies have shown more encouraging results (113). There is clearly scope to develop agents to work in conjunction with Src inhibitors or agents active on multiple cellular signalling pathways including Src inhibition to potentiate the effects. Dietary agents may have the potential to fulfil the latter requirement as it is well established that some bioactive dietary compounds mediate multiple intracellular pathways (32,35,114,115).

1.5. Indoles

Indole-3-carbinol (I3C), also known as 3-indolemethanol or hydroxymethylindole, is a bioactive indole formed from indolyl-methyl-glucosinate, within cruciferous vegetables, when they are cooked or crushed (116). It has been shown to inhibit the development of carcinogen-induced malignancies in rats and Min mouse models (117-121). In low pH conditions, as found in the stomach, I3C is converted into a further bioactive compound, 3,3-diindolylmethane (DIM) (122). DIM accumulates in the nucleus and may account for some of the effects attributed to I3C, particularly as DIM has been shown to spontaneously form from I3C *in vitro* thus rendering a degree of potential uncertainty to interpretation of preclinical data with I3C (116,123).
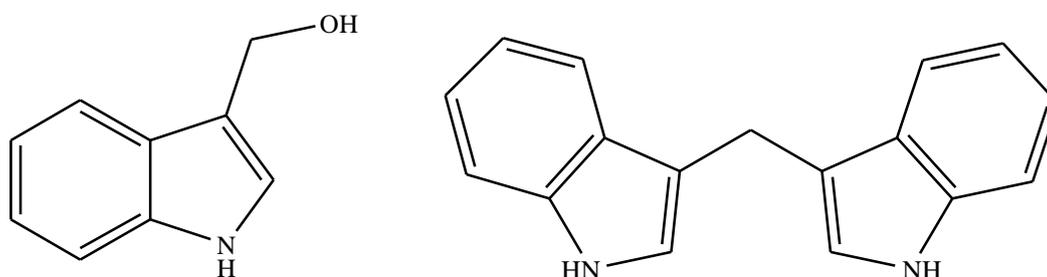
Figure 1.7: Chemical structure of indole-3-carbinol and 3,3-diindoloylmethane

I3C, and more recently DIM, have been the subject of significant research into their potential therapeutic uses and the mechanisms thereof. A number of potential targets have been identified thus far. In terms of their possible chemopreventative use, I3C and DIM have been shown to induce phase I and II enzymes in human and animal models, giving rise to increased metabolism and excretion of carcinogens, thus reducing DNA adduct formation through induction of a range of hepatic enzymes including cytochrome p450, NADPH and NADH reductases (116-118,124-128). An additional cell protective effect of I3C and DIM is that they appear to induce DNA repair mechanisms. DIM has been shown to upregulate GADD153 gene expression and both I3C and DIM upregulate BRCA1 protein activity (116).

Uncontrolled cell proliferation is one of the recognised features of tumours and there is growing evidence that indoles suppress this. Increasing evidence indicates that indoles induce a predominantly G1 cell cycle arrest in carcinomas of the breast and prostate (121,129-135). This may be via inhibition of CDKs acting at G1/S transition as I3C and DIM have been shown to upregulate transcription of the p21 and p27 genes and downregulate CDK6 gene expression via changes in the binding of the Sp1 transcription factor to the gene promoter region (134-138). Additionally I3C and DIM are able to inhibit the phosphorylation of pRb (133,138).

I3C and DIM also exhibit pro-apoptotic actions which contribute to their overall growth inhibitory effect. They have been shown to downregulate Bcl2, suppressing it's inhibitory binding to Bax as well as directly upregulating Bax (130,138,139). It has been proposed that this may at least in part be due to suppression of NF- κB activation (140,141).

EGFR and Src have both been implicated in I3C-induced cell cycle arrest and apoptosis in breast carcinoma cell lines. Specifically EGFR appears to be of greater importance in apoptosis, whereas Src is of greater relevance in cell cycle arrest (142). It has been shown that the combination of I3C and an EGFR inhibitor was effective against a number of breast carcinoma cell phenotypes, at concentrations which are ineffective individually and the combination of I3C and a Src inhibitor was more efficacious than treatment with either agent alone against two breast carcinoma phenotypes. DIM has been shown to induce apoptosis via downregulation of EGFR signalling pathways in breast and lung cancers, these effects occurred irrespective of EGFR mutation status (143). Additionally combination of DIM, with the EGFR inhibitor, erlotinib augmented EGFR downregulation and resultant apoptosis in pancreatic cancer cells (144). The effect of DIM on Src expression and Src mediated pathways has not been explored to date. These findings indicate a potential for therapeutic use of indoles in conjunction with EGFR and Src inhibitors (145,146).

Anderton *et al*. have shown in mice that following oral administration of I3C, both I3C and DIM were detectable in plasma at 15 minutes with I3C dissipating by 1 hour and DIM remaining at detectable levels after 6 hours. They demonstrated that both compounds concentrated in the tissues with concentrations of I3C in the liver and kidney 6 and 4 fold greater than plasma levels respectively. I3C concentrations in the heart and lungs were only slightly elevated relative to plasma levels. Similar distributions were observed with DIM although concentrations were lower but as with plasma levels they were sustained for more than 6 hours (147). In a separate murine study, Anderton *et al*. also demonstrated a rapid rise in plasma and tissue concentrations of DIM following oral administration of an absorption enhanced formulation of DIM (BR-DIM, Bioresponse, USA) with detectable levels persisting

beyond 24 hours post administration. As with I3C, DIM concentrated in the tissues with levels ≥3x plasma concentrations in all tissues examined other than brain. The highest DIM concentrations were detected in the liver with concetrations 8x plasma levels (148). Subsequent data in humans have shown oral administration BR-DIM was well tolerated at doses of up to 200mg, yielded plasma concentrations of up to 104ng/ml and undergoes renal excretion (149,150).

In clinical trials both I3C and DIM have been shown to be associated with minimal, minor side effects (149-151). Thus I3C and DIM may be considered safe potential agents both for chemopreventative and chemotherapeutic use. At present there are ongoing clinical trials into using DIM as a therapeutic agent in cervical intraepithelial neoplasia and prostate carcinoma and chemoprevention in patients with a high genetic risk for breast carcinoma (152).

Despite the extensive preclinical data relating to indoles, DIM especially, in breast and prostate cancer there is a relative paucity of studies regarding indoles in colorectal carcinoma. Within this limitation, recent data has shown a significant potential for DIM in colorectal carcinoma that warrants further investigation. DIM has been shown to reduce colonic inflammation and colitis associated malignancy in murine models (153). In line with this, it has been shown that DIM enhances butyrate induced apoptosis, in mice expressing mutant APC genes, and promotes HDAC degradation, with subsequent promotion of apoptosis, in colorectal carcinoma *in vitro* (154,155).

Given the link between dietary intake of cruciferae with a reduced colorectal cancer incidence, their proven effects in other cancer types and low side effect profile indoles constitute attractive potential agents both for chemoprevention and chemotherapy in

colorectal malignancy meriting further investigation. The potential role of indoles in the treatment of colorectal liver metastases is of particular interest, as in pharmacokinetic studies outlined above the highest concentrations of these agents were found in hepatic tissue (147,148). Furthermore as outlined previously a large proportion of colorectal carcinoma cells are EGFR-dependent and stage-dependent activation of Src is a feature of colorectal carcinoma (119,156). Therefore any effect of I3C or DIM on colorectal carcinoma is likely to be augmented by combination with a Src and/or an EGFR inhibitor.

1.6. Aims and objectives

The overall objective of this project is to develop effective, novel treatments for colorectal carcinoma using indoles.

Using a panel of four phenotypically different colon carcinoma cell lines the effects of I3C and DIM, on cell proliferation, apoptosis and cell cycle arrest, at doses anticipated to be achievable *in vivo,* will be studied. The involvement of EGFR and Src in the response to indoles in colorectal carcinoma will be examined with the aim of potentiating their effect by combination with commercially available EGFR and Src inhibitors. These data will be used to develop a potentially therapeutic combination of agents.

In order to evaluate indole induced cell cycle arrest and apoptosis in colorectal carcinoma hepatic metastases primary cultures will be obtained perioperatively from the resected tissue of patients undergoing elective resection of hepatic colorectal carcinoma metastases at the University Hospitals of Leicester NHS Trust. A

reproducible method for establishing primary cultures will be developed. Cultures established will then be utilised to test the most efficacious treatments identified by the *in vitro* studies.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals

Unless otherwise stated chemicals/materials were sourced from Sigma-Aldrich, UK.

30% Acrylamide

Annexin V Kit

Antibiotic Antimycotic Solution (100x) stabilised

ATPLite Viability assay

AZD0530, Astra-Zeneca

Bio-Rad protein assay reagent

Caspase-Glo assay

DMEM containing 4.5g Glucose

Dried Milk Powder

ECL Detection kit

ECL Hyperfilm

Foetal Calf Serum

Iressa^{TM}, Astra-Zeneca

Page Ruler Plus, Fermentas

PP2

2.1.2 Antibodies

Primary Antibodies against the following proteins:

Actin, Santa Cruz sc-1616

Akt1, Santa Cruz sc-1618

Bax, Calbiochem AM32

Bcl2, Calbiochem AM59

Bcl-XL, Calbiochem AM05

Bid, Santa Cruz sc-6539

Bim, Calbiochem 202000

Cdc2, Cell Signalling #9112

Cdc25A, AbCam ab2357

Cdc25B, BD Pharmingen 610527

Cdc25B, Cell Signalling #9525

Cdc25C, Santa Cruz sc-13138

CDK6, Santa Cruz sc-177

CHK1, Cell Signalling #2345

CHK2 , Cell Signalling #2662

cSrc, Santa Cruz sc-5266

Cyclin B1, Santa Cruz sc-245

Cyclin D1, Santa Cruz sc-8396

Cyclin E, Santa Cruz sc-247

NFκB (p65 subunit), Cell signalling #3034

p16, BD Pharmingen 554070

p21, Santa Cruz sc-397

p27, Santa Cruz sc-1641

PARP, Cell Signalling #9542

phospho Akt (Ser473), Cell Signalling #9271

phospho Cdc2 (Tyr15), Cell Signalling #9111

phospho CHK2 (Thr68), Cell Signalling #2661

phospho Src (Tyr416), Cell Signalling #2101

phospho Src (Tyr527), Cell Signalling #2105

Plk1, Santa Cruz sc-17783

Survivin, Novus NB500-201

α-Tubulin, Oncogene CP06

Secondary Antibodies

Anti-goat, donkey IgG-HRP conjugated, Santa Cruz sc-2020

Anti-mouse, goat IgG-HRP conjugated, Sigma A8924

Anti-rabbit, goat IgG-HRP conjugated, Sigma A8275

2.1.3   Suppliers Addresses

BD Pharmingen, San Diego, California, USA

Bender Medsystems, Vienna, Austria

Bio-Rad, Hemel Hempstead, Hertfordshire, UK

Cell SignalingTechnology, Boston, USA

Fermentas, Ontario, Canada

GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK

Promega, Mannheim, Germany.

Santa Cruz Biotechnology, California, USA

Sigma-Aldrich, Dorset, UK

2.2   Buffers

Cell lysis buffer

This was prepared as a 10x stock consisting of:

200mM Tris (pH7.5)

1.5M NaCl

10mM EDTA – only soluble under alkaline conditions

10% Triton X-100

25mM Sodium pyrophosphate

10mM β-glycerophosphate

10mM Sodium orthovanadate

Made up to 500ml with distilled H_2O.

Immediately prior to use 10x stock was diluted to 1x with distilled H_2O and protease inhibitor cocktail was added to a final concentration of 1x.

Polyacrylamide denaturing running gel

Volumes of water, acrylamide and TEMED varied according to the percentage gel cast as per the table below.

Percentage Gel	Volume distilled H_2O (ml)	Volume 30% acrylamide (ml)	Volume TEMED (μl)
6	5.3	2.0	8
8	4.6	2.7	6
10	4.0	3.3	4
12	3.3	4.0	4
15	2.3	5.0	4

The following did not vary between gel percentages:

2.5ml 1.5M Tris.HCl (pH8.8)

100μl Sodium dodecyl sulphate (SDS)

100μl Ammonium Persulphate (APS)

Gel polymerisation was induced by the addition of the APS and TEMED.

Polyacrylamide denaturing stacking gel (10ml)

6.8ml H_2O

1.7ml 30% acrylamide

1.25ml 1.0M Tris.HCl (pH 6.8)

100μl 10% Sodium dodecyl sulphate (SDS)

100μl 10% Ammonium Persulphate (APS)

10μl TEMED

Gel polymerisation was induced by the addition of the APS and TEMED.

SDS-PAGE running buffer

25mM Tris

192mM Glycine

0.1% Sodium dodecyl sulphate (SDS)

20% Methanol

Running buffer is available as a 10x stock from Geneflow, UK.

Gel stripping buffer

0.2M Glycine

0.4% Sodium dodecyl sulphate (SDS)

pH2.0

4x SDS loading buffer

200mM Tris.HCl (pH 6.8)

400mM Dithiothreitol (DTT)

8% Sodium dodecyl sulphate (SDS)

0.4% Bromophenol blue

40% Glycerol

High concentrations of SDS are poorly soluble, therefore the SDS should be dissolved in the required volume of distilled H_2O in a waterbath heated to 30-40ºC prior to the addition of the other components.

4xSDS loading buffer lacking DTT may be stored at room temperature. However DTT is relatively unstable at room temperature so the appropriate volume should be added to the buffer immediately prior to use.

Western transfer buffer

20mM Tris

150mM Glycine

Transfer buffer is available as a 10x stock from Geneflow, UK.

2.3 Cell Lines

HT29, SW480 and SW620 colorectal carcinoma cell lines originating from ATCC were kindly provided by Dr. Elena Moiseeva (Department of Cancer Studies and Molecular Medicine, University of Leicester). HCT116 wt, p53$^{-/-}$ and p21$^{-/-}$ lines were a kind gift from Dr Bert Vogelstein. HCEC colonic epithelial cells originally from

Nestec, were kindly provided by Mr. Chris Briggs (Department of Cancer Studies and Molecular Medicine, University of Leicester).

HeLa cervical adenocarcinoma cells were kindly provided by Dr. Karen Bowman (Department of Cancer Studies and Molecular Medicine, University of Leicester).

J82 bladder adenocarcinoma cells (ATCC) were kindly provided by Dr. Mai-Kim Cheng (Department of Cancer Studies and Molecular Medicine, University of Leicester).

MDA 468 breast carcinoma cells (ATCC) were kindly provided by Dr Louise Blakemore (Department of Cancer Studies and Molecular Medicine, University of Leicester).

HT29: Human primary colorectal adenocarcinoma

HCT116: Human primary colorectal adenocarcinoma

SW480: Human primary colorectal adenocarcinoma.

SW620: Human colorectal nodal metastasis (from same patient as SW480).

HCEC: Human colonic normal epithelium, SV40 transformed.

HeLa: Human cervical adenocarcinoma.

MDA 468: Human breast carcinoma derived from pleural effusion of patient with metastatic breast carcinoma.

J82: Human primary bladder transitional cell carcinoma

All cell culture procedures were performed in a class II laminar flow cabinet. Cell lines were mycoplasma negative.

2.3.1   Maintenance of cell lines

All cell lines were maintained in DMEM culture medium containing 4.5g/l glucose and supplemented with 10% FCS. Cells were incubated at 37ºC, 5% CO_2 and 100% humidity.

2.3.1.1   Resurrection of cells from liquid nitrogen storage

Cells not required for immediate experimental use were stored in liquid nitrogen.

Cells were removed from liquid nitrogen and warmed, until the storage medium was in liquid phase, in a water bath at 37ºC. The cell suspension was then added to 10 mls of culture medium in a universal tube prior to centrifugation at 1000rpm for 5 minutes. The supernatant was then aspirated to remove the majority of the freezing medium. The pellet was then resuspended in culture medium with 10% FCS and transferred to a fresh culture flask. The cells were incubated for 24 hours or until visibly attached, after which the culture medium was aspirated and replaced with fresh culture medium containing 10% FCS.

Resurrected cells were passaged once prior to experimental use.

2.3.1.2   Passaging of cells

Cells were passaged at 70-80% confluence and a frequency of twice per week.

Cells were washed x3 with 10 mls PBS to remove all culture medium. Trypsin EDTA (TE) (1x) was then added (3 and 5 mls in 75 cm^2 and 175cm^2 flasks respectively). Flasks were then incubated until all cells were detached by a firm tap to the flask, usually 5 to 10 minutes). The TE was then neutralised by the addition of 10 mls medium containing 10% FCS which also served to wash off the detached cells. The

cells were then pelleted from the resultant suspension by centrifugation at 1000rpm for 5 minutes. The supernatant was removed and the pellet re-suspended in 10-15mls of culture medium containing 10% FCS. One ml was then seeded in a fresh flask containing culture medium with 10% FCS (14 and 24 mls in 75 cm^2 and 175cm^2 flasks respectively).

Cells were passaged no more than 40 times following resurrection from liquid nitrogen storage.

2.3.1.3   Preparation of cells for storage in liquid nitrogen

Cells were washed in PBS, detached by trypsinisation and pelleted as for passaging of cells described above. After centrifugation the pellet was resuspended in cell freezing medium consisting of 90% FCS and 10%DMSO. The resultant cell suspension was placed in cryotubes and stored at -20 ºC for 24 hours. After 24 hours the tubes were transferred to liquid nitrogen storage.

2.3.2   Treatment of cells with indoles or inhibitors

Cells were seeded at the required density and incubated for a minimum of 12 hours to allow the cells to adhere prior to treatment.

Stock solutions of the indoles and inhibitors were prepared in DMSO such that the final solutions made up for treatment would contain less than 0.1% DMSO. The stock solutions were aliquoted and frozen. Aliquots were discarded after one cycle of

freezing and thawing. For each experiment an equivalent DMSO control was included.

2.4 Primary cultures

2.4.1 Patient recruitment

Inclusion criteria

- Patients must be over 18 years of age.

- Patients must be of sound mind to give written informed consent

- Patients must have a diagnosis of colorectal liver metastases.

- All patients must have disease amenable to surgical exploration ± resection.

2.4.2 Collection of tissue samples from resected tissue

Fresh tissue samples were collected for immediate culture and for freezing and storage in liquid nitrogen. Tissue samples were obtained from freshly resected hepatic tissue containing metastatic colonic carcinoma. Tissue was transected through the metastasis by the operating surgeon following resection. The central core of the exposed metastatic tissue was excised with a scalpel ensuring that the margin of the metastasis was not breached to prevent any deleterious impact on subsequent hisotological analysis of the resected specimen.

If sufficient tissue was obtained the excised core was divided into two with one half being set aside for immediate culture and the remaining tissue was frozen in nitrogen. If insufficient tissue was available for such a division all of the tissue was cultured.

2.4.3 Freezing and storage of tissue samples in liquid nitrogen

The tissue for freezing in liquid nitrogen was divided into blocks of no greater than 4x4x3mm. Small pieces of cork were prepared with one drop of the adhesive, embedding compound OCT. Isopentane 20mls was placed in a beaker and cooled in liquid nitrogen until syrupy with a layer of solid in the bottom. The tissue blocks were placed onto the OCT coated side of the cork (1 block per cork piece). These were then snap frozen for 30 seconds in the syrupy isopentane using long metal forceps. The frozen blocks were removed from the isopentane and immediately placed in labelled eppendorfs with no more than three blocks per eppendorf. The labelled eppendorfs were placed into the liquid nitrogen.

2.4.4 Transport of tissue

Fresh tissue samples for immediate culture were suspended in culture medium with 10% FCS, in a universal container. The universal was placed on dry ice for transport to the laboratory to prevent further cellular degradation during transfer.

Fresh frozen samples were placed in eppendorfs and transported in a Dewar flask, containing liquid nitrogen.

2.4.5 Preparation of tissue for culture

All tissue was treated as potentially infected and therefore was processed in a class 1 hood. The universal containing the resected tissue was removed from dry ice and the medium allowed warm to room temperature over 5-10 minutes. The tissue was then gently washed with 10 mls of culture medium containing 10% FCS. Culture medium

used for transport of tissue and washing was retained and centrifuged at 1000rpm for 5 minutes to retrieve any floating cells. The supernatant was aspirated and discarded. The pellet was re-suspended in 50:50 Hams F12:DMEM 4500, with gentle agitation to disperse, and transferred to a fresh culture flask. The flask was then incubated at 37ºC, 5% CO_2 and 100% humidity.

The resected tissue was then considered ready for cell dispersal. The subsequent steps were not universal across all specimens as discussed in chapter 3, section 1.2.2.. The tissue was either disaggregated manually or mechanically with a Medimachine™ (BD Biosciences). Where sufficient tissue was obtained equal volumes were dissagregated by each technique. However where there was insufficient tissue for this, manual dissociation was used preferentially.

2.4.5.1. Mechanical tissue disaggregation:

The resected tissue was dissected into blocks of approximately 2mm^3. The Medicon ™ (BD Biosciences) was prerinsed x2 with culture medium. 1ml of culture medium and 3-6 blocks of tissue were then placed in the Medicon. The lid was then placed on the Medicon and it was inserted into the Medimachine. The tissue was then dissagregated with 2 pulses of either 15 or 20 seconds (See chapter 3, section 1.2.2). Following disaggregation the suspension was removed with a syringe via the syringe port. The Medicon was then flushed x3 with 1ml of culture medium and the suspension obtained was retained. This was then resuspended in culture medium in a fresh culture flask for incubation.

2.4.6   Cell culture conditions

The cells were maintained in culture medium supplemented with 10% FCS and 1x antibiotic antimycotic solution (Sigma-Aldrich). The culture media used are discussed in chapter 3. The cells were incubated at 37ºC, 5% CO_2 and 100% humidity. Cells were passaged when at approximately 70% confluence and the culture medium was refreshed every 72-96 hours. All passaged cells were retained and seeded to flasks.

2.5   Assessment of cell proliferation and cell death

2.5.1   Cell viability assay

ATP may be used as a marker for cell viability as it is integral to cell metabolism and decreases markedly in cells undergoing cell death by apoptosis/necrosis. Given the latter it may also be used as a surrogate marker for cell proliferation.

An ATP assay (ATPLite, Perkin Elmer) was used to study the effect of indoles and inhibitors on cell viability and proliferation. The assay functions by chemically lysing the cells enabling quantification of ATP levels on the basis of luminescence caused by the reaction of ATP, an added firefly luciferase and D-luciferin. The light emitted is proportional to ATP concentration.

White 96 well plates (Packard Viewplate-96) were seeded with $1x10^4$ cells/well in 100µl medium. To prevent cross contamination of luminescence at time of assay cells were seeded to alternate lines on the plate. Plates were then incubated overnight. After 12 hours the culture medium was aspirated and replaced with culture medium containing either the treatment solution or DMSO with 8 wells per treatment. Plates

were then incubated for the required time interval prior to assay (12, 24, 48, 72 and 96 hours).

The assay was performed as per the manufacturer's protocol and the luminescence was measured using an Optima FLUOstar Microplate reader (BMG Labtech) at 21ºC. The assays were repeated on three separate occasions.

2.5.2   Cell proliferation assay

In order to validate the results obtained from the ATPLite assay cell proliferation was also measured directly by cell counting.

Cells were seeded on 12 well plates (Nunc) at $1\text{x}10^4$ cells/well in 1ml culture medium. The plates were incubated at 37ºC for 12 hours to allow the cells to adhere. The culture medium was then aspirated and replaced with 1ml culture medium containing either the treatment solution or DMSO with two samples per treatment. The plates were then incubated at 37ºC for 48 hours. The cells were washed x3 with PBS and harvested with 500µl of trypsin which was neutralised with 500µl of culture medium. The resultant single cell suspension was then diluted with 9mls of isoton buffer (Beckman Coulter). Cells were counted using a ZM particle counter (Beckman Coulter). The assays were repeated on three separate occasions.

2.5.3   Cell cycle analysis

Propidium iodide (PI) is a DNA binding dye detectable on flow cytometry. The quantity of bound PI and therefore fluorescent signal is directly proportional to the amount of DNA present in the cell. Flow cytometry analysis of a cell population

prestained with PI allows the proportion in each phase of the cell cycle to be determined.

Cells were seeded onto 9cm^2 culture plates at a density of 1×10^6 per plate in 10 mls of DMEM containing 10% FCS. Cells were then incubated at 37°C for 24 hours prior to treatment. The culture medium was then replaced with 10mls of the treatment medium and the cells were incubated for the required time period.

The cells were then washed x3 with PBS before the addition of 1ml of 1xTE. The cells were then incubated at 37°C for 5 minutes or until cells were fully detached. Five mls culture medium was then added to neutralise the trypsin. The cells were pelleted by centrifugation at 1000rpm for 5 minutes. The supernatant was discarded, the pellet washed with 10mls PBS and centrifuged at 1000rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 2mls ice cold 70% ethanol using a vortex. Samples were incubated for a minimum of 12 hours at 4°C to fix the cells. Fixed samples may be stored for up to 4 weeks prior to analysis.

The samples were centrifuged at 1000rpm for 10 minutes before resuspending the pellet in 800µl PBS. 100µl of Rnase A (1mg/ml) and 100µl of PI (50mg/ml) were added. Samples were incubated overnight at 4°C before analysis of DNA content. Samples were transferred to 5ml Falcon tubes and analysed on a FACScan Flowcytometer (Becton-Dickinson) using the Cell Quest program.

2.5.4 Caspase-Glo assay for apoptosis

The Caspase-Glo 3/7 assay (Promega) was used to study the effect of indoles on

induction of apoptosis. Caspases 3 and 7 play key effector roles in apoptosis and thus can be used as markers of this process. The assay kit contains a luminogenic caspase 3/7 substrate in a reagent optimised for caspase activity, luciferase activity and cell lysis. Caspase cleavage of the substrate liberates free amino-luciferin resulting in the production of light. The luminescence is proportional to the caspase activity.

White 96 well plates (Packard Viewplate-96) were seeded with $1x10^4$ cells/well in 100µl medium. To prevent cross contamination of luminescence at time of assay cells were seeded to alternate lines on the plate. Plates were then incubated overnight. After 12 hours the culture medium was aspirated and replaced with medium containing either the treatment solution or a DMSO control with 4 wells per treatment. Plates were the incubated for the required time interval prior to assay.

The assay was performed as per the manufacturer's protocol and the luminescence was measured using an Optima FLUOstar Microplate Reader (BMG Labtech) at 21ºC. All assays were performed in triplicate.

2.5.5   Annexin V staining for cell death

The Annexin V:FITC apoptosis assay (Bender Medsystems) was used to study the effect of treatment with indoles on cell death. The assay identifies live, apoptotic and necrotic cells within the sample population.

Apoptotic cells undergo a number of changes including the translocation of phosphatidylserine from the inner surface to the outer surface of the cell membrane. Annexin-V is a protein capable of binding to phosphatidylserine and therefore apoptotic cells. In necrotic cells the cellular architecture and membrane is disrupted

thus exposing both surfaces of the cell membrane therefore Annexin-V also binds to necrotic cells. The assay kit contains Annexin-V conjugated to fluoroscein isothiocyanate (FITC) which renders it detectable on flow cytometry.

The assay also contains propidium iodide (PI) which is excluded by intact cellular membranes. Thus PI uptake distinguishes between viable and non-viable cells.

Flow cytometry of treated cells detects three cell populations

1. Cells negative for Annexin V and PI staining – viable cells
2. Cells positive for Annexin V staining negative for PI staining – cells in the early stages of apoptosis
3. Cells positive for both Annexin V and PI staining – cells undergoing necrosis or in the late stages of apoptosis

Cells were seeded onto $9cm^2$ culture plates at a density of $1x10^6$ per plate in 10 mls of DMEM containing 10% FCS. Cells were then incubated at 37ºC for 24 hours prior to treatment. The culture medium was then replaced with 10mls of the treatment medium and the cells were incubated for the required time period.

After incubation the culture medium was aspirated and retained to collect any floating cells. The adherent cells were then harvested by trypsinisation in 1xTE. The floating cell and harvested adherent fractions were combined and pelleted by centrifugation at 1000rpm for 5 minutes. The cell pellet was resuspended in 10 mls of culture medium containing 10% FCS. The cells were then incubated at 37 ºC for 30 minutes to allow the cells to equilibrate and the cell membranes to stabilise following trypsinisation, thus avoiding false positive results for apoptosis. After 30 minutes the cells were

repelleted and suspended in 1ml of 1x annexin binding buffer. 1µl of Annexin V FITC conjugate was added and the cells were incubated for 10 minutes at room temperature. PI was then added to a final concentration of 1.5 µl/ml and the cells were incubated for a further 1 minute at room temperature prior to placement on ice. The cells were analysed on a FACScan Flowcytometer (Becton-Dickinson) using the Cell Quest program.

The assay was performed on three separate occasions for each timepoint and treatment.

2.6 Preparation of cell lysates

2.6.1 Preparation of whole cell lysates

Cells were seeded to 9cm plates at densities of 2 and 1.5 $\times 10^6$ cells per plate for 24 and 48 hour treatments respectively or at 10-20% confluence in $75cm^2$ flasks. The cells were incubated for 12-24 hours to allow them to adhere prior to treatment.

Following the required treatment period the plates were placed on ice and the culture medium was aspirated and retained. The cells were then washed with 5-10mls PBS and the irrigated PBS was combined with the retained medium. The cells were then washed x2 with PBS which was aspirated to dryness. Cell lysis buffer (200-400ul) was added and the plates were incubated on ice for 10 minutes. The plates were then scraped and the collected residue was aspirated and transferred to a cold eppendorf tube.

The previously retained medium was pelleted by centrifugation at 1000rpm and 4ºC for 10 minutes to collect the floating cell fraction. The supernatant was removed and

the pellet washed with 10 mls PBS. The cells were then repelleted. The supernatant was removed and 20µl of cell lysis buffer was added to the pellet before incubation on ice for 10 minutes. The pellet/buffer was then added to the above cold eppendorf tube. The combined fractions were then centrifuged for 10 minutes at 13000rpm and 4ºC. The supernatant, containing the purified cell lysate, was then aspirated and stored at -20ºC.

2.6.2 Quantitative determination of protein concentration using the Bradford assay

In order to ensure equal loading of protein on gels for blotting the protein concentration of the cell lysates was determined before use.

2.6.2.1 Preparation of a standard curve

In order to determine the protein concentration by absorbance a BSA calibration curve was generated.

A stock solution of Bio-Rad staining dye was prepared by diluting 5x Bio-Rad dye in distilled water in a ratio of 1 ml dye to 3.9 mls water. BSA solution (1mg/ml) was diluted to the concentrations in the table below. For each concentration two samples were produced. The required volume of water was added followed by the required volume of BSA solution. Bio-Rad dye stock solution (980µl) was added and the mixture was vortexed for 30 seconds. The mixture was transferred to a plastic cuvette and incubated for 5 minutes at room temperature.

The absorption at 595nm against a distilled water sample was then measured using an Hitachi U-3010 spectrophotometer. For each of the BSA standards if the difference in absorption between the two duplicates was greater than 0.05 the sample was repeated. For determination of the curve the average absorption of the two duplicate samples was used.

Concentration BSA (mg/ml)	Volume 1mg/ml BSA (μl)	Volume water (μl)	Volume Bio-Rad Dye stock solution (μl)	Final volume (μl)
0	0	20	980	1000
0.004	4	16	980	1000
0.008	8	12	980	1000
0.012	12	8	980	1000
0.016	16	4	980	1000
0.02	20	0	980	1000

For the most accurate results the BSA calibration curve should repeated each time the protein concentrations are determined in order to minimise the effects of temperature variations.

2.6.2.2 Measuring the samples

A stock solution of Bio-Rad staining dye was prepared by diluting 5x Bio-Rad dye in distilled water in a ratio of 1 ml dye to 3.98 mls water.

For each whole cell lysate, two samples were prepared. Bio-Rad staining dye stock solution (998µl) was added to an eppendorf. Whole lysate (2 µl) was then added and the mixture vortexed for 30 seconds. The mixture was then transferred to a plastic cuvette and incubated for 5 minutes at room temperature. The absorption at 595nm against a distilled water sample was then measured using a Hitachi U03010 spectrophotometer. Any with a difference in absorbance of greater than 0.05 were repeated. The average of the two duplicates was taken and the BSA calibration curve generated as described above was used to calculate the protein concentration.

2.7 Western blotting

Whole cell lysates of known protein concentration were combined with distilled water and 4x loading buffer to give a final concentration of 1mg/ml protein and 1xSDS. The tops of the eppendorf tubes were punctured with a 22G needle then the eppendorfs were placed in a heating block, preheated to 100 ºC, for 5 minutes.

The samples were loaded onto a polyacrylamide gel made up of a 5% stacking gel and a running gel of percentage determined by the molecular weight of the target protein as per the table below. One well per gel was loaded with 3µl of a pre-stained protein ladder to facilitate protein identification after blotting.

Gel Percentage	Linear range of separation (kD)
15	12-43
10	16-68
7.5	36-94
5	57-212

The samples were electrophoresed in 1x western running buffer for approximately 1 hour at 100V using a Bio-Rad Protean vertical gel system.

The proteins were transferred from the gels to nitrocellulose membrane in 1x western transfer buffer using a Bio-Rad Trans-Blot wet blotting system for 1 hour at 100V. The quality of the membranes following transfer was confirmed by washing in Ponceau Red stain and subsequent visual inspection for bubbles or other anomalies. The membranes were washed x3 in PBST and blocked by immersion in 5% milk or 5% BSA on a rocking plate for 1 hour. The membranes were washed x3 in PBST prior to addition of the primary antibody overnight at 4ºC on a rocking plate. The membranes were then washed x3 in PBST before addition of the relevant secondary antibody for 1 hour at room temperature on a rocking plate. The membranes were then washed a further three times in PBST.

Proteins bands were then visualised by chemiluminscence and exposure to undeveloped photographic film. The membranes were covered in ECL reagent for 1 minute prior to removal of the excess fluid and wrapping in Saran wrap with care to ensure the absence of bubbles under the wrap. The wrapped membranes were placed protein side up in an autoradiographic cassette. These were subsequently exposed to ECL Hyperfilm in a dark room and the film developed using an automated developer.

In order to probe the membranes for a loading control (actin) they were placed in stripping buffer on a shaking plate for 30-60 minutes at room temperature. These were then washed for 10 minutes 3 times in PBST prior to re-blocking and probing with the loading control primary antibody. These were then processed as described above. Actin was used as a loading control as experiments demonstrated that neither I3C nor DIM affected its expression.

When indicated blots were quantified using Quantity One™ (BioRad, USA).

2.7.1 Antibody conditions

Unless otherwise stated the following standard conditions were applied:

- Wells were loaded with 40 µg whole cell lysate

- Membranes were blocked for 1 hour in 5% milk in PBST at room temperature,

- Primary antibodies were suspended in 5% milk in PBST at a concentration of 1 in 1000,

- Secondary antibodies were suspended in 5% milk in PBST at a concentration of 1 in 1000,

- Primary antibodies were incubated overnight at 4 ºC,

- Secondary antibodies were incubated for 1 hour at room temperature.

Primary antibody against	Secondary antibody	Conditions	Molecular weight (kDa)
Actin	Anti-rabbit	Dilute primary antibody 1:4000	43
Akt1	Anti-goat	Standard conditions as stated	62
Bax	Anti-mouse	Dilute primary antibody 1:500	21
Bcl-2	Anti-mouse	Dilute primary antibody 1:500	24-26
Bcl-X_L	Anti-mouse	Standard conditions as stated	34
BID	Anti-goat	Dilute primary antibody 1:2000	22
Bim	Anti-rabbit	Standard conditions as stated	19-24
Cdc2	Anti-rabbit	Dilute primary antibody 1:2000	34

Cdc25A	Anti-mouse	Standard conditions as stated	65
Cdc25B	Anti-mouse	Standard conditions as stated	63
Cdc25C	Anti-mouse	Dilute primary antibody 1:2000	55
CDK6	Anti-rabbit	Standard conditions as stated	37
Chk1	Anti-rabbit	Standard conditions as stated	56
Chk2	Anti-rabbit	Standard conditions as stated	62
Cyclin B1	Anti-mouse	Standard conditions as stated	60
Cyclin D1	Anti-mouse	Dilute primary antibody 1:500	35
Cyclin E	Anti-mouse	Dilute primary antibody 1:500	Single 42 Doublet 50 Hyper-phosphorylated 55 (157)
NF-κB p65	Anti-rabbit	Standard conditions as stated	65
PARP	Anti-rabbit	Standard conditions as stated	Uncleaved 116 Cleaved 85
p16^{INK4}	Anti-mouse	Dilute primary antibody 1:500	16
p21	Anti-rabbit	Standard conditions as stated	21
p27	Anti-mouse	Dilute primary antibody 1:500	27
Phopsho-Akt (Ser473)	Anti-rabbit	Dilute primary antibody 1:1000 in 5% BSA	60
Phospho-cdc2 (Thr161)	Anti-rabbit	Standard conditions as stated	34

Phospho-cdc2 (Tyr15)	Anti-rabbit	Dilute primary antibody 1:2000 in 5% BSA	34
Phospho-Chk2 (Thr68)	Anti-rabbit	Dilute primary antibody 1:500	62
Phospho-Src (Tyr416)	Anti-rabbit	Dilute primary antibody 1:500 in 5%BSA	60
Phospho-Src (Tyr527)	Ani-rabbit	Dilute primary antibody 1:1000 in 5%BSA	60
Plk1	Anti-mouse	Standard conditions as stated	66
cSrc	Anti-mouse	Dilute primary antibody 1:200	60
Survivin	Anti-rabbit	Standard conditions as stated	16.5

2.8 Assessment of cell adhesion

A 0.2% solution of crystal violet in 10% ethanol was prepared and filtered through a 0.22 μm filtration paper. A solubilisation buffer consisting of 250ml 0.2M NaH_2PO_4 (pH4.5), 125ml ethanol and 125ml distilled water was prepared and filtered through a 0.22 μm filtration paper. These stock solutions were stored at room temperature until required.

Four wells per cell line, per treatment on 96 well plates were coated with 80μl of 50μg/ml collagen in 0.02N acetic acid for 12 hours at 4ºC. The plates were washed x3

74

with 150 µl PBS then 8 (4 collagen treated and 4 untreated) wells per cell line per treatment were blocked with 200 µl of 0.1% heat inactivated BSA in serum free medium for 1 hour at 37ºC.

Cells were washed x3 with PBS then harvested with 5mls versene (SW620 and J82) or 1xTE (HT29). Once the cells were detached, the flasks were washed with 10mls serum free medium and the cells pelleted by centrifugation at 1000rpm for 5 minutes. The supernatant was discarded and the pellet was gently agitated before resuspending in serum free culture medium containing 0.1% heat inactivated BSA.

The blocking medium was discarded and the plates were seeded at a density of 3×10^4 cells per well in 200 µl of serum free culture medium containing 0.1% heat inactivated BSA and either 40 µM DIM or an equivalent concentration DMSO.

The cells were then incubated for the required time interval before the assay. Cells were washed x3 with 150 µl serum free medium prior to adding 100 µl 0.2 % crystal violet solution, after which they were incubated for 5 minutes at room temperature before washing x3 with 300 µl serum free medium. Solubilisation buffer (100 µl) was then added and the plates incubated on a shaker at room temperature for 15 minutes. Four empty wells were also included to determine the background absorbance. The absorbance at 550nm was then measured using a FLUOstar Microplate Reader (BMG Labtech). The background absorbance was subtracted from the absorbance in the treated wells to give the final measure of adhesion.

2.9 Statistical analysis

Statistical analyses were undertaken using SPSS 9.0. Data were checked for normal distribution using the Kolmgorov-Smirnov test. Data were assessed for statistical significance using student t-test or one way ANOVA with Bennets test post hoc as appropriate. Results were considered significant if p≤0.05.

Chapter 3

Primary cultures

3.1 Development of reproducible method for establishing ex vivo cell lines

The aim of this section of the project was to develop an effective, reproducible method for *ex vivo* culture of metastatic colorectal tissue. Once established the cultured cell lines would be incubated with chemopreventative agents identified from experiments with immortalised cell lines.

3.1.1 Review of literature

A literature search for articles, which included a description of a technique for establishing primary cultures from colorectal carcinomas, was performed on ISI web of science. The Mesh headings primary culture and colorectal were used with no search restrictions.

There was a paucity of relevant literature however six papers including methodology for establishing primary cultures were identified (158-163) and are summarised in the table below. These papers demonstrated that a low yield of cultured cell lines was to be expected and identified a number of obstacles to establishing lines; these included:

- Infection/microbial contamination
- Stromal overgrowth
- Cell death secondary to mechanical dissociation
- Slow cell growth

There was a wide heterogeneity of the source tissue for the primary cultures between the papers including primary tumours, nodal and distant metastases and in 1 study an

orthotopic murine model. The degree to which they can therefore be extrapolated to hepatic colorectal metastases is debatable.

The precise constituents of the culture media used varied between the papers however the base medium was either RPMI 1640 or a 1:1 solution of Hams F12 and DMEM 4500 and with one exception these were supplemented with 10% FBS. Two of the six papers reported adding antibiotics and antifungal agents to the culture medium (158,159). Only one paper specifically reported using a separate collecting medium, which differed from the culture medium in that FBS was not added (158).

Four papers described the techniques used for tissue dissociation prior to culture and manual dissociation was used in each of these (158,159,162,163). In one study mechanical dissociation was also used although it was only successful in one cell line and resulted in cell death in the remaining lines (41 assuming all lines were prepared by both techniques although this is not confirmed in the text) (159).

Stromal overgrowth was reported as a significant issue when establishing a pure cell line for culture. Four papers stated that differential trypsinisation was used to counter this and one also added Isobutylmethylxanthine (IBMX) to the culture medium to inhibit fibroblast growth (158,159,162,163).

Study	Tumour type	Collecting medium	Growth Medium	Dissociation technique	Technique to prevent stromal overgrowth	Success rate
Douglas-Marie *et al* (2007)	Primary	DMEM + 10% HEPES + sodium pyruvate+penicillin+ streptomycin+gentamycin+ ciprofloxacin+metronidazole+ vancomycin +fungizone	Collecting medium + 10 % FBS After P8 +vancomycin, gentamycin+fungizone only.	Manual	Diffferential trypsinisation	3 of 31 (9.7%)
Farrell *et al* (2000)	Primary, metastases and recurrent		1:1 Hams F12/DMEM +10% FBS+penicillin+streptomycin +amphotericin	Manual or mechanical	Differential trypsinisation Isobutylmethylxanthine in growth medium	9 of 32 (35.5%) primary 0 of 3 (0%) recurrences 2 of 6 (33%) metastases
Flatmark *et al* (2004)	Orthotopic murine model		R10 – RPMI 1640+10% FBS			Not given – 12 lines studied
Gazdar *et al* (1986)	Primary		ACL4 - RPMI 1640 or 1:1 Hams F12/DMEM +10% FBS+insulin+ transferrin+sodium selenite +EGF+ethanolamine+ phosphorylethanolamine+ triiodothyronine+BSA+ glutamine+sodium pyruvate+ 4-(2-hydroxyethyl)-1-piperazine –ethane sulfonic acid buffer			6 of 10 (60%)
Oh *et al* (1999)	Primary and metastases		AR5 – ACL4+5%FBS R10	Manual	Differential trypsinisation	Not given – 12 lines established 6 primary + 6 metastatic
Park *et al* (1987)	Primary and metastases		ACL4 R10	Manual	Differential trypsinisation	ACL4: 2 of 16 primary (12.5%) 3 of 8 metastases (37.5%) R10: 4 of 16 primary (25%) 5 of 8 metastases (62.5%)

Table 3.1 Summary of studies identified in literature search on techniques for establishing *ex vivo* colorectal carcinoma cell cultures

3.1.2   Techniques attempted for culture

3.1.2.1 Selection of culture media

Following the review of the literature detailed in section 3.1.1 the decision was made use 3 culture media for establishing primary cultures. These were:

- 50:50 Hams F12: DMEM 4500 supplemented with 10% FCS

- RPMI supplemented with 10%FCS

- DMEM 4500 supplemented with 10% FCS

The first two media were selected as these had been shown to be effective in the reported studies. DMEM 4500 was also included as it had previously been used successfully within the laboratory to establish primary cultures of breast carcinoma. For specimens where resected tissue was limited RPMI and DMEM:Hams were to be used preferentially as these were the culture media supported by the available literature. Subsequently following a poor overall yield DMEM 4500 with 10% FCS was replaced with McCoys 5A supplemented with 10% FCS in order to continue to develop the culture technique. McCoys 5A had been successfully used in other tissue types and a report of its use in colonic primary cultures was identified (164,165)

The quantity of FCS required for supplementation was assessed. Tissue from a sample was divided between separate flasks containing each of the base culture media and either 2%, 5% or 10% FCS. These were then incubated at 37ºC, 5% CO_2 and 100% humidity. The samples cultured in less than 10% FCS showed evidence of a loss of integrity and cell breakdown by 72 hours. This supported the results of the literature

review and therefore 10% FCS supplementation was utilised throughout the remainder of the project.

As the tissue was potentially infected at source all of the culture media were also supplemented with 1x antibiotic antimycotic solution (Sigma-Aldrich, UK).

3.1.2.2 Selection of techniques for tissue dissociation

The literature review showed a consensus towards manual dissociation and dispersal techniques of cells from the source tissue prior to culture. There were no reports of enzymatic dispersal in the articles reviewed although these techniques have been utilised in other cell types (166). As detailed in section 3.1.1 mechanical dissociation was only reported in one study and in that instance it only yielded one successful line with cell death resulting in the other lines. Conversely one of the investigators (Dr. Elena Moiseeva) had previous experience of successfully establishing primary cultures from breast carcinoma lines using mechanical dispersal techniques.

The decision was therefore made to explore manual and mechanical dispersal techniques initially however in light of the consensus towards manual techniques in the literature this would be used preferentially in instances where tissue was limited.

Manual dissociation was performed by dividing the tissue, into sections of approximately 0.5-1mm^3, using a fresh scalpel and non-tooth forceps. Although ideally the specimens would have been further divided this proved technically unfeasible. The dispersed tissue was then divided into 3 equal volumes which were re-suspended in the three culture media detailed in section 3.1.2.1. and transferred to fresh culture flasks for incubation.

Mechanical dissociation was performed with a Medimachine[tm] (BM Medscience). Tissue blocks were placed in the machine and disaggregated with 2 pulses of either 15 or 20 seconds. Samples for each time interval were obtained from each resection specimen as far as possible. These timings were selected as per the manufacturers recommendations for hepatic and colorectal tissue respectively. As with the manually dissociated samples, the resultant cells were divided and re-suspended in the 3 selected culture media for incubation.

The selected timings were confirmed by a qualitative observation with the first specimen. The tissue was separated into 5 equal volumes and these were disaggregated with 2 pulses of 10, 15, 20, 25 or 30 seconds. The resultant samples were examined and the specimens disaggregated with 25 and 30 second pulses were felt to show evidence of excessive disruption whereas the sample disaggregated for 10 seconds was felt to be inadequately dispersed although this was equivocal and the sample was cultured. Subsequent culture of these samples failed to yield a single viable colony for any time point and therefore the decision was made to proceed using pulse durations of 15 and 20 seconds as recommended in the manufacturer's literature in order to limit the number of techniques being utilised and therefore the volume of tissue required for processing.

3.2 Results

8 patients were recruited and tissue was obtained from 7. 1 patient was excluded as per the protocol as the resected tumour was too small to allow tissue sampling without compromising histological assessment. The patient details for the patients from whom tissue was obtained are summarised in Table 3.2.

Pt	Age (yrs)	Operation	Ischaemic time (mins)
1	62	Open segmentectomy	60
2	57	Open segmentectomy	60
3	60	Laparoscopic segmentectomy	115
4	62	Open segmentectomy	20
5	62	Open biopsy	0
6	66	Open segmentectomy	Unknown
7	75	Open segmentectomy	Unknown

Table 3.2: Details of patients from whom tissue was obtained for establishing *ex vivo* cultures.

From these specimens sufficient tissue was obtained for 64 attempts at culture by a combination the techniques outlined in the preceding sections. The full results for these are listed in table 3.3. From the specimens 11 cell populations were established in culture of which 2 were scattered cell

clusters, that may or may not have subsequently progressed to form confluent populations, 5 were adherent, confluent populations, 2 were floating cell populations and a further 2 were a mix of both adherent and floating cell populations.

Of the solely adherent cell populations 3 were a single cell type and these were morphologically likely to represent fibroblasts. The remaining 2 solely adherent populations both contained 2 morphologically distinct cell types and these were thought potentially represent a mix of fibroblasts and colorectal carcinoma cells. This would be consistent with the issue of fibroblast overgrowth highlighted by the literature review. The 2 floating cell populations did not resemble fibroblasts morphologically and were thought to be the most likely to contain colorectal carcinoma cells. Two flasks were found to contain a mixture of an adherent and a floating cell population. In both of these the adherent cell population were thought to be likely to represent fibroblasts whereas the floating populations were morphologically distinct from the adherent populations. These resembled the other

Patient	Dissociation technique	Culture Medium	Passages	Outcome
1	Mechanical (10 Seconds)	DMEM	0	No growth
		DMEM:Hams F12	0	No growth
		RPMI	0	No growth
	Mechanical (15 Seconds)	DMEM	0	No growth
		DMEM:Hams F12	0	No growth
		RPMI	0	No growth
	Mechanical (20 Seconds)	DMEM	0	No growth
		DMEM:Hams F12	0	No growth
		RPMI	0	No growth
	Manual	DMEM	1	No growth
		DMEM:Hams F12	1	No growth
		RPMI	1	No growth
	Floating	DMEM	0	No growth
		DMEM:Hams F12	0	No growth
		RPMI	0	No growth
2	Manual	DMEM	1	No growth
		DMEM:Hams F12	2	2 adherent cell types
		RPMI	2	No growth
	Floating	DMEM	0	No growth
		DMEM:Hams F12	0	No growth
		RPMI	0	No growth
3	Manual	DMEM	0	No growth
		DMEM:Hams F12	0	No growth
		RPMI	0	No growth
	Floating	DMEM	0	No growth
		DMEM:Hams F12	0	No growth
		RPMI	0	No growth
4	Mechanical (15 Seconds)	DMEM	0	No growth
		DMEM:Hams F12	1	Adherent cell colonies
		RPMI	1	Adherent cell colonies
	Mechanical (20 Seconds)	DMEM	0	No growth
		DMEM:Hams F12	0	No growth
		RPMI	0	No growth
	Manual	DMEM	2	No growth
		DMEM:Hams F12	3	No growth
		RPMI (Sample 1)	2	Floating colonies
		RPMI (Sample 2)	2	Floating colonies
	Floating	DMEM	0	No growth
		DMEM:Hams F12	0	No growth
		RPMI	0	No growth
5	Manual	DMEM:Hams F12	2	Adherent cell population
		RPMI	2	Scattered cells
	Floating	DMEM:Hams F12	0	No growth
		RPMI	0	No growth

		DMEM	0	No growth
6	Mechanical (15 Seconds)	DMEM	0	No growth
		DMEM:Hams F12	0	No growth
		RPMI	0	No growth
	Mechanical (20 Seconds)	DMEM	0	No growth
		DMEM:Hams F12	1	No growth
		RPMI	0	No growth
	Manual	DMEM	1	No growth
		DMEM:Hams F12	2	2 adherent cell types
		RPMI	2	No growth
	Floating	DMEM	0	No growth
		DMEM:Hams F12	0	No growth
		RPMI	0	No growth
7	Mechanical (15 Seconds)	McCoys 5A	1	Scattered Cells
		DMEM:Hams F12	1	No growth
		RPMI	1	No growth
	Manual	McCoys 5A	1	Adherent + floating cell colonies
		DMEM:Hams F12	1	No growth
		RPMI	1	Adherent + floating cell colonies
	Floating	McCoys 5A	0	No growth
		DMEM:Hams F12	0	No growth
		RPMI	0	No growth

Table 3.3: Results of attempts to establish *ex vivo* hepatic metastatic colorectal carcinoma cell culture populations by patient, tissue dissociation technique and culture medium. None of the cell cultures developed evidence of infection therefore this data has not been included. This table demonstrates the poor overall success rate with a maximum of 11 possible colorectal cell populations from 64 preparations.

floating cell populations obtained from other samples and were thought to be consistent with carcinoma cells. It is worth noting that the floating and adherent cell populations derived from the same tissue sample and culture technique are both colorectal carcinoma cell populations as there are reports of investigators deriving two sub-lines from single colorectal primary cultures (165,167).

Tissue typing was not performed due to this section of the project, as explained below, being stopped prior to this so the precise yield of colorectal carcinoma lines is unconfirmed. Overall these results represent a potential maximum yield of 11 from 64 (17.2 %) and a more likely yield of 4 from 64 (6.2%).

The observed cell turnover in all of the cell cultures was very slow, in the order of weeks, with several media changes required prior to the cells achieving sufficient confluence for passaging. Furthermore the total number of cells obtained was low as all of the results reported above were based on culture in 12.5cm^2 flasks.

It is interesting to note that, within this very small population, the tissue samples that had been exposed to a shorter peri-operative ischaemic time appeared to have a higher yield in terms of with establishing a cell line in culture. The two samples giving the highest percentage yield, in terms of number cell populations of any type established relative to number of techniques attempted and therefore flasks seeded, derived from patients 4 (33%) and 5 (50%). These patients had the shortest and no ischaemic times respectively. Conversely the tissue from the patient with the longest recorded ischaemic time, patient 3, did not yield any viable cells. This data may be skewed firstly by the lack of a recorded ischaemic time for patients 6 and 7 and secondly by the small volume of tissue obtained from patient 5. The result of the latter was that only the preparation and culture techniques anticipated to have the highest chance of success were ultilised. This finding would be expected given that ischaemia would be likely to activate cell apoptosis or at least be associated with cellular damage. This finding may be confounded by the apparent trend towards more successful cell culture in tissue obtained later in the project. This, in turn, could be taken to indicate that although the tissue handling and preparation was performed by the same investigator and by the same techniques in each case there may be an improvement in the performance of this process with repetition.

Of the culture media utilised four cultures were successfully established with 50:50 DMEM:Hams F12 mixture + 10 FCS, five with RPMI + 10% FCS and two with

McCoys 5A. No populations were established with DMEM 4500 + 10% FCS. This supports the findings of the literature review although the numbers are too small to draw any definitive conclusions at this stage although it is of note that McCoys was only used for tissue from one patient and thus had the highest percentage yield.

Mechanical dissociation yielded 3 populations from 24 flasks (12.5%) whereas manual dissociation yielded 8 populations from 20 flasks. This data may be skewed by the bias towards manual preparation of samples, where source tissue was limited, on the assumption of higher success rate thus only 4 of 7 patient's tissue was subjected to mechanical dissociation whereas all 7 patients' tissue underwent manual dissociation. In particular manual preparation was used for tissue from patient 5 - the patient noted previously to have undergone no ischaemic time and to have the highest individual percentage yield. Within the limitations of these very small numbers it is also of note that the 3 populations derived from mechanically dissociated tissue were all from samples dissociated with the shorter pulses of 15 seconds. Put together these data imply that minimal tissue preparation and therefore minimisation of potential physical cell damage is more likely to result in establishing a viable cell culture. This finding might be anticipated as physical cell damage or loss of cell integrity would lead to cell cycle arrest and activation of cell apoptosis.

The floating cells obtained from the transport medium and initial washing of the resected tissue failed to yield any viable cells when the flasks were examined at 72-96 hours. Possible reasons for this include:

- Presence of necrotic tissue within the resected metastasis which may not be apparent macroscopically but would be more likely to be separated from the

underlying viable tissue through the process of washing or immersion in transport medium.

- Damaged cells/cells undergoing apoptosis are more likely to be poorly adherent to adjacent cells than undamaged, viable cells.

- Loss of cell integrity during processing of tissue/removal of specimen

- Ischaemic damage during resection

Alternatively the failure to establish a single *ex vivo* culture from the floating cells may reflect the overall poor yield demonstrated throughout this project and as might be anticipated given the data in the literature.

As reported above the cell turnover demonstrated by the *ex vivo* cultures was very slow with clear implications for the proposed experiments dependent on cell turnover such as analysis for cell cycle arrest. Furthermore the yield in both in terms of number of cells obtained per cell line and number of established lines was far lower than initially had been anticipated and therefore it was unlikely that a large enough panel of cell lines to obtain significant results would be established within the time limits of the project. These reasons in conjunction with the impact this section of the project was making on time for experiments on immortalised cell lines led to the decision being taken to focus on the latter experiments.

3.3 Potential developments and improvements

Although the data is insufficient to draw any definitive conclusions a number of key issues were identified:

- Early indications are that a prolonged ischaemic time during the initial resection appears to decrease the likelihood of obtaining viable cells in culture.

- Fibroblast overgrowth is a potential problem

- Extent of tissue disruption during preparation may inversely correlate with likelihood of establishing a cell population in culture.

- Cell turnover demonstrated was very slow potentially limiting the experimental use of the cell lines as well as making it difficult to obtain a large enough cell population to utilise.

Although the project was stopped at this stage the anticipated next stages in development of the technique would have been:

- Tissue typing of established cell cultures using epidermal cellular markers cytokeratins 8 and 18 to identify if the cells were of epithelial origin and therefore likely to represent metastatic tissue rather than fibroblasts or hepatocytes.

- Exploring feasibility of obtaining tissue with short or no ischaemic time – this would entail a review of the ethical approval for the project.

- Enzymatic disaggregation with collagenase and hyalurodinase digestion

- Fibroblast depletion by passing the suspensions through antibody coated beads

- Separation of mixed adherent cell populations by differential tryspinisation

- Separation of mixed floating and adherent cell populations for further culture

- Inhibition of fibroblast overgrowth using inhibitors such as Isobutylmethylxanthine (IBMX)

- Addition of growth factors IGF-1 and EGF to the cell culture medium to stimulate cell growth. Depending on results consider addition of VEGF and PDGF.

Chapter 4

## Indole-3-carbinol in colorectal carcinoma

As outlined in detail in the introduction there is epidemiological evidence that dietary intake of vegetables, including those of the genus cruciferae, influences the risk of colorectal carcinogenesis (16,17). The indoles, a group of bioactive compounds found in cruciferous vegetables have been proposed as contributing to this effect. Indole-3-carbinol (I3C) has been shown to exhibit pro-apoptotic effects and induce cell cycle arrest in malignant cell lines. EGFR and Src have been implicated mechanistically in these effects (142). Pharmacological data shows that the greatest concentrations of I3C and its bioactive metabolites are found in the liver, with levels 6x plasma concentrations, following oral ingestion indicating a role in the treatment or prevention of hepatic metastases (147).

Although there has been extensive research into the effect of indoles in other carcinoma types there is a relative paucity of data relating to the effects of indoles in colorectal carcinoma. The aim of this section of the project, therefore, was to investigate using a panel of immortalised cell lines the effect of I3C, both as a sole agent and in conjunction with inhibition of EGFR and Src, in colorectal carcinoma.

4.1   Effect of I3C on colorectal carcinoma cell viability

As outlined in the methods a panel of 4 immortalised colorectal carcinoma cell lines (HT29, HCT116, SW480 and SW620) weas utilised for *in vitro* experiments. Treatment with I3C resulted in a dose dependent decrease in cell viability (ATPLite) versus DMSO control after 48 hours incubation (Figure 4.1). Statistically significant decreases were yielded by doses of 200 µM and above. This indicates a response at

doses higher than are anticipated to be achievable *in vivo* (147).
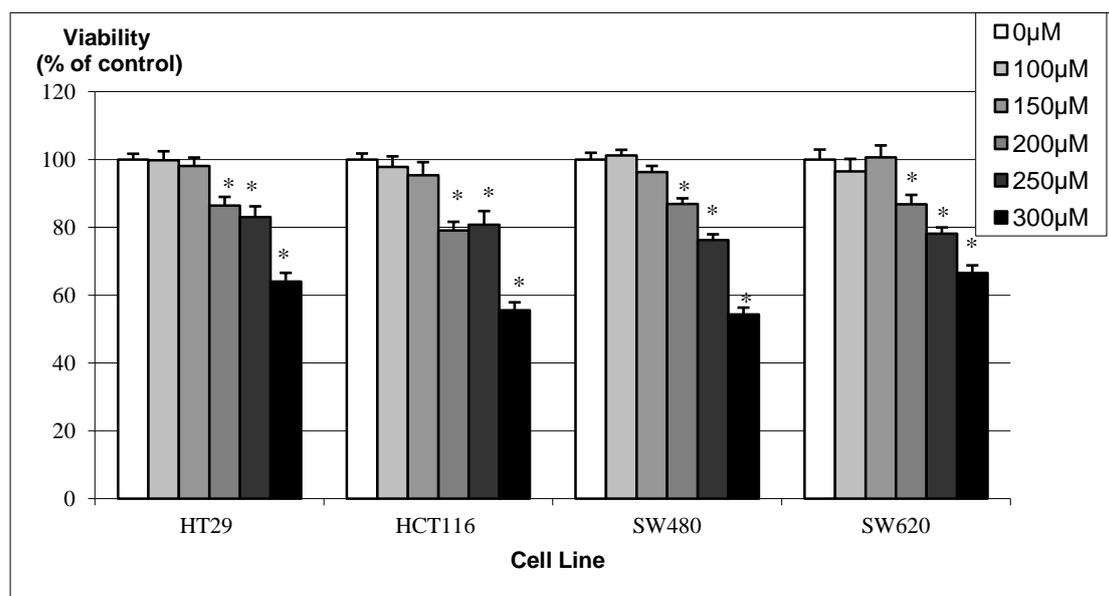
Figure 4.1: Cell viability (ATPLite) in 4 colorectal carcinoma cell lines.
I3C induced decreased cell viability, at 48 hours, in a dose dependent manner.
Significant decreases were observed at doses of 200 µM and above which are
likely to be unachievable *in vivo.* Results normalised to DMSO control. n=24 over
3 repetitions *=p<0.05 versus DMSO control

4.1.1   Effect of I3C on apoptosis in colorectal carcinoma
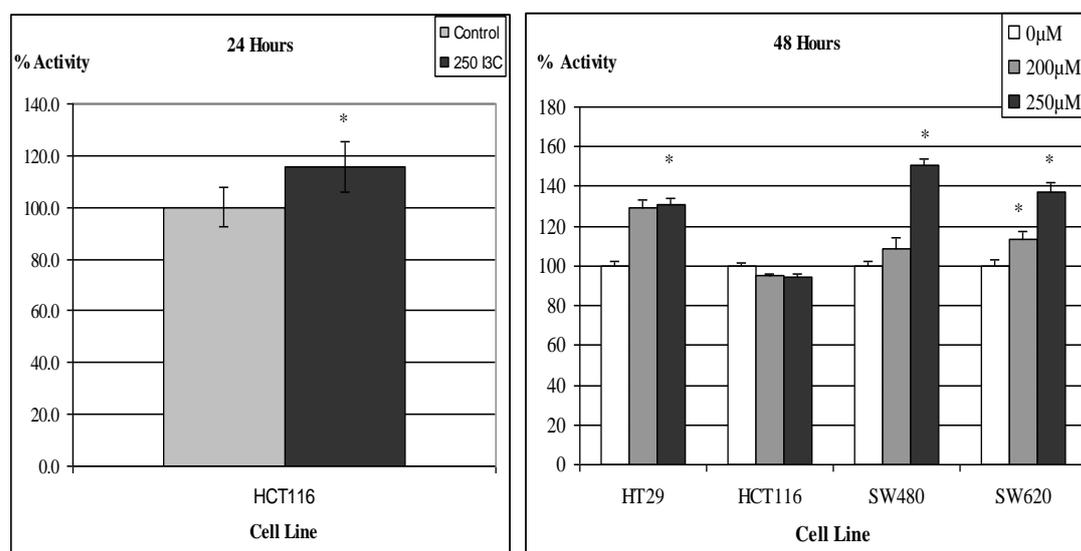

Figure 4.2: Caspase 3/7 activity (Caspase-Glo) in 4 colorectal carcinoma cell
lines.
Increased caspase activity, indicating induction of apoptosis, was observed
following treatment with 250 µM I3C in 3 of 4 cell lines at 48 hours and in the
remaining cell line, HCT116, after 24 hours treatment. Results normalised to
DMSO control. n=24 over 3 repetitions
*=p<0.05 versus DMSO control

Treatment with 250 μM I3C induced apoptosis in the panel of colorectal carcinoma cell lines evidenced by increased activation of caspases 3 and 7 (Caspase-Glo). In 3 of 4 cell lines statistically significant caspase activation was observed at 48 hours. However, these increases were small (130.8-150.4% of DMSO control). The fourth cell line, HCT116, exhibited a small but significant increase in caspase 3/7 activation following 24 hours treatment (120% of control) (Figure 4.2).

4.2 Effect of I3C with receptor inhibitors in colorectal carcinoma

As detailed in the introduction EGFR and Src have both been implicated in I3C induced cell cycle arrest and apoptosis in breast carcinoma cell lines (143). The combination of I3C and an EGFR inhibitor was effective at concentrations which are ineffective individually and the combination of I3C and a Src inhibitor was more efficacious than either agent individually against two breast carcinoma phenotypes. Phenotyping of the panel of four colorectal carcinoma cell lines confirmed overexpression of EGFR in all bar one (SW620) and Src overexpression in all four (Figure 4.3). I3C was therefore combined with inhibitors of EGFR and Src to ascertain if combination therapy would yield reductions in cell viability at I3C doses anticipated to be achievable *in vivo.*
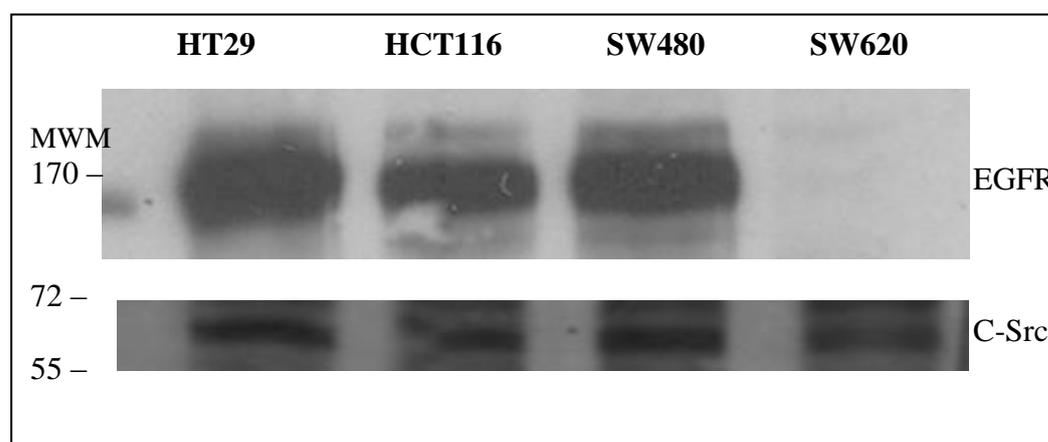

Figure 4.3: EGFR and Src expression in four colorectal carcinoma cell lines. Western blotting showed EGFR was overexpressed in 3 of 4 lines and Src was overexpressed in all four lines.

4.2.1 Effect of I3C with EGFR inhibition on colorectal carcinoma cell viability

Treatment with 150 µM I3C failed to yield a decrease in cell viability in the panel of four colorectal carcinoma cell lines (data presented in chapter 4.1). Combination with the commercially available EGFR inhibitor, ZD1839 (Iressa, Astra Zeneca UK), resulted in decreased viability following 48 hours treatment in 3 of the 4 colorectal cell lines relative to both DMSO control and ZD1839 as a sole agent (p<0.05 vs DMSO control in HT29 and vs DMSO control and ZD1839 in HCT116 and SW480) (Figure 4.4).

As would be anticipated from cell phenotyping SW620 failed to exhibit a response to EGFR inhibition. This data demonstrated that doses of I3C which are subtherapeutic
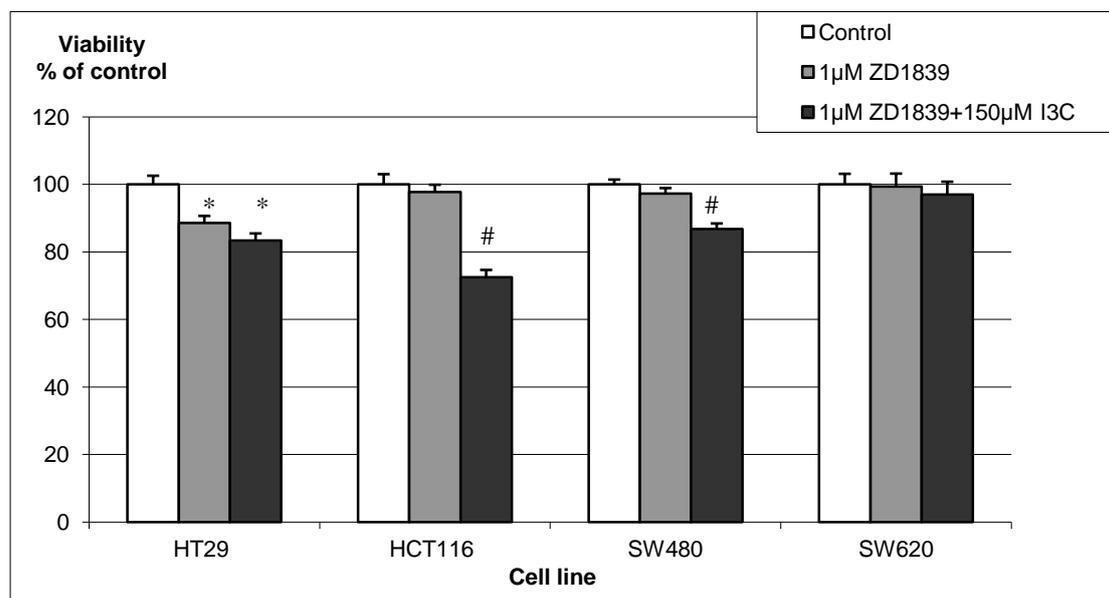
Figure 4.4: Cell viability (ATPLite) in 4 colorectal carcinoma cell lines after 48 hours treatment with a combination of 1 µM ZD1839 (Iressa, Astra Zeneca UK) +150 µM I3C versus DMSO control and 1 µM ZD1839 alone. An increased response was observed with combination treatment in 3 of 4 cell lines .As anticipated from cell phenotyping SW620 does not exhibit an increased response with EGFR inhibition. Results normalised to DMSO control. n=24 over 3 repetitions
* = p<0.05 vs DMSO control   # = p<0.05 vs DMSO control and ZD1839

in isolation can be efficacious in combination with EGFR inhibition although the effect is restricted to cells which constitutively overexpress EGFR.

4.2.2 Effects of I3C with Src inhibition on colorectal carcinoma cell viability

Doses of 100 and 150 μM I3C, which had proved subtherapeutic as a sole agent (data presented in section 4.1) in combination with 5 μM PP2, a commercially available selective Src inhibitor, induced loss of cell viability in the panel of four cell lines (67.6-89.6% of control and 67.0-93.0% of DMSO control for combinations incorporating 100 and 150 μM I3C respectively). These decreases were greater than that achieved with PP2 alone ($p < 0.05$) (Figure 4.5). These data demonstrated that doses of I3C which are subtherapeutic in isolation can be efficacious in combination with Src inhibition.
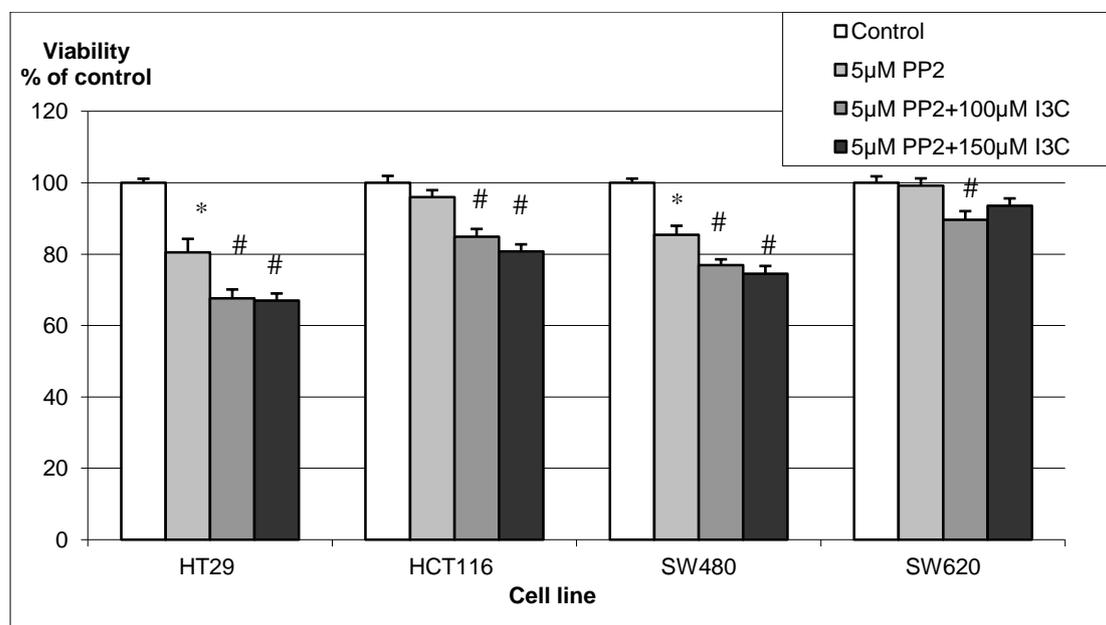
Figure 4.5: Cell viability (ATPLite) in 4 colorectal carcinoma cell lines.
An increased response was observed after treatment for 48 hours with a combination of 5 μM PP2 with 100 μM or 150 μM I3C, relative to DMSO control and 5 μM PP2 alone, in all 4 cell lines demonstrating that doses of I3C which are subtherapeutic in isolation can be efficacious in combination with Src inhibition. Results normalised to DMSO control. n=24 over 3 repetions. * = $p < 0.05$ vs DMSO control   # = $p < 0.05$ vs DMSO control and ZD1839

96

I3C was also combined with AZD0530 (Astra Zeneca, UK) a Src inhibitor under development.

There were no definitive data regarding therapeutic dosing for AZD0530 in colorectal tissues. Guidance from Astra Zeneca indicated that concentrations greater than 1.0 µM were potentially achievable (168). In preclinical studies 50 µg/kg AZD0530 proved insufficient to inhibit tumour volume in mice inoculated with HT29 colorectal carcinoma cells (169) whereas 1 µM AZD0530 induced apoptosis and inhibited cell growth in non-small cell lung carcinoma *in vitro* (170). Dose response data for AZD0530 in colorectal carcinoma cells was therefore obtained.

Treatment with AZD0530 resulted in a dose dependent decrease in cell viability (ATPLite) versus DMSO control after 48 hours incubation. Statistically significant decreases were yielded by doses of $\geq$0.25 µM in two of four cell lines, by doses of $\geq$0.5 µM in one cell line and $\geq$1.0 µM in the fourth cell line (Figure 4.6). On the basis
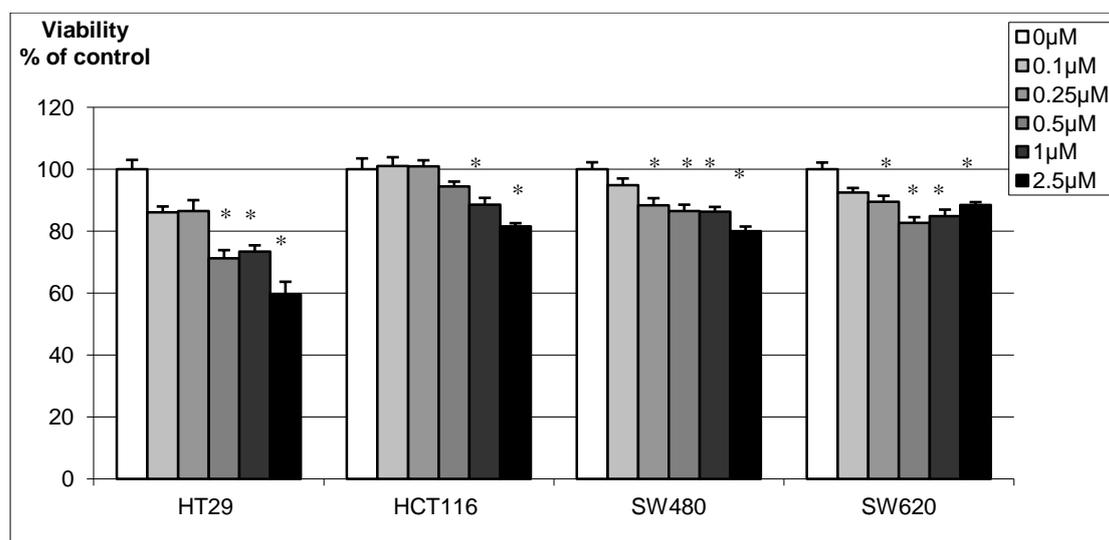
Figure 4.6: Cell viability (ATPLite) in four colorectal carcinoma cell lines. A dose related decrease in cell viability was observed following 48 hours treatment with increasing doses of Src inhibitor AZD0530 (Astra Zeneca, UK). Doses $\geq$1.0 µM yielded significant decreases in all four cell lines. Results normalised to DMSO control. n=8 *=$p<0.05$ versus DMSO control.
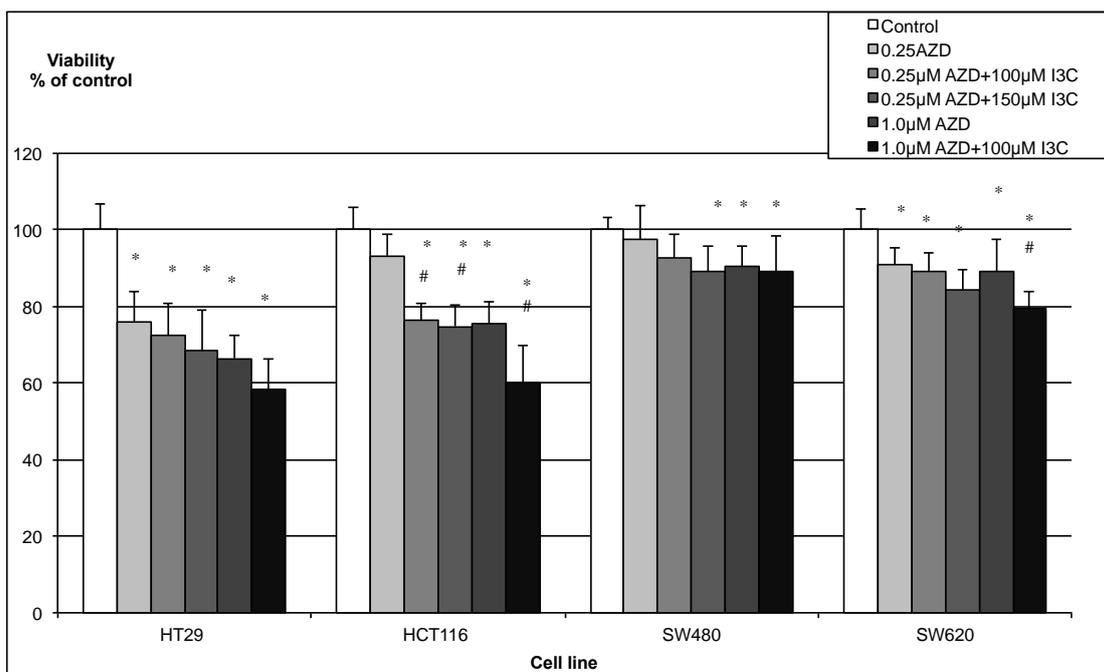
Figure 4.7: Cell viability (ATPLite) in four colorectal carcinoma cell lines.
A trend towards greater decreases in cell viability was observed following 48 hours treatment with a combination of AZD0530 and I3C, at doses which were sub-therapeutic in isolation, relative to AZD0530 alone though only statistically significant in HCT116. Results normalised to DMSO control. n=24 over 3 repetitions. * = $p<0.05$ vs DMSO control # = $p<0.05$ vs DMSO control and AZD0530.

of these data 1.0 μM was deemed to be therapeutic and 0.25 μM was deemed to be

sub-therapeutic.

Treatment for 48 hours with a combination of AZD0530 and I3C at doses deemed

subtherapeutic as sole agents (0.25 μM AZD0530 and either 100 or 150 μM I3C)

showed a trend towards greater decreases in cell viability than monotherapy in the

panel of four colorectal cell lines (Figure 4.7). Only HCT116 reached statistical

significance. 48 hours treatment with a combination of a subtherapeutic I3C dose (100

μM) and a therapeutic AZD0530 (1.0 μM) induced increased loss of cell viability in

the panel of four colorectal carcinoma cell lines (58.2-89.0% of DMSO control) in

comparison with AZD0530 monotherapy (66.4-90.3% of control). These reached

statistical significance in HCT116 and SW620 (Figure 4.8).
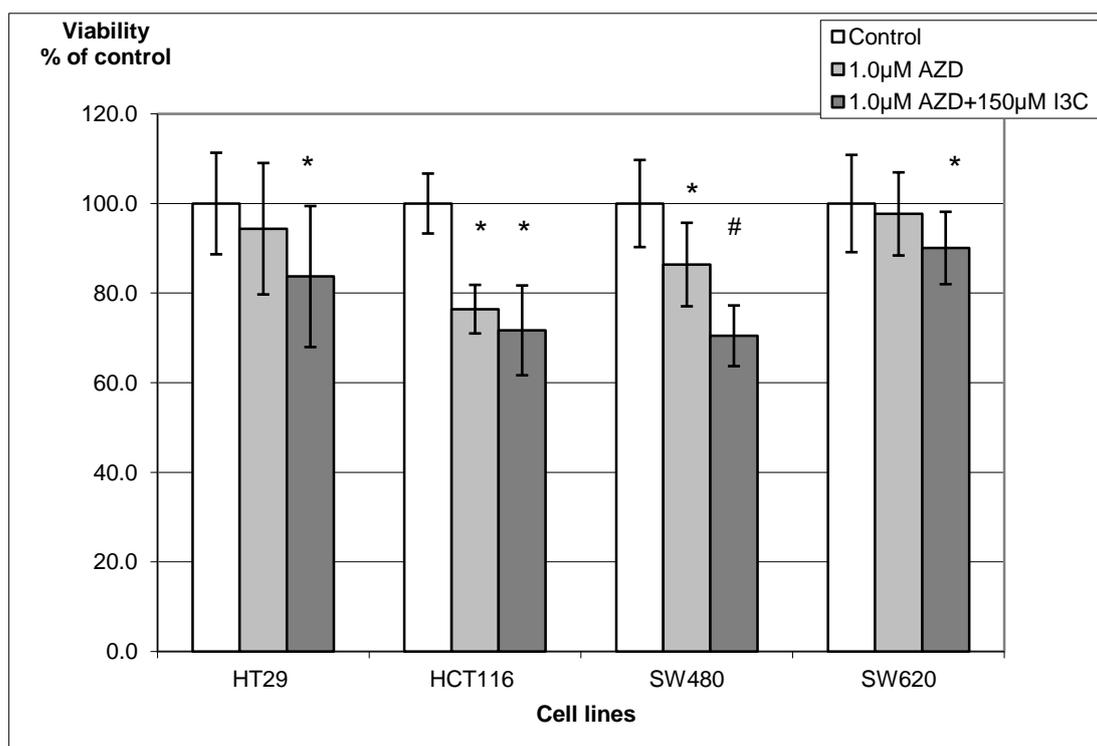
Figure 4.8: Cell viability (ATPLite) in 4 colorectal carcinoma cell lines. Greater decreases in cell viability were observed following 48 hours treatment with a combination of AZD0530 and I3C compared with AZD0530 alone, indicating that doses of I3C likely to be achievable *in vivo* but subtherapeutic in isolation can be efficacious in combination with Src inhibition. Results normalised to DMSO control. *= $p < 0.05$ vs DMSO control   # = $p < 0.05$ vs DMSO control and AZD0530 alone

The decrease in viability in response to combination therapy with 150 μM I3C and 1.0 μM AZD0530 was progressive and sustained over extended time points (72 and 96 hours) in all the cell lines (Figure 4.9). These data, with the data obtained using PP2, indicate that doses of I3C likely to be achievable *in vivo* but subtherapeutic in isolation can be efficacious in combination with Src inhibition.

Treatment with a combination of 1.0 μM AZD0530 and 150 μM I3C showed a slight trend ($p > 0.05$) towards increased caspase 3/7 activation and therefore induction of apoptosis at 12 hours (Figure 4.10).
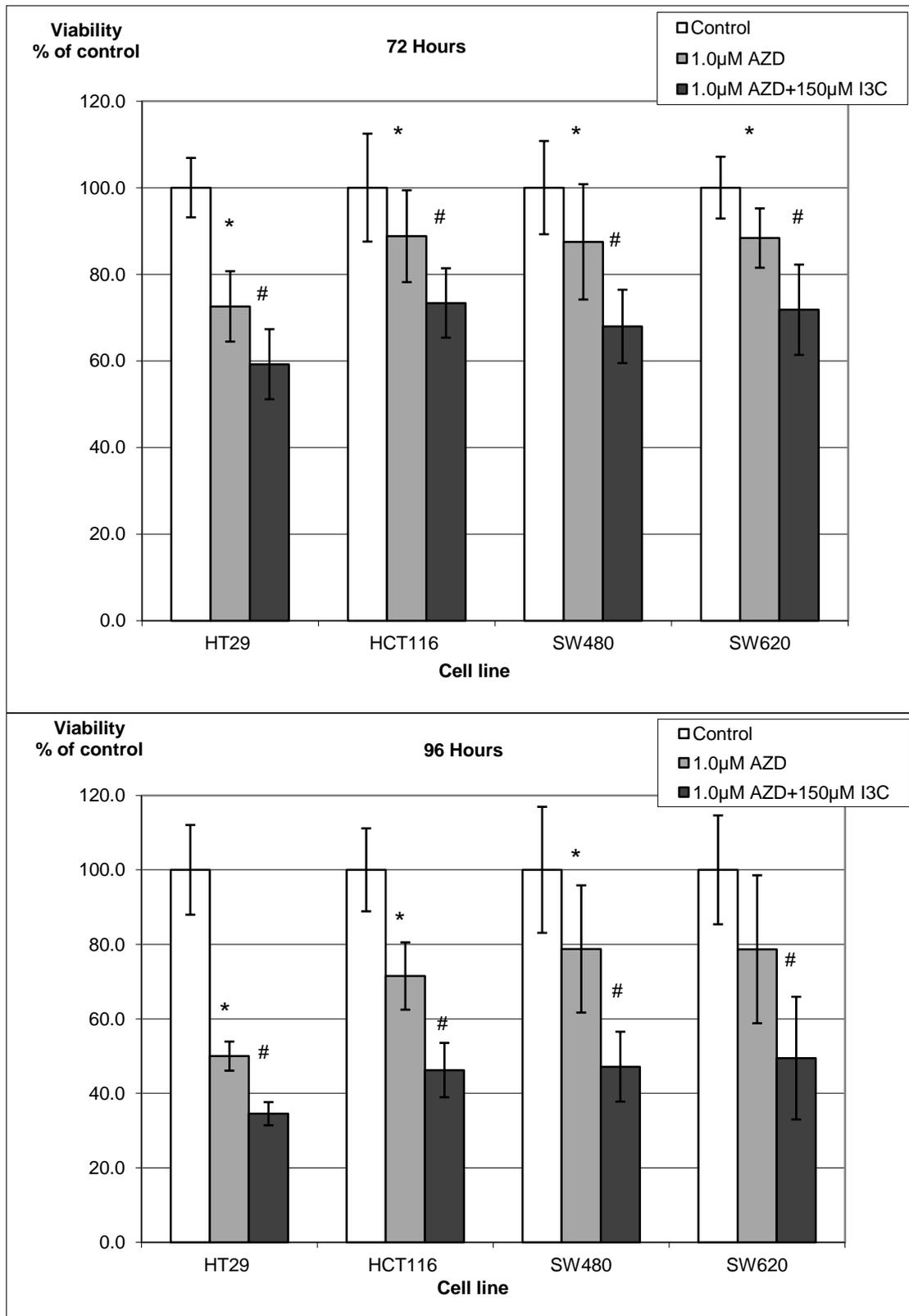
Figure 4.9: Cell viability (ATPLite) in four colorectal carcinoma cell lines. A combination of AZD0530 and I3C induced progressive loss of cell viability compared with AZD0530 alone, following 72 and 96 hours treatment, indicating that doses of I3C likely to be achievable *in vivo* but subtherapeutic in isolation can be efficacious in combination with Src inhibition. Results normalised to DMSO control.

*= p<0.05 vs DMSO control   # = p<0.05 vs DMSO control and AZD0530 alone
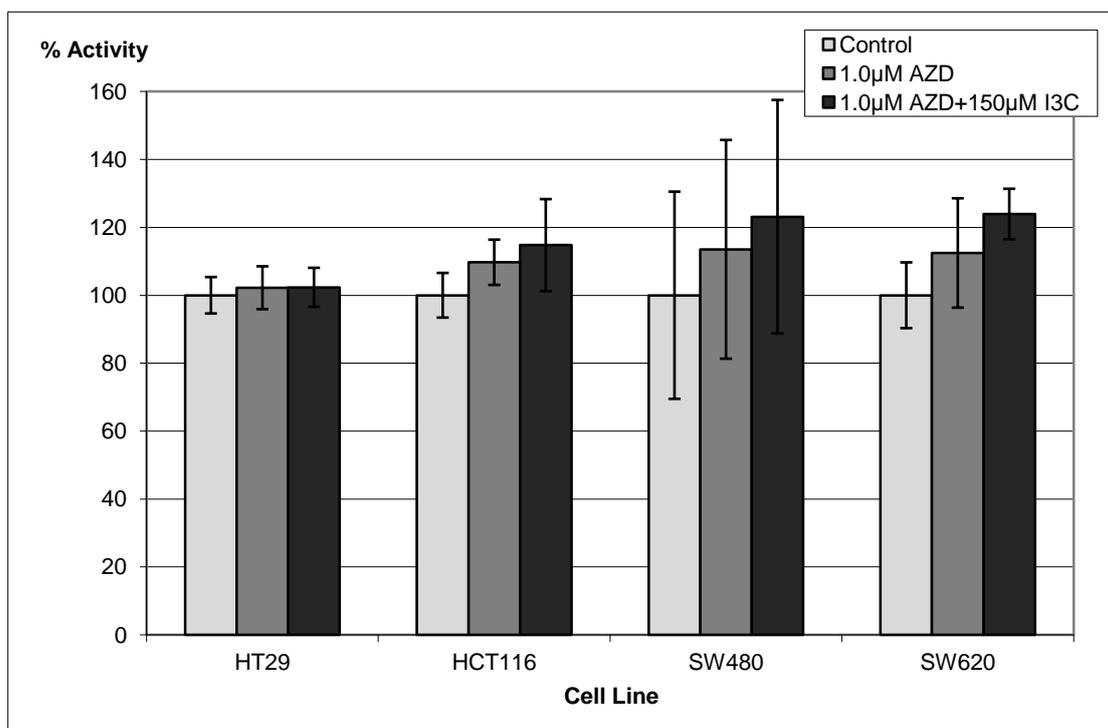
Figure 4.10: Caspase 3/7 activity (Caspase-Glo) in 4 colorectal carcinoma cell lines.
A non-significant trend towards increased activation, and therefore induction of apoptosis was observed following 12 hours treatment with AZD0530 and I3C. Results normalised to DMSO control.

At this stage in the project data showing the comparative effects on cell viability with I3C and DIM (detailed in chapter 5) led to the decision to focus subsequent research on the latter compound and therefore caspase 3/7 activation at subsequent timepoints was not assessed.

4.3 Conclusion

In summary it was demonstrated that although treatment with I3C yielded a loss of cell viability and induction of apoptosis in colorectal carcinoma cells this only occurs with doses unlikely to be achievable in vivo ($\geq$200 µM) and is therefore of limited clinical relevance. This data contrasts with data in breast carcinoma in Tag mice at which effects were observed with doses of 100 µM I3C (171). The effects of I3C can

be augmented by combination with inhibitors of EGFR and Src in line with cell phenotyping however the effects remain modest. Putting these limited data together with the pharmacokinetic data that indicates a relatively short half-life for I3C *in vivo* (147) means that I3C is unlikely to constitute an attractive, potential chemopreventative nor chemotherapeutic agent for colorectal carcinoma.

Chapter 5

3,3 Diindolylmethane in colorectal carcinoma

As outlined in detail in the introduction there is epidemiological evidence that dietary intake of vegetables, including those of the genus cruciferae, influences the risk of colorectal carcinogenesis (16,17). The indoles, a group of bioactive compounds found in cruciferous vegetables have been proposed as contributing to this effect. 3,3 diindolylmethane (DIM), an acid condensation product of I3C, has been shown to exhibit pro-apoptotic effects and induce cell cycle arrest in malignant cell lines (130,133-135).

Although there has been extensive research into the effect of indoles in other carcinoma types there is a relative paucity of data relating to the effects of indoles in colorectal carcinoma. Pharmacological data shows that the greatest concentrations of DIM are found in the liver, with levels 8x plasma concentrations, following oral ingestion making it an attractive proposition for the treatment or prevention of colorectal hepatic metastases (147). The aim of this section of the project, therefore, was to investigate using a panel of immortalised cell lines the effect of DIM in colorectal carcinoma.

There is data on the role of Src in I3C mediated apoptosis and cell cycle arrest. It has been demonstrated that combination of I3C with Src inhibition was more efficacious than either agent alone. To date there are no data on the effect of DIM on Src expression however there is a degree of uncertainty in the interpretation of preclinical data with I3C as DIM has been shown to form spontaneously from I3C *in vitro* . A further aim of this project was therefore to explore the effect of DIM on Src

expression and Src mediated pathways and to investigate whether combination of DIM with a Src inhibitor could augment any potential therapeutic effect.

5.1 Effect of DIM on colorectal carcinoma cell viability

As described in the methods a panel of four immortalised colorectal carcinoma cell lines (HT29, HCT116, SW480 and SW620) were utilised for *in vitro* experiments.

Treatment with DIM resulted in a dose dependent decrease in cell viability (ATPLite) versus DMSO control after 48 hours incubation (Figure 5.1). Statistically significant decreases were yielded by doses of 25 µM and above in three of four cell lines and by doses of 40 µM in the fourth cell line, SW620. These data indicate significant effects at levels anticipated to be achievable *in vivo*.

Previous research with resveratrol, a potential chemopreventative, polyphenolic phytoalexin derived from grapes, demonstrated direct inhibition of firefly luciferase, a
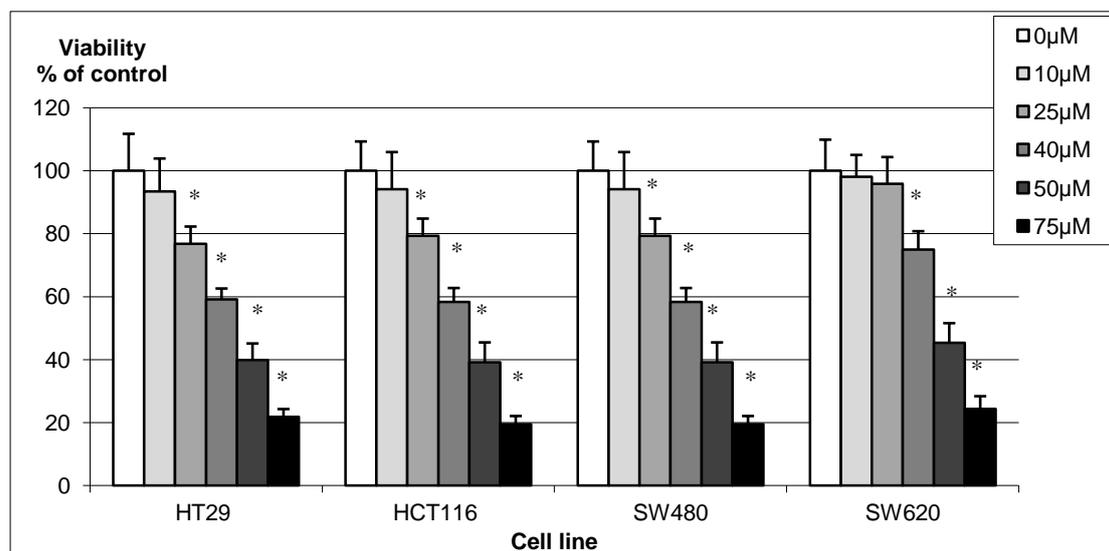
Figure 5.1: Cell viability (ATPLite) in 4 colorectal carcinoma cell lines.
A dose related decrease in cell viability was observed following 48 hours treatment with DIM. Results normalised to DMSO control. n=8 *denotes $p < 0.05$ versus DMSO control

component of several luminescence assays including ATPLite (172). The ATPLite viability assay was therefore validated for use with DIM by comparing it with an alternative measure of viability using a Coulter Cell Counter (Figure 5.2). These data confirm statistically significant decreases in cell viability following 48 hours treatment in all four cell lines with 40 μM DIM. Although the results were not identical between the two assays the difference relative to the demonstrated decreases was such that this was taken to validate ATPLite as a viability assay and as a surrogate measure for cell number for DIM treatments.

On the basis of the data from the ATPLite viability assay 40 μM DIM was selected for further experiments as this demonstrated a significant response in all the cell lines
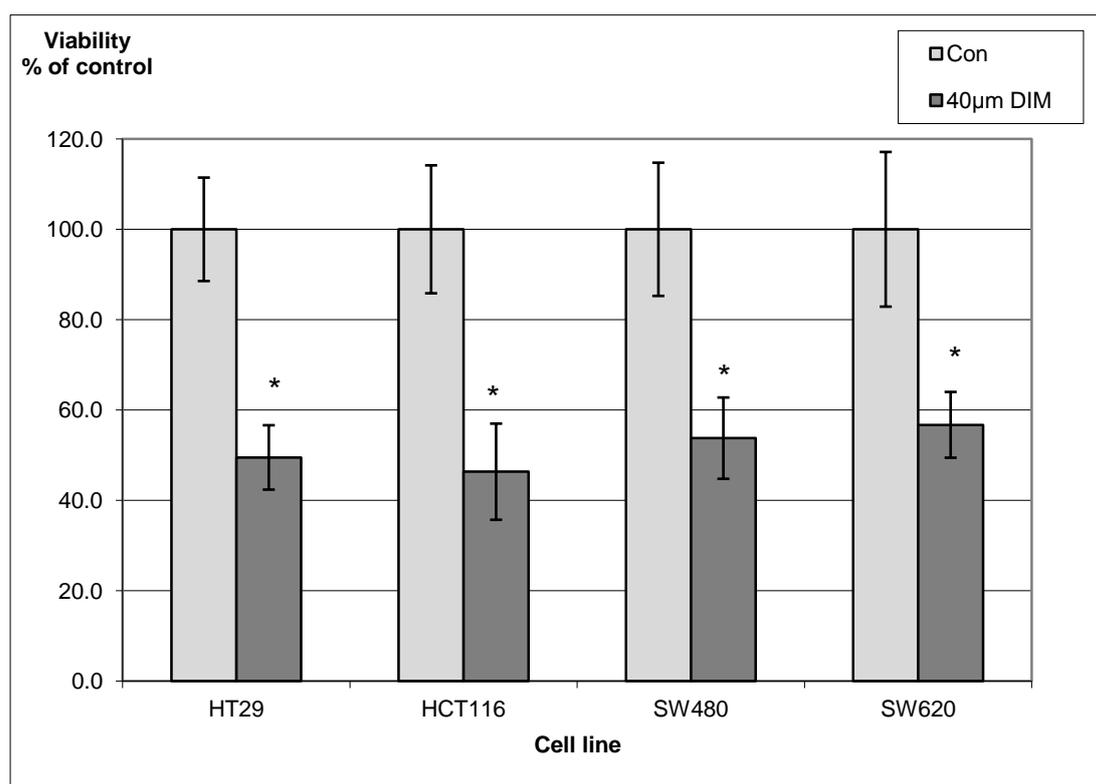
Figure 5.2: Cell viability (Coulter Cell Count) in 4 colorectal carcinoma cell lines.
A significant decrease in viability was observed following treatment, for 48 hours, with DIM in all 4 cell lines. This validated the data obtained using the ATPLite assay. Results normalised to DMSO control. n=6 over 3 repetitions *= $p < 0.05$ vs DMSO control.
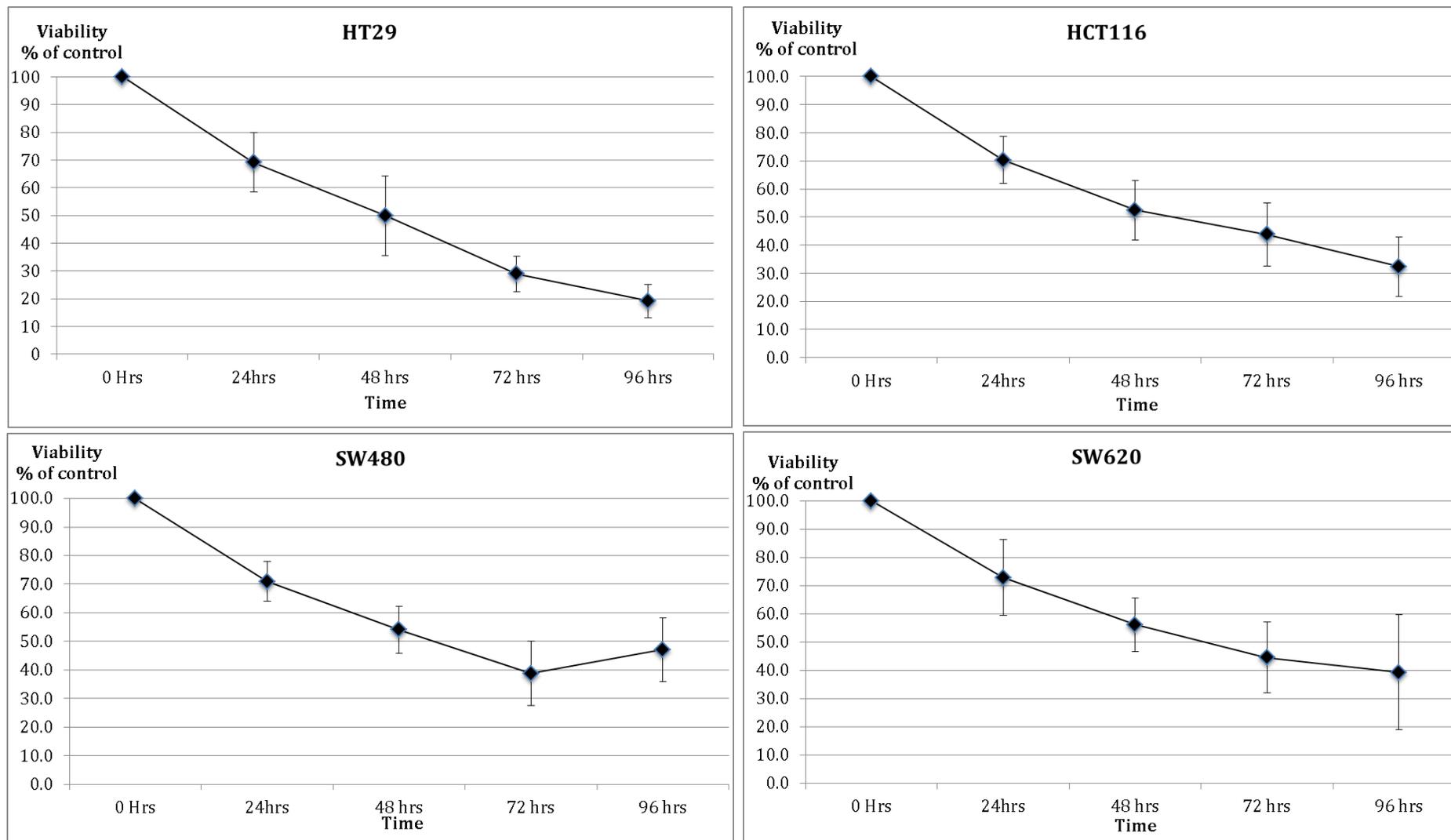
Figure 5.3: Cell viability (ATPLite) over time in 4 colorectal carcinoma cell lines.
A progressive and sustained decrease in cell viability was observed following treatment with 40 µM DIM. Results normalised to DMSO control. n=24 over 3 repetitions. *denotes p<0.05 versus DMSO control

in the panel and furthermore was a dose anticipated to be achievable *in vivo*(148,173).

The decrease in viability in response to treatment with DIM was progressive and sustained over extended time points all the cell lines, with exception of one cell line, yielding cell populations of 69.2-72.8% of control and 19.1-47.0% of control at 24 and 96 hours respectively (Figure 5.3). SW480 failed to exhibit a progressive viability decrease at the longest treatment time point (96 hours) and in SW620 the cell viability decrease between the 72 and 96 hour timepoints failed to achieve statistical signifcance.

5.1.1   Effect of DIM on cell death in colorectal carcinoma

Treatment with 40μM DIM induced apoptosis evidenced by increased activation of caspases 3 and 7 (Caspase-Glo). In 3 of 4 cell lines statistically significant caspase activation occurred by 12 hours. However these increases were small (112-131% of control). There was not a pronounced increase (155-368% of control) until the 24 hour time point. The fourth cell line, HT29, also demonstrated increased caspase activation (217% of control) but only at the 48 hour time point (Figure 5.4).

FACS analysis with annexin V and propidium iodide (PI) staining confirmed increased cell death in all cell lines in the panel following treatment with 40μM DIM at both 24 and 48 hours (Table 5.5). Early apoptosis was significantly increased in 2 and 3 cell lines at 24 and 48 hours respectively. Necrosis/late apoptosis was increased in 3 and 4 cell lines at 24 and 48 hours respectively. Given that one cell line did not demonstrate increased early apoptosis at either time point these data may indicate that treatment with DIM induces cell death through both necrosis and apoptosis or,
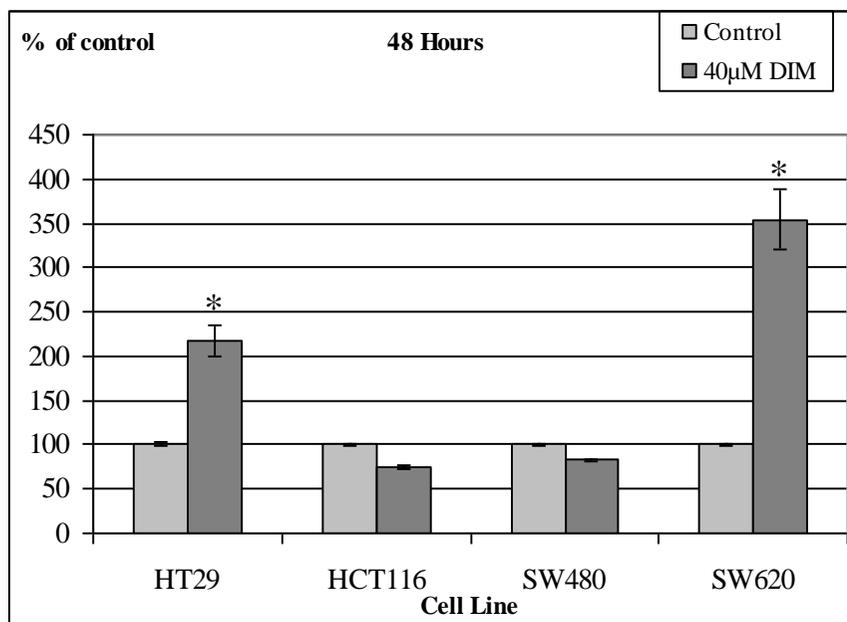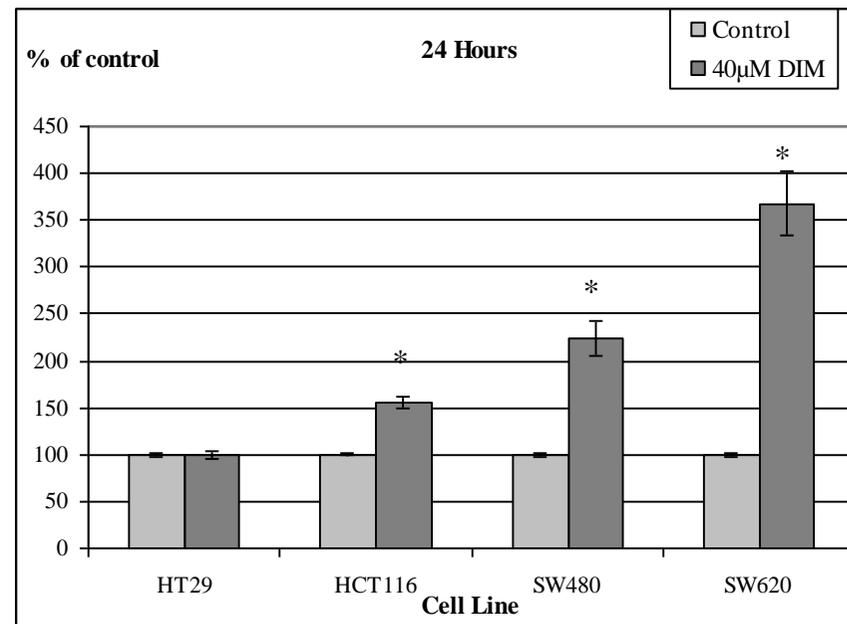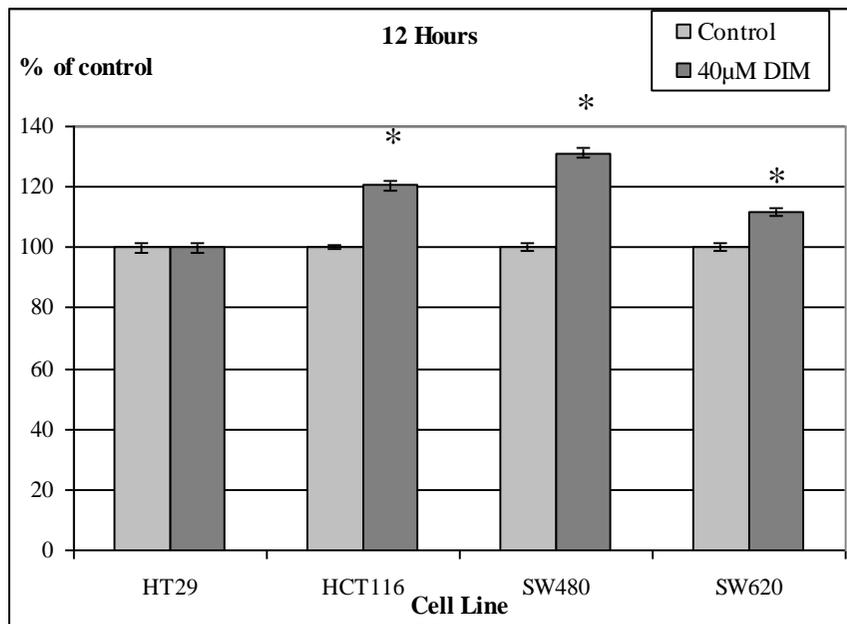
Figure 5.4: Caspase 3/7 activity in 4 colorectal carcinoma cells lines.

Increased caspase 3/7 activity, indicating apoptosis, was observed at 12 hours and increasing at 24 hours in 3 of 4 cell lines following treatment with DIM. This increase was sustained in a single line (SW620) at 48hours. The remaining cell line (HT29) demonstrated increased caspase 3/7activity at 48 hours. Results normalised to DMSO control.

n=24 over 3 repetitions.*= $p < 0.05$ versus DMSO control.
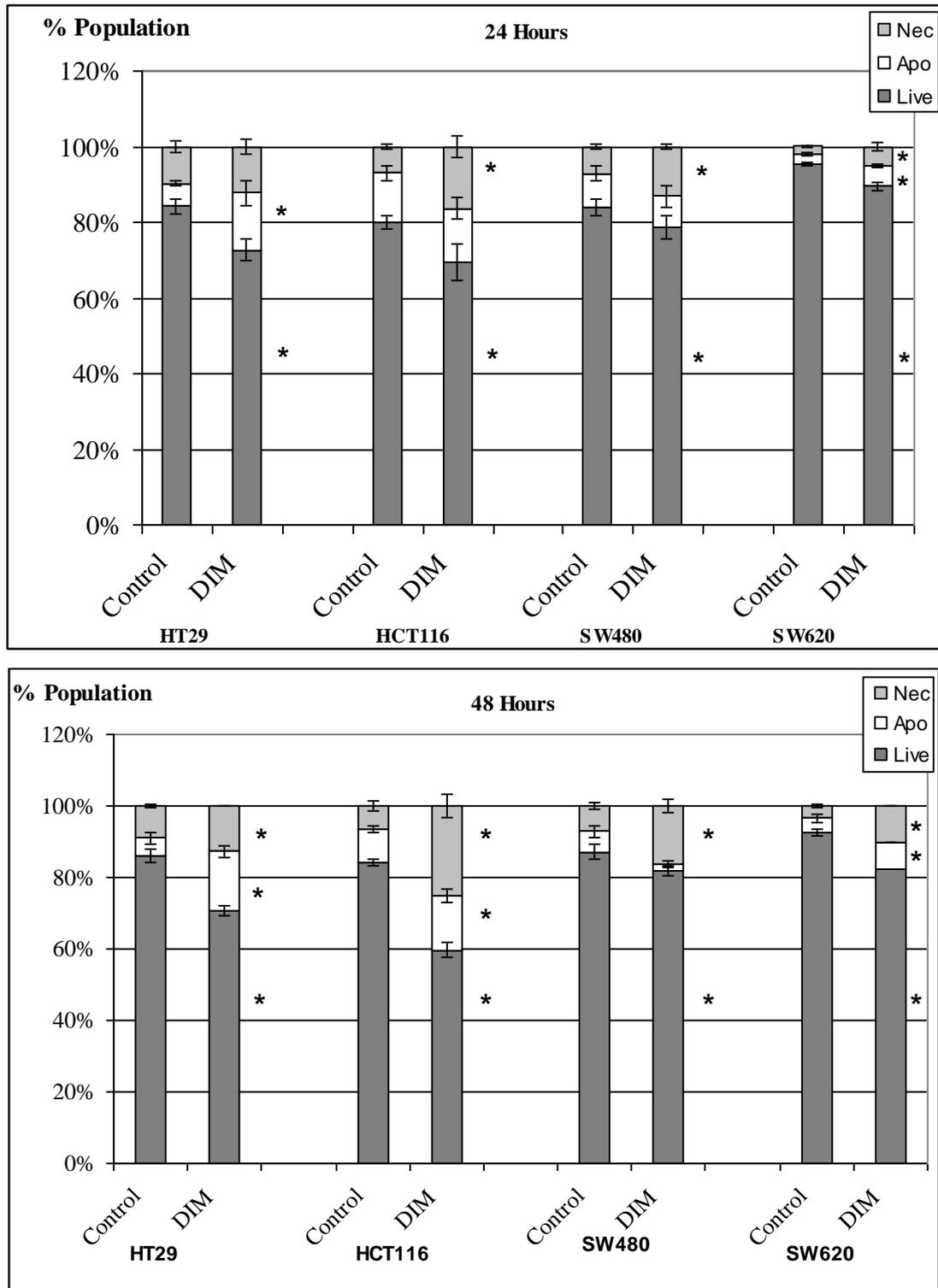
Figure 5.5: Annexin assay for 4 colorectal carcinoma cell lines.
Increased cell death was observed in all four cell lines after 24 and 48 hours treatment with DIM. At 24 hours treatment early apoptosis (apo) was increased in 2 lines (HT29 and SW620) and necrosis/late apoptosis (nec) increased in 3 lines (HCT116, SW480 and SW620). At 48 hours treatment early apoptosis was increased in all lines except SW480 and necrosis/late apoptosis was increased in all cell lines. n=6 over 3 repetitons . * denotes p<0.05 versus DMSO control.

alternatively, may reflect the rate of the apoptotic process in these cells as both late apoptosis and necrosis yield cells positive for both annexin V and PI staining.

5.1.2   Effect of DIM on cell cycle arrest in colorectal carcinoma
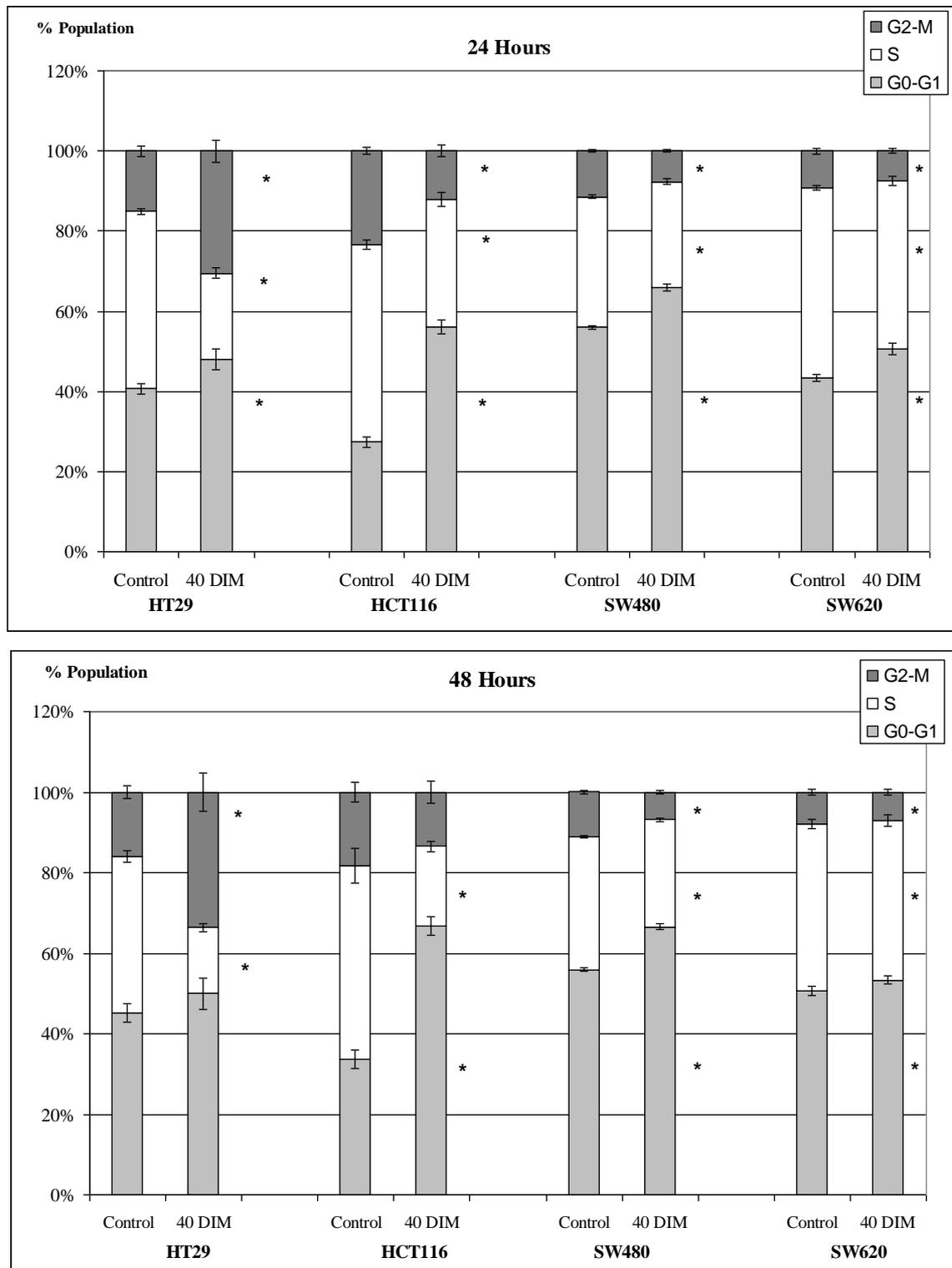
Figure 5.6: Cell cycle analysis (FACS) in 4 colorectal carcinoma cell lines. Following treatment with 40 μM DIM, three of the four cell lines demonstrated G0-1 cell cycle arrest at 24 and 48 hours, whereas HT29 demonstrated G2-M phase arrest at both time points. n=9 over 3 repetitions * denotes p<0.05 versus DMSO control

Treatment with 40 µM DIM induced cell cycle arrest in the panel of colorectal carcinoma cell lines on FACS analysis at 24 and 48 hours incubation. Three of the four cell lines (HCT116, SW480 and SW620) exhibited arrest at the G1-S checkpoint whereas the fourth cell line (HT29) arrested at the G2-M checkpoint (Figure 5.6).

5.2 Effect of DIM on cellular adhesion in colorectal carcinoma
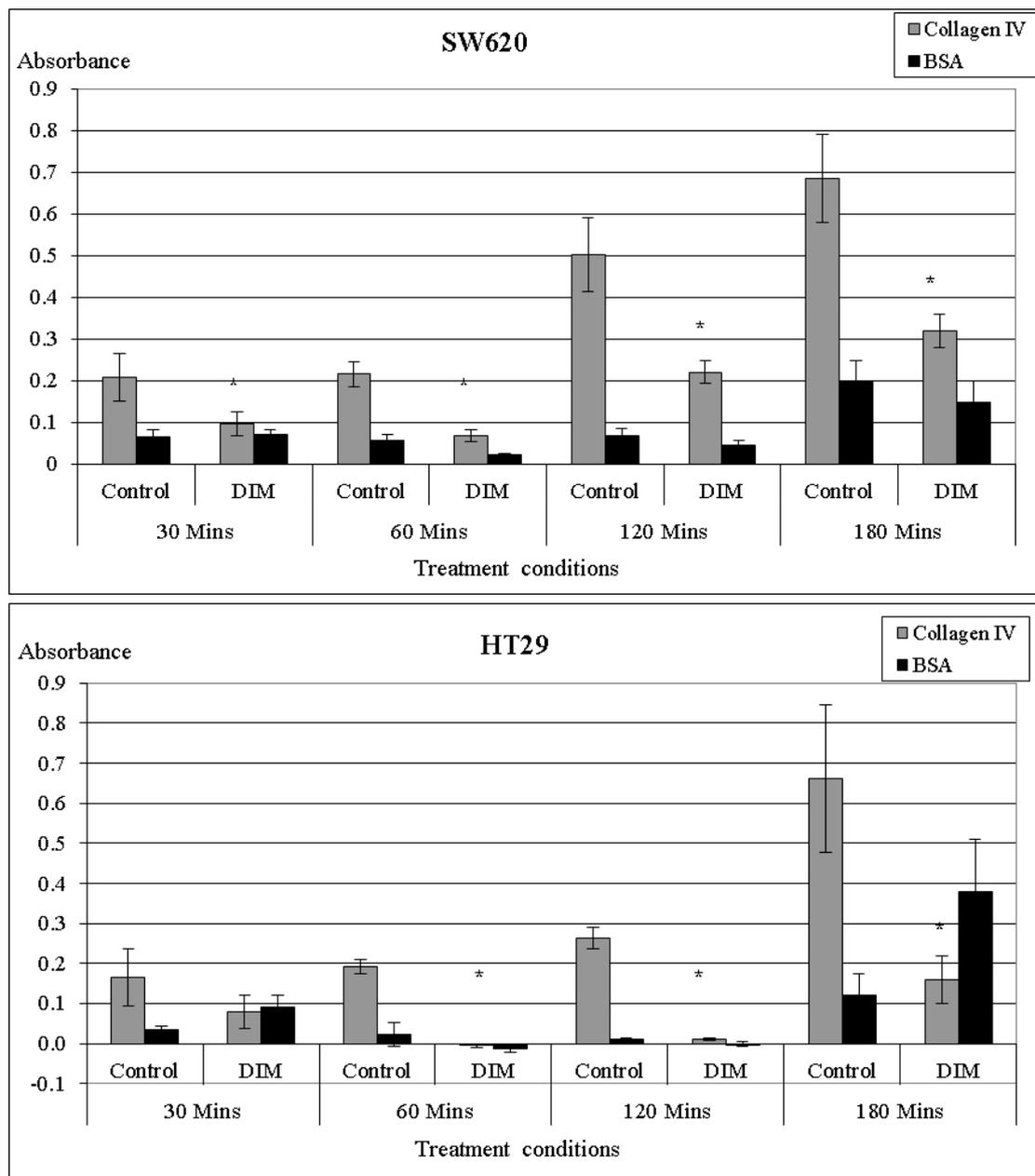
Figure 5.7: Cell adhesion over time in colorectal carcinoma cell lines, SW620 and HT29.
Adhesion is proportional to relative absorbance at 550nm. Inhibition of cell adhesion was observed, following treatment with 40 µM DIM, at all time points in SW620 and at all time points except 30 minutes in HT29. * denotes p<0.05 vs DMSO

112

The effect of treatment with DIM on cellular adhesion, and therefore on the potential formation of tumour metastases, was examined. HT29 and SW620 cell lines were selected for the assay. HT29 as it had previously demonstrated a high sensitivity to the effects of DIM in both cell viability and cell cycle arrest experiments and SW620 as it was originally derived from a colorectal carcinoma lymph node metatstasis.

Treatment with 40 μM DIM yielded a greater than 50% decrease (53.3-68.3%) in relative adhesion versus DMSO control at all four time points (30, 60, 120 and 180 minutes) in SW620 cells ($p < 0.05$) thus demonstrating a significant and sustained inhibition. The maximal effect was seen at 60 minutes incubation (Figure 5.7).

The HT29 cells also demonstrated a sustained inhibition of cellular adhesion across all four time points with a greater than 50% decrease (51.8-100%) in relative adhesion versus DMSO control, although this failed to reach statistical significance at 30 minutes incubation (Figure 5.7).

5.3   DIM and Src inhibition using AZD0530 in colorectal carcinoma

DIM was combined with AZD0530 (AstraZeneca, UK) a Src inhibitor undergoing clinical trials prior to full commercial release to ascertain if the effect on carcinoma cell viability could be augmented through combination therapy. As previously described a dose of 1.0 μM of AZD0530 was utilised, as per the manufacturers guidance.

5.3.1   DIM with Src inhibition using AZD0530 and effects on colorectal carcinoma cell viability

Treatment with a combination of 40 μM DIM and 1.0 μM AZD0530 yielded increased loss of cell viability versus DMSO control, DIM and AZD0530 as single
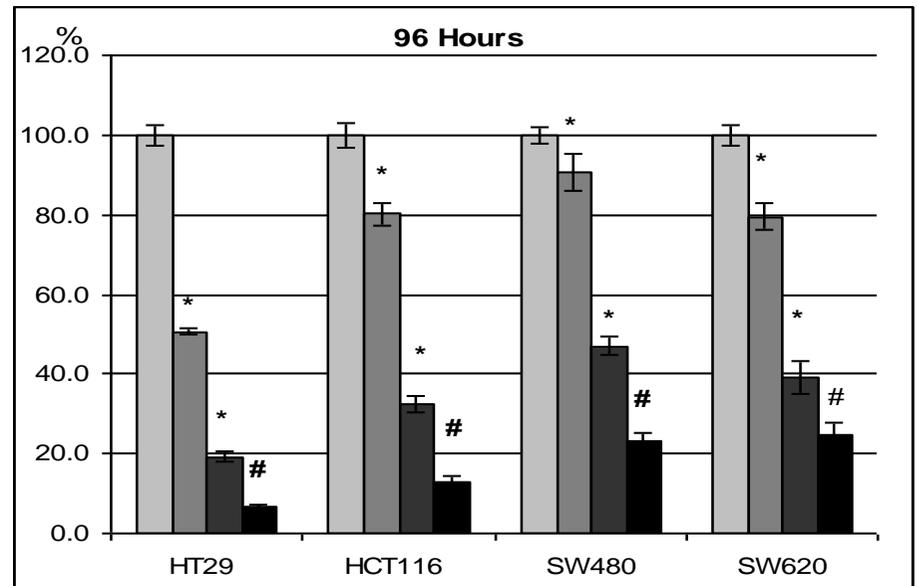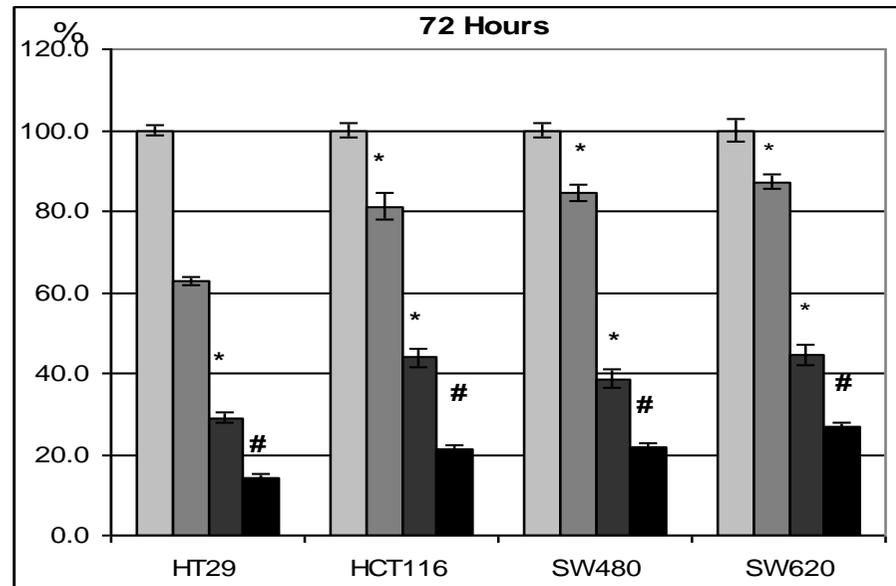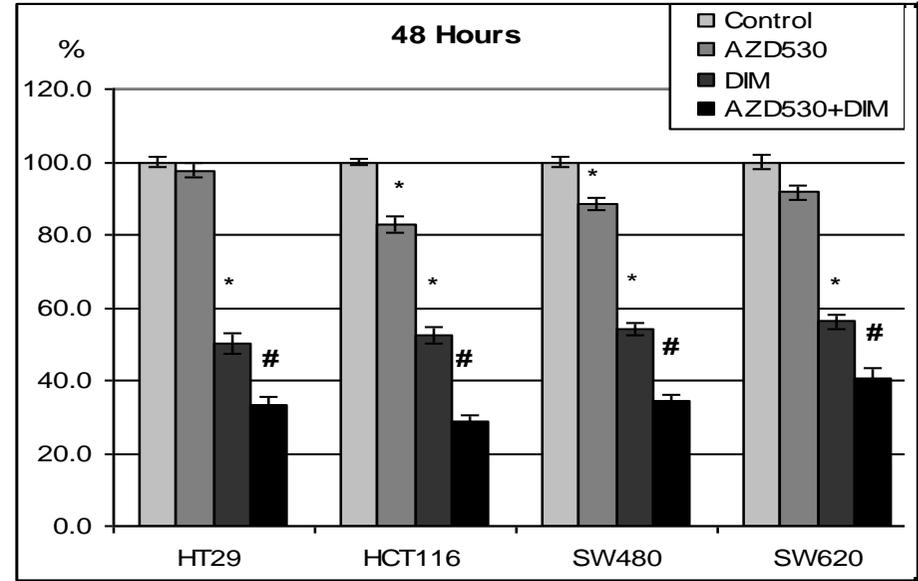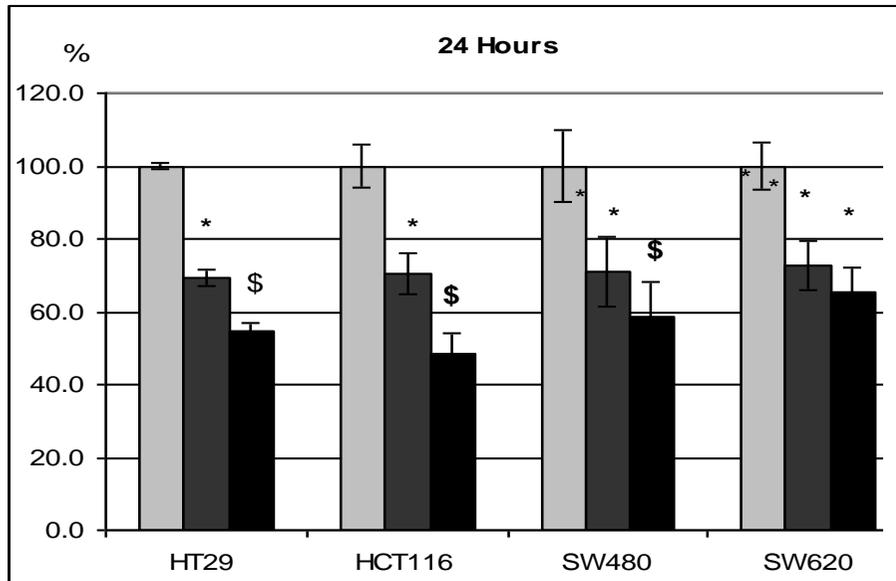
Figure 5.8 (Previous page): Cell viability (ATPLite) in 4 colorectal carcinoma cell lines.
Greater decreases in cell viability were observed following treatment with a combination of 1.0 μM AZD0530 and 40 μM DIM relative to DIM and AZD0530, as sole agents. The decrease with combination treatment was sustained and progressive over time (24-96 hours). Note no data for AZD0530 as sole agent at 24 hours. n=24 over 3 repetitions. Results normalised to DMSO control. *denotes $p < 0.05$ versus DMSO control $ denotes $p < 0.05$ versus DMSO control and DIM # denotes $p < 0.05$ versus DMSO control, AZD0530 and DIM as sole agents

agents at 48 hours incubation. These effects were progressive and sustained across 48, 72 and 96 hour timepoints yielding cell populations of 28.5-69.6% and 6.6-29.9% of control at 48 and 96 hours treatment respectively (Figure 5.8). As previously for data obtained using the ATPLite assay this data was validated with Coulter Cell Count data (not shown).

5.3.2   DIM and Src inhibition using AZD0530 and effects on cell death in colorectal carcinoma
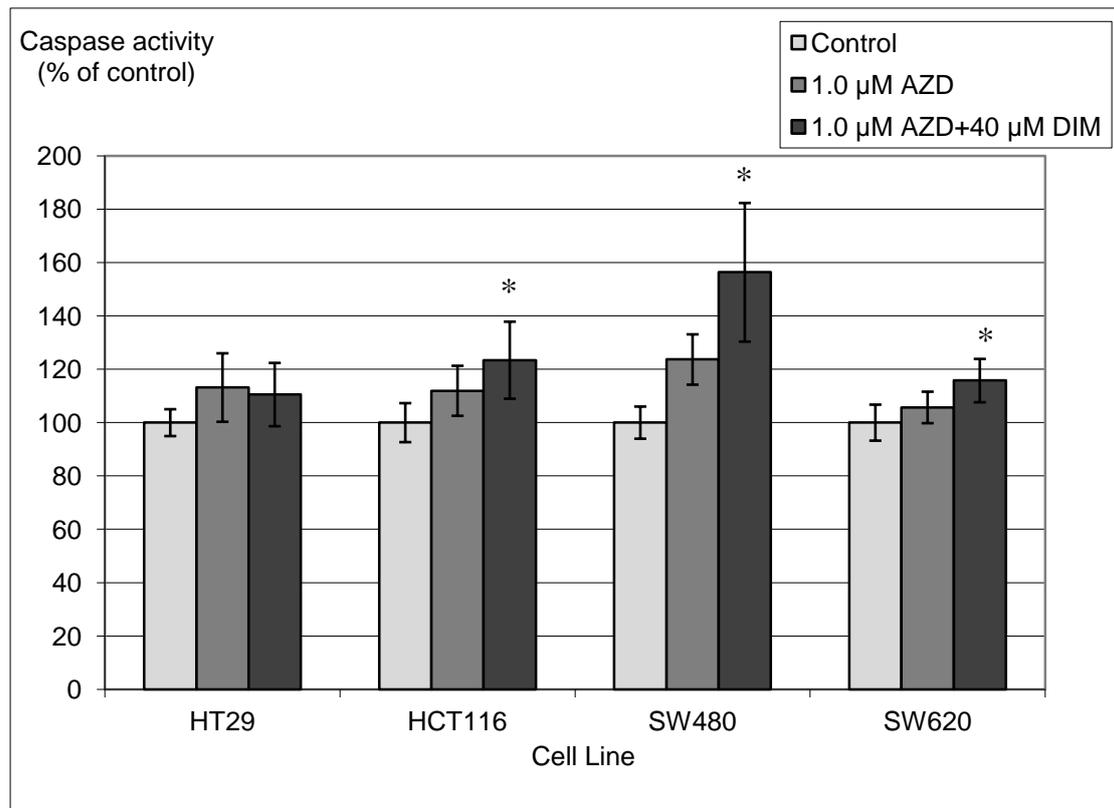
Figure 5.9: Caspase 3/7 activity (Caspase-Glo) in 4 colorectal carcinoma cell lines. A small increase in caspase activity, indicating induction of apoptosis, was observed in 3 of the 4 cell lines following 12 hours treatment with a combination 1.0μM AZD0530 and 40μM DIM relative to AZD0530 alone and DMSO control. n=24 over 3 repetitions.   * denotes $p < 0.05$ versus DMSO control and AZD0530.
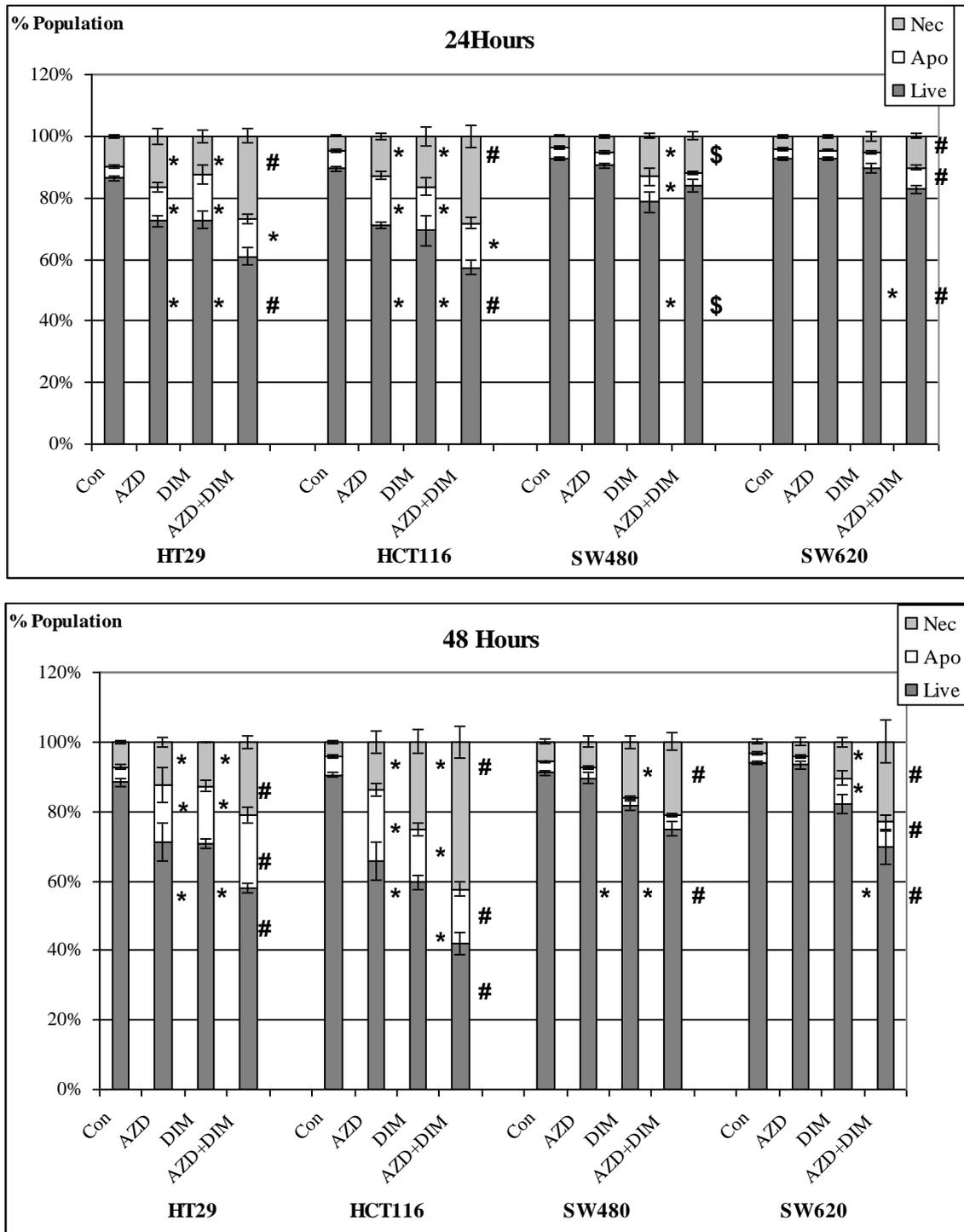
Figure 5.10: Annexin assay (FACS) in 4 colorectal carcinoma cell lines.
Increased cell death was observed in all four cell lines following treatment, for 24 and 48 hours, with 1.0 μM AZD0530 and 40 μM DIM in combination versus both AZD0530 and DIM, as sole agents, and DMSO control, with the exception of SW480 following 24 hours treatment.
n=6 over 3 repetitions * = p<0.05 versus DMSO control $=p<0.05 versus DMSO control and AZD0530. #=p<0.05 versus DMSO control, DIM and AZD0530 as sole agents.

Treatment with a combination of 40 μM DIM and 1.0 μM AZD0530 induced apoptosis in the panel of colorectal carcinoma cell lines evidenced by increased activation of caspases 3 and 7 (Caspase-Glo) at 12 hours (Figure 5.9). In 3 of 4 cell lines (HCT116, SW480 and SW620) these small increases (111-156% of DMSO control) were statistically significant versus both DMSO control and AZD0530 monotherapy.

FACS analysis with annexin V and propidium iodide (PI) staining confirmed increased cell death by apoptosis and late apoptosis/necrosis, versus DMSO control and both AZD0530 and DIM as sole agents, in the panel of four colorectal carcinoma cell lines, with one exception, following treatment with a combination of 1.0μM AZD0530 and 40μM DIM at both 24 and 48 hours (Figure 5.10) (p<0.05). SW480 failed to demonstrate increased cell death relative to cells treated with DIM alone after 24 hours. However an increase in cell death by apoptosis and late apoptosis/necrosis was exhibited following 48 hours treatment (p<0.05).

5.4 DIM, AZD0530 and protein expression in colorectal carcinoma

On the basis of the results of the cell cycle analysis and cell death experiments in response to treatment with DIM the decision was made to focus on HT29 and HCT116 for subsequent experiments into cellular protein expression. Specifically these two cell lines were selected as firstly they yielded the greatest sensitivity to treatment, in terms of change in cell viability. Thus one would anticipate a greater change in protein expression would be demonstrated and secondly HT29 demonstrated arrest at a different cell cycle phase in comparison with the other three

cell lines, thus a different mechanism may be responsible. Concentrations of 40 μM DIM and 1.0 μM AZD0530 were utilised for the reasons outlined in previous sections.

Initially, the influence of p53 status on response to DIM was examined. Treatment with a range of doses of DIM (0-75 μM) for 48 hours yielded a small, but statistically significant, reduction in the viability decrease in an HCT116 p53 knockout cell line in comparison with an HCT116 wild type cell line at the highest dose examined (80.9% of control versus 86.7%). No other statistically significant changes in the response were observed although a slight reduction in the decrease was also observed at 25 μM (Figure 5.12). This demonstrates that the effect of DIM on overall cell viability occurs, at least predominantly, independent of p53 mediated pathways.
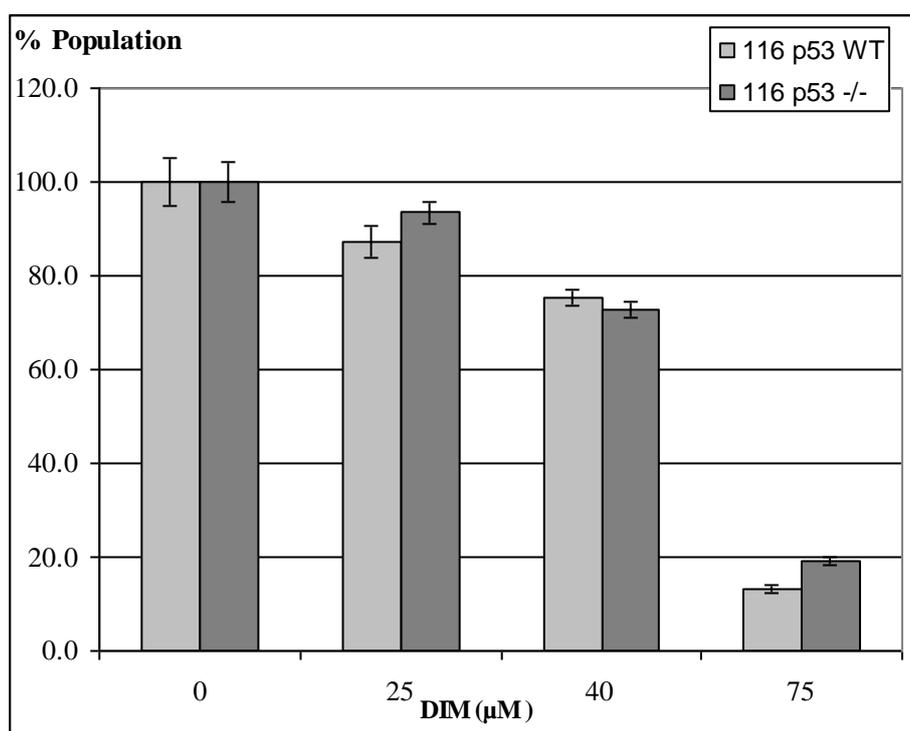
Figure 5.11: Cell viability (ATPLite) in HCT116 p53 wild type (WT) and HCT116 p53 knockout (-/-).
Following 48 hours treatment with DIM at increasing doses, a decreased reduction in cell viability was observed in HCT116 p53 -/- relative to HCT116 p53 WT with 75 μM DIM only. These data indicate that the effect of DIM on colorectal carcinoma cell viability predominantly occurs independent of p53. Results normalised to DMSO control. n=8 * = $p<0.05$ versus HCT116 p53 WT

118

Western blotting was performed to establish the effects of treatment with DIM and AZD0530 on cellular protein expression. Treatment intervals of 24 and 48 hours were used and blots were loaded in the following order unless otherwise stated in the accompanying text:

24 Hours Treatment				48 Hours Treatment			
DMSO Control	AZD0530	DIM	AZD0530+ DIM	DMSO Control	AZD0530	DIM	AZD0530+ DIM

Unless otherwise stated results are reported for both time points. Where both cell lines demonstrated a similar response to treatment, a representative blot from only one of the two lines is included for illustration and this is stated in the figure legend. For each blot the actin loading control is shown immediately below.
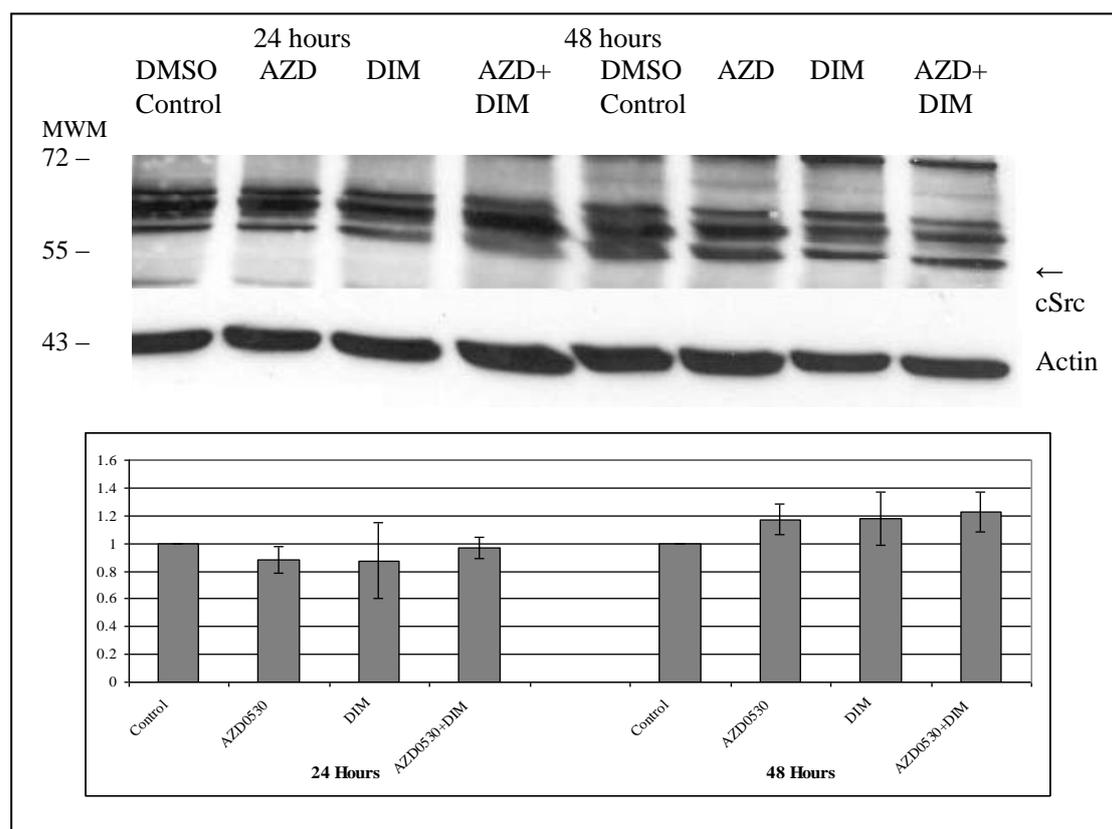
Figure 5.12: cSrc (molecular weight 60kDa) expression in HT29 colorectal carcinoma cells.
No change in protein expression was exhibited following treatment for 24 and 48 hours with AZD0530 and DIM. Similar results were obtained with HCT116 cells.
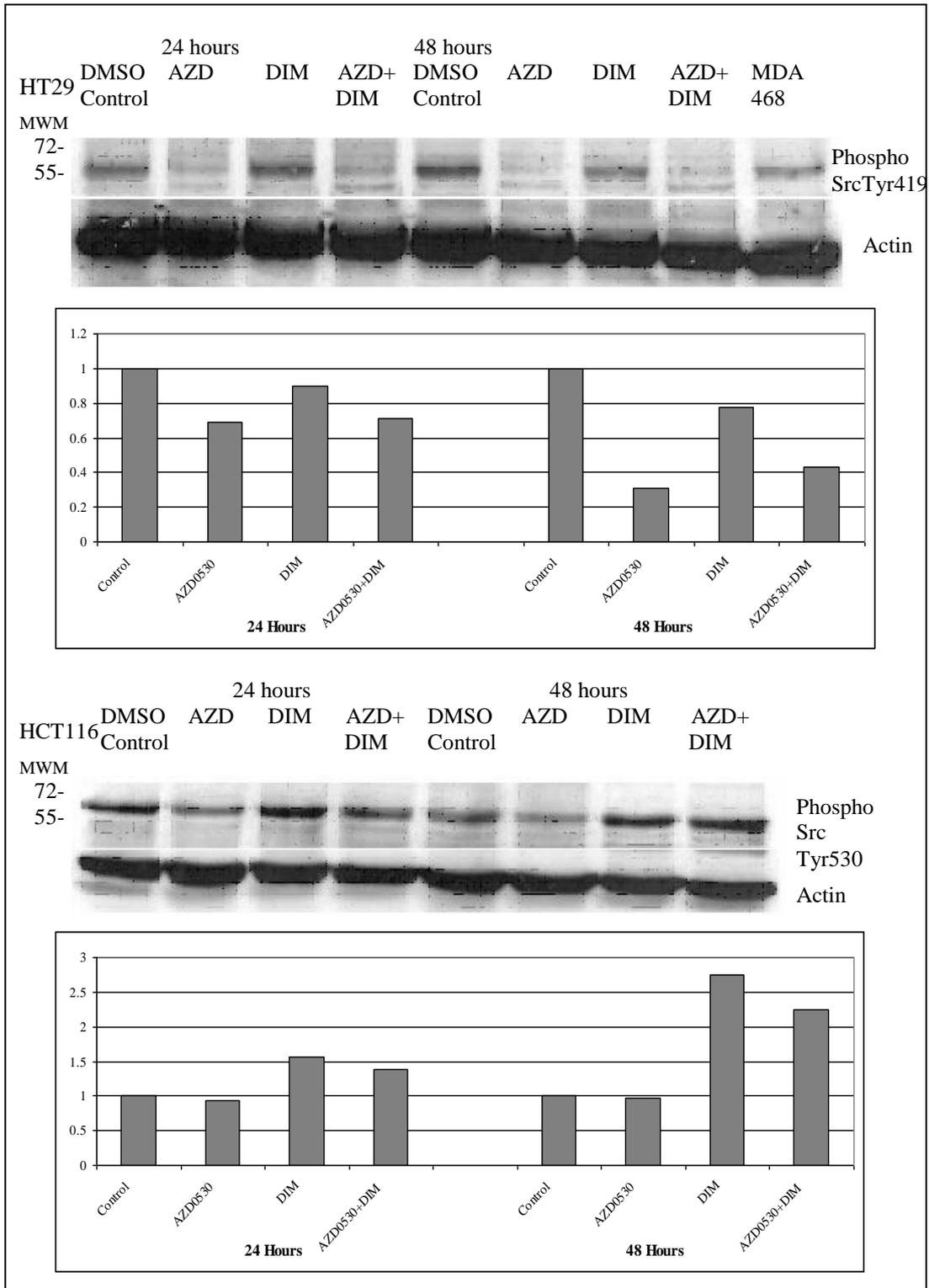
Figure 5.13: Src tyrosine 419 and tyrosine 530 phosphorylation status in HT29 and HCT116 colorectal carcinoma cell lines.

Western blots demonstrate: 1) decreased phosphorylation at tyrosine 419, indicating downregulation of Src in HT29 colorectal carcinoma cells following treatment with AZD0530 and DIM; and 2) increased phosphorylation at tyrosine 530, indicating downregulation of Src, in response to treatment with DIM in HCT116 colorectal carcinoma cells. These data show that AZD0530 and DIM act in a complimentary fashion in down regulating Src in colorectal carcinoma cell lines. Similar results were obtained with both cell lines. MDA468 was included as a positive control for phospho-Src (Tyr419).

n=2 (Src (Tyr530)) and 1 (PhosphoSrc (Tyr 419))

Treatment with DIM and AZD0530 both alone and in combination did not yield a significant change in the expression of the oncogene cSrc (Figure 5.12). cSrc activity is regulated through tyrosine phosphorylation at two sites with opposing effects: phosphorylation at Tyr419 upregulates the enzyme activity, whereas phosphorylation at Tyr530 is inhibitory. Both DIM and AZD0530 yield decreases in the upregulatory phosphorylation at Tyr419, although this effect is greater with AZD0530 however treatment with DIM also increases inhibitory phosphorylation at Tyr530 (Figure 5.13). Thus DIM and AZD0530 may act in a complimentary fashion in downregulation of Src activity. These data are based on single blots for Src phosphorylated at Tyr419 and two blots for Src phosphorylated at Tyr530.

5.4.1 DIM, AZD0530 and effects on cell cycle protein expression in colorectal carcinoma

An HCT116 p21 knockout cell line treated with 40 μM DIM yielded increased G1 cell cycle arrest (46.8% versus 36.7%)(p,0.05) and a small increase in G2-M arrest relative to DMSO control though the latter did not achieve statistical significance (p=0.09). However there was a decrease in G1 cell cycle arrest compared (46.8% versus 58.6%) to HCT116 wild type (Figure 5.14) (p<0.05).

An HCT116 p53 knockout cell line also yielded increased cell cycle arrest versus DMSO control (49.6% versus 32.1%) but a decrease versus the wild type cell line (49.6% versus 58.6%) (Figure 5.15).

These data indicate that cell cycle arrest in response to treatment with DIM is probably partially mediated via p21 and p53 pathways. It is of note that the decrease
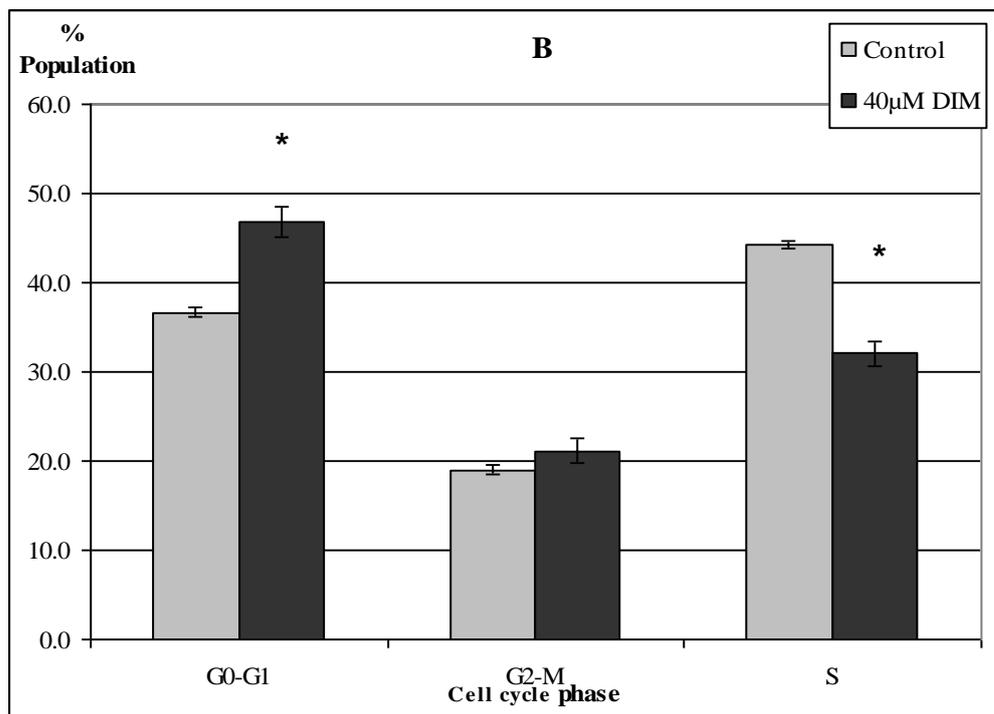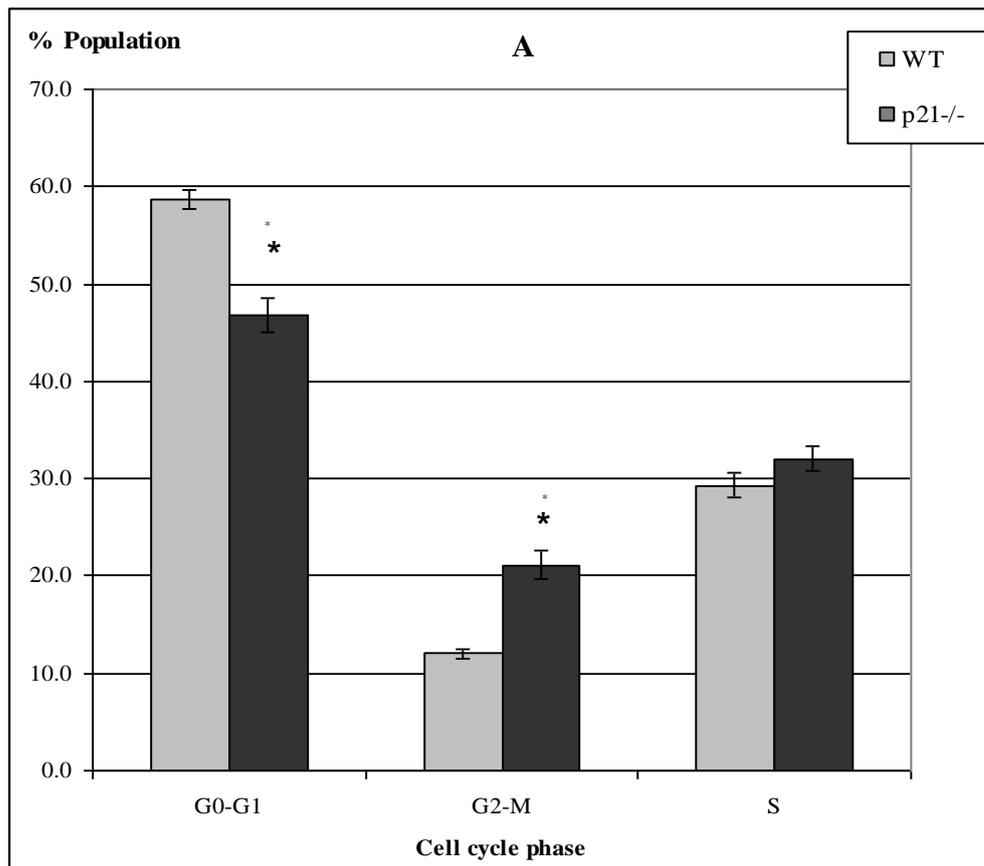
Figure 5.14: Cell cycle analysis (FACS) for HCT116 wild type (WT) and HCT116 p21 knockout (-/-).

Decreased G1 cell cycle arrest was observed following 48 hours treatment with DIM in HCT116 p21 -/- relative to HCT116 p21 WT (A) however G1 cell cycle arrest was increased in HCT116 p21 -/- following 48 hours treatment with DIM relative to DMSO control (B). These data indicate that DIM induced cell cycle arrest in colorectal carcinoma is probably partially mediated via p21. n=12 *= p<0.05

122
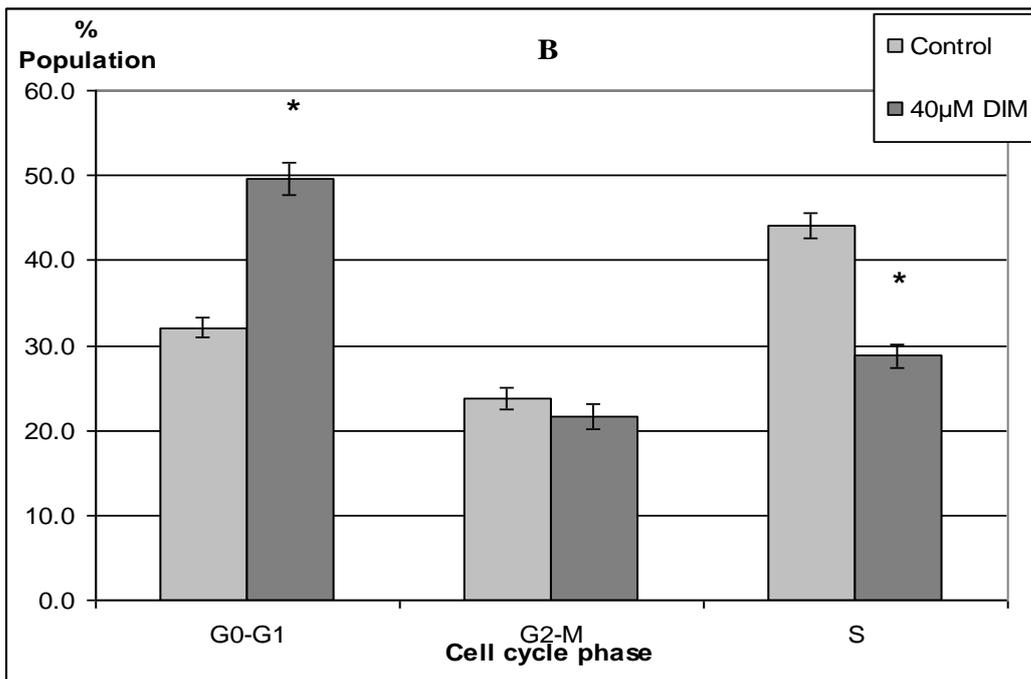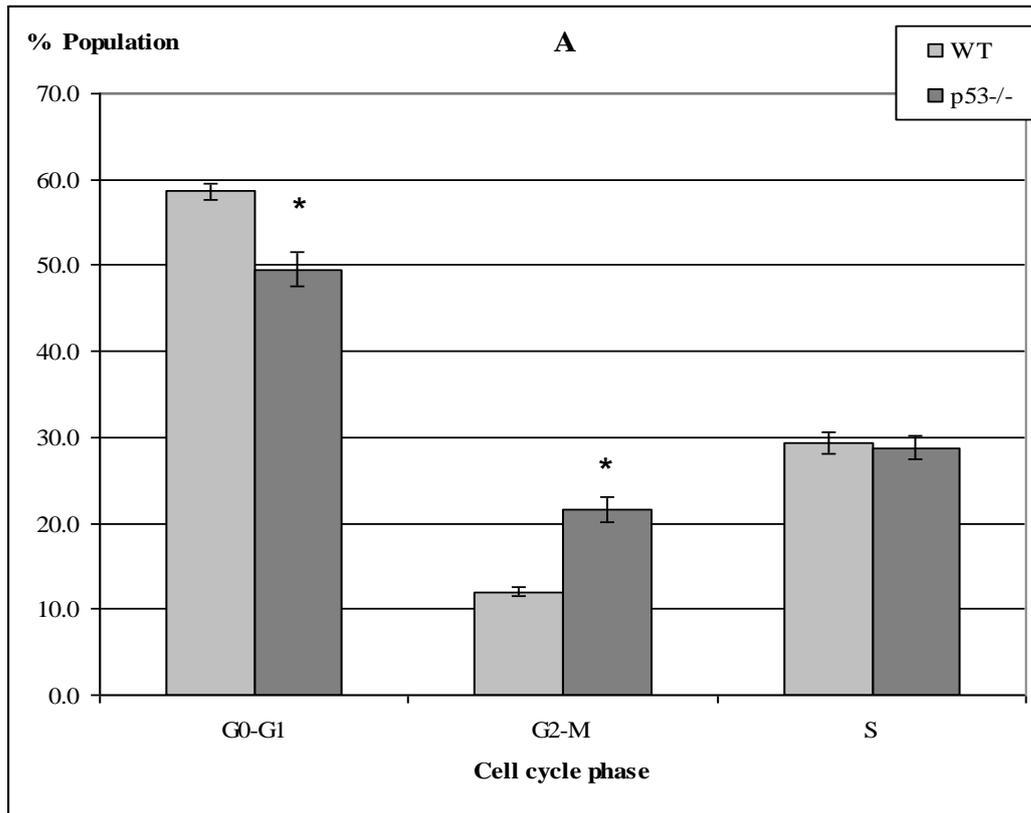
Figure 5.15: Cell cycle analysis (FACS) for HCT116 wild type and HCT116 p53 knockout.

Decreased G1 cell cycle arrest was observed following 48 hours treatment with DIM in HCT116 p53 -/- relative to HCT116 p53 WT (A) however G1 cell cycle arrest was increased in HCT116 p53 -/- following 48 hours treatment with DIM relative to DMSO control (B). These data indicate that DIM induced cell cycle arrest in colorectal carcinoma is probably partially mediated via p53. n=12 *= $p<0.05$

The data reported in section 5.1.2 showed that treatment with DIM induced G2/M phase cell cycle arrest in HT29 cells and G1/S phase cell cycle arrest HCT116 and the other colorectal carcinoma cell lines examined.

PLK1 is involved in promoting progression through the G2-M cell cycle checkpoint via phosphorylation of cdc25C at serine 198, resulting in translocation of cdc25C from the cytoplasm to the nucleus, where it is able to interact with cdc2 and cyclin B1 thus decreases in PLK1 expression could contribute to G2-M arrest. Treatment with DIM decreased PLK1 expression, both alone and in combination with AZD0530. Blot quantification established that this was an additive effect although insufficient to reach statistical significance (Figure 5.16). In conjunction with the potential effect
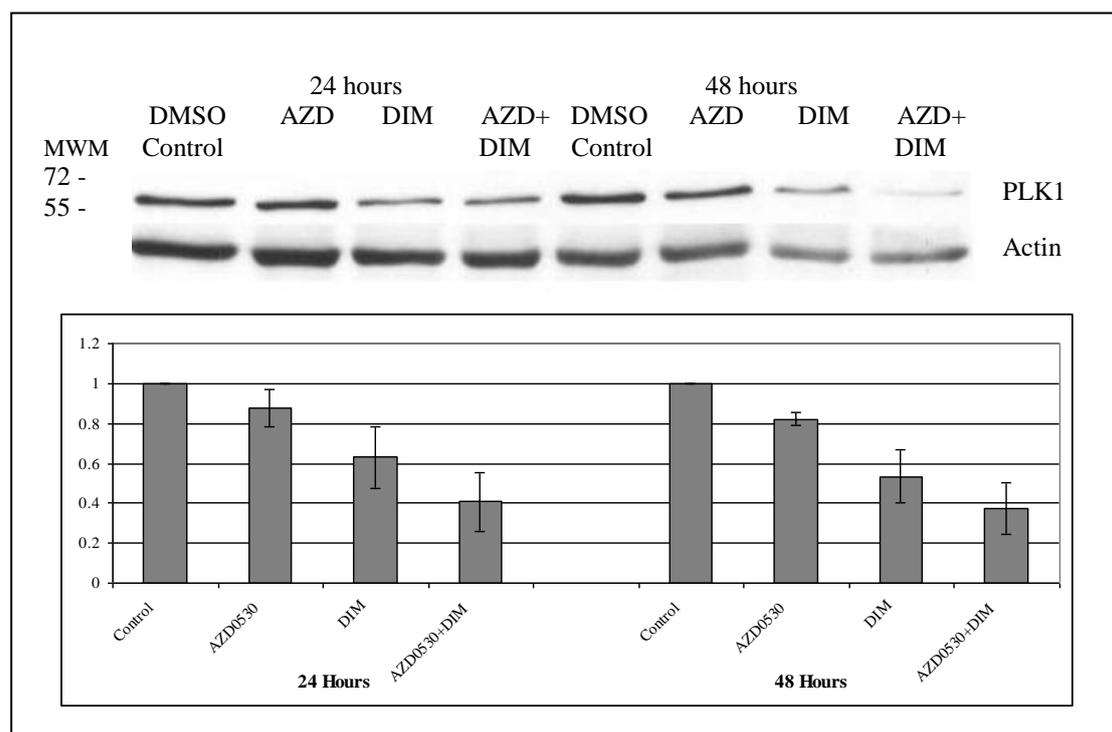
Figure 5.16: PLK1 expression in HCT116 colorectal carcinoma cells.
Western blotting demonstrated PLK1 was downregulated following treatment for 24 and 48 hours with DIM and AZD530 as sole agents and in combination, potentially promoting G2/M cell cycle arrest and cell death. Similar results were observed in HT29 cells. n=3
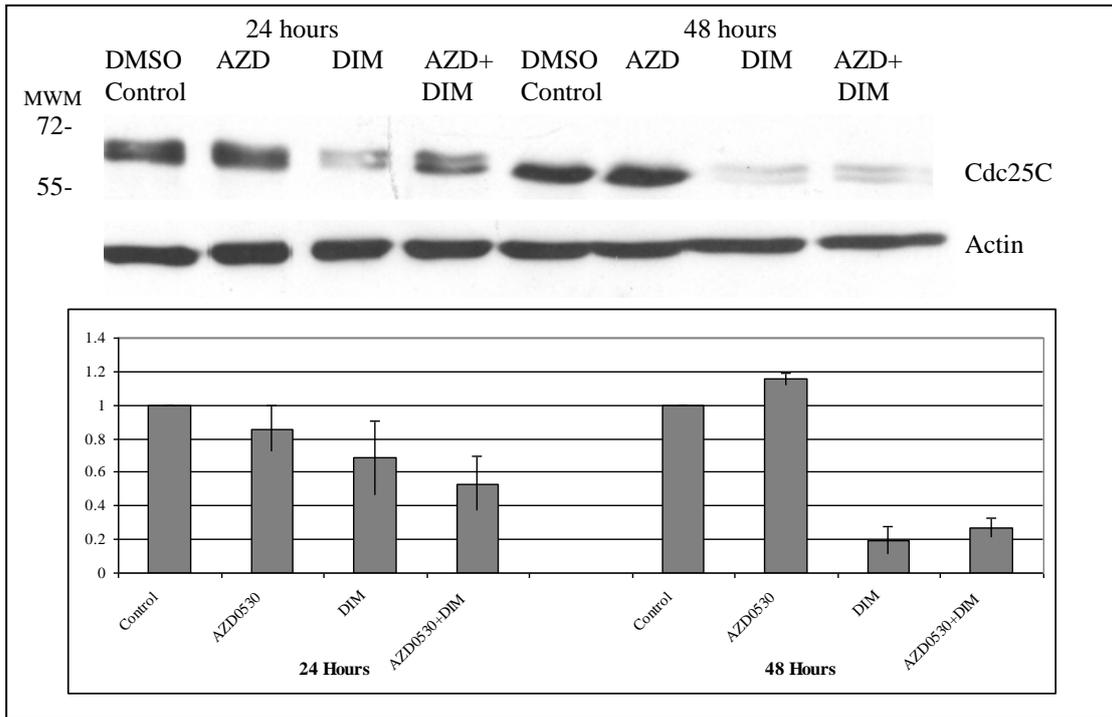
Figure 5.17: Cdc25C expression in HCT116 colorectal carcinoma cells. Following treatment with DIM for 24 and 48 hours downregulation of Cdc25C was observed promoting G2/M cell cycle arrest. Similar results were observed in HT29 cells. n=3
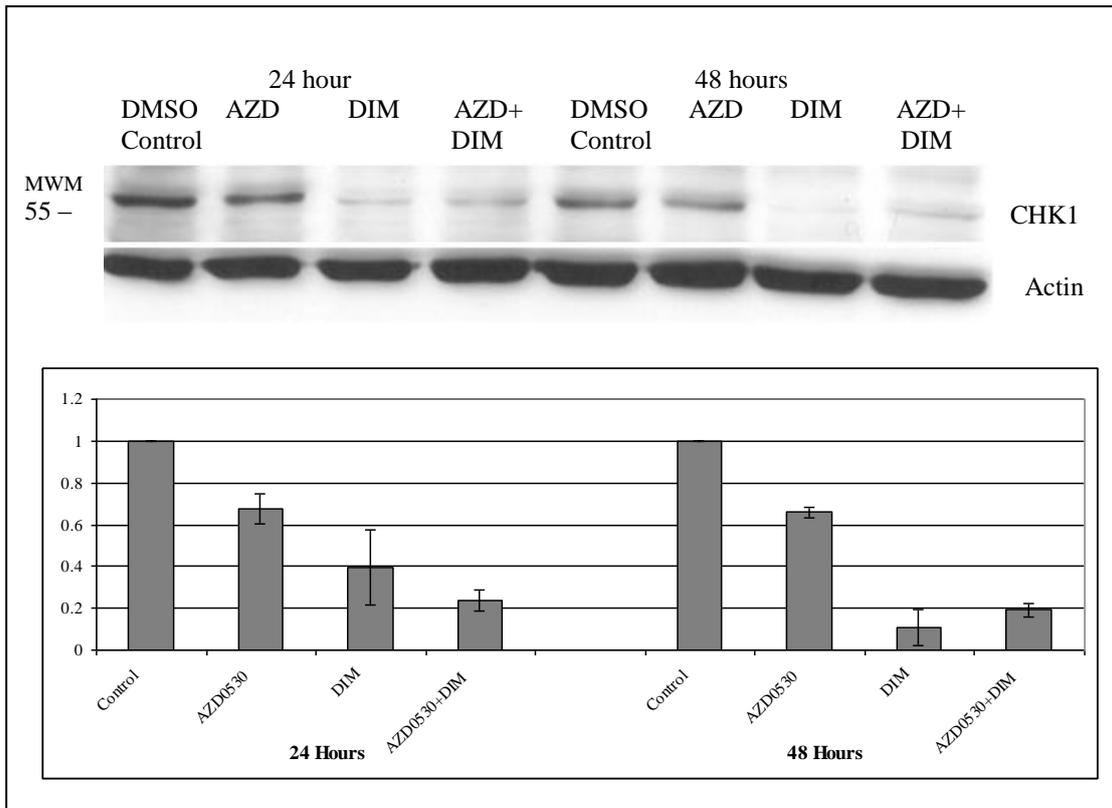
Figure 5.18: CHK1 expression in HCT116 colorectal carcinoma cells. Western blotting demonstrated CHK1 was downregulated following treatment for 24 and 48 hours with DIM and AZD530 as sole treatments and in combination. Similar results were observed in HT29 cells. n=3

HT29

24 hours

48 hours

MWM
72-

55-

DMSO Control | AZD | DIM | AZD+ DIM | DMSO Control | AZD | DIM | AZD+ DIM

CHK2

Actin

1.4
1.2
1
0.8
0.6
0.4
0.2

Control | AZD0530 | DIM | AZD0530+DIM | Control | AZD0530 | DIM | AZD0530+DIM

24 Hours | **48 Hours**

HT29

24 hours

48 hours

DMSO Control | AZD | DIM | AZD+ DIM | DMSO Control | AZD | DIM | AZD+ DIM

MWM
72-

55-

Phospho-CHK2

Actin

3
2.5
2
1.5
1
0.5
0

Control | AZD0530 | DIM | AZD0530+DIM | Control | AZD0530 | DIM | AZD0530+DIM

24 Hours | **48 Hours**

HCT116

24 hours

48 hours

DMSO Control | AZD | DIM | AZD+ DIM | DMSO Control | AZD | DIM | AZD+ DIM

MWM
72-

55-

Phospho-CHK2

Actin

1.2
1
0.8
0.6
0.4
0.2
0

Control | AZD0530 | DIM | AZD0530+DIM | Control | AZD0530 | DIM | AZD0530+DIM

24 Hours | **48 Hours**

126

Figure 5.19 (Previous page): CHK2 expression in HT29 and phospho-CHK2 (Thr68) expression in HT29 and HCT116 colorectal carcinoma cells.
 Following treatment with AZD0530 and DIM for 24 and 48 hours CHK2 expression did not change on western blotting in HT29 or HCT116 (not shown) but increased phosphorylation was observed at 48 hours in HT29, implying increased CHK2 activation, but not in HCT116. This difference may account for the difference in the phases of cell cycle arrest observed in these cell lines in response to treatment with DIM. n=3

of decreased PLK1 expression on cdc25C nuclear translocation treatment with DIM

also resulted in decreased total levels of cdc25C at 24 and 48 hours (Figure 5.17).

Cdc25C activity is also regulated via inhibitory phosphorylation at serine 216 by the

checkpoint kinases CHK1 and CHK2 in response to stimuli such as DNA damage.

CHK1 expression, in both HT29 and HCT116, is decreased in response to treatment

with DIM (Figure 5.18) thus promoting cdc25C activity and therefore progression

through the G2/M checkpoint. CHK2 expression is unchanged in either cell line in

response to treatment with DIM however DIM induces increased phosphorylation at

threonine 68 in HT29 cells, after 48 hours, yielding increased CHK2 activation and

therefore G2/M arrest. This phosphorylation and resultant upregulation is absent in

HCT116 cells (Figure 5.19) and this may in part account for the discrepancy in the

phases of cell cycle arrest exhibited by cell lines examined.

The critical final steps for progression through the G2/M checkpoint are

dephosphorylation of cdc2 at two sites (threonine 14 and tyrosine 15) and binding to

cyclin B1. Cdc25C is responsible for dephosphorylation of cdc2(174). Treatment with

DIM yielded decreases in total level of cdc2 and in the level of tyrosine 15

phosphorylated cdc2 (Figure 5.20). The latter data could indicate an increase in, or

maintenance of pre-treatment, cdc2 activity, however, it is likely to reflect the
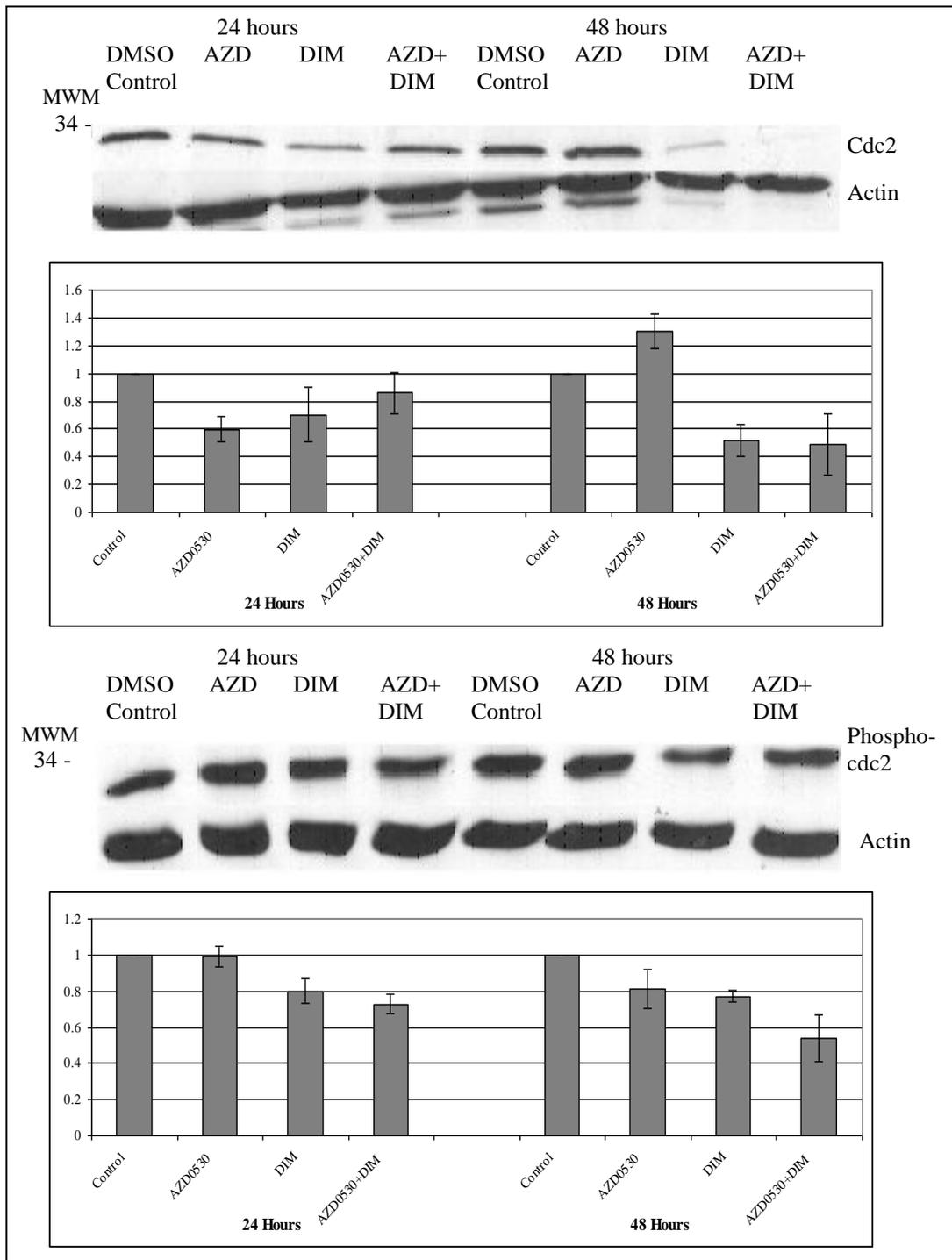
Figure 5.20: Cdc2 and phospho-cdc2 (Tyr15) expression in HT29 colorectal carcinoma cells.

Decreased levels of cdc2 and phosphorylated cdc2 were observed on western blotting following treatment with DIM, both as a sole agent and in combination with AZD0530, for 24 and 48 hours. Decreases in cdc2 lead to G2/M arrest. Dephosphorylation of cdc2 activates it however the fall in phosphorylated cdc2 may represent the fall in total cdc2 in this scenario. Similar results were observed with HCT116. n=3

decrease in total cdc2 in response to DIM treatment. The decrease in cdc2 expression with DIM was increased by combination with AZD0530. DIM treatment also yielded a decrease in cyclin B1 expression (Figure 5.21) further promoting G2-M arrest.

At the G1-S cell cycle checkpoint the cyclin dependent kinase cdk6 associates with cyclin D1 and p27. The resultant complex phosphorylates the retinoblastoma (Rb) protein thus inhibiting its activity leading to release of E2F transcription factor and ultimately cell cycle progression. Treatment with DIM down regulated cdk6 after 48 hours treatment (Figure 5.22) and therefore potentially promoted G1-S cell cycle arrest. DIM also downregulated cyclin D1 in HT29 cells but the effect was equivocal in HCT116 with changes in expression observed in 1 of 3 blots only (Figure 5.23). Akt activity has been proposed as an upstream mediator of cdk6 and cyclin D downregulation in response to DIM in prostate cancer. DIM failed to induce changes in expression of either Akt or its phosphorylated form in the colorectal carcinoma cell
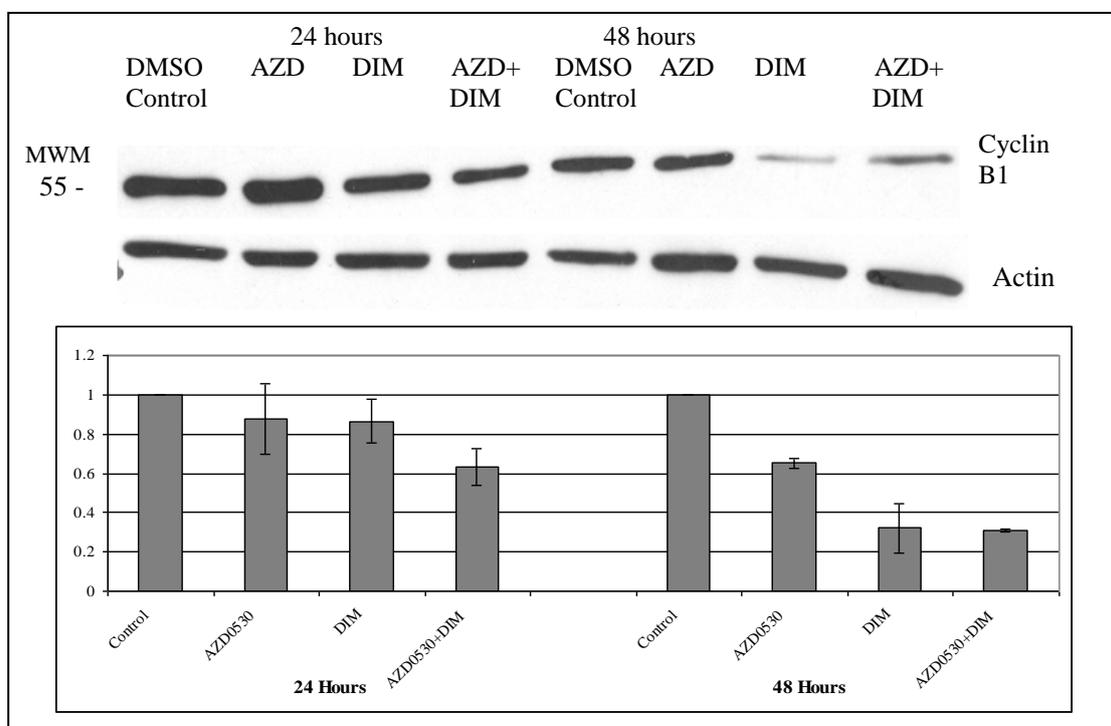
Figure 5.21: Cyclin B1 expression in HCT116 colorectal carcinoma cells. Decreased levels of cyclin B1 were observed on western blotting following treatment with DIM for 24 and 48 hours. Similar results were observed with HT29. n=3
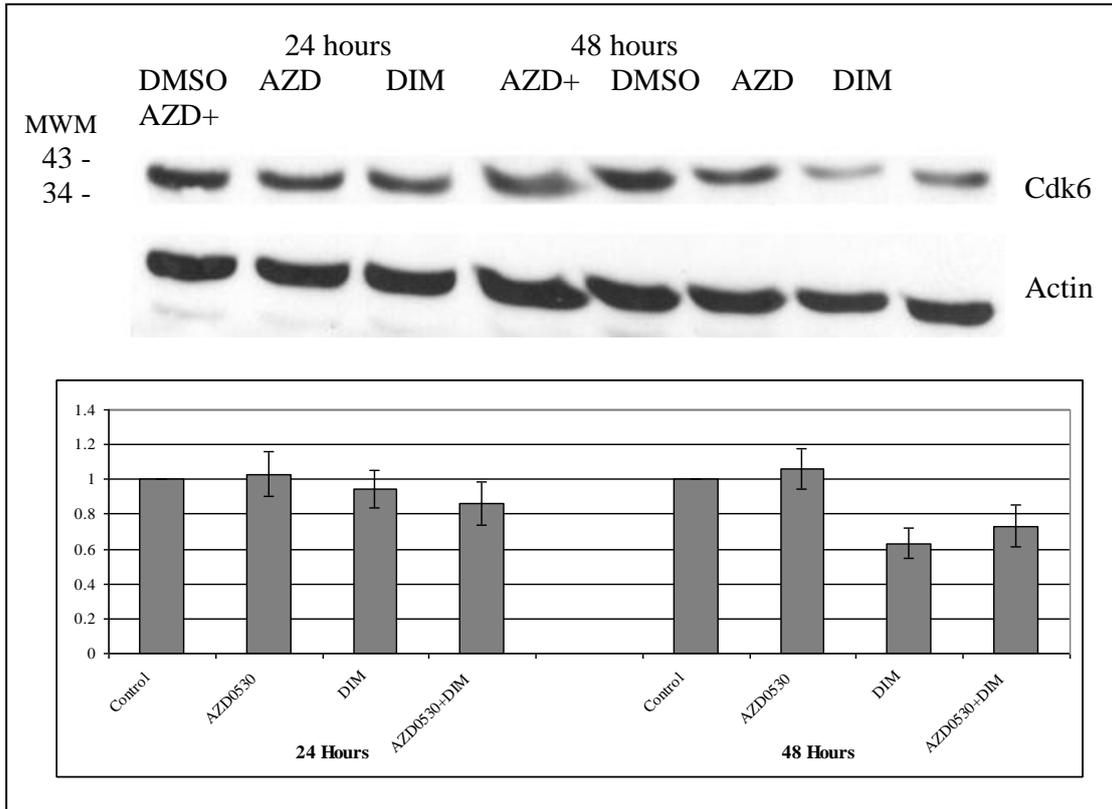
Figure 5.22: Cdk6 expression in HCT116 colorectal carcinoma cells.
Decreased levels of cdk6 were observed on western blotting following 48 hours treatment with DIM. Similar results were observed with HT29. n=3
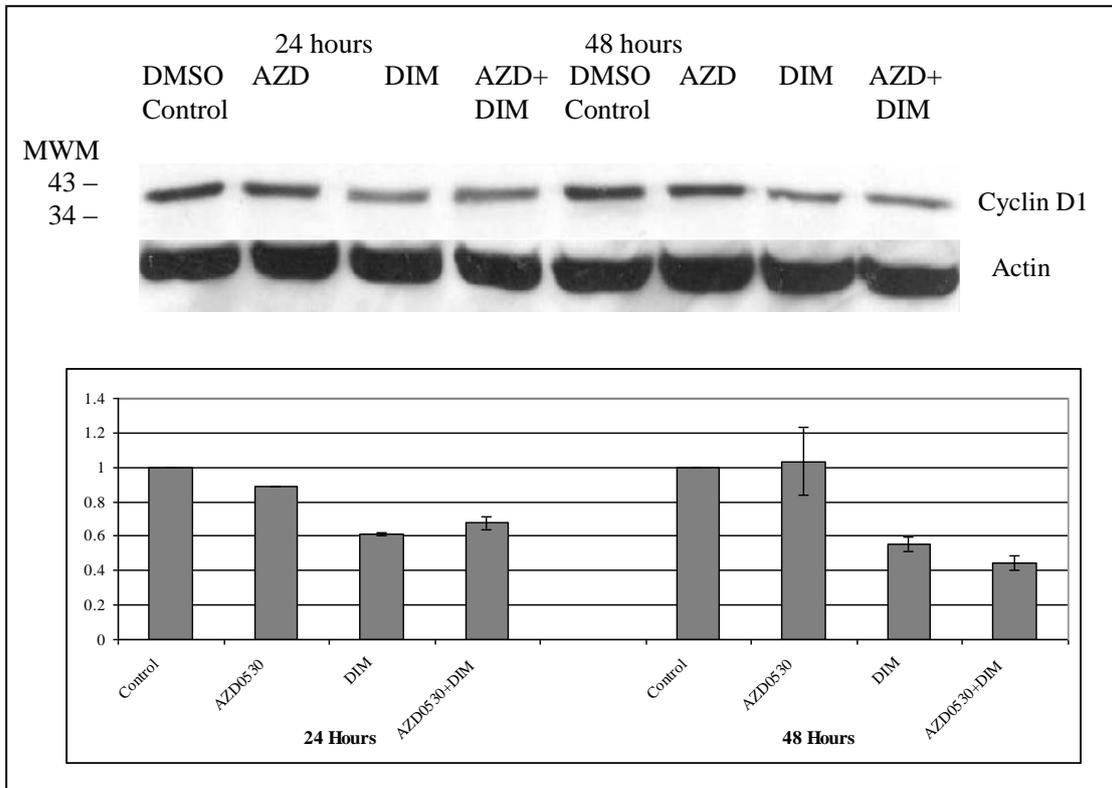
Figure 5.23: Cyclin D1 expression in HT29 colorectal carcinoma cells.
Decreased levels of cyclin D1 were observed on western blotting following 48 hours treatment with DIM. Similar results were observed with HCT116. n=3

lines (Data not shown).

Under favourable conditions for cell cycle progression Rb protein is further
phosphorylated by cyclin E promoting progress to S phase. DIM treatment yielded a
small decrease in cyclin E expression (Figure 5.24) thus further promoting cell cycle
arrest through Rb activation. The antibody against cyclin E exhibits three bands on
blotting (singlet 42kDa, doublet 50 kDa, hyperphosphorylated form 55kDa) (157) and
decreases were observed in all bands. These changes in expression at the G1-S
checkpoint are regulated upstream through the activity of p16 (inhibitory), p21
(inhibitory) and cdc25A (promoter). p16 was not reliably detected on blotting in this
instance. Cdc25A expression did not alter in response to DIM or AZD0530 (data not
shown). Expression of p21 was increased in response to DIM in HCT116 in one of
two blots though the expression in the other blot was high thus rendering
interpretation difficult (Figure 5.25). The data presented earlier indicating partial
dependence on p21 expression for G1-S arrest in HCT116 cells would support the
hypothesis that p21 expression is increased with resultant downstream promotion of
G1-S arrest.
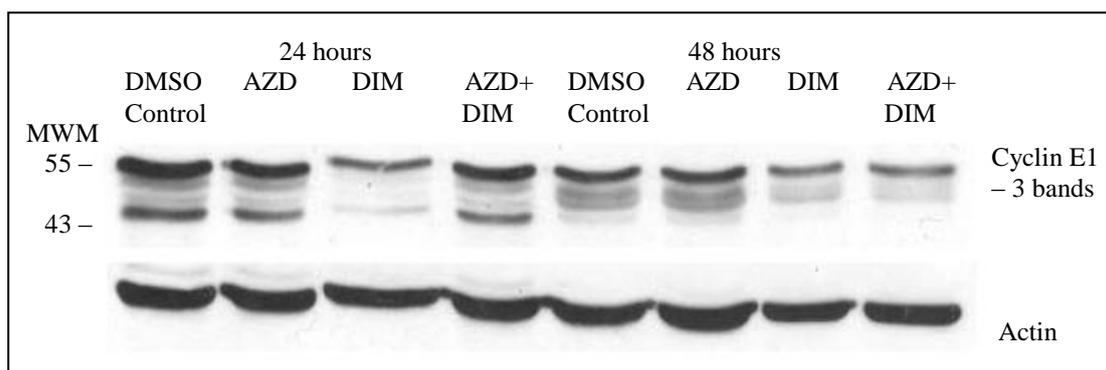
Figure 5.24: Cyclin E1 expression in HT29 colorectal carcinoma cells.
Decreased levels of cyclin E were observed on western blotting following treatment
for 24 and 48 hours with DIM. Similar results were observed with HCT116. The
cyclin E antibody yields three bands (singlet 42kDa, doublet 50 kDa,
hyperphosphorylated form 55kDa). n=3
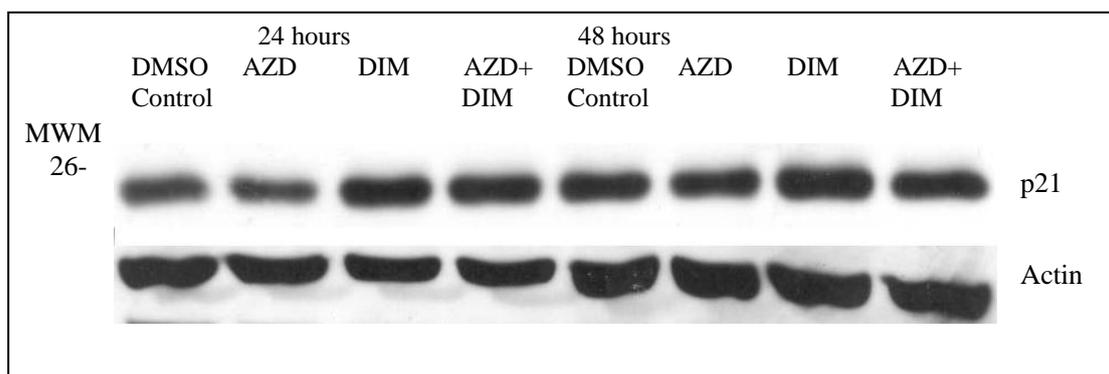
Figure 5.25: p21 expression in HCT116 colorectal carcinoma cells.
Increased levels of p21 were observed on western blotting following treatment with DIM, for 24 and 48 hours, in 1 of 2 blots. The remaining blot showed high expression throughout. The data presented in Figure 5.16 in combination with this data would support the involvement of, and therefore upregulation of, p21 in DIM induced GI arrest in HCT116. n=3.

5.4.2 DIM, AZD0530 and effects on apoptotic protein expression in colorectal carcinoma

Cell death is induced by a diverse range of proteins and molecular pathways. Indoles, including DIM, have been shown to affect a number of these in a range of malignant cell types (130,138-141).

DIM has been shown to inhibit activation and translocation of nuclear factor kappa B p65 (NfκB p65), an inhibitor of apoptosis in breast and pancreatic tumours (140,141). Treatment with DIM failed to demonstrate a similar inhibition in either of the colorectal carcinoma cell lines examined (data not shown).

Survivin, a member of the inhibitory apoptosis protein (IAP) family frequently overexpressed in tumour cell lines, inhibits caspase activation and therefore negatively regulates apoptosis (175,176). Treatment with DIM and AZD0530 led to downregulation of survivin expression both as sole agents and in combination (Figure
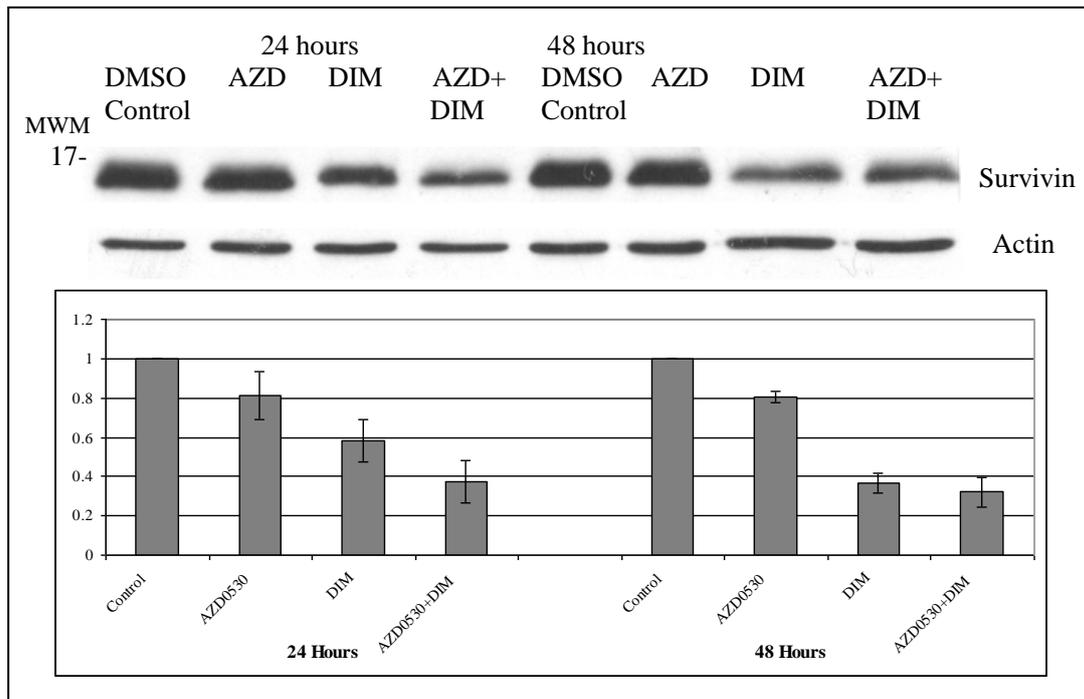
Figure 5.26: Survivin expression in HT29 colorectal carcinoma cells. Decreased survivin expression was observed on western blotting following with DIM alone or in combination with AZD0530 for 24 an 48 hours. Similar results were observed with HT29. n=3

5.26). The effect of combining the two agents was additive, on blot quantification, though not statistically significant.

DIM has been shown, in various cancer cell types, to downregulate antiapoptotic members of the Bcl-2 protein family, including Bcl-2 and Bcl-XL, and upregulate proapoptotic members, including Bax, Bid and Bim (130,139). In the colorectal carcinoma lines tested there was no demonstrable change in the expression of these proteins in response to treatment with DIM or AZD0530 (blots not shown). These data indicate that the previously reported cell death occurs independently of these pathways in colorectal carcinoma cells.

As reported in chapter 5.4.2 a decrease in PLK1 expression is induced by treatment with DIM and AZD0530, potentially promoting G2-M arrest via inhibition of nuclear

translocation of cdc25C. Decreases in PLK1 expression are also implicated in the induction of apoptosis (177,178)

CHK2 activation, as demonstrated in response to treatment with DIM in chapter 5.4.2. is also associated with p53 independent induction of apoptosis and cell death by senescence (179).

5.5   DIM and AZD0350 treatment in normal colorectal cells

In order for DIM and AZD0350 to be of clinical use as treatments for colorectal carcinoma either in combination or as sole agents it is necessary for there to be significantly greater therapeutic response to treatment in malignant cells than in adjacent, normal, healthy tissue. The drugs also need to be adequately tolerated by patients with an acceptable side effect profile.

BR-DIM is currently marketed as a dietary supplement as such has been since before 1994. It therefore has not been required to undergo testing for FDA approval. MHRA approval has also not been required. However, it has been demonstrated to be tolerated with minimal side effects in two small studies (150,151). AZD0350 has passed phase II preclinical trials and is in use in phase III trials. It has therefore undergone testing in vivo for toxicity.

To support the specific purposes of this project however the effect of these agents on SV40 transformed human colonic epithelial cells (HCEC) *in vitro* was examined, as a surrogate for normal epithelial cells (Figure 5.27). Sole treatment with AZD0350 or DIM resulted in a reduction in cell viability (ATPLite) of 0% and 12.2% versus

DMSO control at 24 hours and 0.9% and 15.3% at 48 hours respectively. Combination treatment with both agents had a greater effect on cell viability with decreases in cell viability versus DMSO control of 13.2% and 29.3% at 24 and 48 hours respectively.
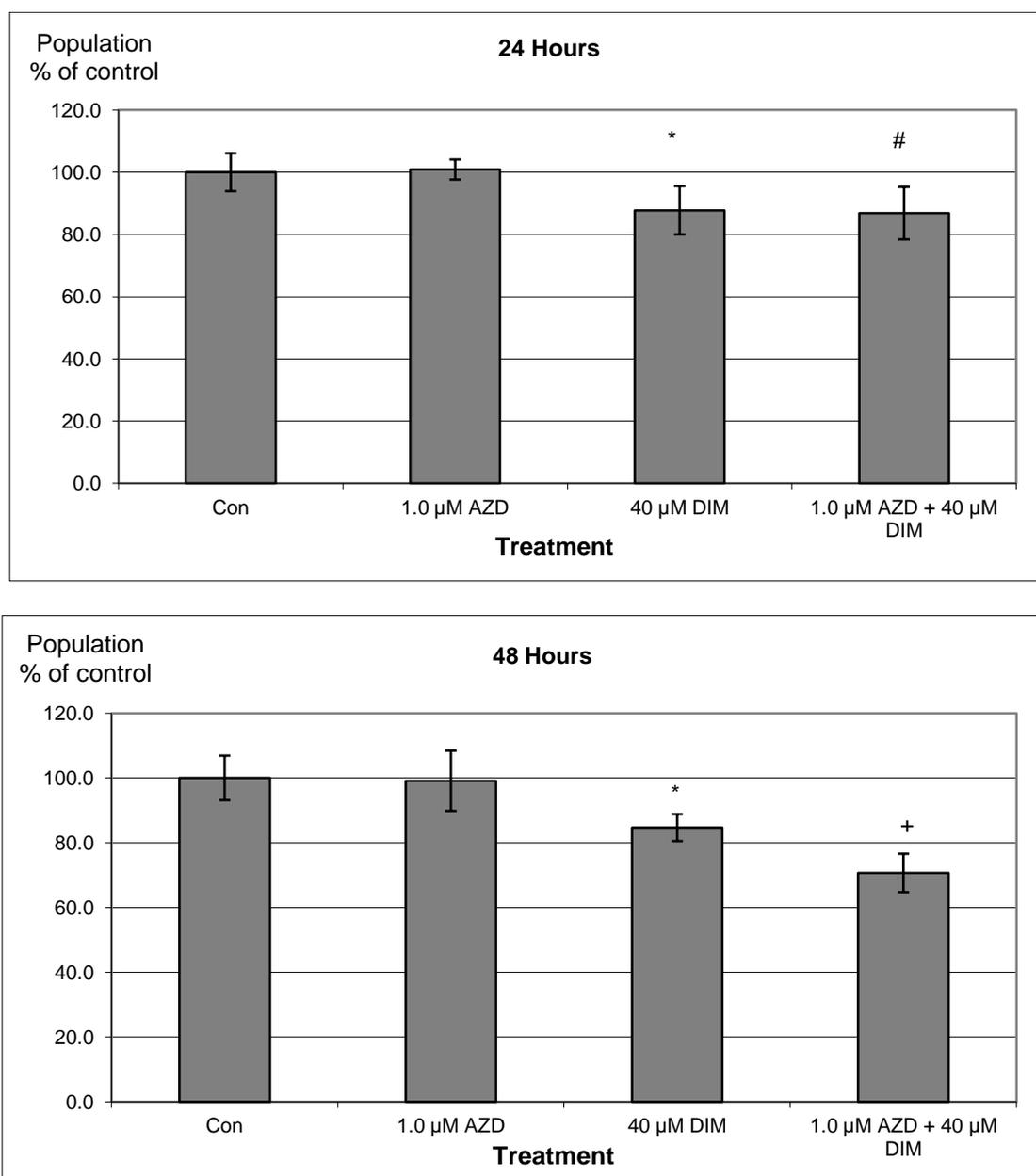
Figure 5.27: Cell viability (ATPLite) in HCEC cells.
Decreases in cell viability were observed following 48 hours with DIM alone and in combination with AZD0530 though these decreases were less than previously recorded in malignant colorectal cell lines indicating a degree of selectivity for malignant cells with the treatments. Results normalised to DMSO control. n=24 over 3 repetitions.
* = p<0.05 vs DMSO control
+ = p<0.05 vs DMSO control and AZD0530 alone
# = p<0.05 vs DMSO control, AZD0530 alone and DIM alone

The minimal change observed with treatment with AZD0350 did not achieve statistical significance. Treatment with DIM, as a sole agent, only demonstrated a relatively mild effect. Combination therapy exhibits a more significant effect. When compared to the effect of the same treatment on the panel of 4 immortalised colorectal carcinoma cell lines however, in which decreases of 59.4-66.9% at 48 hours were observed, there does appear to be a substantially greater toxicity in the malignant cell lines indicating a degree of selectivity, as required for therapeutic use.

5.6 Conclusion

In summary we demonstrated that DIM induced dose related loss of cell viability in colorectal carcinoma cells at doses of 25-75 µM (GI50 45 µM) that was progressive and sustained. These data contrast with findings in breast carcinoma in Tag mice which were sensitive only to doses of 75 µM (171).

Treatment with 40 µM DIM induced apoptosis in all four cell lines as demonstrated on FACS analysis and by caspase 3/7 activation, in line with findings in other carcinoma types (180-182). The data did not exhibit the striking rises in apoptosis that the decreases in cell viability would suggest although large late apoptosis/necrosis populations observed on FACS analysis may indicate DIM induction of necrosis. This needs further data for clarification.

Apoptosis in response to DIM independent of p53 status and was associated with decreased expression of PLK1 and survivin. The latter finding was in line with data

reported in prostate carcinoma cancer (183). Conversely expression of NFκB and the Bcl-2 family of apoptotic proteins were unchanged.

Treatment with 40 μM DIM induced G1 cell cycle arrest in 3 colorectal carcinoma cell lines and G2/M arrest in the remaining colorectal cell line (HT29). G1 arrest was partially mediated by p21 and to a lesser degree by p53. At the GI cell cycle checkpoint DIM treatment was associated with decreased expression of cdk6 and cyclins D1 and E. In contrast with findings in other cell lines Cdc25A and Akt statuses were unchanged (134,184,185). At the G2/M checkpoint DIM was associated with activatory phosphorylation of Chk2 (in HT29 only), inhibitory phosphorylation of cdc2 and decreased expression of cdc2, cdc25C, cyclin B1 and PLK1.

Treatment with DIM was associated with inhibitory changes in Src phosphorylation status. We also demonstrated that the effects of DIM on cell viability and number of the mechanistic effects could be augmented by combination treatment with the Src inhibitor AZD0530.

These effects on cell viability in response to DIM and AZD0530 showed selectivity for colonic malignant cells relative to normal colonic epithelial cells. These findings will be discussed in greater depth in chapter 6 however they clearly demonstrate promise for DIM as a chemopreventative and chemotherapeutic agent in colorectal carcinoma.

Chapter 6

Discussion

Colorectal carcinoma is the third most common cancer in the UK and is the second most common cause of cancer related death, accounting for 10% of cancer related mortality. Epidemiological studies have shown a link between a diet low in vegetables, especially cruciferous vegetables, and an increased risk of colorectal cancer (16,17). The indoles have been identified as a group of bioactive compounds that may be responsible for these effects.

The primary aim of this project was to establish, using immortalised cell lines, whether treatment with the dietary indole indole-3-carbinol (I3C) or its acid condensation product, 3,3 diindolymethane, would prove efficacious in the treatment of colorectal carcinoma and if any effects demonstrated could be potentiated through inhibition of EGFR or Src.

The second aim was to establish primary cultures from liver tissue of patients undergoing hepatic resections for colorectal metastases. Any cell lines successfully established in ongoing culture would then be utilised for experiments into cell viability in response to treatment regimes developed in the immortalised cell lines.

6.1   Establishing primary cultures from colorectal carcinoma tissue

As discussed in detail in chapter 3 attempts to establish primary cultures from resected colorectal hepatic metastases had a low yield and a number of potential obstacles were identified. Though data interpretation is limited by the number of resection specimens obtained, early indications were that manual dissociation is likely to have a greater success rate. Consistent with the literature, fibroblast overgrowth is likely to be an issue. The other potential issues identified were exposure of the

resected tissue to prolonged ischaemia and the slow cell growth observed.

Since the completion of this aspect of the project further literature on the subject has been published with concomitant refinements in the techniques for establishment of *in vitro* cultures. These papers also identified further obstacles to successful establishing cultures from resected tissue.

Oikonomou *et al.* (186) successfully established two cell lines in culture from primary colorectal tumours. However, consistent with our experience, they observed that after 24 hours incubation, the majority of cells were non-viable and where viable cultures were obtained, cellular turnover was such that cultures could be maintained for four weeks prior to passaging becoming necessary. Furthermore, as in the previously reviewed literature, fibroblast contamination was identified in the majority of samples. Percol gradient purification was utilised to obtain purified colorectal carcinoma cells with limited success. They also found that protein expression varied between the source tissue *in vivo* and subsequently established cultures *in vitro* thus consideration should be given to establishing comparative oncogene expression by immunohistochemistry and/or western blot analysis prior to interpreting experimental results obtained from any cultures successfully established.

Dalerba *et al.* (187) observed that primary cultures established from colorectal tumours often display a short life span *in vitro* characterised by progressive telomere shortening and irreversible cell cycle arrest after a defined and reproducible number of passages in a manner resembling cellular senescence in non-malignant differentiated tissue *in vivo*. They demonstrated that this characteristic, which differs from the source primary tumours *in vivo*, was due to loss of human telomerase reverse

transcriptase (hTERT) expression and re-constitution of expression via retroviral gene transfer reversed the effect with resultant cell line immortalisation. This technique was not attempted in this project and any further studies to establish primary cultures from colorectal hepatic metastatic tissue would potentially need to incorporate a technique to maintain hTERT expression to improve the yield.

Contrary to the prevailing trends, Broquet *et al.* (188) reported a high yield of 71% overall in establishing *in vitro* cultures from resected colorectal carcinoma tissue. It is of note given the potential issues, identified in chapter 3, with peri-operative ischaemia, that 30 specimens were obtained from resected liver metastases, although the success rate by anatomical location of the source tissue is not given in the paper. Fibroblast overgrowth was successfully inhibited in all cases by precoating culture plates with polyHEMA.

Other than Brouquet *et al.* (188) only one paper reported overall success rates in establishment of cultures which, with a rate of 23%, was consistent with the previously discussed literature (189). Of the four papers one utilised manual dissociation (186), two manual dissociation with subsequent enzymatic digestion (188,189) and one mechanical dissociation (187). These trends are consistent with previously published techniques and correlate with our experience that within the small numbers of our data set manual dissociation appeared to yield a greater number of viable cells in culture. Three groups used DMEM as the culture medium, 2 supplemented with 10% FCS (186,187) and one did not state inclusion of FCS (188), and one MEM supplemented with 10% FCS (189). Antibiotic and antimycotic solutions were used all in cases however Brouquet *et al.* (188) reported that their yield increased to 100% following the addition of gentamycin to the protocol.

Overall, with the exception of Brouquet *et al.* (188) the prevailing trend in the literature is that establishing primary cultures from resected colorectal carcinoma tissue remains challenging with a comparatively low yield. We identified a number of refinements to the technique we described in chapter 3 that may increase our yield and given the literature, indications are that focus should be on manual and enzymatic dissociation techniques. On the basis of the experience of Brouquet *et al.* (188) precoating culture flasks/plates with polyHEMA should be considered in the first instance as should immortalisation by activation of hTERT. The addition of gentamycin to the antibiotic/antimycotic solution used is not indicated at this stage as no bacterial infections were observed in our samples, suggesting the antimicrobial cover was adequate. These adaptions might address some of the issues identified in chapter 3 although, if this project is continued, consideration should be given to reviewing the ethical approval with respect to potentially obtaining tissue with little or no ischaemic exposure.

6.2 Indoles in treatment of colorectal carcinoma

As outlined in the introduction and chapter 5, Anderton *et al.* (147) demonstrated that in mice levels of DIM in the liver were 8-10 times plasma concentrations following oral administration of the increased bioavailability form of DIM (BR-DIM, Bioresponse, USA) and it was anticipated that concentrations of 40 µM would be achievable in the liver of humans. Reed *et al.* (150) subsequently demonstrated, after the commencement of this project, that a well tolerated oral dose of 200 µg BR-DIM yielded average peak plasma concentrations of up to 3.2 µM (104 ng/ml) thus concentrations in the liver may lie in the range of 25-41 µM which indicates that

average concentrations are likely to be slightly lower than the concentrations used for experiments throughout this thesis. However, given we have shown that DIM yields significant reductions in colorectal carcinoma cell viability down to concentrations of 10 μM, the achievable average concentration remains likely to lie well within the therapeutic range of DIM (see figure 5.1). There are no data for colonic tissue concentrations of DIM following oral administration. However, Anderton *et al.* showed concentrations of ≥3x plasma concentrations in all the tissues examined with the exception of brain tissue (148). Colonic tissue concentrations may follow a similar pattern although this is clearly supposition. Furthermore, given that DIM is relatively poorly absorbed, it would be anticipated that faecal concentrations, and therefore environmental conditions for colonic mucosa, are likely to be relatively high in comparison with plasma. These hypotheses could easily be tested by measuring faecal concentration of DIM post oral ingestion and colonic mucosa could be readily obtained endoscopically following administration.

Given the doses expected to be achievable *in* vivo of DIM and I3C (100-150 μM) (147,148,150), DIM showed far greater therapeutic effect on cell viability than I3C in colorectal carcinoma cells with significant responses to doses of 10-25 μM and 200 μM respectively. These data contrast strikingly with previously published data in breast carcinoma in which I3C and DIM induced apoptosis in primary Tag mouse mammary tumours at doses of 100 μM and 75 μM respectively (171). Treatment with DIM as a sole agent clearly yields a profound decrease in cell viability in colorectal carcinoma and this effect was shown to be sustained and progressive over time. DIM also demonstrated a significantly greater effect on, and therefore selectivity for, malignant colorectal tissue. This correlates with findings in other tumour types

including prostate and pancreatic carcinoma (190,191). This data in combination with the very low side effect profile of DIM (150,151) indicate potential for DIM as both a chemopreventative and a chemotherapeutic agent in colorectal carcinoma.

The effect of DIM on colorectal cancer cell viability is mediated through a combination of induction of cell cycle arrest and initiation of cell death.

There is evidence of induction of apoptosis by DIM both on Annexin V/propidium iodide FACS data and caspase 3/7 activation. However these assays did not indicate the dramatic increases one might anticipate given the changes in cell viability over similar time frames although the contribution from cell cycle arrest may account for some of the apparent shortfall. The FACS data revealed large populations in the late apoptosis/necrosis population raising the possibility that DIM may induce both apoptosis and necrosis. It is conceivable that DIM also induces cell death through promotion of cellular senescence as Chk2 activation was clearly demonstrated in this study and Chk2 activation has been reported to induce cell senescence(192). However, to date, DIM has not been reported to induce cell senescence in any cell line. DIM induction of apoptosis correlates with data in a range of other carcinoma types including thyroid, prostate, and pancreatic (180-182). Conversely DIM has not been reported to induce necrosis in isolation, although it has been reported in combination with taxotere (183). The mode of induced cell death in response to DIM treatment thus warrants further elucidation.

DIM induced G1 arrest in three of four colorectal carcinoma cell lines examined and G2-M arrest in the fourth (HT29). The latter finding has since been confirmed by Choi *et al* although they demonstrated both G1 and G2-M arrest occurring in HT29 in response to DIM (193). The reason for the discrepancy between the cell lines in terms of the points of cell cycle arrest is undefined, although possiblities are discussed in the text below. It is of note that the point of DIM induced cell cycle arrest occurring in other cancer lines is predominantly G1 (133-135,180) although Kandala and Srivastava demonstrated DIM induction of G2-M arrest in three ovarian carcinoma cell lines (194).

At the G1 checkpoint we demonstrated DIM yields decreased expression of the kinase cdk6 and its associated cyclin, cyclin D1. Under normal cell conditions these form a complex which in turn inhibits pRb protein, an inhibitor of cell cycle progression, by phosphorylation yielding downstream promotion of progression through G1. Thus, DIM induced downregulation should increase pRb activation resulting in G1 arrest. These findings correlate with published data in breast and prostate cancer (134,185). We also demonstrated that DIM decreased cyclin E expression, further promoting G1 phase arrest as cyclin E is critical for transition from G1 to S through its role, in a complex with cdk2 in inhibitory phosphorylation of p27. Any upstream effectors of these changes remain unclear. Cdc25A is the most striking candidate to mediate these and this has been shown to be the case in breast carcinoma (134,184) however as stated in section 5.4.1 we showed that cdc25A expression did not change in response to DIM in colorectal carcinoma. Garikapatay *et al.* (185) proposed downregulation of Akt and phosphorylated Akt as the upstream mediators of changes in cyclin D and cdk6 expression in prostate cancer. However we found that in colon carcinoma these

remained unchanged (data reported but not shown). Additionally p21 has been implicated in DIM induced G1 arrest (135,193,195). Western blotting was inconclusive as to the role of p21 in the cellular response to DIM in colorectal carcinoma cells as the results were equivocal between a rise in p21 expression and a background high expression. Comparison of the response in terms of cell cycle arrest between HCT116 p21 wild type and p21 -/- cell lines supports the hypothesis that p21 is integral to G1 arrest in this scenario as the HCT116 p21 -/- line demonstrated a significant decrease in the G1 population relative to wild type. Furthermore the HCT116 p21 -/- demonstrated a small increase in G2/M arrest relative to DMSO control though this did not achieve statistical significance. Putting these data together it is likely, but not conclusive, that increased p21 expression is involved in determining and mediating G1 arrest in colorectal carcinoma cells in response to DIM. In contrast HCT116 p53 -/- cells demonstrated a smaller decrease in G1 arrest in comparison with HCT116 p53 wild type cells, although significant G1 arrest still occurred, and did not show a significant increase in G2/M arrest relative to DMSO controls in response to treatment with DIM. These data indicate that p53 is also likely to partially mediate G1 arrest in response to DIM. However, as p21 knockout had a greater effect and p21 is downstream of p53 it may well be that p21 is activated by multiple pathways in this scenario and being closer to the final effectors in any, as yet to be fully defined, cascade has a more critical role.

The overall effects of DIM at the G2/M checkpoint are summarised in figure 6.1. At the G2-M checkpoint DIM activates Chk2 through phosphorylation at threonine 68 in HT29 colorectal carcinoma cells, although total cellular levels of Chk2 remained unchanged. Choi *et al* did not remark upon Chk2 status in their study of DIM induced
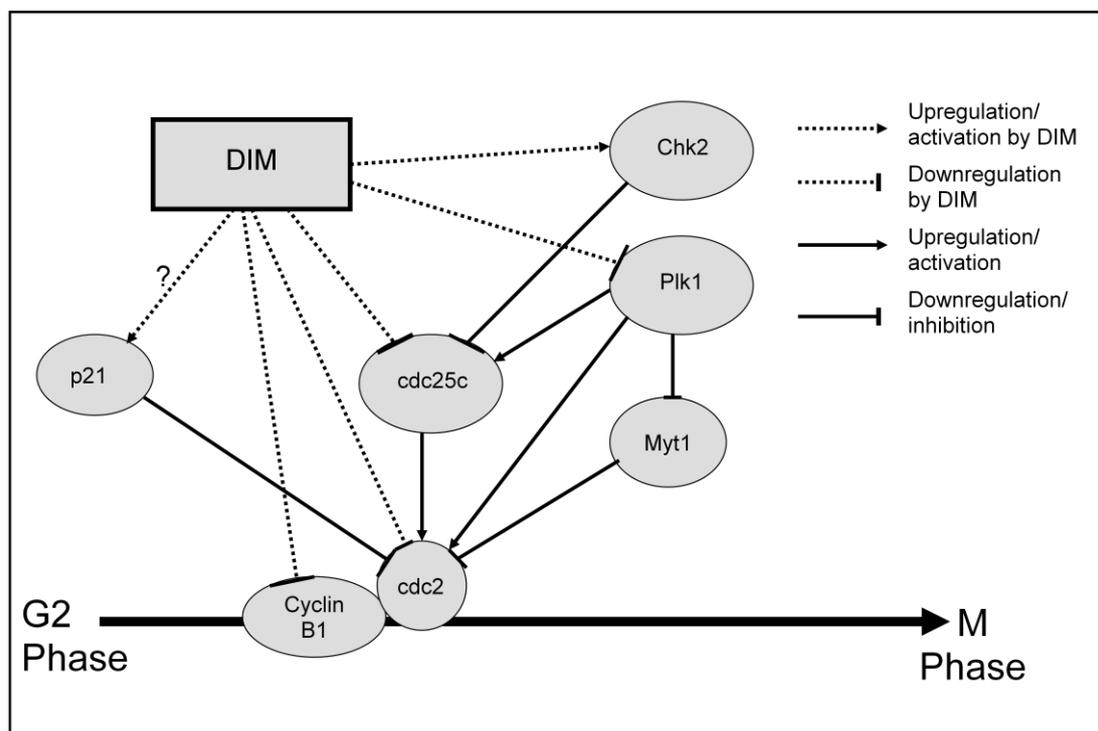
Figure 6.1: Simplified diagram of actions of DIM at the G2/M checkpoint in colorectal carcinoma. Dotted lines indicate effects of DIM, solid lines indicate effects of cell proteins. DIM promotes CHK2 activity via phosphorylation and inhibits expression of PLK1, cdc25c, cdc2 and cyclin B1. Data indicates DIM may upregulate p21 but this was not conclusive.

cell cycle arrest in HT29 (193)**.** Chk2 activation leads to inhibition of cdc25C phosphatase through phosphorylation at serine 216 (196). We demonstrated that DIM also induced a decrease in total cdc25C expression further potentiating the Chk2 mediated inhibition of cdc25C in response to DIM. The increased phosphorylation of Chk2 and decreased cdc25C expression in response to DIM correlates with the findings of Kandala and Srivastava in ovarian carcinoma cell lines undergoing DIM induced G2/M arrest, although they also demonstrated increased total Chk2 expression (194). It is of note that recently there has been extensive debate as to the relevance of Chk2 in initiation and propagation of G2/M arrest with some authors proposing that it is redundant and G2/M arrest is mediated via p53/p21 and/or Chk1 pathways (197-201), whereas Stracker *et al*. (202) found that Chk2 activity augmented the Chk1 mediated pathway and Rheinhart *et al.* (203) and Toetcher *et al.*

(204) showed that Chk2 activation initiated arrest independently of the other pathways. Furthermore Taylor and Stark ascertained that Chk2 activation not only directly promoted G2/M arrest, it also promoted p53 activation thus indirectly promoting mitotic arrest (205). Additionally Toettcher *et al* found that although p53 activation initiated G2/M arrest, Chk2 activation was required for sustained arrest leading to DNA repair or induction of cell death (204). Our data indicate that Chk2 activity is not redundant in G2/M arrest as we demonstrated Chk2 activation and G2/M arrest occurring without activation of p21 or Chk1 in HT29 cells. Conversely HCT116 cells which did not exhibit Chk2 activation also failed to undergo G2/M arrest. These data are not conclusive and would require further experiments with Chk2 knockout lines for validation.

In this study it was shown that DIM decreased expression of the mitotic kinase PLK1. This novel finding has a number of significant implications for cell cycle arrest and induction of apoptosis. PLK1 mediates the activity of a number of key proteins at the G2/M cell cycle checkpoint. PLK1 activates cdc25C and promotes its nuclear translocation (206). Thus decreases in PLK1 in response to DIM will further potentiate the decrease in cdc25C activity and expression described above. PLK1 also phosphorylates cyclin B1 resulting in nuclear translocation where it complexes with cdc2 (cdk1), a critical step in checkpoint progression (207). Plk1 also promotes cyclin B1/cdc2 complexing via activatory phosphorylation of cdc2 and downregulation of the cdc2 inhibitory kinase myt1 (208-211). Therefore DIM induced downregulation of PLK1 promotes G2/M arrest via alteration of a range of cell cycle proteins. PLK1 also has a role in cell cycle progression through G1 via inhibition of p53 (212) and therefore decreased PLK1 expression may contribute to DIM induced G1 arrest

observed in the HCT116, SW480 and SW620 cell lines although Liu and Erikson demonstrated that PLK1 depletion in HeLa cells induced G2/M arrest with subsequent apoptosis indicating G2 as the predominant phase of PLK1 activity (213).

PLK1 downregulation, as outlined previously, is also associated with induction of apoptosis although the downstream mechanism of this remains unclear (209,214-216). It has been proposed that this may be mediated via survivin although this is controversial. Zili *et al.* showed that PLK1 depletion induced G2-M arrest and apoptosis via downregulation of survivin activity. Conversely Colnaghi *et al.* found that activatory phosphorylation of survivin was critical for induction of cell cycle arrest. However TRAIL induced apoptosis occurred independently of PLK1 (217,218). Ando *et al.* (212) demonstrated that PLK1 inhibited the proapoptotic effects of p53 and Komatsu *et al* (214) showed that PLK1 regulated apoptosis in liver tumour cells via modulation of TAp63, a member of the p53 protein family, and PLK1 depletion yielded increased activity of downstream effectors of p53 including PUMA, p21 and 14-3-3.

PLK1 downregulation by DIM is of particular relevance in colorectal carcinoma as PLK1 is commonly overexpressed in colorectal cancers but not in normal colonic mucosa and overexpression is associated with a worse prognosis (219,220). DIM downregulation of PLK1 is of particular clinical interest as, whilst it has been demonstrated that silencing of PLK1 expression leads to cell death in malignant cells, non-malignant cells appear to be spared which may contribute to the greater impact of

DIM on colorectal cancer cell viability relative to normal colorectal epithelium (HCEC) cells shown in this thesis (215).

In addition to the effects mediated via PLK1 downregulation described above DIM further promotes G2-M arrest through decreased expression of cyclin B1 and cdc2. As outlined previously, complexing and subsequent nuclear translocation of cyclin B1 with cdc2 is the critical final stage cell progression through the G2-M checkpoint. These findings correlate in part with those of Choi *et al* in HT29 cells in that they also demonstrated decreased cyclin B1 expression. However they found that cdc2 levels were unchanged although activity was decreased (193). In support of our findings Kandala and Srivastava (194) and Jin (184) found similar changes in ovarian and breast carcinoma cells respectively.

We demonstrated that DIM treatment downregulates survivin expression in colorectal carcinoma cells. This is in line with data obtained in prostate cancer (183). This is potentially of particular significance from a therapeutic point of view and may account for a number of the observed effects of DIM. Survivin, a member of the inhibitory apoptosis protein family, is rarely expressed in differentiated adult tissues but is commonly overexpressed in malignant tissues and is believed to contribute significantly to cancer progression (221-223). It inhibits apoptosis, in response to diverse apoptotic stimuli, through formation of a complex with XIAP leading to promotion of XIAP stability and thus synergistic inhibition of caspase 9 and inactivation of the mitochondrial protein, SMAC (224,225). Additionally it has been proposed that survivin is likely to contribute to progression of malignant, and

therefore aberrant, cells through the G2-M checkpoint. This is on the basis that expression of survivin is increased tenfold during G2-M relative to either the preceding or subsequent phases (226) and inhibition of survivin led to delayed metaphase (227). Combining these factors DIM suppression of survivin is likely to contribute to both the induction of cellular apoptosis exhibited in all the four cell colorectal carcinoma cell lines and also to the G2-M arrest in HT29 cells.

Kawasaki *et al.* (228) and Sarela *et al.* (229) established that high levels of survivin are associated with a poor prognosis in colorectal tumours which in addition to the above mechanisims may be related to radioresistance. Rodel *et al.* (230) showed that survivin expression was related to resistance to radiotherapy. Furthermore survivin expression has been associated with resistance to chemotherapy agents, including Taxol and Paclitaxel, with a concordant sensitisation with survivin inhibition (231-233). DIM therefore is of particular clinical interest as it may have potential for treating a poor prognosis patient group both as a chemotherapeutic agent in itself and also in conjunction with other chemotherapy agents and radiotherapy as a chemosensitising and radiosensitising agent.

DIM induced survivin downreguation may also be relevant from a chemopreventative perspective as Kawasaki *et al.* (234) found that the likelihood of survivin overexpression increased in line with the degree of dysplasia present in colonic polyps, indicating a potential mechanism for DIM to suppress progression to invasive malignancy in premalignant colonic tissue.

A number of other proteins have been implicated in the pro-apoptotic effects of DIM in other cancer types. Amongst these, NFκB p65 (RelA) has been reported to mediate DIM induced apoptosis in breast and prostate cancer as well as decreased angiogenesis and invasion in the latter; (141,235,236), however in colon carcinoma we showed that NFκB p65 expression remained unchanged (data reported but not shown). Downregulation of the antiapoptotic protein Bcl-2 and complimentary upregulation of the proapoptotic protein Bax in response to DIM have been reported in breast cancer (130,139) however, in prostate cancer DIM induced apoptosis occurs independently of either of these mediators (237). Our data indicate that in colon carcinoma DIM induced apoptosis was independent of both Bcl-2 and Bax (data reported but not shown). These findings partially correlate with the findings of Ji *et al* who found that Bax expression was unchanged in HCT116 and HT29 and Bcl-2 expression was unchanged in HCT116, but decreased in HT29 following administration of DIM (238). Downregulation of Bcl-XL has been implicated in DIM induced apoptosis in murine models of prostate carcinoma (239) though our data did not replicate these changes in colon carcinoma. We also demonstrated that Bid and Bim levels did not change significantly in response to DIM. Cytochrome c release following DIM induced disruption of the mitochondrial membrane has been proposed as a mechanism by which DIM induces apoptosis independent of p53 and the Bcl-2 protein family (240) and Ji *et al* found an increase in cytosolic concentrations of cytochrome c consistent with this hypothesis. This potential mechanism provides an attractive explanation for the absence of changes in pro and antiapoptotic proteins reported here although as described previously the downstream effectors of PLK1 in apoptosis still require elucidation and may prove relevant to this scenario.

We presented early data indicating DIM is likely to decrease Src activity through inhibitory phosphorylation at tyrosine 530 and decreased activatory phosphorylation at tyrosine 419. The finding of decreased tyrosine 419 phosphorylation supports the data indicating increased tyrosine 530 phosphorylation as the latter causes the Src protein to adopt the inactive, closed conformation which in turn prevents autophosphorylation at tyrosine 419. Beyond the potential effects on cell proliferation downregulation of Src is of particular clinical relevance as in theory it will decrease the metastatic potential of a treated tumour through stabilisation of cellular adhesion and decreases in VEGF mediated endothelial permeability. Additionally Src downregulation is relevant to possible chemopreventative use of DIM as stage related increases in Src activity have been implicated in colorectal carcinogenesis (95,96).

We have also presented preliminary data indicating that DIM substantially decreases cellular adhesion in colorectal carcinoma. These promising data suggest that DIM has the potential to decrease formation of metastases *in vivo*. These correlate with the reported effect of administration of Brussels sprout juice in colorectal carcinoma (241) and tie in with findings of decreased metastases, with DIM treatment, from thyroid and breast tumours (143,242,243).

As mentioned previously in this section we demonstrated that although administration of DIM induced a small but statistically significant decrease in cell viability in HCEC cells, used as an immortalised surrogate for normal colonic mucosa, this effect was significantly less than the effect observed in the malignant cell lines at the same time point (decreases of 15% versus 34-41% of control for HCEC and the panel of four

153

colorectal carcinoma lines respectively). It is worth noting that HCEC cells have undergone immortalisation by SV40 transformation and this means that they display some characteristics more consistent with malignant cell lines than normal tissue in particular SV40 transformation has been shown to alter p53 and Rb expression and activity in cultured cells lines (244,245). The impact of this on our data is likely to be limited as we demonstrated that DIM induced apoptosis independent of p53 status and DIM induced cell cycle arrest was only partially mediated via p53. Furthermore these data correlate with findings in other tissue types (190,191,246). Thus, we conclude that DIM demonstrates a degree of selectivity for malignant tissue in its effect, supporting its potential as a therapeutic agent.

6.3 Indoles and kinase inhibitors

We clearly demonstrated that combining I3C with Src and EGFR inhibitors and DIM with the Src inhibitor AZD0530 can significantly potentiate their effects on cell viability *in vitro.* These effects occurred in line with oncogene overexpression on cell phenotyping and thus appropriate combinations are anticipatable *in vivo* on the basis of histological staining of resected tissue.

Since the completion of this project Astra Zeneca have withdrawn AZD0530 on the basis of the OVERT1 trial in patients with advanced ovarian malignancy (247). However the results achieved using indole-3-carbinol and Src inhibition with either AZD0530 or PP2 were similar. Furthermore, both the upregulation of inhibitory tyrosine phosphorylation of Src and the downregulation of activatory tyrosine phosphorylation by DIM are partial. Therefore, it is likely that combination with an

alternative Src inhibitor would also yield increased therapeutic effect. It would be interesting to combine DIM with other Src inhibitors to confirm or disprove this hypothesis.

6.4   Conclusions and scope for further research

In summary, the dietary indole, DIM is clearly established as a well tolerated chemopreventative agent that is commercially available as a dietary supplement. We have established that it has a high efficacy, *in vitro,* as a therapeutic agent for colorectal carcinoma both as sole agent and in combination with Src inihibition, with significant loss of cell viability in treated cells through increased cell death and induction of cell cycle arrest. Furthermore there are strong indications that DIM decreases cellular adhesion in colorectal carcinoma cells potentially decreasing the risk of formation of metastases. Given that DIM concentrates in the liver following oral administration this is potentially of particular relevance as colorectal tumours predominantly metastasise to the mesenteric lymph nodes and the liver. DIM exhibits selectivity for malignant cells in its effects which in combination with its low side effect profile makes it an attractive prospect for further development as a therapeutic agent both in the treatment of primary colorectal carcinoma and in hepatic metastases, given the aforementioned hepatic drug concentration.

In line with the findings of another group we found that DIM induced apoptosis in colorectal carcinoma appears to occur independently of the Bcl-2 protein family and also that it does not appear to be mediated via NfκB p65 nor Akt as is the case in other cell types.

We demonstrated DIM downregulates PLK1 and survivin as well as activating Chk2; changes which are likely to mediate the induced apoptosis and cell cycle arrest. Downregulation of PLK1 and survivin indicate that DIM may have a role in the treatment of patient group with a poor prognosis.

Although indicative data have been described in this thesis, the specific intracellular pathways of action of DIM underlying the effects on cell death, and to a lesser extent cell cycle arrest, in colorectal carcinoma still merit further elucidation. This may yet disclose additional prospective targets for combination therapy further potentiating the cytotoxic effects.

It would be interesting to progress with DIM to 3D and primary cultures and murine models in order to further elucidate any effects on invasion, migration and metastasis.

Overall, although any potential combinations with alternative Src inhibitors would need further preclinical assessment to confirm additive effects, these data clearly demonstrate promise for DIM as a chemopreventative and chemotherapeutic agent in colorectal carcinoma and are sufficient to support taking DIM forward as a treatment for colorectal carcinoma into murine studies.

Bibliography

(1) Cancer Research UK. UK Cancer mortality statistics. 2007; Available at: http://info.cancerresearchuk.org/cancerstats/mortality/?a=5441. Accessed January, 2007.

(2) Cancer Research UK. UK Cancer incidence statistics. 2007; Available at: http://info.cancerresearchuk.org/cancerstats/incidence/?a=5441. Accessed January, 2007.

(3) Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011 Mar 4;144(5):646--674.

(4) Doll R, Peto R. The causes of cancer: Quantitative estimates of avoidable risks of cancer in the United States today. J Natl Cancer Inst 1981;66(6):1191-1308.

(5) Davenport MP, Ward RL, Hawkins NJ. The null oncogene hypothesis and protection from cancer. J Med Genet 2002 JAN;39(1):12-15.

(6) Cancer Research UK. Cancer Stats Factsheet Large Bowel (Colorectal) Cancer. 2007; Available at: http://info.cancerresearchuk.org/images/publicationspdfs/factsheet_bowel_apr 2007.pdf. Accessed January, 2007.

(7) Takayama T, Miyanishi K, Hayashi T, Sato Y, Nirrsu Y. Colorectal cancer: genetics of development and metastasis. J Gastroenterol 2006 MAR 2006;41(3):185-192.

(8) Fearon ER, Vogelstein B. A Genetic Model for Colorectal Tumorigenesis. Cell 1990 JUN 1 1990;61(5):759-767.

(9) Jones S, Chen W, Parmigiani G, Diehl F, Beerenwinkel N, Antal T, et al. Comparative lesion sequencing provides insights into tumor evolution. Proc Natl Acad Sci U S A 2008 MAR 18 2008;105(11):4283-4288.

(10) Rustgi AK. The genetics of hereditary colon cancer. Genes Dev 2007 OCT 15 2007;21(20):2525-2538.

(11) Salovaara R, Loukola A, Kristo P, Kaariainen H, Ahtola H, Eskelinen M, et al. Population-based molecular detection of hereditary nonpolyposis colorectal cancer. Journal of Clinical Oncology 2000 JUN 2000;18(11):2193-2200.

(12) Lynch HT, Lynch PM, Lanspa SJ, Snyder CL, Lynch JF, Boland CR. Review of the Lynch syndrome: history, molecular genetics, screening, differential diagnosis, and medicolegal ramifications. Clin Genet 2009 JUL 2009;76(1):1-18.

(13) Poulogiannis G, Frayling IM, Arends MJ. DNA mismatch repair deficiency in sporadic colorectal cancer and Lynch syndrome. Histopathology 2010 JAN;56(2):167-179.

(14) Muzny DM, Bainbridge MN, Chang K, Dinh HH, Drummond JA, Fowler G, et al. Comprehensive molecular characterization of human colon and rectal cancer. Nature 2012 JUL 19;487(7407):330-337.

(15) Scottish Intercollegiate Guidelines Network. Diagnosis and management of colorectal cancer. A national clinical guideline. Available at: http://www.sign.ac.uk/pdf/sign126.pdf. Accessed 7th July, 2014.

(16) Freedman AN, Michalek AM, Marshall JR, Mettlin CJ, Petrelli NJ, Black JD, et al. Familial and nutritional risk factors for p53 overexpression in colorectal cancer. Cancer Epidemiology Biomarkers & Prevention 1996 APR 1996;5(4):285-291.

(17) Bray F, Sankila R, Ferlay J, Parkin DM. Estimates of cancer incidence and mortality in Europe in 1995. Eur J Cancer 2002;38(1):99-166.

(18) Haslam AC, Pearson JM, Bisset DL, Michie HR, Hobbiss JH. A Dukes/Jass combination - is it more discriminating? Colorectal Disease 2006 JUN 2006;8(5):418-422.

(19) Rae LC, Gibberd RW. Survival of patients with colorectal cancer detected by a community screening program. Med J Aust 2000 JAN 3 2000;172(1):13-15.

(20) Biasco G, Derenzini E, Grazi G, Ercolani G, Ravaioli M, Pantaleo MA, et al. Treatment of hepatic metastases from colorectal cancer: Many doubts, some certainties. Cancer Treat Rev 2006 MAY;32(3):214-228.

(21) Jatzko GR, Lisborg PH, Stettner HM, Klimpfinger MH. Hepatic Resection for Metastases from Colorectal-Carcinoma - a Survival Analysis. Eur J Cancer 1995;31A(1):41-46.

(22) Doll R, Peto R. The Causes of Cancer - Quantitative Estimates of Avoidable Risks of Cancer in the United-States Today. J Natl Cancer Inst 1981 1981;66(6):1191-&.

(23) Jimenez-Lara AM. Colorectal cancer: Potential therapeutic benefits of Vitamin D. Int J Biochem Cell Biol 2007;39(4):672-677.

(24) Dorai T, Aggarwal BB. Role of chemopreventive agents in cancer therapy. Cancer Lett 2004 NOV 25;215(2):129-140.

(25) Kolonel LN, Altshuler D, Henderson BE. The multiethnic cohort study: Exploring genes, lifestyle and cancer risk. Nature Reviews Cancer 2004 JUL;4(7):519-527.

(26) Hirohata T, Nomura AMY, Hankin JH, Kolonel LN, Lee J. An Epidemiologic-Study on the Association between Diet and Breast-Cancer. J Natl Cancer Inst 1987 APR;78(4):595-600.

(27) Freedman ND, Schatzkin A, Leitzmann MF, Hollenbeck AR, Abnet CC. Alcohol and head and neck cancer risk in a prospective study. Br J Cancer 2007 MAY 7;96(9):1469-1474.

(28) Hashibe M, Boffetta P, Janout V, Zaridze D, Shangina O, Mates D, et al. Esophageal cancer in Central and Eastern Europe: Tobacco and alcohol. International Journal of Cancer 2007 APR 1;120(7):1518-1522.

(29) Zhang SMM, Lee IM, Manson JE, Cook NR, Willett WC, Buring JE. Alcohol consumption and breast cancer risk in the Women's Health Study. Am J Epidemiol 2007 MAR 15;165(6):667-676.

(30) Manson MM. Cancer prevention - the potential for diet to modulate molecular signalling. Trends Mol Med 2003 JAN;9(1):11-18.

(31) Hayes JD, McMahon M. Molecular basis for the contribution of the antioxidant responsive element to cancer chemoprevention. Cancer Lett 2001 DEC 28;174(2):103-113.

(32) Surh YJ. Cancer chemoprevention with dietary phytochemicals. Nature Reviews Cancer 2003 OCT;3(10):768-780.

(33) Manson MM. General characteristics of chemopreventative agents. 2007 February.

(34) Wu X, Patterson S, Hawk E. Chemoprevention - History and general principles. Best practice & research.Clinical gastroenterology 2011 2011-Aug;25(4-5):445-59.

(35) Gescher A, Pastorino U, Plummer SM, Manson MM. Suppression of tumour development by substances derived from the diet - mechanisms and clinical implications. Br J Clin Pharmacol 1998 JAN;45(1):1-12.

(36) Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD editors. Molecular biology of the cell. 3rd ed.: Garland Publishing Inc.; 1994.

(37) Hames D, Hooper N. Instant Notes Biochemistry. 3rd ed.: Taylor and Francis Group; 2005.

(38) Ferrari S. Protein kinases controlling the onset of mitosis. Cellular and Molecular Life Sciences 2006 APR;63(7-8):781-795.

(39) Nyberg KA, Michelson RJ, Putnam CW, Weinert TA. Toward maintaining the genome: DNA damage and replication checkpoints. Annu Rev Genet 2002;36:617-656.

(40) Smits VAJ, Medema RH. Checking out the G2. Biochimica Et Biophysica Acta-Gene Structure and Expression 2001 MAY 28;1519(1-2):1-12.

(41) Walworth NC. Cell-cycle checkpoint kinases: checking in on the cell cycle. Curr Opin Cell Biol 2000 DEC;12(6):697-704.

(42) Zhou BBS, Elledge SJ. The DNA damage response: putting checkpoints in perspective. Nature 2000 NOV 23;408(6811):433-439.

(43) Vermeulen K, Van Bockstaele DR, Berneman ZN. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. Cell Prolif 2003 JUN;36(3):131-149.

(44) Ekholm SV, Reed SI. Regulation of G(1) cyclin dependent kinases in the mammalian cell cycle. Curr Opin Cell Biol 2000 DEC;12(6):676-684.

(45) Johnson DG, Walker CL. Cyclins and cell cycle checkpoints. Annu Rev Pharmacol Toxicol 1999;39:295-312.

(46) Loyer P, Trembley JH, Katona R, Kidd VJ, Lahti JM. Role of CDK. Cell Signal 2005 SEP;17(9):1033-1051.

(47) Satyanarayana A, Kaldis P. Mammalian cell-cycle regulation: several Cdks, numerous cyclins and diverse compensatory mechanisms. Oncogene 2009 AUG 20 2009;28(33):2925-2939.

(48) De Boer L, Oakes V, Beamish H, Giles N, Stevens F, Somodevilla-Torres M, et al. Cyclin A/cdk2 coordinates centrosomal and nuclear mitotic events. Oncogene 2008 JUL 17 2008;27(31):4261-4268.

(49) Meyerson M, Harlow E. Identification of G(1) Kinase-Activity for Cdk6, a Novel Cyclin-D Partner. Mol Cell Biol 1994 MAR 1994;14(3):2077-2086.

(50) Xu W, Ji J. Dysregulation of CDK8 and Cyclin C in tumorigenesis. Journal of Genetics and Genomics 2011 OCT 2011;38(10):439-452.

(51) Ren SJ, Rollins BJ. Cyclin C/Cdk3 promotes Rb-dependent G0 exit. Cell 2004 APR 16 2004;117(2):239-251.

(52) Loyer P, Busson A, Trembley JH, Hyle J, Grenet J, Zhao W, et al. The RNA Binding Motif Protein 15B (RBM15B/OTT3) Is a Functional Competitor of Serine-Arginine (SR) Proteins and Antagonizes the Positive Effect of the CDK11(p110)-Cyclin L2 alpha Complex on Splicing. J Biol Chem 2011 JAN 7 2011;286(1):147-159.

(53) Fu TJ, Peng JM, Lee G, Price DH, Flores O. Cyclin K functions as a CDK9 regulatory subunit and participates in RNA polymerase II transcription. J Biol Chem 1999 DEC 3 1999;274(49):34527-34530.

(54) Simone C, Bagella L, Bellan C, Giordano A. Physical interaction between pRb and cdk9/cyclinT2 complex. Oncogene 2002 JUN 13 2002;21(26):4158-4165.

(55) Caldon CE, Musgrove EA. Distinct and redundant functions of cyclin E1 and cyclin E2 in development and cancer. Cell Division 2010 JAN 17 2010;5:2.

(56) Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Molecular Biology of the cell. Fourth ed. New York: Garland Science; 2002.

(57) Peters J. The anaphase promoting complex/cyclosome: a machine designed to destroy. Nature Reviews Molecular Cell Biology 2006 SEP 2006;7(9):644-656.

(58) Taylor SS, Scott MIF, Holland AJ. The spindle checkpoint: a quality control mechanism which ensures accurate chromosome segregation. Chromosome Research 2004 2004;12(6):599-616.

(59) Hotchkiss RS, Strasser A, McDunn JE, Swanson PE. MECHANISMS OF DISEASE Cell Death. N Engl J Med 2009 OCT 15 2009;361(16):1570-1583.

(60) Vakifahmetoglu H, Olsson M, Zhivotovsky B. Death through a tragedy: mitotic catastrophe. Cell Death Differ 2008 JUL;15(7):1153-1162.

(61) Rufini A, Tucci P, Celardo I, Melino G. Senescence and aging: the critical roles of p53. Oncogene 2013 OCT 24;32(43):5129-5143.

(62) Vermeulen K, Berneman ZN, Van Bockstaele DR. Cell cycle and apoptosis. Cell Prolif 2003 JUN;36(3):165-175.

(63) Gewirtz DA. Autophagy and Senescence in Cancer Therapy. J Cell Physiol 2014 JAN;229(1):6-9.

(64) Salama R, Sadaie M, Hoare M, Narita M. Cellular senescence and its effector programs. Genes Dev 2014 JAN 15;28(2):99-114.

(65) Golstein P, Kroemer G. Cell death by necrosis: towards a molecular definition. Trends Biochem Sci 2007 JAN;32(1):37-43.

(66) Enders G editor. Cell cycle deregulation in cancer. New York: Springer; 2010.

(67) Surova O, Zhivotovsky B. Various modes of cell death induced by DNA damage. Oncogene 2013 AUG 15;32(33):3789-3797.

(68) Eriksson D, Stigbrand T. Radiation-induced cell death mechanisms. Tumor Biol 2010 AUG;31(4):363-372.

(69) Kerr JFR, Wyllie AH, Currie AR. Apoptosis - Basic Biological Phenomenon with Wide-Ranging Implications in Tissue Kinetics. Br J Cancer 1972;26(4):239-&.

(70) Turner P, McLennan A, Bates A, White M. Instant Notes Molecular Biology. 3rd ed.: Taylor and Francis Group; 2005.

(71) Nahle Z, Polakoff J, Davuluri RV, McCurrach ME, Jacobson MD, Narita M, et al. Direct coupling of the cell cycle and cell death machinery by E2F. Nat Cell Biol 2002 NOV;4(11):859-864.

(72) Jin ZY, El-Deiry WS. Overview of cell death signaling pathways. Cancer Biology & Therapy 2005 FEB;4(2):139-163.

(73) Damgaard RB, Gyrd-Hansen M. Inhibitor of apoptosis (IAP) proteins in regulation of inflammation and innate immunity. Discovery medicine 2011 2011-Mar;11(58):221-31.

(74) Mehan S, Meena H, Sharma D, Sankhla R. JNK: A Stress-Activated Protein Kinase Therapeutic Strategies and Involvement in Alzheimer's and Various Neurodegenerative Abnormalities. Journal of Molecular Neuroscience 2011 MAR 2011;43(3):376-390.

(75) Plotnikov A, Zehorai E, Procaccia S, Seger R. The MAPK cascades: Signaling components, nuclear roles and mechanisms of nuclear translocation. Biochimica Et Biophysica Acta-Molecular Cell Research 2011 SEP 2011;1813(9):1619-1633.

(76) Krajarng A, Nakamura Y, Suksamrarn S, Watanapokasin R. alpha-Mangostin Induces Apoptosis in Human Chondrosarcoma Cells through Downregulation of ERK/JNK and Akt Signaling Pathway. J Agric Food Chem 2011 MAY 25 2011;59(10):5746-5754.

(77) Salminen A, Ojala J, Kaarniranta K. Apoptosis and aging: increased resistance to apoptosis enhances the aging process. Cellular and Molecular Life Sciences 2011 MAR 2011;68(6):1021-1031.

(78) Butenko ZA. Apoptosis regulating oncogenes in the mechanisms of lymphoma- and carcinogenesis. Experimental Oncology 1995 SEP 1995;17(3):165-171.

(79) Feng W, Xiao J, Zhang Z, Rosen DG, Brown RE, Liu J, et al. Senescence and apoptosis in carcinogenesis of cervical squamous carcinoma. Modern Pathology 2007 SEP 2007;20(9):961-966.

(80) Wang XW. Role of p53 and apoptosis in carcinogenesis. Anticancer Res 1999 NOV-DEC 1999;19(6A):4759-4771.

(81) Ekshyyan O, Aw TY. Apoptosis: A key in Neurodegenerative disorders. Current Neurovascular Research 2004 OCT 2004;1(4):355-371.

(82) Michel PP, Lambeng N, Ruberg M. Neuropharmacologic aspects of apoptosis: Significance for neurodegenerative diseases. Clin Neuropharmacol 1999 MAY-JUN 1999;22(3):137-150.

(83) Vandenabeele P, Galluzzi L, Vanden Berghe T, Kroemer G. Molecular mechanisms of necroptosis: an ordered cellular explosion. Nature Reviews Molecular Cell Biology 2010 OCT 2010;11(10):700-714.

(84) Wheeler DL, Iida M, Dunn EF. The Role of Src in Solid Tumors. Oncologist 2009 2009;14(7):667-678.

(85) Said N, Theodorescu D. Pathways of metastasis suppression in bladder cancer. Cancer Metastasis Rev 2009 DEC 2009;28(3-4):327-333.

(86) Parsons SJ, Parsons JT. Src family kinases, key regulators of signal transduction. Oncogene 2004 OCT 18 2004;23(48):7906-7909.

(87) Summy JM, Gallick GE. Src family kinases in tumor progression and metastasis. Cancer Metastasis Rev 2003 DEC 2003;22(4):337-358.

(88) Talamonti MS, Roh MS, Curley SA, Gallick GE. Increase in Activity and Level of Pp60c-Src in Progressive Stages of Human Colorectal-Cancer. J Clin Invest 1993 JAN 1993;91(1):53-60.

(89) Sgroi DC. Breast Cancer Src Activity: Bad to the Bone. Cancer Cell 2009 JUL 7 2009;16(1):1-2.

(90) Myoui A, Nishimura R, Williams PJ, Hiraga T, Tamura D, Michigami T, et al. C-src tyrosine kinase activity is associated with tumor colonization in bone and lung in an animal model of human breast cancer metastasis. Cancer Res 2003 AUG 15 2003;63(16):5028-5033.

(91) Park SI, Zhang J, Phihips KA, Araujo JC, Najjar AM, Volgin AY, et al. Targeting src family kinases inhibits growth and lymph node metastases of prostate cancer in an orthotopic nude mouse model. Cancer Res 2008 MAY 1 2008;68(9):3323-3333.

(92) Irby RB, Mao W, Coppola D, Kang J, Loubeau JM, Trudeau W, et al. Activating SRC mutation in a subset of advanced human colon cancers. Nat Genet 1999;21(2):187-190.

(93) Chen J. Is Src the key to understanding metastasis and developing new treatments for colon cancer? Nature Clinical Practice Gastroenterology & Hepatology 2008 JUN 2008;5(6):306-307.

(94) Cartwright CA, Kamps MP, Meisler AI, Pipas JM, Eckhart W. Pp60c-Src Activation in Human-Colon Carcinoma. J Clin Invest 1989 JUN 1989;83(6):2025-2033.

(95) Cartwright CA, Meisler AI, Eckhart W. Activation of the Pp60c-Src Protein-Kinase is an Early Event in Colonic Carcinogenesis. Proc Natl Acad Sci U S A 1990 JAN 1990;87(2):558-562.

(96) Kline CLB, Jackson R, Engelman R, Pledger WJ, Yeatman TJ, Irby RB. Src kinase induces tumor formation in the c-SRC C57BL/6 mouse. International Journal of Cancer 2008 JUN 15 2008;122(12):2665-2673.

(97) Aligayer H, Boyd DD, Heiss MM, Abdalla EK, Curley SA, Gallick GE. Activation of Src kinase in primary colorectal carcinoma: an indicator of poor clinical prognosis. Cancer 2002 2002-Jan-15;94(2):344-51.

(98) Watanabe T, Tsuda M, Tanaka S, Ohba Y, Kawaguchi H, Majima T, et al. Adaptor Protein Crk Induces Src-Dependent Activation of p38 MAPK in Regulation of Synovial Sarcoma Cell Proliferation. Molecular Cancer Research 2009 SEP 2009;7(9):1582-1592.

(99) Ciuffini L, Castellani L, Salvati E, Galletti S, Falcone G, Alema S. Delineating v-Src downstream effector pathways in transformed myoblasts. Oncogene 2008 JAN 17 2008;27(4):528-539.

(100) Liu ZJ, Falola J, Zhu XD, Gu Y, Kim LT, Sarosi GA, et al. Antiproliferative effects of Src inhibition on medullary thyroid cancer. Journal of Clinical Endocrinology & Metabolism 2004 JUL 2004;89(7):3503-3509.

(101) Jallal H, Valentino M, Chen G, Boschelli F, Ali S, Rabbani SA. A Src/Abl kinase inhibitor, SKI-606, blocks breast cancer invasion, growth, and metastasis in vitro and in vivo. Cancer Res 2007 FEB 15 2007;67(4):1580-1588.

(102) Mukhopadhyay D, Tsiokas L, Zhou XM, Foster D, Brugge JS, Sukhatme VP. Hypoxic Induction of Human Vascular Endothelial Growth-Factor Expression through C-Src Activation. Nature 1995 JUN 15 1995;375(6532):577-581.

(103) Weis S, Cui JH, Barnes L, Cheresh D. Endothelial barrier disruption by VEGF-mediated Src activity potentiates tumor cell extravasation and metastasis. J Cell Biol 2004 OCT 25 2004;167(2):223-229.

(104) Kline CLB, Olson TL, Irby RB. Src activity alters alpha 3 integrin expression in colon tumor cells. Clin Exp Metastasis 2009 FEB 2009;26(2):77-87.

(105) Frame MC, Fincham VJ, Carragher NO, Wyke JA. V-SRC'S hold over actin and cell adhesions. Nature Reviews Molecular Cell Biology 2002 APR 2002;3(4):233-245.

(106) Brabek J, Constancio BS, Shin NY, Pozzi A, Weaver AM, Hanks SK. CAS promotes invasiveness of Src-transformed cells. Oncogene 2004 SEP 23 2004;23(44):7406-7415.

(107) Cortes-Reynosa P, Robledo T, Macias-Silva M, Wu SV, Salazar EP. Src kinase regulates metalloproteinase-9 secretion induced by type IV collagen in MCF-7 human breast cancer cells. Matrix Biology 2008 APR 2008;27(3):220-231.

(108) Kuo LY, Chang HC, Leu TH, Maa MC, Hung WC. Src oncogene activates MMP-2 expression via the ERK/Sp1 pathway. J Cell Physiol 2006 JUN 2006;207(3):729-734.

(109) Avizienyte E, Wyke AW, Jones RJ, McLean GW, Westhoff MA, Brunton VG, et al. Src-induced de-regulation of E-cadherin in colon cancer cells requires integrin signalling. Nat Cell Biol 2002 AUG;4(8):632-638.

(110) Griffiths GJ, Koh MY, Brunton VG, Cawthorne C, Reeves NA, Greaves M, et al. Expression of kinase-defective mutants of c-Src in human metastatic colon cancer cells decreases Bcl-x(L) and increases oxaliplatin- and Fas-induced apoptosis. J Biol Chem 2004 OCT 29 2004;279(44):46113-46121.

(111) Wheeler DL, Iida M, Kruser TJ, Nechrebecki MM, Dunn EF, Armstrong EA, et al. Epidermal growth factor receptor cooperates with Src family kinases in acquired resistance to cetuximab. Cancer Biology & Therapy 2009 APR 15 2009;8(8):696-703.

(112) Kopetz S. Targeting SRC and epidermal growth factor receptor in colorectal cancer: rationale and progress into the clinic. Gastrointestinal cancer research : GCR 2007 2007;1(4 Suppl 2):S37-41.

(113) Mayer EL, Krop IE. Advances in Targeting Src in the Treatment of Breast Cancer and Other Solid Malignancies. Clinical Cancer Research 2010 JUL 15 2010;16(14):3526-3532.

(114) Thomson AM, Sherratt PJ, Neal GE, Manson MM, James RFL, Hayes JD. Regulation of class alpha, mu, pi, sigma and theta glutathione S-transferases in rat liver by cancer chemopreventive drugs and model enzyme inducing agents. Clinical Chemistry & Enzymology Communications 1999;8(4-6):315-333.

(115) Thomasset SC, Berry DP, Garcea G, Marczylo T, Steward WP, Gescher AJ. Dietary polyphenolic phytochemicals - Promising cancer chemopreventive agents in humans? A review of their clinical properties. International Journal of Cancer 2007 FEB 1;120(3):451-458.

(116) Kim YS, Milner JA. Targets for indole-3-carbinol in cancer prevention. J Nutr Biochem 2005 FEB;16(2):65-73.

(117) Guo D, Schut AJ, Davis CD, Snyderwine EG, Bailey GS, Dashwood RH. Protection by chlorophyllin and indole-3-carbinol against 2-amino-1-methyl-6-phenylimidazo4,5-bpyridine (PhIP)-induced DNA adducts and colonic aberrant crypts in the F344 rat. Carcinogenesis 1995;16(12):2931-2937.

(118) Plate AYA, Gallaher DD. Effects of indole-3-carbinol and phenethyl isothiocyanate on colon carcinogenesis induced by azoxymethane in rats. Carcinogenesis 2006 01 FEB 2006;27(2):287-292.

(119) Xu H, Yu Y, Marciniak D, Rishi AK, Sarkar FH, Kucuk O, et al. Epidermal growth factor receptor (EGFR)-related protein inhibits multiple members of the EGFR family in colon and breast cancer cells. Molecular Cancer Therapeutics 2005;4(3):435-442.

(120) Xu M, Orner GA, Bailey GS, Stoner GD, Horio DT, Dashwood RH. Post-initiation effects of chlorophyllin and indole-3-carbinol in rats given 1,2-

dimethylhydrazine or 2-amino-3-methyl-imidazo4,5-fquinoline. Carcinogenesis 2001;22(2):309-314.

(121) Xu M, Orner GA, Bailey GS, Stoner GD, Horio DT, Dashwood RH. Erratum: Post-initiation effects of chlorophyllin and indole-3-carbinol in rats given 1,2-dimethylhydrazine or 2-amino-3-methyl-imidazo4,5-f quinoline (Carcinogenesis (2001) vol. 22 (309-314)). Carcinogenesis 2001;22(4):685.

(122) Grose KR, Bjeldanes LF. Oligomerization of Indole-3-Carbinol in Aqueous Acid. Chem Res Toxicol 1992 MAR-APR 1992;5(2):188-193.

(123) Bradlow HL, Zeligs MA. Diindolylmethane (DIM) Spontaneously Forms from Indole-3-carbinol (I3C) During Cell Culture Experiments. In Vivo 2010 JUL-AUG 2010;24(4):387-391.

(124) He YH, Friesen MD, Ruch RJ, Schut HAJ. Indole-3-carbinol as a chemopreventive agent in 2-amino-1-methyl-6-phenylimidazo[4, 5-b]pyridine (PhIP) carcinogenesis: Inhibition of PhIP-DNA adduct formation, acceleration of PhIP metabolism, and induction of cytochrome P450 in female F344 rats. Food and Chemical Toxicology 2000 JAN;38(1):15-23.

(125) Kishida T, Beppu M, Nashiki K, Izumi T, Ebihara K. Effect of dietary soy isoflavone aglycones on the urinary 16a-to-2-hydroxyestrone ratio in C3H/HeJ mice. Nutrition & Cancer 2000;38(2):209-214.

(126) Lee S-, Kim J-, Yamaguchi K, Eling TE, Baek SJ. Indole-3-carbinol and 3,3'-diindolylmethane induce expression of NAG-1 in a p53-independent manner. Biochemical & Biophysical Research Communications 2005 04 MAR 2005;328(1):63-69.

(127) Renwick AB, Mistry H, Barton PT, Mallet F, Price RJ, Beamand JA, et al. Effect of some indole derivatives on xenobiotic metabolism and xenobiotic-

induced toxicity in cultured rat liver slices. Food and Chemical Toxicology 1999 JUN 1999;37(6):609-618.

(128) Sanderson JT, Slobbe L, Lansbergen GWA, Safe S, van den Berg M. 2,3,7,8-tetrachlorodibenzo-p-dioxin and diindolylmethanes differentially induce cytochrome P450 1A1, 1B1, and 19 in H295R human adrenocortical carcinoma cells. Toxicological Sciences 2001 MAY 2001;61(1):40-48.

(129) Moiseeva EP, Heukers R, Manson MM. EGFR and Src are involved in indole-3-carbinol-induced death and cell cycle arrest of human breast cancer cells. Carcinogenesis 2007 FEB;28(2):435-445.

(130) Hong C, Firestone GL, Bjeldanes LF. Bcl-2 family-mediated apoptotic effects of 3,3 '-diindolylmethane (DIM) in human breast cancer cells. Biochem Pharmacol 2002 MAR 15;63(6):1085-1097.

(131) Cover CM, Hsieh SJ, Tran SH, Hallden G, Kim GS, Bjeldanes LF, et al. Indole-3-carbinol inhibits the expression of cyclin-dependent kinase-6 and induces a G1 cell cycle arrest of human breast cancer cells independent of estrogen receptor signaling. J Biol Chem 1998 13 FEB 1998;273(7):3838-3847.

(132) Hudson EA, Howells LM, Gallacher-Horley B, Fox LH, Gescher A, Manson MM. Growth inhibitory effects of the chemopreventive agent indole-3-carbinol are increased in combination with the polyamine putrescine in the SW480 colon tumour cell line. BMC Cancer 2003 3:ate of Pubaton: 14 JAN 2003.

(133) Vivar OI, Lin C, Firestone GL, Bjeldanes LF. 3,3 '-Diindolylmethane induces a G(1) arrest in human prostate cancer cells irrespective of androgen receptor and p53 status. Biochem Pharmacol 2009 SEP 1;78(5):469-476.

(134) Chang XF, Tou JC, Hong CB, Kim HA, Riby JE, Firestone GL, et al. 3,3'-Diindolylmethane inhibits angiogenesis and the growth of transplantable human breast carcinoma in athymic mice. Carcinogenesis 2005 APR 2005;26(4):771-778.

(135) Hong CB, Kim HA, Firestone GL, Bjeldanes LF. 3,3 '-Diindolylmethane (DIM) induces a G(1) cell cycle arrest in human breast cancer cells that is accompanied by Sp1-mediated activation of p21(WAF1/CIP1) expression. Carcinogenesis 2002 AUG 2002;23(8):1297-1305.

(136) Cram EJ, Liu BD, Bjeldanes LF, Firestone GL. Indole-3-carbinol inhibits CDK6 expression in human MCF-7 breast cancer cells by disrupting Sp1 transcription factor interactions with a composite element in the CDK6 gene promoter. J Biol Chem 2001 JUN 22;276(25):22332-22340.

(137) Firestone GL, Bjeldanes LF. Indole-3-carbinol and 3-3'-diindolylmethane antiproliferative signaling pathways control cell-cycle gene transcription in human breast cancer cells by regulating promoter-Sp1 transcription factor interactions. J Nutr 2003 JUL;133(7):2448S-2455S.

(138) Chinni SR, Li YW, Upadhyay S, Koppolu PK, Sarkar FH. Indole3-carbinol (I3C) induced cell growth inhibition, G1 cell cycle arrest and apoptosis in prostate cancer cells. Oncogene 2001 MAY 24;20(23):2927-2936.

(139) Rahman KMW, Li YW, Wang ZW, Sarkar SH, Sarkar FH. Gene expression profiling revealed survivin as a target of 3,3 '-diindolylmethane-induced cell growth inhibition and apoptosis in breast cancer cells. Cancer Res 2006 MAY 1 2006;66(9):4952-4960.

(140) Takada Y, Andreeff M, Aggarwal BB. Indole-3-carbinol suppresses NF-kappa B and I kappa B alpha kinase activation, causing inhibition of expression of NF-kappa B-regulated antiapoptotic and metastatic gene products and

enhancement of apoptosis in myeloid and leukemia cells. Blood 2005 JUL 15;106(2):641-649.

(141) Rahman KMW, Ali S, Aboukameel A, Sarkar SH, Wang Z, Philip AP, et al. Inactivation of NF-kappa B by 3,3 '-diindolylmethane contributes to increased apoptosis induced by chemotherapeutic agent in breast cancer cells. Molecular Cancer Therapeutics 2007 OCT 2007;6(10):2757-2765.

(142) Moiseeva EP, Heukers R, Manson MM. EGFR and Src are involved in indole-3-carbinol-induced death and cell cycle arrest of human breast cancer cells. Carcinogenesis 2007 FEB;28(2):435-445.

(143) Rahimi M, Luang K, Tang CK. 3,3′-Diindolylmethane (DIM) Inhibits the Growth and Invasion of Drug-Resistant Human Cancer Cells Expressing EGFR Mutants. Cancer Letters, Cancer Lett 2010 September 1;295(1):59-68.

(144) Ali S, Banerjee S, Ahmad A, El-Rayes BF, Philip PA, Sarkar FH. Apoptosis-inducing effect of erlotinib is potentiated by 3,3 '-diindolylmethane in vitro and in vivo using an orthotopic model of pancreatic cancer. Molecular Cancer Therapeutics 2008 JUN 2008;7(6):1708-1719.

(145) Moiseeva EP, Howells LM, Fox LH, Hudson EA, Manson MM. Transient activation of Src and EGFR may contribute to indole-3 carbinol-induced cell death in MDA-MB-468 breast cells. Cancer Epidemiology Biomarkers & Prevention 2004 NOV;13(11):1881S-1881S.

(146) Moiseeva EP, Fox LH, Howells LM, Temple LAF, Manson MM. Indole-3-carbinol-induced death in cancer cells involves EGFR downregulation and is exacerbated in a 3D environment. Apoptosis 2006;11(5):799-812.

(147) Anderton MJ, Manson MM, Verschoyle RD, Gescher A, Lamb JH, Farmer PB, et al. Pharmacokinetics and tissue disposition of indole-3-carbinol and its

acid condensation products after oral administration to mice. Clinical Cancer Research 2004 01 AUG 2004;10(15):5233-5241.

(148) Anderton MJ, Manson MM, Verschoyle R, Gescher A, Steward WP, Williams ML, et al. Physiological modeling of formulated and crystalline 3,3' - diindolylmethane pharmacokinetics following oral administration in mice. Drug Metabolism & Disposition 2004;32(6):632-638.

(149) Reed GA, Arneson DW, Putnam WC, Smith HJ, Gray JC, Sullivan DK, et al. Single-dose and multiple-dose administration of indole-3-carbinol to women: Pharmacokinetics based on 3,3 '-diindolylmethane. Cancer Epidemiology Biomarkers & Prevention 2006 DEC;15(12):2477-2481.

(150) Reed GA, Sunega JM, Sullivan DK, Gray JC, Mayo MS, Crowell JA, et al. Single-Dose Pharmacokinetics and Tolerability of Absorption-Enhanced 3,3-Diindolylmethane in Healthy Subjects. Cancer Epidemiol Biomarkers Prev 2008;17(10):2619-2624.

(151) Dalessandri KM, Firestone GL, Fitch MD, Bradlow HL, Bjeldanes LF. Pilot Study: Effect of 3,3′-Diindolylmethane Supplements on Urinary Hormone Metabolites in Postmenopausal Women With a History of Early-Stage Breast Cancer. Nutr Cancer 2004;50(2):161-167.

(152) US National Institute of Health. ClinicalTrials.Gov. Available at: http://clinicaltrials.gov/ct2/results?term=diindolylmethane&recr=&rslt=&type =&cond=&intr=&outc=&lead=&spons=&id=&state1=&cntry1=&state2=&cnt ry2=&state3=&cntry3=&locn=&gndr=&rcv_s=&rcv_e=&lup_s=&lup_e=. Accessed January 5th, 2012.

(153) Kim YH, Kwon H, Kim DH, Shin EK, Kong Y, Park JHY, et al. 3,3 '-Diindolylmethane Attenuates Colonic Inflammation and Tumorigenesis in Mice. Inflamm Bowel Dis 2009 AUG 2009;15(8):1164-1173.

(154) Li Y, Li X, Guo B. Chemopreventive Agent 3,3 '-Diindolylmethane Selectively Induces Proteasomal Degradation of Class I Histone Deacetylases. Cancer Res 2010 JAN 15 2010;70(2):646-654.

(155) Bhatnagar N, Li X, Chen Y, Zhou X, Garrett SH, Guo B. 3,3 '-Diindolylmethane Enhances the Efficacy of Butyrate in Colon Cancer Prevention through Down-Regulation of Survivin. Cancer Prevention Research 2009 JUN 2009;2(6):581-589.

(156) Frame MC. Src in cancer: Deregulation and consequences for cell behaviour. Biochimica et Biophysica Acta - Reviews on Cancer 2002 21 JUN 2002;1602(2):114-130.

(157) Santa Cruz Biotechnology. CyclinE (HE12): Antibody sc-247 datasheet. 2011; Available at: http://datasheets.scbt.com/sc-247.pdf. Accessed 28th December, 2011.

(158) Dangles-Marie V, Pocard M, Richon S, Weiswald LB, Assayag F, Saulnier P, et al. Establishment of human colon cancer cell lines from fresh tumors versus xenografts: Comparison of success rate and cell line features. Cancer Res 2007;67(1):398-407.

(159) Farrell TM, Pettengill OS, Longnecker DS, Cate CC, Cohn KH. Growing colorectal tumors: minimizing microbial and stromal competition and assessing in vitro selection pressures. Cytotechnology 2000;34(3):205-211.

(160) Flatmark K, Maelandsmo GM, Martinsen M, Rasmussen H, Fodstad O. Twelve colorectal cancer cell lines exhibit highly variable growth and metastatic capacities in an orthotopic model in nude mice. Eur J Cancer 2004;40:1593-1598.

(161) Gazdar AF, Oie HK. Growth of Cell-Lines and Clinical Specimens of Human Non-Small Cell Lung-Cancer in a Serum-Free Defined Medium. Cancer Res 1986;46(11):6011-6011.

(162) Oh J, Ku J, Yoon K, Kwon H, Kim W, Park H, et al. Establishment and characterization of 12 human colorectal-carcinoma lines. Int J Cancer 1999;81:902-910.

(163) Park JG, Oie HK, Sugarbaker PH, Henslee JG, Chen TR, Johnson BE, et al. Characteristics of Cell-Lines Established from Human Colorectal-Carcinoma. Cancer Res 1987;47(24):6710-6718.

(164) Arnold LF, Baram P. In Vitro Culture of Periodontal Ligament Cells. J Dent Res 1972;51:953-959.

(165) Brattain MG, Marks ME, McCombs J, Finely W, Brattain DE. Characterization of human colon carcinoma cell lines isolated from a single primary tumour. Br J Cancer 1983;47:373-381.

(166) Gomm JJ, Coope RC, Browne PJ, Coombes RC. Separated human breast epithelial and myoepithelial cells have different growth factor requirements in vitro but can reconstitute normal breast lobuloalveolar structure. J Cell Physiol 1997;171:11-19.

(167) McBain JA, Weese JL, Meisner LF, Wolberg WH, Willson JKV. Establishment and characterization of human colorectal cancer cell lines. Cancer Res 1984;44:5813-5821.

(168) Astra-Zeneca U. Guidance for use of drug AZD0530. 2007.

(169) Logie A, Martin PD, Partridge EA, Byatt SL, Whittaker RD, Green TP. Pharmacokinetics, tissue distribution and anti-tumor activity of the Src/Abl kinase inhibitor AZD0530 in a rat xenograft model.[Abstract]. Proc Amer Assoc Cancer Res 2005 April 16th - 20th;46:Abstract #5989.

(170) Helfrich B, Frederick B, Raben D, Bunn PA. The dualspecific   Src/Abl kinase inhibitor AZD0530 inhibits in vitro   growth and induces apoptosis in non-small-cell lung cancer   lines. Eur J Cancer 2006;Suppl. 4:177.

(171) Moiseeva EP, Verschoyle R, Manson MM. Susceptibility to indole-3-carbinol is related to Src in primary Tag mouse mammary tumours and EGFR/Her2 overexpression in human breast cancer cell lines [Abstract]. In: National Cancer Research Institute (NCRI) Cancer Conference 2007; Birmingham UK 2007 30 Sep -3 Oct 2007;.

(172) Bakhtiarova A, Taslimi P, Elliman SJ, Kosinski PA, Hubbard B, Kavana M, et al. Resveratrol inhibits firefly luciferase. Biochem Biophys Res Commun 2006;351(2):481-484.

(173) Astra Zeneca. Guidance for use of drug AZD0530. 2007.

(174) Gautier J, Solomon MJ, Booher RN, Bazan JF, Kirschner MW. cdc25 is a specific tyrosine phosphatase that directly activates p34cdc2. Cell, Cell 1991 1991 Oct 4;67(1):197-211.

(175) Sah NK, Khan Z, Khan GK, Bisen PS. Structural, functional and therapeutic biology of survivin. Cancer Letters 2006 8 December 2006;244(2):164-171,.

(176) Tamm I, Wang Y, Sausville E, Scuderio DA, Vigna N, Oltersdorf T, et al. IAP-Family Protein Survivin Inhibits Caspase Activity and Apoptosis Induced by Fas (CD95), Bax, Caspases, and Anticancer Drugs. Cancer Research, Cancer Res 1998 December 1, 1998;58:5315-5320.

(177) Reagan-Shaw S, Ahmad N. Silencing of polo-like kinase (PLK) 1 via siRNA causes induction of apoptosis and impairment of mitosis machinery in human prostate cancer cells: implications for the treatment of prostate cancer. The Faseb Journal, FASEBJ 2005 April 1, 2005;19(6):611-613.

(178) Zhu J, Lan H, Hong S, Hu G, Ai Q, Yang Z, et al. Polo-like kinase 1 siRNA-607 induces mitotic arrest and apoptosis in human nasopharyngeal carcinoma cells. African Journal of Biotechnology, Afr J Biotechnol 2011 13 July, 2011;10(35):6809-6815,.

(179) Chen CR, Wang W, Rogoff HA, Li X, Mang W, Li CJ. Dual Induction of Apoptosis and Senescence in Cancer Cellsby Chk2 Activation: Checkpoint Activation as a Strategy against Cancer. Cancer Res 2005 July 15, 2005;65(14):6017-6021.

(180) Tadi K, Chang Y, Ashok B, Chen Y, Moscatello A, Schaefer S, et al. 3,3 '-Diindolylmethane, a Cruciferous Vegetable Derived Synthetic Anti-Proliferative Compound in Thyroid Disease. Biochem Biophys Res Commun 2005 NOV 25;337(3):1019-1025.

(181) Khwaja FS, Wynne S, Posey I, Djakiew D. 3,3 '-Diindolylmethane Induction of p75(NTR)-Dependent Cell Death via the p38 Mitogen-Activated Protein Kinase Pathway in Prostate Cancer Cells. Cancer Prev Res 2009 JUN;2(6):566-571.

(182) Abdelrahim M, Newman K, Vanderlaag K, Samudio I, Safe S. 3,3 '-Diindolylmethane (DIM) and its derivatives induce apoptosis in pancreatic cancer cells through endoplasmic reticulum stress-dependent upregulation of DR5. Carcinogenesis 2006 APR;27(4):717-728.

(183) Rahman KMW, Banerjee S, Ali S, Ahmad A, Wang Z, Kong D, et al. 3,3 '-Diindolylmethane Enhances Taxotere-Induced Apoptosis in Hormone-Refractory Prostate Cancer Cells through Survivin Down-regulation RID E-8747-2010 RID A-6809-2011. Cancer Res 2009 MAY 15;69(10):4468-4475.

(184) Jin Y. 3,3 '-Diindolylmethane inhibits breast cancer cell growth via miR-21-mediated Cdc25A degradation. Mol Cell Biochem 2011 DEC;358(1-2):345-354.

(185) Garikapaty VPS, Ashok BT, Tadi K, Mittelman A, Tiwari RK. 3,3 '-Diindolylmethane Downregulates Pro-Survival Pathway in Hormone Independent Prostate Cancer. Biochem Biophys Res Commun 2006 FEB 10 2006;340(2):718-725.

(186) Oikonomou E, Kothonidis K, Zografos G, Nasioulas G, Andera L, Pintzas A. Newly established tumourigenic primary human colon cancer cell lines are sensitive to TRAIL-induced apoptosis in vitro and in vivo. Br J Cancer 2007 JUN 26 2007;97(1):73-84.

(187) Dalerba P, Guiducci C, Poliani PL, Cifola I, Parenza M, Frattini M, et al. Reconstitution of human telomerase reverse transcriptase expression rescues colorectal carcinoma cells from in vitro senescence: Evidence against immortality as a constitutive trait of tumor cells. Cancer Res 2005 MAR 15 2005;65(6):2321-2329.

(188) Brouquet A, Taleb P, Lot A, Beauchet A, Julie C, Prevost G, et al. A Model of Primary Culture of Colorectal Cancer and Liver Metastasis to Predict Chemosensitivity. J Surg Res 2011 APR 2011;166(2):247-254.

(189) Pastor DM, Poritz LS, Olson TL, Kline CL, Harris LR, Koltun WA, et al. Primary cell lines: false representation or model system? a comparison of four human colorectal tumors and their coordinately established cell lines. International journal of clinical and experimental medicine 2010 2010 Feb 22;3(1):69-83.

(190) Li Y, Chinni S, Sarkar F. Selective growth regulatory and pro-apoptotic effects of DIM is mediated by Akt and NF-kappaB pathways in prostate cancer cells. Front Biosci 2005 JAN 1;10:236-243.

(191) Banerjee S, Wang Z, Kong D, Sarkar FH. 3,3 '-Diindolylmethane Enhances Chemosensitivity of Multiple Chemotherapeutic Agents in Pancreatic Cancer RID A-6809-2011. Cancer Res 2009 JUL 1;69(13):5592-5600.

(192) Chen C, Wang W, Rogoff H, Li X, Mang W, Li C. Dual induction of apoptosis and senescence in cancer cells by Chk2 activation: Checkpoint activation as a strategy against cancer. Cancer Res 2005 JUL 15;65(14):6017-6021.

(193) Choi HJ, Lim DY, Park JHY,. Induction of G1 and G2/M cell cycle arrests by the dietary compound 3,3'-diindolylmethane in HT-29 human colon cancer cells. BMC Gastroenterology 2009;9:39-50.

(194) Kandala PK, Srivastava SK. Activation of Checkpoint Kinase 2 by 3,3 '-Diindolylmethane Is Required for Causing G(2)/M Cell Cycle Arrest in Human Ovarian Cancer Cells. Mol Pharmacol 2010 AUG;78(2):297-309.

(195) Gong YX, Sohn H, Xue L, Firestone GL, Bjeldanes LF. 3,3 '-diindolylmethane is a novel mitochondrial H+-ATP synthase inhibitor that can induce p21(Cip1/Waf1) expression by induction of oxidative stress in human breast cancer cells. Cancer Res 2006 MAY 1 2006;66(9):4880-4887.

(196) Blasina A, Van de Weyer I, Laus M, Luyten W, Parker A, McGowan C. A human homologue of the checkpoint kinase Cds1 directly inhibits Cdc25 phosphatase. Curr Biol 1999 JAN 14;9(1):1-10.

(197) Ahn J, Urist M, Prives C. Questioning the role of checkpoint kinase 2 in the p53 DNA damage response. J Biol Chem 2003 JUN 6;278(23):20480-20489.

(198) Jallepalli P, Lengauer C, Vogelstein B, Bunz F. The Chk2 tumor suppressor is not required for p53 responses in human cancer cells. J Biol Chem 2003 JUN 6;278(23):20475-20479.

(199) Jin J, Ang XL, Ye X, Livingstone M, Harper JW. Differential roles for checkpoint kinases in DNA damage-dependent degradation of the Cdc25A protein phosphatase RID H-1709-2011. J Biol Chem 2008 JUL 11;283(28):19322-19328.

(200) Lossaint G, Besnard E, Fisher D, Piette J, Dulic V. Chk1 is dispensable for G2 arrest in response to sustained DNA damage when the ATM/p53/p21 pathway is functional. Oncogene 2011 OCT;30(41):4261-4274.

(201) Zhang W, Poh A, Fanous AA, Eastman A. DNA damage-induced S phase arrest in human breast cancer depends on Chk1, but G(2) arrest can occur independently of Chk1, Chk2 or MAPKAPK2. Cell Cycle 2008 JUN 1;7(11):1668-1677.

(202) Stracker TH, Usui T, Petrini JHJ. Taking the time to make important decisions: The checkpoint effector kinases Chk1 and Chk2 and the DNA damage response. DNA Repair 2009 SEP 2;8(9):1047-1054.

(203) Reinhardt HC, Yaffe MB. Kinases that control the cell cycle in response to DNA damage: Chk1, Chk2, and MK2. Curr Opin Cell Biol 2009 APR;21(2):245-255.

(204) Toettcher JE, Loewer A, Ostheimer GJ, Yaffe MB, Tidor B, Lahav G. Distinct mechanisms act in concert to mediate cell cycle arrest. Proc Natl Acad Sci U S A 2009 JAN 20;106(3):785-790.

(205) Taylor W, Stark G. Regulation of the G2/M transition by p53. Oncogene 2001 APR 5;20(15):1803-1815.

(206) Toyoshima-Morimoto F, Taniguchi E, Nishida E. Plk1 promotes nuclear translocation of human Cdc25C during prophase. EMBO Rep 2002 APR;3(4):341-348.

(207) Toyoshima-Morimoto F, Taniguchi E, Shinya N, Iwamatsu A, Nishida E. Polo-like kinase 1 phosphorylates cyclin B1 and targets it to the nucleus during prophase. Nature 2001 MAR 8;410(6825):215-220.

(208) Zhang Z, Su W, Feng C, Yu D, Cui C, Xu X, et al. Polo-like kinase 1 may regulate G2/M transition of mouse fertilized eggs by means of inhibiting the phosphorylation of Tyr 15 of Cdc2. Mol Reprod Dev 2007 OCT;74(10):1247-1254.

(209) Reagan-Shaw S, Ahmad N. Silencing of polo-like kinase (Plk) 1 via siRNA causes induction of apoptosis and impairment of mitosis machinery in human prostate cancer cells: implications for the treatment of prostate cancer. FASEB J 2005 JAN;19(1):611-+.

(210) Wells N, Watanabe N, Tokusumi T, Jiang W, Verdecia M, Hunter T. The C-terminal domain of the Cdc2 inhibitory kinase Myt1 interacts with Cdc2 complexes and is required for inhibition of G(2)/M progression. J Cell Sci 1999 OCT;112(19):3361-3371.

(211) Josue Ruiz E, Vilar M, Nebreda AR. A Two-Step Inactivation Mechanism of Myt1 Ensures CDK1/Cyclin B Activation and Meiosis I Entry. Current Biology 2010 APR 27;20(8):717-723.

(212) Ando K, Ozaki T, Yamamoto H, Furuya K, Hosoda M, Hayashi S, et al. Polo-like kinase 1 (Plk1) inhibits p53 function by physical interaction and phosphorylation. J Biol Chem 2004 JUN 11;279(24):25549-25561.

(213) Liu X, Erikson R. Polo-like kinase (Plk)1 depletion induces apoptosis in cancer cells. Proc Natl Acad Sci U S A 2003 MAY 13;100(10):5789-5794.

(214) Komatsu S, Takenobu H, Ozaki T, Ando K, Koida N, Suenaga Y, et al. Plk1 regulates liver tumor cell death by phosphorylation of TAp63. Oncogene 2009 OCT 15 2009;28(41):3631-3641.

(215) Liu X, Lei M, Erikson R. Normal cells, but not cancer cells, survive severe Plk1 depletion. Mol Cell Biol 2006 MAR;26(6):2093-2108.

(216) Zhu J, Lan H, Hong S, Hu G, Ai Q, Yang Z, et al. Polo-like kinase 1 siRNA-607 induces mitotic arrest and apoptosis in human nasopharyngeal carcinoma cells. African Journal of Biotechnology 2011 JUL 13 2011;10(35):6809-6815.

(217) Colnaghi R, Wheatley SP. Liaisons between Survivin and Plk1 during Cell Division and Cell Death. J Biol Chem 2010 JUL 16 2010;285(29):22592-22604.

(218) He Z, Wu J, Dang H, Lin H, Zheng H, Zhong D. Polo-like kinase 1 contributes to the tumorigenicity of BEL-7402 hepatoma cells via regulation of Survivin expression. Cancer Lett 2011 APR 28 2011;303(2):92-98.

(219) Takahashi T, Sano B, Nagata T, Kato H, Sugiyama Y, Kunieda K, et al. Polo-like kinase 1 (PLK1) is overexpressed in primary colorectal cancers. Cancer Sci 2003 FEB;94(2):148-152.

(220) Weichert W, Kristiansen G, Schmidt M, Gekeler V, Noske A, Niesporek S, et al. Polo-like kinase 1 expression is a prognostic factor in human colon cancer. World Journal of Gastroenterology, World J Gastroenterol 2005;11(36):5644-5650.

(221) Altieri DC. Validating survivin as a cancer therapeutic target. Nature Reviews Cancer 2003 JAN 2003;3(1):46-54.

(222) Li FZ. Survivin study: What is the next wave? J Cell Physiol 2003 OCT 2003;197(1):8-29.

(223) Velculescu VE, Madden SL, Zhang L, Lash AE, Yu J, Rago C, et al. Analysis of human transcriptomes. Nat Genet 1999 DEC 1999;23(4):387-388.

(224) Du CY, Fang M, Li YC, Li L, Wang XD. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell 2000 JUL 7 2000;102(1):33-42.

(225) Dohi T, Okada K, Xia F, Wilford CE, Samuel T, Welsh K, et al. An IAP-IAP complex inhibits apoptosis. J Biol Chem 2004 AUG 13 2004;279(33):34087-34090.

(226) Altieri DC, Marchisio C. Survivin apoptosis: An interloper between cell death and cell proliferation in cancer. Laboratory Investigation 1999 NOV 1999;79(11):1327-1333.

(227) Giodini A, Kallio MJ, Wall NR, Gorbsky GJ, Tognin S, Marchisio PC, et al. Regulation of microtubule stability and mitotic progression by survivin. Cancer Res 2002 MAY 1 2002;62(9):2462-2467.

(228) Kawasaki H, Altieri DC, Lu CD, Toyoda M, Tenjo T, Tanigawa N. Inhibition of apoptosis by survivin predicts shorter survival rates in colorectal cancer. Cancer Res 1998 NOV 15 1998;58(22):5071-5074.

(229) Sarela AI, Macadam RCA, Farmery SM, Markham AF, Guillou PJ. Expression of the antiapoptosis gene, Survivin, predicts death from recurrent colorectal carcinoma. Gut 2000 MAY 2000;46(5):645-650.

(230) Rodel C, Haas J, Groth A, Grabenbauer GG, Sauer R, Rodel F. Spontaneous and radiation-induced apoptosis in colorectal carcinoma cells with different intrinsic radiosensitivities: Survivin as a radioresistance factor. International Journal of Radiation Oncology Biology Physics 2003 APR 1 2003;55(5):1341-1347.

(231) Pennati M, Binda M, Colella G, Zoppe' M, Folini M, Vignati S, et al. Ribozyme-mediated inhibition of survivin expression increases spontaneous and drug-induced apoptosis and decreases the tumorigenic potential of human prostate cancer cells. Oncogene 2004 JAN 15 2004;23(2):386-394.

(232) Zaffaroni N, Pennati M, Colella G, Perego P, Supino R, Gatti L, et al. Expression of the anti-apoptotic gene survivin correlates with taxol resistance in human ovarian cancer. Cellular and Molecular Life Sciences 2002 AUG 2002;59(8):1406-1412.

(233) Zhang M, Mukherjee N, Bermudez RS, Latham DE, Delaney MA, Zietman AL, et al. Adenovirus-mediated inhibition of survivin expression sensitizes human prostate cancer cells to paclitaxel in vitro and in vivo. Prostate 2005 AUG 1 2005;64(3):293-302.

(234) Kawasaki H, Toyoda M, Shinohara H, Okuda J, Watanabe I, Yamamoto T, et al. Expression of survivin correlates with apoptosis, proliferation, and angiogenesis during human colorectal tumorigenesis. Cancer 2001 JUN 1 2001;91(11):2026-2032.

(235) Kong D, Li Y, Wang Z, Banerjee S, Sarkar FH. Inhibition of angiogenesis and invasion by 3,3 '-diindolylmethane is mediated by the NF-kappa B downstream target genes MMP-9 and uPA that regulated bioavailability of VEGF in prostate cancer. Cancer Res 2007 APR 1 2007;67(7):3310-3319.

(236) Rahman KMW, Sarkar FH. Inhibition of nuclear translocation of nuclear factor-kappa B contributes to 3,3 '-diindolylmethane-induced apoptosis in breast cancer cells. Cancer Res 2005 JAN 1 2005;65(1):364-371.

(237) Nachshon-Kedmi M, Yannai S, Haj A, Fares FA. Indole-3-carbinol and 3,3 '-diindolylmethane induce apoptosis in human prostate cancer cells. Food and Chemical Toxicology 2003 JUN 2003;41(6):745-752.

(238) Kim EJ, Park SY, Shin H, Kwon DY, Surh Y, Park JHY. Activation of caspase-8 contributes to 3,3 '-diindolylmethane-induced apoptosis in colon cancer cells. J Nutr 2007 JAN 2007;137(1):31-36.

(239) Cho HJ, Park SY, Kim EJ, Kim J, Park JHY. 3,3 '-Diindolylmethane Inhibits Prostate Cancer Development in the Transgenic Adenocarcinoma Mouse Prostate Model. Mol Carcinog 2011 FEB 2011;50(2):100-112.

(240) Nachshon-Kedmi M, Yannai S, Fares FA. Induction of apoptosis in human prostate cancer cell line, PC3, by 3,3 '-diindolylmethane through the mitochondrial pathway. Br J Cancer 2004 OCT 4 2004;91(7):1358-1363.

(241) Smith TK, Lund EK, Clarke RG, Bennett RN, Johnson IT. Effects of Brussels sprout juice on the cell cycle and adhesion of human colorectal carcinoma cells (HT29) in vitro. J Agric Food Chem 2005 MAY 18 2005;53(10):3895-3901.

(242) Kim EJ, Shin M, Park H, Hong JE, Shin H, Kim J, et al. Oral Administration of 3,3 '-Diindolylmethane Inhibits Lung Metastasis of 4T1 Murine Mammary Carcinoma Cells in BALB/c Mice. J Nutr 2009 DEC 2009;139(12):2373-2379.

(243) Rajoria S, Suriano R, George A, Shanmugam A, Schantz SP, Geliebter J, et al. Estrogen Induced Metastatic Modulators MMP-2 and MMP-9 Are Targets of 3,3 '-Diindolylmethane in Thyroid Cancer. Plos One 2011 JAN 18 2011;6(1):e15879.

(244) Ali S, DeCaprio J. Cellular transformation by SV40 large T antigen: interaction with host proteins. Semin Cancer Biol 2001 February;11(1):15--23.

(245) Technau A, Wolff A, Sauder C, Birkner N, Brandner G. p53 in SV40-transformed DNA-damaged human cells binds to its cognate sequence but fails to transactivate target genes. Int J Oncol 2001 February;18(2):281--286.

(246) Sun SS, Han J, Ralph WM, Chandrasekaran A, Liu K, Auborn KJ, et al. Endoplasmic reticulum stress as a correlate of cytotoxicity in human tumor cells exposed to diindolylmethane in vitro. Cell Stress Chaperones 2004 MAR 2004;9(1):76-87.

(247) AstraZeneca Clinical Trials. Available at: http://www.astrazenecaclinicaltrials.com/other-drug-products/discontinued-products/AZD05301/?dateRange=all. Accessed December 6, 2011.