CURCUMIN-INDUCED G_2/M CELL CYCLE ARREST IN COLORECTAL CANCER CELLS

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L. M. Blakemore MSci Hons (Glasgow), MSc (Leicester)

Department of Biochemistry

University of Leicester

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Abstract

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L. M. Blakemore

Curcumin, a diet-derived chemopreventive and chemotherapeutic agent has been shown to induce G_2/M cell cycle arrest, and previous studies suggested that microtubule depolymerisation may be linked to M-phase arrest. However, mechanisms involved have not been elucidated and often non-physiological concentrations of curcumin were used. The goal of this study was to characterise in more detail curcumin-induced cell cycle arrest using a panel of human colorectal cancer cell (CRC) lines, HT-29, SW480, HCT116 $p53^{+/+}$, HCT116 $p53^{-/-}$ and HCT116 $p21^{-/-}$.

Using physiologically relevant concentrations of curcumin (5-10μM), achievable in the gut tissue following oral ingestion, cell cycle analysis showed that treatment for 12 hours results in significant G_2/M arrest in all five cell lines. Curcumin treatment significantly increased the number of cells in M phase in 4 out of the 5 lines tested for this duration, and those with microsatellite instability (HCT116) were found to have a higher mitotic index than those with chromosomal instability. Pre-treatment with caffeine abrogated mitotic arrest in these cell lines, indicating the involvement of the ATM/ATR kinases. Activating phosphorylation of the Chk1 kinase was increased and total protein levels of CDC25C reduced, further implicating the DNA damage pathway in the induction of arrest. Higher levels of HSP70 were also found, indicating proteotoxic stress such as proteasomal inhibition.

Image analysis revealed impaired mitotic progression, and significantly higher levels of mitotic spindle abnormalities following curcumin treatment. Aurora B mislocalisation and significantly lower levels of centrosomal separation were found in the HCT116 $p53^{+/+}$ line. Furthermore, the high levels of pH2A.X staining seen in curcumin-treated mitotic but not interphase cells suggest that aberrant mitosis may result in DNA damage. This proteotoxic and genotoxic stress incurred following curcumin treatment may contribute to the upregulation of NKG2D ligands on the cell surface, leading to CRC lysis and enhancement of the anti-cancer immune response.

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List of Abbreviations

5-FU	5-fluorouracil
53BP1	p53-binding protein 1
8-oxo-dA	8-oxo-deoxyadenosine
8-oxo-dG	8-oxo-deoxyguanosine
APC	Adenomatous polyposis coli
APC/C	Anaphase promoting complex/cyclosome
APS	Ammonium persulfate solution
ATL	Adult T cell leukemia
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related protein
ATRIP	ATR-interacting protein
BAX	Bcl-2-associated X protein
BRCA1	Breast cancer type 1 susceptibility protein
BRCT	BCRA1 carboxyl-terminal
BSA	Bovine serum albumin
CCNSC	Cancer Chemotherapy National Service Center
CD	Cluster of differentiation

CDC	Cell division cycle
CDK	Cyclin-dependent kinase
CDT1	CDC10-dependent transcript 1
CHFR	Checkpoint with FHA and RING-finger domains
Chk	Checkpoint kinase
CIN	Chromosomal instability
CKI	Cyclin kinase inhibitor
COX	Cyclooxygenase
CPC	Chromosomal passenger complex
CPT	Camptothecin
CRC	Colorectal cancer
CRUK	Cancer Research UK
DAPI	4', 6-Diamidino-2-phenylindole
DATS	Diallyl trisulphate
DBH	Debromohymenialdisine
DCFH-DA	2',7'-Dichlorofluorescein
DIM	3,3'-Diindolylmethane
DMSO	Dimethyl sulfoxide

DSB	Double-strand break
dsDNA	Double-stranded DNA
DNA-PK	DNA-activated protein kinase
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ECGC	Epigallocatechin gallate
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol tetraacetic acid
EtOH	Ethanol
FACS	Fluorescence activated cell sorting
FAP	Familial adenomatous polyposis
FCS	Foetal calf serum
FHA	Forkhead-associated
FITC	Fluorescein isothiocyanate
FOLFOX	5-FU and oxaliplatin
FOLFIRI	5-FU and folinic acid
G_1	Gap 1 phase of the cell cycle
G_2	Gap 2 phase of the cell cycle

GTP	Guanosine triphosphate
HEC-1	Highly expressed in cancer-1
HER	Human epidermal growth factor receptor
HNPCC	Hereditary nonpolyposis colorectal cancer
hr	Hour (s)
HSC70	Heat shock cognate 70
HSP70	Heat shock protein 70
HTLV-1	Human T-lymphotropic virus type I
HU	Hydroxyurea
IGF-1	Insulin-like growth factor 1
IGFBP-3	Insulin-like growth factor-binding protein 3
IGF-R1	Insulin-like growth factor receptor 1
INCENP	Inner centromere protein
INK4	Inhibitors of CDK4
kDa	Kilodalton (s)
KNL-1	Kinetochore NuL-1
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homologue
KSP	Kinesin spindle protein

M	Mitotic phase of the cell cycle
mAb	Monoclonal antibody
MAD2	Mitotic arrest deficient 2
MAPK	Mitogen-activated protein kinase
MCC	Mitotic checkpoint complex
MCM	Mini-chromosome maintenance
MDC1	Mediator of DNA damage checkpoint protein 1
Mdm2	Murine double minute
MICA/MICB	MHC class I polypeptide related sequence A/B
MIN	Microsatellite instability
MCAK	Mitotic centromere-associated kinesin
MMP	Matrix metalloproteinase
MMR	Mismatch repair
MLH1	MutL homologue 1
MLH3	MutL homologue 3
MPF	Mitosis promoting factor
mRNA	Messenger ribonucleic acid
MYT1	Myelin transcription factor 1

NBS1	Nijmegen breakage syndrome 1
NICE	National Institute for Health and Clinical Excellence
NMR	Nuclear magnetic resonance
NSAIDS	Non-steroidal anti-inflammatory drugs
NuMA	Nuclear mitotic apparatus
ORC	Origin of recognition complex
pAb	Polyclonal antibody
PARP	Poly-ADP ribose polymerase
PBS	Phosphate buffered saline
PBS-T	PBS-Tween
PCNA	Proliferating cell nuclear antigen
pH2A.X	Phospho-histone H2A.X
pH3	Phospho-histone H3
PI	Propidium iodide
PI3K	Phosphoinositide 3 kinase
PI3KCA	PI3K catalytic subunit
PIKK	Phosphoinositide 3-kinase related protein kinase
PIPES	1,4-Piperazinediethanesulfonic acid

Plk1	Polo-like kinase 1
Pre-RC	Pre-replication complex
PTIP	Pax transactivation domain interacting protein
RCF2	Retinoblastoma control factor 2
RDS	Radio-resistant DNA synthesis
RFC	Replication C factor
RING	Really interesting new gene
ROS	Reactive oxygen species
RPA	Replication protein A
S	Synthesis phase of the cell cycle
SAC	Spindle assembly checkpoint
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	Small interferingRNA
TEMED	Tetramethylethylenediamine
TopBP1	Topo II-binding protein 1
Topo I	Topoisomerase I
Topo II	Topoisomerase II
TNFα	Tumour necrosis factor-α

TNM	Tumour, node, metastasis
TRAIL	Tumour necrosis factor related apoptosis inducing ligand
ULBP	UL-16 binding protein
US FDA	United States Food and Drug Administration
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
Wt	Wild-type
XIAP	X-linked inhibitor of apoptosis protein

Contents

List of Figures

List of Tables

Chapter 1 Introduction

1.1 Colorectal Cancer

1.1.1 Colorectal Cancer Incidence

In 2004, colorectal cancer (CRC) was found to be the seventh leading cause of death in high-income countries, accounting for 0.27 million deaths worldwide (WHO, 2008). In the United Kingdom of Great Britain and Northern Ireland (UK), online data published by Cancer Research UK (CRUK) in 2007 showed that CRC is the second deadliest cancer, after lung cancer. In the UK, the survival rates of those diagnosed with CRC have doubled over the past 30 years, from approximately 25% to 50% from the mid 1970s to the mid 2000s (ONS, Coleman, 2007, Rachet, 2009, Richard, 2008). The increase in survival is attributed to two factors: earlier diagnosis and improved treatment. Table 1 illustrates the importance of early diagnosis of CRC prior to advanced disease progression (Dukes' A is the lowest grading of cancer, Dukes' D the most advanced).

Patients presenting with low grade cancers have significantly higher survival rates than cancers diagnosed with more extensive disease. There have been improved methods for the management of colorectal cancer in the fields of chemotherapy, radiation and surgery. However, many types of chemotherapy still have severe side effects. Due to its limited toxicity, curcumin is being investigated for use alone or in combination with chemotherapeutic agents for the treatment of CRC.

Table 1 Percentage of Cases and 5 Year Relative Survival (%) by Dukes' Stage at Diagnosis for CRC Patients

Dukes' stage at diagnosis	Percentage of cases	Five-year relative survival
A	8.7%	93.2%
B	24.2%	77.0%
C	23.6%	47.7%
D	9.2%	6.6%
Unknown	34.3%	35.4%

Patients that are diagnosed at an earlier stage of CRC (Dukes A and B) have a better prognosis than those who present with more advanced disease (Dukes C and D). Data compiled from patients diagnosed between 1996-2002, England (CRUK).

1.1.2 Molecular Mechanisms of Colorectal Cancer

Studies from meta-analysis have shown an association between microsatellite instability (MIN) and chromosomal instability (CIN) status regarding prognosis in CRC (Walther et al., 2008). The MIN tumour phenotype is associated with inactivation of the mismatch repair (MMR) system, the primary function of which is to monitor DNA during the replication process and eliminate base-base mismatches or insertion-deletion loops that occur following DNA polymerase slippage (Peltomaki, 2001). In humans there are at least six MMR genes required to encode proteins for this process including *MSH2*, *MSH6*, *MutL homologue 1 and 3* (*MLH1*, *MLH3*), *PMS1*, *PMS2*, and mutations in any of these genes can affect the integrity of the MMR system. A defective MMR system can increase the number of genome-wide mutations that in turn increase the risk of oncogenic transformation. The majority of mutations arise in the *MSH2* and *MLH1* genes, and as they encode the proteins that are the most vital for the MMR system, mutations in these genes can result in tumours with a high degree

of microsatellite instability. In addition, mutations in genes encoding DNA damage signalling proteins such as DNA-PK (DNA-activated protein kinase) and ATR (ataxia telangiectasia and Rad3-related protein), and double strand break repair proteins such as RAD50, alongside mutations in mismatch repair genes have been found in tumours with microsatellite instability (Miquel et al., 2007). Germline mutations of one of the mismatch repair genes, often *MSH2* or *MLH1* are strongly associated with hereditary nonpolyposis colorectal cancer (HNPCC), which accounts for approximately 5% of all CRC cases (Bronner et al., 1994, Fishel et al., 1993, Jacob et al., 2001, Leach et al., 1993, Nicolaides et al., 1994). HNPCC patients have an inherited predisposition to colorectal cancer and extracolonic epithelial-derived tumours that is autosomally dominant. Research has shown that for the tumourigenesis associated with MMR gene defects to occur, both alleles of the relevant MMR gene must be inactivated (Liu et al., 1995). Microsatellite instability occurs in more than 90% of HNPCC tumours and is seen in approximately 15-25% of sporadic colorectal cancers.

Chromosomal instability is defined as changes in the chromosome structure or number in tumour cells. This can be evaluated in a clinical setting by performing analysis of the cell cycle distribution using FACS (fluorescence activated cell sorting) DNA analysis (Walther et al., 2008). The presence of unusual peaks in place of the usual diploid 2N/4N peaks in a DNA histogram is indicative of aneuploidy or polyploidy and confirms chromosomal instability. More detailed evaluation of chromosomal instability can be performed using integrative genomics such as spectral karyotyping, high resolution array-based comparative genomic hybridisation and global gene expression profiling (Camps et al., 2009, Knutsen et al., 2010). This allows detailed

analysis of specific architectural changes within the genome and can give a profile of abnormal biological pathways within the tumour.

A meta-analysis evaluating the prognostic importance of MIN status found that MIN positive tumours had a better prognosis than MIN negative tumours. CIN is more common in CRC than MIN and is present in approximately 65-70% of these cancers. CIN has been associated with worse survival in patients diagnosed with more advanced colorectal cancer, and it has been suggested that CIN, and MIN status should be examined as prognostic markers in all clinical trials (Walther et al., 2008). There are CRC tumours that do not clearly fall into either the MIN or CIN category. Firstly, there are rare colorectal cancers that are both MIN and CIN positive. Secondly, it has been found that approximately 25% of CRC tumours are both CIN and MIN negative (Goel et al., 2003).

Familial adenomatous polyposis (FAP) is an inherited disease, and individuals with FAP have a predisposition to colorectal cancer. Germiline mutations in the tumour suppressor *adenomatous polyposis coli* (*APC*) gene were found in FAP patients (Groden et al., 1991, Joslyn et al., 1991, Kinzler et al., 1991, Miyoshi et al., 1992 Nishisho et al., 1991). Furthermore, mutations in the *APC* gene occur in over 60% of sporadic colorectal cancers (Powell et al., 1992). The APC protein is involved in regulation of the β-catenin protein. Both APC and β-catenin are involved in the Wnt signalling pathway which is frequently deregulated in colorectal cancer (Papkoff et al., 1996, Reifenberger et al., 2002). Loss of tumour suppressor and checkpoint proteins such as APC, p21, p53, and mutations in oncogenes such as the *ras* genes are proposed to be key steps in the transition of benign tumours (adenomas) to malignant

tumours (carcinomas) in CRC (Fearon and Vogelstein, 1990, Polyak et al., 1996). Accumulation of these mutations as tumourigenesis progresses in the 'adenoma-carcinoma sequence' genetic model of colorectal cancer has been extensively studied. Mutations can arise due to deficiencies in DNA repair genes. For example the substitution of V599E (replacement of valine with glutamate at residue 599) in the *BRAF* gene, is frequently found in MMR-deficient cancers (Rajagopalan et al., 2002). Multiple allelic deletions were found in individual chromosomes and the authors proposed that this may have arisen from abnormal chromosomal segregation in cancer cells, and such allelic loss of tumour suppressor genes may contribute to CRC (Fearon and Vogelstein, 1990). Mutations of the tumour suppressor and checkpoint protein p53 have been found in 40-50% of colorectal carcinomas (Baker et al., 1989). Other types of genetic mutations have been found to result in the activation of oncogenes such as the epidermal growth factor receptor (EGFR). Overexpression of EGFR via amplification or increased copy number has been found in 10-15% of colorectal cancers (Sauer et al., 2005, Shia et al., 2005).

Large population studies have shown that activating mutations in oncogenes *KRAS* (*v-Ki-ras2 Kirsten rat sarcoma viral oncogene homologue*) or *BRAF* are present in 32-37% and 10-17% of CRC cases respectively (Brink et al., 2003; de Vogel et al., 2009, Ogino et al., 2009, Rajagopalan et al., 2002, Samowitz et al., 2000). These genes encode proteins involved in the RAS/MAPK signalling pathway, and activation can lead to uncontrolled proliferation of cancer cells. Similar studies have found mutations in the *PI3KCA (phosphoinositide 3 kinase catalytic subunit)* gene in 15% of colorectal cancers (Barault et al., 2008, Nosho et al., 2008b). The *PI3KCA* gene encodes the catalytic

subunit of the PI3K (phosphoinositide 3 kinase) protein that is involved in the PI3K/AKT pathway. PI3K is one of the major targets of Ras, and the PI3K/AKT pathway is involved in many cellular processes such as cell growth, differentiation, survival and intracellular trafficking.

Chronic inflammation can contribute to tumourigenesis, and patients with inflammatory bowel diseases such as ulcerative colitis and Crohn's disease have an increased incidence of colorectal cancer (Bernstein et al., 2001). Inflammation can also contribute to FAP progression and treatment with non-steroidal anti-inflammatory drugs can slow the growth of colorectal adenomas in patients with FAP (first shown in (Waddell et al., 1989), although the efficacy of NSAIDS (non-steroidal anti-inflammatory drugs) may also be due to inflammation independent-pathways such as β-catenin accumulation and Wnt signalling (Boon et al., 2004).

1.1.3 Current Treatments for Colorectal Cancer

Treatment for bowel cancer is dependent on the grade and location of the cancer. The Dukes' system of colorectal cancer has traditionally been used to grade colorectal cancer (Dukes, 1932). In Dukes' A there is invasion into but not through the bowel wall, and surgery is generally used to remove the cancer. Dukes' B indicates that there is invasion through the muscle wall, but no lymph nodes are affected. Dukes' C means that the cancer has spread to at least one lymph node in the area. For Dukes' B and C bowel cancer surgery may be used to remove the cancer and local lymph nodes. Chemotherapy may be used before surgery to shrink the tumour, and this is referred to as first line or neo-adjuvant therapy. Following surgery, patients receive a course of

chemotherapy to prevent recurrence of the tumour and this is referred to as adjuvant chemotherapy. Dukes' D is used to define CRC with widespread metastases to the liver or lung. Dukes' D is also referred to as advanced bowel cancer, and this is usually not curable. A combination of surgery, chemotherapy, and radiotherapy is used to control the symptoms and spread of advanced bowel cancer. The TNM (tumour, node, metastasis) grading system is now replacing the Dukes' classification system in the clinical setting. The T stages describe the size of the tumour, the N stages the involvement of lymph nodes, and the M stages metastases (Sobin, 1997). The criteria for the new TNM system alongside the modified Dukes' system is shown in table 2.

Table 2 Staging of Colorectal Cancer

UICC	**TNM**	**Modified Dukes'**
Stage 0	Carcinoma in situ	A
Stage I	No nodal involvement, no distant metastasis Tumour invades submucosa (T1, N0, M0) Tumour invades muscularis propria (T2, N0, M0)	
Stage II	No nodal involvement, no distant metastasis Tumour invades into subserosa (T3, N0, M0) Tumour invades into other organs (T4, N0, M0)	B
Stage III	Nodal involvement, no distant metastasis 1 to 3 regional lymph nodes involved (any T, N1, M0) 4 or more regional lymph nodes involved (Any T, N2, M0)	C
Stage IV	Distant metastases (any T, any N, M1)	D

This table compares the Dukes and UIC staging system to the criteria for the newer tumour, node, metastasis (TNM) system for grading CRC (CRUK, 2004).

Surgery is the main form of treatment for 80% of patients with colorectal cancer (NICE, 2004). Depending on the grade of cancer, patients may also receive chemotherapy before or after treatment. Research has shown that following surgery, patients who underwent a six month course of chemotherapy had significantly lower levels of recurrence and higher survival rates (Dube et al., 1997). The main therapy that has been used to treat colorectal cancer is the anti-metabolite 5-fluorouracil (5-FU), and this is commonly given alongside folinic acid (leucovorin) to improve efficacy. 5-FU is a uracil analogue, and was first synthesised by a group in the 1950s (Heidelberger et al., 1957).

Oxaliplatin (Eloxatin) interferes with DNA synthesis, and is commonly used to treat advanced colorectal cancer. The anti-cancer activity of oxaliplatin was first shown in a screen of the effects of platinum-based drugs on the mouse leukemia cell line L1210 (Kidani et al., 1980). The combination therapy of oxaliplatin with 5-FU and folinic acid is referred to as FOLFOX. The agent irinotecan (Campto) is used in combination with 5-FU and folinic acid (known as FOLFIRI) in first line treatment of colorectal cancer, or alone as second line therapy (NICE, 2005). Irinotecan is a topoisomerase I inhibitor and prevents DNA decatenation or 'unwinding'. The type I and type II topoisomerases (topo I, topo II) are enzymes required for altering DNA topography and play a particularly important role within the cell cycle (Nitiss, 2009). The topo I and topo II enzymes induce temporary single-strand breaks, and double-strand breaks respectively to alter DNA structure. Irinotecan can form stable complexes with DNA and topo I, and during DNA replication these stable complexes can obstruct advancing replication forks, resulting in double-strand breaks and inhibition of DNA replication

(Liu et al., 2000a). Irinotecan and topotecan (used in the treatment of cervical, ovarian and small cell lung cancer) are synthetic analogues of the natural agent camptothecin. Camptothecin was isolated from the leaves of the *Captotheca acuminate* Decne (Nyssaceae) tree, in the 1960s during a screen of natural agents by a group directed by the late Dr Monroe Wall (Wall and Wani, 1996). Some of the agents isolated by this screen were tested at the Cancer Chemotherapy National Service Center (CCNSC), and the *Camptotheca* extracts were shown to have high antitumour potential in the Ca-755 mouse mammary carcinoma model. It was only later in 1985 that camptothecin (CPT) was shown to act via inhibition of the topo I enzyme (Hsiang et al., 1985). This led to the synthesis of many new CPT analogues that were tested for their ability to act on the topo I enzyme and their antitumour activity in mouse models of leukemia (Hsiang et al., 1989, Jaxel et al., 1989, Wall et al., 1986, Wani et al., 1987a, Wani et al., 1987b, Wani et al., 1986). Around this time, CPT compounds were shown to be highly effective in inhibiting the growth of human colorectal cancer xenographs in nude mice (Giovanella et al., 1989). This led to further clinical trials of CPT and analogues alone or in combination with agents such as cisplatin, and the development of the current range of related compounds approved for chemotherapy today.

The status of MMR genes has been shown to influence the sensitivity of colorectal cancer cell lines to the CPT analogue irinotecan (Jacob et al., 2001). Further clinical research has shown that patients that have advanced colorectal cancers exhibiting features of microsatellite instability (MSI^+) with mutations in the *BAX* gene, that encodes a pro-apoptopic protein (Bcl-2-associated X protein, Bax), and the *MSH6* and *MSH3* mismatch repair genes respond better to irinotecan (Fallik et al., 2003).

Monoclonal antibody (mAb) therapies designed to inhibit the growth and blood supply of cancer cells are undergoing clinical trials in the treatment of colorectal cancer, and have been approved for management of this disease in the United States of America (Cercek and Saltz, 2008). Bevacizumab targets cells expressing vascular endothelial growth factor (VEGF), and cetuximab targets cells expressing the epidermal growth factor receptor (EGFR). However, there is limited evidence that patients with colorectal cancer benefit from monoclonal antibody treatments (Cercek and Saltz, 2008). Two recent clinical trials measured the effects of cetuximab combination therapy with FOLFOX (OPUS trial) or FOLFIRI (CRYSTAL trial) compared to FOLFOX or FOLFIRI treatment alone in patients with metastatic colorectal cancer (Bokemeyer et al., 2009, Van Cutsem et al., 2007). The response to treatment was measured by the overall response rate in the OPUS trial and progression-free survival in the CRYSTAL trial. A significant improvement compared to FOLFOX/FOLFIRI treatment alone was found in patients receiving additional cetuximab with tumours bearing a wild-type version of the KRAS gene in both trials. The OPUS trial found that patients with colorectal tumours carrying a mutant version of the *KRAS* gene had a significantly lower overall response rate when receiving the combination of FOLFOX and cetuximab compared to FOLFOX alone. As a result screening for *KRAS* gene status is now being conducted prior to enrolment in anti-EGFR therapy trials. Clinical resistance to anti-EGFR therapies such as cetuximab and panitumumab has also been found in patients with mutant versions of the *BRAF*, *PIK3CA* and *NRAS* genes in colorectal tumours (De Roock et al., 2010, Di Nicolantonio et al., 2008, Sartore-Bianchi et al., 2009). Mutations in the *BRAF* and *KRAS* genes have previously been found to be mutually exclusive (Rajagopalan et al., 2002). Therefore, screening for *KRAS* status alone may not be

enough to identify the patients that would directly benefit from this therapy, as those wild-type *KRAS* may have a mutant version of *BRAF*, or other response-related genes. The development of molecular markers to elucidate which patients would benefit from this type of therapy is essential to improve the treatment of CRC (Cercek and Saltz, 2010). While bevacizumab and cetuximab are approved for treatment of metastatic colon cancer by the United States Food and Drug Administration (US FDA), they are not currently approved by the National Institute for Health and Clinical Excellence (NICE) in the treatment of CRC in the UK (NICE, 2007).

Other chemotherapeutic agents in use or being developed for the treatment of CRC include anti-mitotic agents. These compounds target the mitotic phase of the cell cycle and include anti-tubulin agents, that either hyper-stabilise or destabilise the mitotic spindle, or inhibitors of key cell cycle proteins involved in the coordination of mitosis. There are numerous clinical trials that have recently been completed or are ongoing, investigating the effect of anti-mitotic agents such as paclitaxel, vincristine and docetaxel in the treatment of advanced CRC (ClinicalTrials.gov Identifiers: NCT00598247, NCT00024401, NCT00625573, NCT00034190, NCT00427570, NCT00003543, NCT00288444). Paclitaxel is another agent discovered during a plant screening program for anticancer compounds and was originally isolated from the *Taxus brevifolia* tree (Wani et al., 1971). Initial studies showed that this agent acted as a spindle poison, blocking mitosis and inducing G_2/M cell cycle arrest (Fuchs and Johnson, 1978). Paclitaxel acts by stabilising microtubules and preventing their depolymerisation back to tubulin (Manfredi et al., 1982). This is in contrast to many other plant-derived spindle poisons such as maytansine, vincristine, colchicine, and

phyllodotoxin that act by binding to soluble tubulin and inhibit the polymerisation of tubulin to form microtubules (Wall and Wani, 1996). Table 3 has a summary of these agents and their approved clinical uses.

Table 3 Naturally Occurring Anti-mitotic Chemotherapeutic Drugs in Clinical Use

Plant	Agent (s)	Synthetic analogues, derivatives	Microtubule Mechanism of action	Approved Uses (cancer types)
Taxus brevifolia	Paclitaxel	Docetaxel	Stabiliser	Lung, ovarian, breast, prostate
Catharanthus roseus	Vincristine	Vinblastine, Vinorelbine	De-stabiliser	Lung, follicular lymphoma, leukemia, Non-Hodgkin's lymphoma, breast
Podophyllum peltatum	Podophyllotoxin	Etoposide, teniposide, etophos	De-stabiliser	Lung, testicular, lymphoma, non-lymphocytic leukemia
Colchicum autumnale	Colchicine	ZD6126	De-stabiliser	Completed Phase I Clinical trials (Solid tumours: colon, renal and ovarian) (LoRusso et al., 2008)

(Adapted from (Jackson et al., 2007))

Many of these agents that target the microtubules have severe side-effects, and the cytoskeleton of resting cells can be perturbed. New types of anti-mitotic drugs that target key proteins involved in mitosis aim to improve the therapeutic index of anti-mitotic drugs by targeting only dividing cells (Jackson et al., 2007). Mitotic kinases

such as Aurora A and Polo-Like Kinase 1 (Plk1) are frequently overexpressed in colorectal cancer, and have been implicated in adenoma to carcinoma progression (Baba et al., 2009, Sillars-Hardebol et al., 2010, Takahashi et al., 2003). Clinical trials are ongoing to investigate the efficacy of Plk inhibitors such as ON 01910 in the treatment of colorectal cancer and other solid tumours (Jimeno et al., 2008). However, severe side effects of mitotic kinase inhibitors have been discovered in Phase I and Phase II clinical trials. Several clinical trials investigating the effects of Aurora inhibitors such as AZD1152 and MLN8054 in the treatment of advanced solid tumours have been terminated due to high levels of neurotoxicity (Williams, 2009). A phase II clinical trial to investigate the effects of inhibitors of the kinesin spindle protein (KSP) on advanced or metastatic colorectal cancer is ongoing (NCT00103311).

1.2 Anti-Cancer Properties of Curcumin

1.2.1 Curcumin as a Chemopreventive and Chemotherapeutic Agent

Chemopreventive agents are defined as chemicals used to reverse, halt or prevent the process of cellular transformation (Hong and Sporn, 1997, Kelloff et al., 1997). These anti-oncogenic compounds are found naturally in plants or are synthesised, such as small molecule inhibitors. Curcumin is found in turmeric, a spice derived from the rhizome of *Curcuma Longa*, a member of the ginger family. Curcumin and other curcuminoids give turmeric its distinctive yellow colour. Curcumin has been used in traditional medicine for centuries, especially in China, India and Indonesia. Today curcumin is one of the most extensively studied chemopreventive agents. A chemotherapeutic agent is defined as any chemical used to treat disease, mainly

cancer. Chemopreventive agents generally have limited side-effects, can be taken indefinitely on a daily basis, and are non-toxic. In contrast, chemotherapeutic agents are generally used for a limited duration and can have severe and wide-ranging side effects.

Animal studies have shown that curcumin inhibits colon carcinogenesis in carcinogen-induced models of cancer (Huang et al., 1992, Rao et al., 1995, Rao et al., 1993). Curcumin was also shown to inhibit adenoma development in the Min/+ mouse, a model of FAP (Perkins et al., 2002). Phase I clinical trials investigating the effects of curcumin on patients with colorectal cancer showed that oral doses of 3.6g daily were well tolerated, and that curcumin treatment resulted in inhibition of prostaglandin E_2 (PGE_2) formation (Sharma et al., 2004, Sharma et al., 2001).

Research investigating the molecular mechanisms of the inhibition of colorectal carcinogenesis in cancer cell lines has found that curcumin treatment has pleiotropic effects on cell signalling pathways including inhibition of inflammation, angiogenesis, cell growth, metalloproteinase production, and promotion of apoptosis and cell death. Curcumin is an anti-inflammatory agent and treatment results in the inhibition of NF-κB activation and the expression of cyclooxygenase 2 (COX-2) in colorectal cancer cells (Plummer et al., 1999). Pre-treatment with curcumin can also prevent VEGF-mediated upregulation of COX-2 (Binion et al., 2008). Curcumin selectively inhibits COX-2 but not cyclooxygenase 1 (COX1), down-regulating both COX-2 mRNA (messenger ribonucleic acid) and total protein expression (Goel et al., 2001). COX-2 overexpression has been linked to colorectal carcinogenesis, and knock-out of COX-2 in mouse models of FAP protects against colorectal tumour formation (Kargman et al., 1995, Oshima et al.,

1996). Curcumin treatment mediates downregulation of COX-2 via inhibition of the transcription factor NF-κB in colorectal cancer cells (Plummer et al., 1999). The inhibition of NF-κB activation by curcumin is central to many of this agent's anti-inflammatory and pro-apoptopic properties (Aggarwal et al., 2006). Under normal conditions, the NF-κB protein is sequestered in an inactive form in the cytoplasm by the inhibitory IκB proteins. However, this process can become deregulated in cancer cells, and constitutively active NF-κB is linked to colorectal carcinogenesis (Lind et al., 2001, Sakamoto et al., 2009). NF-κB activation can lead to the increased transcription of anti-apoptopic proteins such as the Bcl-2 family member, Bcl-xL and the X-linked inhibitor of apoptosis protein (XIAP) (Chen et al., 2000, Lin et al., 2004). Treatment with curcumin can inhibit the expression of these anti-apoptopic target genes in colorectal cancer cells and results in apoptosis (Collett and Campbell, 2006). Curcumin mediated apoptosis also involves the pro-apoptopic Bcl-2 family member, Bax (Rashmi et al., 2003). In Bax knock-out colorectal cancer cells curcumin-induced activation of caspase 8, 9 and 3 was blocked, as was PARP (Poly-ADP ribose polymerase) cleavage and the release of cytochrome C from mitochondria. However, the *BAX* gene is frequently mutated in colorectal cancers with MSI, conferring a selective growth advantage to tumour cells (Ionov et al., 2000). Other mechanisms of curcumin-mediated apoptosis in colorectal cancer cells involve upregulation of death receptor expression. Curcumin treatment can upregulate total protein levels of the membrane-bound death receptor 5, contributing to tumour necrosis factor apoptosis-inducing ligand (TRAIL)-mediated cell death (Jung, 2006).

Curcumin treatment can halt the proliferation of colorectal cancer cells via cell cycle checkpoint activation, and EGFR inhibition (Chen and Xu, 2005, Chen et al., 1999, Jaiswal et al., 2002, Moragoda et al., 2001, Reddy et al., 2006, van Erk, 2004). Furthermore, curcumin has been shown to downregulate matrix metalloproteinase 3 (MMP3) in primary colorectal myofibroblasts, and to reduce TNF-α (tumour necrosis factor-α) induced upregulation of MMP3, and fibroblast growth factor-2 upregulation of MMP1 and MMP3 in a colonic subepithelial myofibroblast line (Bamba et al., 2003, Epstein et al., 2010, Yasui et al., 2004). Downregulation of cellular production of MMPs by curcumin could contribute to the inhibition of the progression and metastasis of colorectal cancer. Taken together these *in vitro* findings provide a strong rationale for the use of curcumin as a therapeutic agent in the treatment of colorectal cancer.

Further studies investigating the effects of the combination of curcumin with conventional chemotherapeutic agents in colorectal cancer cell lines provide further evidence to support the use of this agent in combination therapy. A study comparing the effects of curcumin and oxaliplatin treatment in colorectal adenocarcinoma cell lines showed that the combination significantly enhanced the anti-proliferative and pro-apoptopic effects of oxaliplatin (Howells et al., 2007). Curcumin enhanced oxaliplatin-mediated caspase 8, 9 and caspase 3/7 activity in these studies in both the HT-29 p53 mutant and HCT116 p53 wild-type lines. Additional research in these lines showed that curcumin treatment in combination with FOLFOX resulted in greater inhibition of proliferation and promotion of apoptosis than treatment with FOLFOX alone (Patel et al., 2008). The combination of curcumin and FOLFOX decreased the

expression and activating phosphorylation of EGFR and other members of this receptor family, human epidermal growth factor receptor (HER)-2, and HER-3. The growth inhibition of these cells was also linked to negative regulation of insulin-like growth factor 1 (IGF-1) by insulin-like growth factor-binding protein 3 (IGFBP3), preventing IGF-1 binding to the insulin-like growth factor receptor 1 (IGF-R1). The finding that combination treatment increased total IGFBP-3 levels was important, as low serum levels of IGFBP-3 are found in high risk colorectal adenocarcinomas (Renehan et al., 2004). IGF-R1 and EGFR signalling can activate the serine/threonine kinase Akt, increasing COX-2 expression via NF-κB expression (St-Germain et al., 2004). Western blotting analysis showed that levels of total and phospho-Akt and total COX-2 were significantly decreased in colorectal cancer cells treated with FOLFOX and curcumin (Patel et al., 2008). Similar results were found by this group when colorectal cancer cells that had survived treatment with FOLFOX alone were treated with a combination of curcumin and FOLFOX, indicating that combining curcumin with standard chemotherapeutic agents could help kill chemoresistant CRC cells (Patel et al., 2010). These findings were supported by a study investigating the effects of the combination of curcumin and FOLFOX on FOLFOX-chemoresistant CRC cells (Yu et al., 2009). CRC cells that were resistant to FOLFOX displayed higher levels of cluster of differentiation (CD) markers associated with metastasis and the CRC stem cell phenotype such as CD133, CD44 and CD166 (Dalerba et al., 2007). Treating these cells with a combination of curcumin and FOLFOX resulted in a decrease in total protein levels of these markers and concomitant reduced anchorage dependent colony formation and the disintegration of colonospheres. In summary, these investigations show that curcumin in combination with standard CRC chemotherapeutic agents can

improve the growth inhibitory and apoptopic properties of these agents and can prevent the development of chemoresistant CRC cells.

There are currently several phase I and phase II clinical trials underway testing curcumin as a chemopreventive agent. These trials are aimed at preventing the occurrence or recurrence of cancer in high risk patients, such as patients with recently resected adenomatous polyps, aberrant crypt foci, or those that are undergoing colorectal endoscopy or surgery (NCT00927485, NCT00365209, NCT00973869). Five phase I and II clinical trials testing the potential of curcumin as a chemotherapeutic agent are ongoing. Two of these trials are testing curcumin in combination with another agent, either capecitabine and radiation therapy for rectal cancer, or in combination with Ashwagandha extract for the treatment of advanced osteosarcoma (NCT00745134, NCT00689195). The other three trials are investigating the effects of curcumin on advanced pancreatic cancer, cutaneous T-cell lymphoma, or non-small cell lung cancer (NCT00094445, NCT00969085, NCT01048983).

Table four compares the relative doses of curcumin found in the diet, dietary supplements, or doses currently used in ongoing clinical trials. While there is epidemiological evidence that curcumin in the diet may contribute to lower levels of gastro-intestinal neoplasia, it is unlikely that concentrations that are generally used in colorectal cancer cell lines to investigate the anti-cancer properties of curcumin would be achievable in the gut without ingestion of tablets or sticks containing purified curcumin.

Table 4 Comparing the Relative Amounts of Curcumin Found in the Diet, Supplements, and Doses Used in Clinical Trials

Curcumin Intake	**Weight of curcumin ingested**
In cooked food	Up to 0.15g per day ((Sharma et al., 2005))
In turmeric tablets sold as a dietary supplement (Bio Health Tumeric Rhizome capsules)	One tablet up to three times per day: 0.010-0.030g
In curcumin tablets sold as a dietary supplement (Protek Tumeric caplets, Just Vitamins Ltd.)	One to two tablets daily: 0.475-0.95g
In curcumin/tumeric and piperine tablets sold as a dietary supplement (Doctor's Best Curcumin C3 Complex® with BioPerine)	Two tablets daily provides: 1.33-1.52g curcumin 100mg piperine
Curcumin tablets/sticks for clinical trials (Such as Curcumin C3 Complex® Tablets, Sabinsa Co.)	Range tested: 4-8g daily†

*Calculations based on the estimation of 2% curcumin in turmeric rhizome tablets (up to 3% curcumin can be found in the turmeric rhizome).

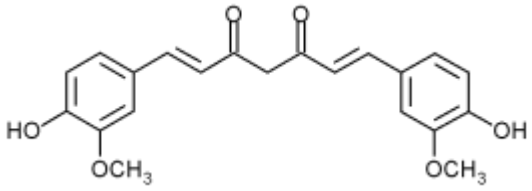
† A recently registered clinical trial NCT00696085 to test curcumin in the treatment of cutaneous T-cell lymphoma plans will start with 8g/day for the first 30 days, then increase the dose to 12g/day for months 2-6. This study is not yet open for participant recruitment.

Human clinical studies that have gathered pharmacokinetic data on the bioavailability of curcumin have shown that serum levels of curcumin usually peak 1 to 2 hours following consumption and gradually decline within 12 hours (Cheng et al., 2001). While no serum levels of curcumin could be detected after 1 hour following a daily dose of 2g curcumin per day, serums levels of curcumin were detectable following doses of 4-8g of curcumin per day (Shoba et al., 1998). Doses of 4, 6, and 8g of

curcumin per day resulted in peak serum levels of 0.51 ± 0.11, 0.63 ± 0.06, and 1.77 ± 1.87μM. Phase I clinical studies have shown that concentrations of 7.7 ± 1.8 and 12.7 ± 5.7 nmol/g have been found in colorectal tumour tissue and the normal mucosa of patients taking 3.6g curcumin a day for a week prior to surgery (Garcea et al., 2005). Yet, in this study while curcumin levels in the plasma were above the levels of detection (around 0.3nmol/L) they were below the limits of quantitation (3nmol/L).

1.2.2 Curcumin Structure and Metabolism

A



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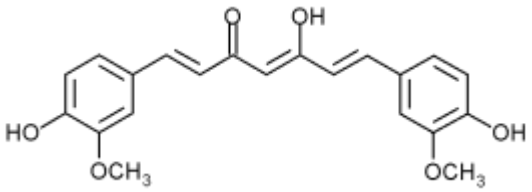


Figure 1 Chemical Structure of Curcumin

Curcumin is shown in its (A) keto and (B) enol forms. In both the solid state and in solvents such as DMSO (dimethyl sulfoxide), curcumin exists as keto-enol tautomers (Mague et al., 2004, Payton et al., 2007, Tonnesen et al., 1982).

Curcumin, 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, is a hydrophobic polyphenol. X-ray crystal structure analyses and NMR (nuclear magnetic resonance) spectroscopy have shown that in both the solid state and in solvents such as DMSO (dimethyl sulfoxide), curcumin exists as keto-enol tautomers as opposed to

β-diketone tautomers, as shown in Figure 1 (Mague et al., 2004, Payton et al., 2007, Tonnesen et al., 1982). The hydrogen binding capabilities between these two groups differ significantly, and may alter the selectivity of protein binding. Curcumin is a photosensitive compound, and degrades rapidly in light and under basic pH conditions (Lin et al., 2000, Tonnesen et al., 1986). Under acidic conditions the degradation of curcumin is much slower, permitting higher levels of stability in the gastrointestinal tract, where the pH is between 1-6 (Villegas et al., 2008, Wang et al., 1997).

The development of curcumin as a chemopreventive and chemotherapeutic agent has been hampered by the low aqueous solubility and minimal bioavailability of this compound. To combat these problems various delivery approaches are being developed including liposomal and micellar systems, phospholipid complexes, the PEGylation of curcumin, and the use of 'adjuvants' such as piperine to enhance bioavailability (Reviewed by Anand et al., 2008).

1.3 The Cell Cycle and its Checkpoints

1.3.1 The Cell Cycle

The hallmarks of cancer are defined as: self-sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, sustained angiogenesis, tissue invasion and metastasis, and limitless replicative potential (Hanahan and Weinberg, 2000). Halting the unregulated cellular proliferation or 'limitless replicative potential' of cancer cells is one of the main targets of chemotherapy.

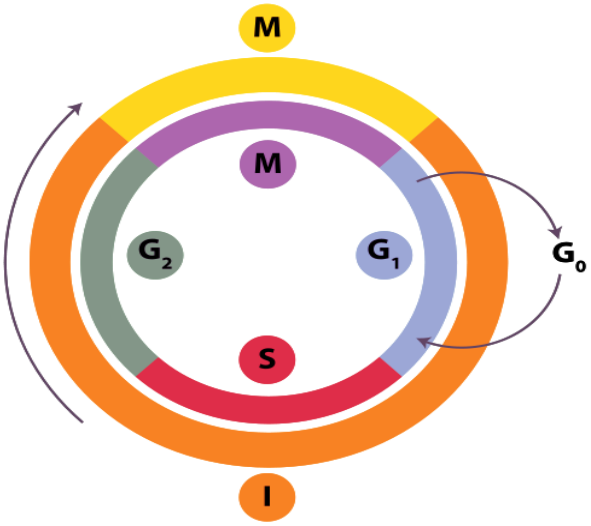


Figure 2 Stages of the Cell Cycle

The four phases of the cell cycle, G_1, S, G_2, and M phase illustrated alongside the resting or quiescent G_0 phase that lies out with the cell cycle. The term interphase describes cycling cells in phases G_1, S and G_2, and mitosis (M phase) is distinct from interphase. Waves of specific CDK activity regulate progression through the cell cycle Early G_1 is dependent on CDK4 or CDK6 and in late G_1 entry to S phase is controlled by CDK2 complexes. CDK2 and CDK1 activity regulate progression from S phase, G_2 and to early mitosis. Nuclear localisation of cyclin B-CDK1 complexes marks the start of mitosis. (Ford, 2004).

There are four phases of the cell cycle, the first gap phase, G_1, the DNA synthesis phase, S, the second gap phase, G_2 and mitosis. Cycling from one phase to the next is controlled by cyclin-dependent kinases (CDKs) and regulatory cyclin subunits (Ford, 2004). Progression in early G_1 is dependent on cyclin D-CDK4 or cyclin D-CDK6 complexes. In late G_1 entry to S phase is controlled by cyclin E-CDK2 complexes. Waves of cyclin A-CDK2 and cyclin A-CDK1 activity regulate progression from S phase, G_2 and early mitosis. Nuclear localisation of cyclin B-CDK1 complexes marks the start of mitosis. Resting, or quiescent cells are said to be in G_0. Throughout the cell cycle checkpoints exist to allow for repair to any damage incurred during division, and to prevent replication in the presence of genomic instability. DNA damage can activate

checkpoint signalling, activating checkpoint proteins p53 and p21, which can inhibit the activity of the heterodimeric CDK-cyclin complexes. Checkpoint activation temporarily prevents cell cycle progression to allow time for repair or for the apoptopic program to proceed. However, many cancers have developed faulty checkpoint signalling. Losing the ability to initiate cell cycle arrest via checkpoint proteins can have disastrous consequences for genomic integrity and can lead to mutations that cause further deregulation of the cancer cell. Chemotherapeutic agents can force cancer cells to arrest during cell division, by interfering with metabolic pathways, the DNA replication process or the assembly of the mitotic spindle. There are three main cell cycle checkpoints, two occurring at the transitional stages between the phases G_1/S, and G_2/M, and one during mitosis.

1.3.2 DNA Damage Checkpoint Signalling

The DNA damage checkpoints throughout the cell cycle are dependent on DNA damage network signalling. The main components involved in the DNA damage signalling network are outlined in Table 5. These have been classified into four main groups, sensors of DNA damage, mediators, transducers and effector proteins. DNA damage can be 'sensed' by proteins such as Rad9, Rad1 and Hus1, that bind together to form a complex (the 9-1-1 complex) with a similar structure to the proliferating cell nuclear antigen-like (PCNA-like) sliding clamp (Shiomi et al., 2002). The chromatin-binding Rad17 protein can form complexes with the replication C factor subunit (RFC) proteins, forming an RFC-related complex, which acts as a clamp loader. Following DNA damage, the 9-1-1 complex is recruited to the site of damage by the RFC-related

complex (Kondo et al., 2001, Melo et al., 2001). This facilitates phosphorylation of the chromatin-bound complexes mediated by the ataxia telangiectasia and Rad-3-related (ATR) and ataxia telangiectasia mutated (ATM) kinases. DNA damage can also be detected by ATR-interacting protein (ATRIP) binding directly to replication protein A (RPA) coated single stranded DNA complexes and the subsequent recruitment of ATR to these sites (Zou and Elledge, 2003). RPA is required both for the recruitment of ATRIP and ATR, but also for the subsequent recruitment of checkpoint kinase 1 (Chk1).

Table 5 Classification of Proteins Involved in the DNA Damage Network by Function

Function	Class	Protein
Sensors	RFC1-like	Rad17, RFC2-5
	PCNA-like	Rad9, Rad1, HUS1
Mediators	BRCT domain-containing	BRCA1, 53BP1, TopBP1, MDC1, PTIP
	DSB recognition/repair (The MRN complex)	Mre11, NBS1, Rad50
Transducers	Phosphatidylinositol 3 kinase-like kinase (PIKK)	ATR, ATM
	PIKK binding protein	ATRIP
	Protein kinase	Chk1, Chk2, Plk1
Effectors	Transcription factor	p53, p21
	Phosphatase	CDC25A, B, C
	Protein kinase	CDKs, CDC7

Adapted from (Niida and Nakanishi, 2006). Abbreviations used in this table: 53BP1, p53-binding protein 1, ATM, ataxia telangiectesia-mutated, ATR, ataxia telangiectesia and Rad3 related, ATRIP, ATR-interacting protein, BRCA1, breast cancer type 1 susceptibility protein, CDC7, cell division cycle 7, CDC25A/B/C, cell division cycle 25 A/B/C, CDK, cyclin-dependent kinase, Chk1, Checkpoint kinase 1, Chk2, Checkpoint kinase 2, DSB, double-strand break, BRCT domain, BRCA1 carboxyl-terminal domain, NBS1 Nijmegen breakage syndrome 1, Plk1, Polo-like kinase 1, PTIP, pax transactivation domain interacting protein, MDC1, mediator of DNA damage checkpoint protein 1, RCF2, retinoblastoma control factor 2, TopBP1, topo II-binding protein 1.

The DNA damage mediators play a central role in checkpoint activation and repair. Once DNA damage such as double-strand breaks has been detected, members of the phosphoinositide 3-kinase related protein kinase family (PIKK), principally ATM, phosphorylate histone H2A.X (Lukas et al., 2004, Lukas et al., 2003). This phosphorylation event acts as an epigenetic signal or 'beacon' either side of the site of the double strand break, by modifying the chromatin in the region (Rogakou et al., 1999, Stucki and Jackson, 2006). Recruitment of mediator proteins that contain phospho-peptide binding BRCA1 carboxyl-terminal (BRCT) domains, such as 53BP1, BRCA1, MDC1, NBS1, PTIP and TopBP1, to sites of DNA damage is dependent on histone H2A.X phosphorylation (Carney et al., 1998, Goldberg et al., 2003, Jowsey et al., 2004, Lou et al., 2003, Schultz et al., 2000, Scully et al., 1997, Stewart et al., 2003, Xu et al., 2004, Yamane et al., 2002). The mediator of DNA damage checkpoint protein 1 (MDC1) is the main interaction partner for phosphorylated histone H2A.X (pH2A.X), and is required for recruitment of other mediators such as NBS1, 53BP1, BRCA1 and phosphorylated ATM to foci following DNA damage (Lou et al., 2006). The MRN complex contains Mre11, Rad50 and Nbs1 and is involved in DNA repair and the transduction of DNA damage signalling, and interacts with both MDC1 and ATM at sites of pH2A.X foci (Goldberg et al., 2003, Falck et al., 2005, You et al., 2005). The MRN complex is also thought to be involved in the initial recognition and recruitment of ATM to sites of DNA damage to phosphorylate histone H2A.X (Stucki and Jackson, 2006). Upon completion of DNA repair, H2A.X is dephosphorylated, and this step is essential for DNA damage checkpoint recovery (Chowdhury et al., 2005, Keogh et al., 2006).

Signals generated by sensor proteins following DNA damage are rapidly transduced to the ATM and ATR protein kinases, members of the PIKK family (Abraham, 2001). ATM is generally activated following double-stranded DNA (dsDNA) damage, such as chromosomal breaks, and ATR is generally activated following single stranded DNA (ssDNA) damage, such as lesions generated by stalled replication forks (Harrison and Haber, 2006). However, *in vivo* both of these proteins can be activated following double-stranded breaks (DSBs), with ATM activated initially, followed by ATR activation (Jazayeri et al., 2006, Myers and Cortez, 2006). ATM is activated following the detection of blunt DSBs, and this may involve the MRN complex, or changes in chromatin structure (Dupre et al., 2006, Lee and Paull, 2005). The MRN complex may then contribute to early-stage end resection of the DNA, which is then taken over by other exonucleases and DNA helicases (Gravel et al., 2008, Mimitou and Symington, 2008, Rhind, 2009 Zhu et al., 2008). As resection proceeds during DNA repair, it creates a long overhang of ssDNA, deactivating ATM and activating ATR (Choi et al., 2007, MacDougall et al., 2007, Yoo et al., 2007).

It has been shown in yeast cells that a single DSB can induce cell cycle arrest for 12-14 hours at the G_2/M checkpoint (Lee et al., 1998). In mammalian cells, activation of the ATM and ATR kinases results in the transduction of checkpoint signalling by downstream effector proteins, including the checkpoint kinases, Chk1 and Chk2. Substrates of ATM include the transcription factor p53, the p53 regulatory protein Mdm2 (murine double minute), Chk2, BRCA1, NBS1 and Rad17 (Banin et al., 1998, Bao et al., 2001, Matsuoka et al., 2000, Maya et al., 2001, Wu et al., 2000, Xu et al., 2002). ATR works together with ATRIP, Rad17 and Hus1, to activate Chk1 via phosphorylation

following DNA damage (Cortez et al., 2001, Liu et al., 2000b, Weiss et al., 2002, Zou et al., 2002). In addition, inhibition of Plk1 occurs following DNA damage via an ATM/ATR dependent pathway and this is linked to checkpoint activation (Smits et al., 2000, van Vugt et al., 2001).

Throughout the cell cycle, the CDC25 (cell division cycle 25) protein phosphatases remove inhibitory residues on the cyclin dependent kinases (CDKs) to facilitate progression through each phase. Activation of the checkpoint kinases Chk1 and Chk2, results in negative regulation of the CDC25 phosphatases via direct inhibition, nuclear exclusion and proteolytic degradation, leading to inactivation of the CDKs and cell cycle arrest (Bartek and Lukas, 2003, Blasina et al., 1999, Falck et al., 2001, Furnari et al., 1999, Graves et al., 2000, Kastan, 2001, Lopez-Girona et al., 2001, Peng et al., 1997a, Uto et al., 2004).

1.3.3 Regulating Entry into the Cell Cycle

The availability of growth factors and hormones regulates entry into the cell cycle. During G_1 phase there is a 'restriction point' (R), and for cells to pass this regulatory point they must have reached a certain size or critical mass (Ford, 2004). Any cells that do not attain this critical mass leave the cell cycle and return to the resting phase G_0. Passing R commits cells to completing the cell cycle. The retinoblastoma protein (Rb) is essential for regulating the restriction point in the cell cycle. Rb was the first tumour suppressor, or tumour 'regulatory' gene to be described (Murphree and Benedict, 1984). The activation of the Rb protein is regulated via phosphorylation by the cyclin D-dependent kinases, CDK4 or CDK6. This in turn can be controlled by the cyclin kinase

inhibitors (CKI). The INK4 (inhibitors of CDK4) family of CKIs, including the $p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$ and $p19^{Inc4d}$ specifically inhibit CDK4 and CDK6 activity. The CIP/KIP CKI inhibitors such as $p21^{CIP1}$, $p27^{Kip1}$ and $p57^{Kip2}$ can inhibit all cyclin/CDK complexes involved in the cell cycle. The levels of growth factors can regulate the levels of CKIs during G_1. While the Rb protein is hypo-phosphorylated during early G_1, it can bind to and sequester the transcription factor E2F in the cytoplasm, preventing its nuclear translocation. E2F is required for the transcription of genes necessary for cell cycle progression, involved in DNA replication, nucleotide synthesis, CDKs and cyclins, and their regulators, the CKIs. Low levels of growth factors will maintain Rb in a hypo-phosphorylated state via CKI-mediated cyclin-CDK inhibition, retaining E2F in the cytoplasm. In contrast, growth factor sufficiency will prevent CKI inhibition of the cyclin-CDK complexes, and will result in phosphorylation and inactivation of the Rb protein, allowing E2F nuclear translocation and transcriptional activity. Inactivation of the Rb protein occurs in two stages, the initial inactivation by cyclin D-CDK4/CDK6 complexes in G_1, followed by the complete inactivation by cyclin E-CDK2 complexes in late G_1. For cells to proceed from G_1 to S phase, they must pass the 'restriction point' via the CDK-Rb-E2F pathway. A recent study has shown that colorectal adenomas and adenocarcinomas frequently overexpress the hyper-phosphorylated inactive version of the Rb protein, and this may contribute to the uncontrolled proliferation of these CRCs (Ayhan et al., 2010).

1.3.4 The G_1/S Checkpoints

The checkpoint at the boundary between G_1 and S phase allows cells to halt the cycle and repair any DNA damage prior to chromosomal replication, and prevents transmission of mutations to daughter cells. Activation of this checkpoint is largely dependent on activation of p53 by either ATM or ATR. Mdm2 is an E3 ligase that regulates p53 activation by direct binding, nuclear export of p53, and by targeting this transcription factor for proteasomal degradation via ubiquitination (Honda et al., 1997, Momand et al., 1992). Phosphorylation of p53 on residue Ser15 by the ATM and ATR kinases permits dissociation from Mdm2, preventing its proteolytic destruction (Banin et al., 1998, Canman et al., 1998, Lakin et al., 1999, Tibbetts et al., 1999). ATM and DNA-PK can also directly phosphorylate Mdm2 on residues Ser123, and Ser17 respectively, contributing to p53 release and activation (Khosravi et al., 1999, Maya et al., 2001, Mayo et al., 1997). The checkpoint kinases Chk1 and Chk2 can phosphorylate p53 on additional sites such as Ser20, Ser15 and Ser23, contributing to p53 protein stability following DNA damage (Shieh et al., 2000).

After activation, p53 is involved in the transcription of a number of genes, including the $p21^{CIP1}$ protein. This CKI is central to induction of the G_1/S checkpoint, and an important factor in the regulation of the cell cycle by p53 (Dulic et al., 1994, Eldeiry et al., 1994). Inhibition of the cyclin-cyclin dependent kinase complexes cyclin E-CDK2 during G_1/S and cyclin A-CDK2 during S phase is dependent on p53-mediated transcription of p21 (Eldeiry et al., 1994).

An alternative mechanism to prevent entry into S phase following DNA damage that is independent of ATM/ATR or checkpoint kinase signalling involves a protein encoded

by cell division cycle 10 (CDC10)-dependent transcript 1 (CDT1) (Higa et al., 2003). CDT1 is part of the pre-replication complex that is required to licence DNA origins prior to DNA synthesis (Nishitani et al., 2000). The pre-replication complex (pre-RC) is comprised of the six subunit origin of recognition complex (ORC, subunits ORC1-6), origin loading factors CDC6/CDC18 (cell division cycle 6/18) and CDT1, and the mini-chromosome maintenance proteins (MCM, MCM2-MCM7) (Diffley, 1996). If DNA damage occurs in G_1, the cullin 4-ROC1 E3 ligase and the Cop9 signalosome target CDT1 for proteolytic destruction, thus inactivating the pre-replication complex and preventing entry into S phase (Higa et al., 2003).

Once cells have progressed to S phase, checkpoint activation can be triggered via DNA damage or disruption of DNA synthesis (Li and Zou, 2005). Detection of DNA lesions via stalled replication forks can occur during S phase, and cell cycle arrest permits time for repair of damage. Cells lacking a functional S phase checkpoint undergo radioresistant DNA synthesis (RDS) following exposure to ionising radiation, and cells isolated from patients with ataxia telangiectasia, Nijmegen-breakage syndrome, ataxia telangiectasia-like disorder and Fanconi anemia exhibit RDS (Painter, 1981, Stewart et al., 1999, Taniguchi et al., 2002, Young and Painter, 1989). The activity of CDC25A is required to remove inhibitory residues from cyclin E-CDK2 or cyclin A-CDK2 for S phase progression and inhibition of this phosphatase by Chk1 or Chk2 after DNA damage results in S phase arrest (Falck et al., 2001, Sorensen et al., 2003, Zhao et al., 2002). Halting the activity of CDK2 in S phase impairs loading of the Cdc45 origin binding factor onto chromatin, which is required for origin unwinding and the

recruitment of DNA polymerases to the pre-RC during chromosomal replication (Walter and Newport, 2000).

Downstream targets of ATM also include BRCA1, NBS1, and SMC1 during S phase arrest, contributing to checkpoint activation (Kim et al., 2002, Lim et al., 2000, Wu et al., 2000, Xu et al., 2002). While the mechanisms are not fully understood, SMC1 can interact with BRCA1, and BRCA1 is known to bind the MRN complex containing the NBS1 protein (Antoccia et al., 2008, Yazdi et al., 2002, Zhong et al., 1999). It has been proposed that the MRN complex may serve to stabilise replication forks, and BRCA1 may localise to these MRN complexes to prevent end processing during S phase arrest (Durant and Nickoloff, 2005).

1.3.5 The DNA Damage G_2/M Checkpoint

The G_2/M checkpoint ensures that any damage or errors accumulated during the DNA replication process have been repaired prior to mitosis, and the main signalling pathways involved are outlined in Figure 3. For a cell in late G_2 to enter mitosis activation of the cyclin B-CDK1 complex, or mitosis promoting factor (MPF) is required, and induction of the G_2/M checkpoint is ultimately dependent on inactivation of these cyclin-CDK complexes prior to mitosis (Gould and Nurse, 1989). The CDC25C phosphatase activates cyclin B-CDK1 complexes by removing inhibitory phosphate residues on Tyr14 and Tyr15 on CDK1 (Gautier et al., 1991, Millar et al., 1991, Smythe and Newport, 1992, Solomon et al., 1992). Phosphorylation of an additional regulatory site, Thr161, is required for the optimal catalytic activity of CDK1 (Krek and Nigg, 1991, Norbury et al., 1991, Solomon et al., 1992).

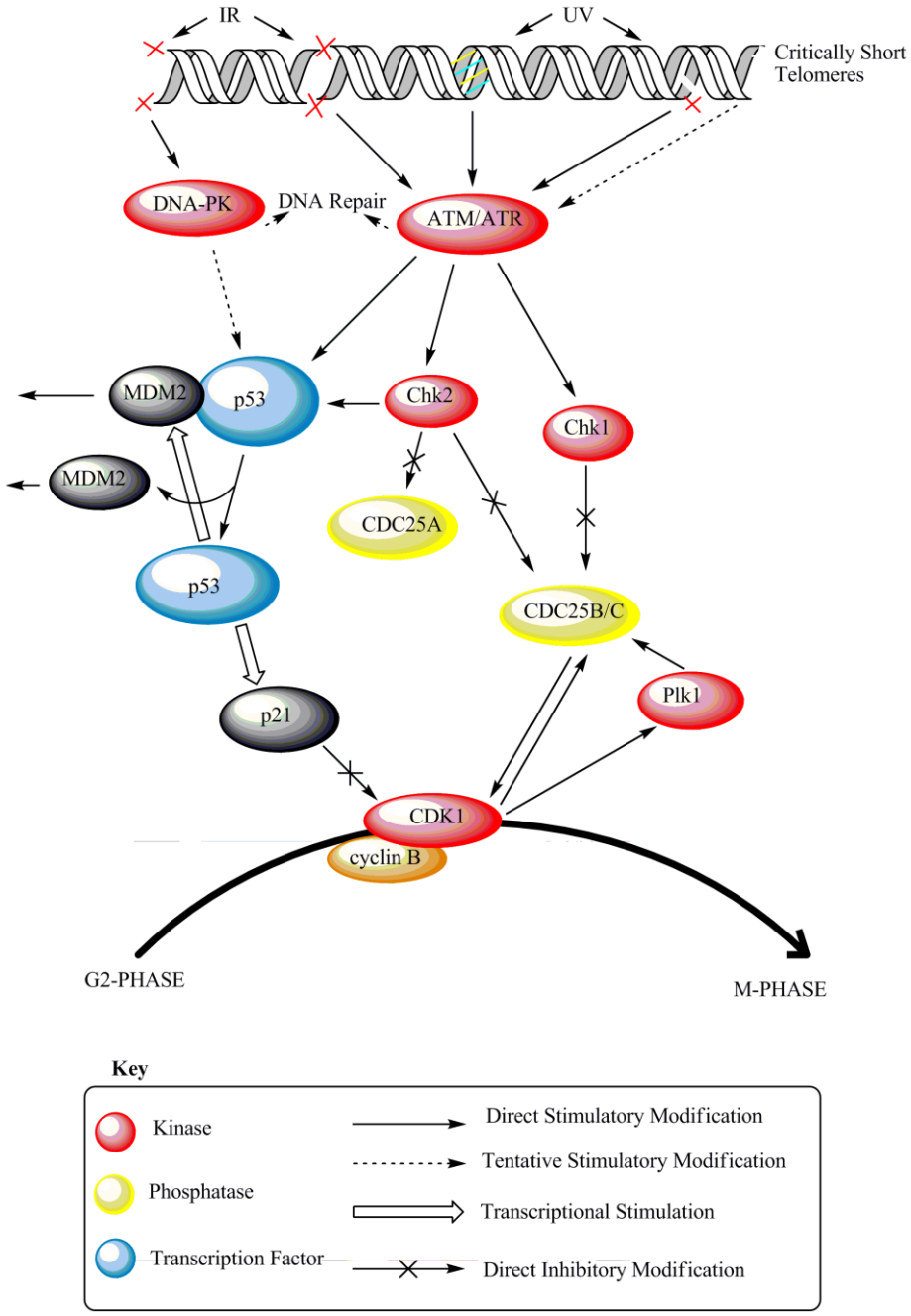


Figure 3 DNA Damage Signalling at the G_2/M Checkpoint

Genotoxic stress induced by irradiation (IR), ultraviolet light (UV) and critically short telomeres can result in DNA damage. The ATM/ATR proximal kinases and DNA-PK relay DNA damage signals detected by sensor molecules to downstream kinases such as Chk1 and Chk2. The Chk kinases can modulate the activity of p53 and p21, and can alter the activity, localisation, and proteasomal degradation of the CDC25 phosphatases. DNA damage signalling ultimately leads to G_2/M arrest by inhibition of CDK-cyclin complexes (Adapted from Cell Signaling).

If Chk1 is activated by ATR, it can negatively regulate CDC25C via phosphorylation on Ser216, leading to nuclear export by the 14-3-3 protein, reduced levels of active cyclin B-CDK1 complexes, and checkpoint arrest (Lopez-Girona, 1999, Peng et al., 1997b). The ATR-Chk1-CDC25C signalling pathway is one of the central mechanisms for G_2/M checkpoint activation. However ATM-Chk2-CDC25C signalling may act tandem with this pathway to contribute to arrest (Li and Zou, 2005, Matsuoka et al., 2000).

The CDC25A and CDC25B phosphatases also contribute to the G_2/M transition, and regulate the assembly of cyclin B-CDK1 complexes (Timofeev et al., 2010). Inhibition of CDC25B blocks cells in late G_2, and it has been proposed that this protein may act as a 'starter phosphatase' to initiate activation of pools of CDC25C and cyclin B-CDK1 complexes required for entry into mitosis (Lammer et al., 1998). Similarly, knock-down of CDC25A via RNA interference (siRNA) resulted in a delay in both G_1/S and G_2/M transitions, showing that this phosphatase is involved in regulating the activity of both the cyclin E-CDK2 and cyclin B-CDK1 complexes respectively (Mailand et al., 2002).

Wee1 and MYT1 (myelin transcription factor 1) can negatively regulate the activity of CDK1 during late G_2 via inhibitory phosphorylation of residues Thr14 and Tyr15, and retention of CDK1 in the cytoplasm (Heald et al., 1993, Liu et al., 1997, Liu et al., 1999, McGowan and Russell, 1993, Mueller et al., 1995, Parker et al., 1992, Parker and Piwnica-Worms, 1992). Activity of the Wee1 protein is required to induce G_2/M arrest following irradiation in yeast cells, and in yeast and *Xenopus* systems Chk1 can positively regulate Wee1 (Lee et al., 2001, Oconnell et al., 1997, Raleigh and O'Connell, 2000, Rowley et al., 1992). In humans, activation of BRCA following DNA damage leads to Chk1 activation, and increased levels of the Wee1 kinase,

contributing to G_2/M arrest (Yarden et al., 2002). Further studies have shown that siRNA knock down of Chk1, MYT1 or Wee1 reduces the number of cells undergoing G_2/M arrest following DNA damage (Wang et al., 2004). The mitotic kinase Plk1 has many roles in the G_2/M transition, including the regulation of CDC25B nuclear localisation to initiate activation of cyclin B-CDK1 complexes and CDC25C (Lobjois et al., 2009). Plk1 controls Wee1 destruction and Chk2 inactivation at the onset of mitosis following DNA damage induced arrest, and can negatively regulate the activity of MYT1 during mitosis to ensure the optimal activity of the MPF (Nakajima et al., 2003, van Vugt et al., 2010, van Vugt et al., 2004). The activity of Plk1 is reduced following DNA damage at the G_2/M checkpoint and during mitosis (Jang et al., 2007, Lee et al., 2010, Smits et al., 2000). Moreover, ATM/BRCA1/Chk1 signalling following DNA damage leads to reduced transcription of Plk1 and contributes to G_2/M arrest (Ree et al., 2003).

Research investigating DNA damage-induced arrest has shown that while G_1/S checkpoint arrest is abrogated in cells lacking the p21 or p53 proteins, G_2/M arrest can still occur, but the duration of this arrest is reduced (Brugarolas et al., 1995, Bunz et al., 1998, Deng et al., 1995, Fan et al., 1995, Waldman et al., 1995). Cells lacking p53 were able to sustain G_2/M arrest for a longer duration than those lacking p21 (Bunz et al., 1998). It was proposed that this was largely due to the ability of p21 to inactivate cyclin B-CDK1 complexes, even if the inhibition of CDK1 is less powerful than the inhibition of other CDKs such as CDK2, CDK4 and CDK6 (Harper et al., 1995, Xiong et al., 1993). While p53 is the main transcriptional regulator for p21 following DNA damage, there are other transcriptional regulators such as the SMAD proteins and Sp1

that may be active in cells lacking p53, thus permitting these cells to sustain G_2/M arrest for longer than those lacking p21 (Macleod et al., 1995, Michieli et al., 1994, Moustakas and Kardassis, 1998).

G_2/M checkpoint induction was shown to be dependent on Rad17 activation following DNA damage (Bao et al., 2001). Later studies have shown that complexes containing Rad17, such as the Rad17-Rfc2-5 complexes, and the 9-1-1 complex are essential for the detection of certain types of DNA damage and the subsequent activation of the proximal kinase ATR (Delacroix et al., 2007, Parrilla-Castellar et al., 2004, Zou et al., 2003).

1.3.6 The Antephase and Decatenation Checkpoints

Antephase is referred to as the period in late G_2, immediately prior to chromosome condensation at the onset of mitosis (Sluder, 2004). Others have referred to this stage as early prophase, where initial chromosome condensation may be visible, but cyclin B-CDK1 complexes are still cytoplasmic, the nuclear envelope is intact, and the cell is not yet 'fully committed' to mitosis (Mikhailov et al., 2005, Mikhailov et al., 2004). The 'antephase' checkpoint has been proposed to occur immediately prior to mitosis and is viewed as being distinct from the DNA damage signalling-mediated G_2/M checkpoint (Chin and Yeong, 2010, Matsusaka and Pines, 2004). Key proteins involved in antephase arrest are the 'checkpoint with FHA (forkhead-associated) and RING (really interesting new gene)-finger domains' (CHFR) protein and the p38 kinase (Scolnick and Halazonetis, 2000). Antephase arrest has been associated with the exclusion of cyclin B from the nucleus and the proteolytic destruction of Plk1 by the ubiquitin ligase CHFR

(Kang et al., 2002, Summers et al., 2005). This ensures the inactivation of cyclin-CDK complexes and prevents entry into mitosis. The process by which antephase checkpoint signalling is initiated remains unclear, but studies have shown that this is independent of ATM signalling (Mikhailov et al., 2004). Exposure of cells to spindle poisons, topo II poisons and histone deacetylase inhibitors can arrest cells in antephase (Chaturvedi et al., 2002, Matsusaka and Pines, 2004, Mikhailov et al., 2004, Scolnick and Halazonetis, 2000).

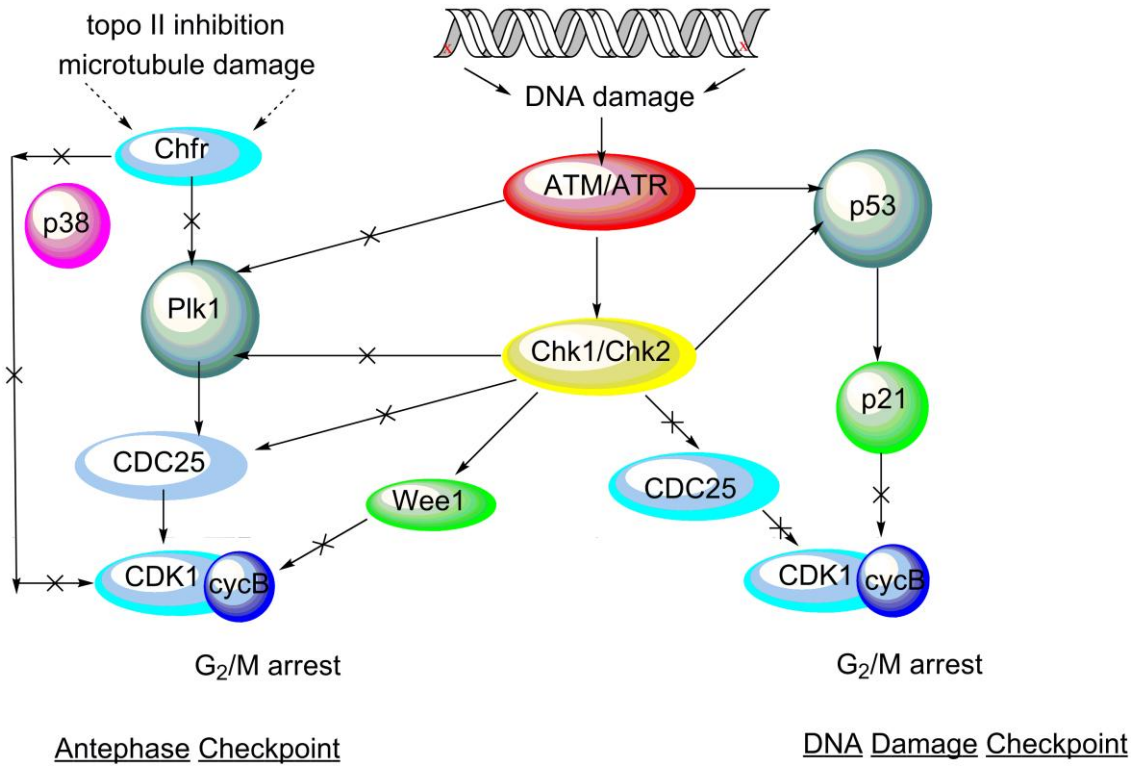


Figure 4 Antephase and DNA Damage G_2/M Checkpoints

The antephase and DNA damage checkpoints both occur at the G_2/M boundary but have distinct mechanisms of signalling. While the ATM/ATR kinases can act upon proteins involved in the antephase checkpoint, their activity is not necessary for activation, instead, signalling is dependent upon p38. In contrast, activation of the DNA damage checkpoint at the G_2/M boundary is dependent upon ATM/ATR signalling.

During interphase, sister chromatid entanglements and nonreplicative catenations can be acquired, and effective decatenation or disentanglement must take place to ensure correct chromosome segregation in mitosis (Gimenez-Abian et al., 2000). Topo II enzymes act to decatenate chromosomes by transiently breaking one DNA double-helix to allow another DNA double-helix to pass through, and then resealing the break (Damelin and Bestor, 2007). Studies have revealed a DNA damage-independent 'decatenation checkpoint' in late G_2, triggered by decatenation failure, for example as a result of the catalytic inhibition of the topo II enzymes (Downes et al., 1994). Further studies are required to elucidate whether G_2 delay as a result of the inhibition of decatenation is a result of the antephase checkpoint that is triggered by stresses such as aberrant chromatin topology and spindle damage (Damelin and Bestor, 2007).

1.3.7 Mitosis and the Spindle Assembly Checkpoint

Mitosis can be divided into six stages: prophase, prometaphase, metaphase, anaphase, telophase and cytokinesis (Sluder, 2004). The central checkpoint during mitosis is the spindle assembly checkpoint (SAC). Failure of chromosomes to attach to the mitotic spindle correctly can activate the SAC and this prevents the metaphase to anaphase transition (Musacchio and Salmon, 2007). The cyclin-CDKs that drive mitosis are cyclin B-CDK1 complexes, or cyclin A-CDK complexes, which are also known as the MPF. Early studies have shown that histone H1 is a target of the MPF (Arion et al., 1988, Labbe et al., 1988). More recent studies have shown that histone H3 is a target of the mitotic kinase Aurora B (Adams et al., 2001, Giet and Glover, 2001, Goto et al., 2002). The phosphorylation of both histones H1 and H3 during mitosis is linked to the

regulation of chromosomal condensation and dynamic mobility of chromatin (Contreras et al., 2003, Goto et al., 1999, Gurley et al., 1978, Paulson and Taylor, 1982, Wei et al., 1999). The highest order of chromosome condensation and folding within the cell cycle peaks during metaphase (Li et al., 1998, Perez-Cadahia et al., 2010).

In early prophase cyclin-CDK complexes are activated in the cytoplasm, and it is thought that cells are irreversibly 'committed' to mitosis once these active complexes enter the nucleus (Mikhailov et al., 2005). The translocation of these complexes to the nucleus is linked to the phosphorylation and subsequent masking of a cytoplasmic retention signal on cyclin B (Li et al., 1997). Nuclear envelope breakdown is estimated to begin around 10 minutes after the nuclear import of the MPF, and the proteolytic destruction of cyclin A begins at this point (Furuno et al., 1999). Asters of microtubules form around the centrosomes or 'spindle poles', and depending on the cell line, separation of the centrosomes can be seen by late prophase (Sluder, 2004). The separation of the centrosomes is a complex process involving force-generating mechanisms generated by microtubule dynamics (Brust-Mascher and Scholey, 2002, Sharp et al., 2000, Whitehead et al., 1996). The MPF may contribute to the nucleation of microtubules around the spindle poles, and the phosphorylation of centrosomal proteins (Nakamura et al., 2010, Vandre et al., 1984).

Nuclear envelope breakdown involves the dispersal of nucleoli and takes approximately 1-2 minutes, marking the beginning of prometaphase (Sluder, 2004). During prometaphase cells must assemble a bipolar mitotic spindle, chromosomes must attach correctly to the mitotic spindle and align correctly at the spindle equator. Failure to do so can result in activation of the spindle assembly checkpoint in

metaphase, and if this checkpoint is compromised, aberrant mitosis will occur (Ho et al., 2008, Perera et al., 2007, Rieder et al., 1994). Spindle polarity is achieved by the formation of radial asters of microtubules following the separation of the centrosomes, and cells with one centrosome form monopolar spindles, and those with more than two form multipolar spindles (Bajer, 1982, Heneen, 1975, Sluder et al., 1997, Sluder and Begg, 1985). In addition, failure to separate centrosomes correctly can result in the formation of a monopolar spindle (Lim et al., 2009). Capture of microtubules by chromosomes is mediated by kinetochores located on each side of the centromeric region of the chromosome (Rieder, 1982). Attachment occurs sequentially with one kinetochore joining astral microtubules first (mono-orientated chromosome) followed by its 'sister' kinetochore (bi-orientated chromosome) (Rieder and Alexander, 1990, Rieder et al., 1994). Microtubules that are attached to chromosomes in this fashion form the 'kinetochore fibres' of the mitotic spindle and permit chromosomal alignment on the spindle equator to form the metaphase plate (Compton, 2006, McEwen et al., 1997). The nuclear mitotic apparatus protein (NuMA) is important for this process and is thought to help stabilise the kinetochore fibres of the mitotic spindle (Haren et al., 2009).

The mitotic checkpoint is controlled by the mitotic checkpoint complex (MCC) which is comprised of the CDC20 (cell division cycle 20), BubR1, Bub3 and MAD2 (mitotic arrest deficient 2) proteins (Sudakin et al., 2001). The MCC 'senses' chromosomes that are not correctly aligned on the metaphase plate, and initiates checkpoint signalling that results in cell cycle arrest (Braastad et al., 2004). One unattached kinetochore is sufficient to activate the SAC (Rieder et al., 1995). The MCC activates the SAC by

regulating the activity of the anaphase promoting complex/cyclosome (APC/C) (Morgan, 1999, Zachariae and Nasmyth, 1999, Zich and Hardwick, 2009). The APC/C is an E3 ligase that coordinates the timely destruction of proteins via proteasomal degradation during mitosis (Gmachl et al., 2000). Targets of the APC/C during mitosis include securin, which is required to maintain the cohesion of sister chromatids, cyclin B, and other mitotic kinases such as Plk1, Aurora A and Aurora B (Castro et al., 2005, Eckerdt and Strebhardt, 2006, Honda et al., 2000, Littlepage, 2002, Stewart and Fang, 2005). The mitotic cyclin-CDK complexes are also the targets of the APC and their destruction is required for mitotic exit (Gmachl et al., 2000, Sudakin et al., 1995). The CDC20 protein is the 'initiator' of APC/C activity and is inhibited by the SAC proteins throughout mitosis until kinetochore attachment is complete (Hwang et al., 1998, Sudakin et al., 2001). Structural analysis has revealed that the SAC proteins act by hiding the CDC20 binding site on the APC/C and locking the APC/C into a 'closed' state to prevent the ubiquitination of target proteins (Herzog et al., 2009).

Once kinetochore attachment is completed APC/C is activated and destruction of cyclin B and securin begins (Clute and Pines, 1999, Hagting et al., 2002). This is followed by sister chromatid separation, which marks the beginning of anaphase. The sister chromatids then move towards each spindle pole, and the distance between the spindle poles increases (Sluder, 2004, Waters et al., 1993). Inactivation of CDK1 by the APC/C is required for this process to be completed correctly and to successfully exit mitosis (Wheatley et al., 1997, van Zon et al., 2010)}.

Telophase and cytokinesis are the final stages of mitosis. Once chromosomes have been drawn to opposing spindle poles, nuclear envelopes reform around the

separated chromosomes. Following this a cleavage furrow in the centre of the spindle can be seen, a result of activation of the cleavage apparatus which is comprised of a contractile ring of actin-myosin around the circumference of the mitotic spindle (Fishkind and Wang, 1993, Oegema and Mitchison, 1997). Contraction of the actin-myosin ring culminates in severance of the microtubules between the two daughter cells, and membrane trafficking and fusion to the abscission site completes mitosis (Barr and Gruneberg, 2007, O'Halloran, 2000, Skop et al., 2001).

1.3.8 Regulation of Mitosis by the Chromosomal Passenger Complex

While the cyclin A-CDK1 and cyclin B-CDK1 complexes are crucial for entry and commitment to mitosis, the chromosomal passenger complex (CPC) is required to regulate complex mitotic processes (Ruchaud et al., 2007). The CPC is comprised of four core units, the Aurora B kinase and three non-enymatic subunits, inner centromere protein (INCENP), survivin and borealin (Adams et al., 2000, Cooke et al., 1987, Gassmann et al., 2004, Honda et al., 2003, Klein et al., 2006, Sampath et al., 2004, Terada et al., 1998). The CPC is dynamic throughout mitosis and regulates key events by localising at the chromosome arms and centromeres in prophase, the centromeres in prometaphase and metaphase, the spindle midbody in anaphase and at the cleavage furrow in telophase (Earnshaw and Bernat, 1991, Ruchaud et al., 2007).

In late G_2 the CPC member Aurora B begins to phosphorylate histone H3 near the centromeres, this phosphorylation spreads over the chromosome during condensation in prophase and is considered to be a hallmark of Aurora B activity

(Crosio et al., 2002, Giet and Glover, 2001, Hsu et al., 2000, Monier et al., 2007, Murnion et al., 2001). Studies have shown that the CPC is also important in regulating mitotic spindle formation, via inhibitory phosphorylation of microtubule depolymerases such as the mitotic centromere-associated kinesin (MCAK) and Kif2a (Knowlton et al., 2009, Moore and Wordeman, 2004). The CPC play a role in regulating kinetochore attachment to the mitotic spindle, and can 'sense' aberrant tension from incorrectly attached kinetochores, and may act to release these attachments by interactions with MCAK (Andrews, 2004, Knowlton et al., 2006, Zhang et al., 2007b). The CPC can also regulate kinetochore attachments via phosphorylation of the KMN complex which is comprised of KNL-1 (kinetochore NuL-1), the Mis12 complex and HEC-1 (highly expressed in cancer-1) (Cheeseman et al., 2006, Welburn et al., 2010).

Chromosomes that are correctly orientated on the mitotic spindle with one attachment on each kinetochore to microtubules emanating from opposing spindle poles are said to be amphitelic or bi-orientated. A syntelic attachment can occur when both kinetochores are connected to microtubules emanating from the same spindle pole. Merotelic attachments arise when one of the kinetochores forms attachments to microtubules from both spindle poles. Many of these attachments can occur during prometaphase, and the CPC can correct these attachments through its microtubule destabilising ability (Vader et al., 2008). In addition, the CPC can serve to stabilise the SAC until all chromosomes have established amphitelic attachments, and relocation of the CPC from centromeres to the spindle midbody during anaphase may assist deactivation of the SAC (Hauf et al., 2003, Morrow et al., 2005, Vazquez-Novelle and Petronczki, 2010).

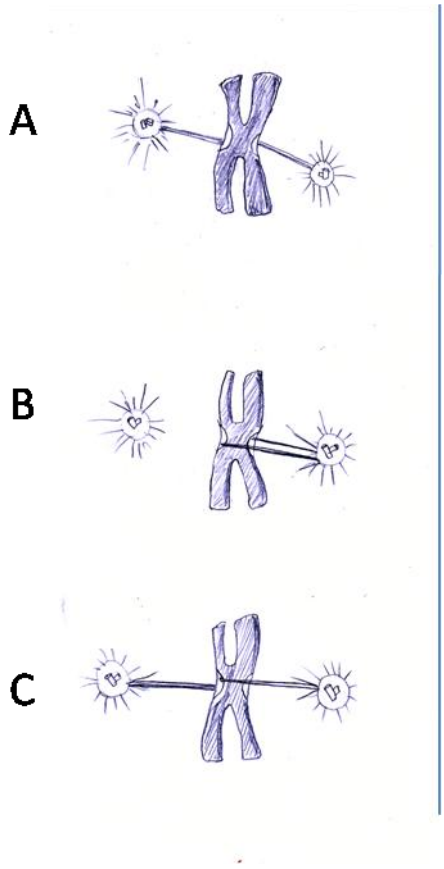


Figure 5 Chromosomal Attachment to the Mitotic Spindle

Chromosomes that have formed correct attachments to the mitotic spindle are referred to as amphitelic (A). These bi-orientated attachments are required for accurate chromosome separation during mitosis. Incorrect attachments can occur during kinetochore capture in prometaphase and must be corrected to satisfy the spindle checkpoint. Syntelic attachments arise when both kinetochores on one chromosome bind to microtubules emanating from one spindle pole (B). When one kinetochore forms attachments to microtubules emanating from both spindle poles, these are referred to as merotelic attachments (C). The CPC is involved in correcting syntelic and merotelic attachments during prophase and does so by 'sensing' abnormal tension at kinetochores and modifying the stability of microtubule attachments accordingly. Diagram adapted from (Ruchaud et al., 2007).

For chromosomes to have separated correctly during anaphase, the SAC must have been inactivated for cleavage of cohesin, and sister chromatids must be have been

fully decatenated by topo II (Wang et al., 2010). While it remains unclear what role the CPC may play in regulating decatenation of sister chromatids, a link between topo II and Aurora B activity has been shown (Coelho et al., 2008). The activity of the CPC is also important for cytokinesis, and it has been proposed that the Aurora B-regulated NoCut or abscission checkpoint ensures that cytokinesis only occurs after chromosomes have separated beyond the midbody, and no chromatin can be 'sensed' in the zone of cleavage (Mendoza et al., 2009, Norden et al., 2006, Steigemann et al., 2009).

1.4 Epithelial Cell Expression of NKG2D Ligands in Colorectal Cancer

1.4.1 An Introduction to the NKG2DL

NKG2D ligands (NKG2DL) are expressed on the epithelial cell surface, and play a key role in immune cell-mediated lysis of transformed and infected CRC cells (Ebert and Groh, 2008). The NKG2DL are a group of damage-associated ligands that are upregulated on epithelial cells during conditions of stress (Eagle and Trowsdale, 2007). Known NKG2DL include MICA-B (MHC Class I polypeptide related sequence A and B), and ULBP1-3 (UL-16 binding proteins) (Gonzalez et al., 2008). NKG2DL are recognised by the activating immune receptor NKG2D, which is expressed by natural killer cells (NK cells), natural killer T cells (NKT cells), γδ T cells and αβ CD8 T cells, and engagement can result in immune cell activation and epithelial cell lysis (Ebert and Groh, 2008, Raulet, 2004).

The importance of these ligands in anti-tumour immunity has been highlighted by a study showing that NKG2D deficient mice are defective in tumour surveillance in models of spontaneous malignancy (Guerra et al., 2008). NKG2DL are induced at an early stage of tumorigenesis and are said to be an intrinsic sensor of oncogenic transformation (Unni, 2008).

1.4.2 Chemotherapy and the NKG2DL

Upregulation of NKG2DL has been linked to activation of the DNA damage pathway in an ATM and Chk1 –dependent manner (Gasser et al., 2005, Soriani et al., 2009). Radiation and treatment with conventional chemotherapeutics such as doxorubicin, melphalan, and the proteasomal inhibitor bortezomib have been shown to increase cell surface expression of NKG2DL such as MICA/MICB, and ULBP1-3 in cancer cells (Armeanu et al., 2008, Butler et al., 2009, Kim et al., 2006, Soriani et al., 2009). Of note, the highest levels of MICB cell surface expression were found in cells undergoing G_2/M arrest following treatment with doxorubicin and melphalan (Soriani et al., 2009).

1.5 Introduction to Cell Lines Used in This Study

1.5.1 MIN and CIN CRC Lines

One of the cell lines used in the study, the HCT116 colorectal cancer cell line has been defined as presenting with MIN. However, due to chromosomal imbalances revealed by spectral karyotyping it is possible that this line is a rare MIN positive and CIN positive cell line (Mohr and Illmer, 2005). A more recent study has shown that this

near diploid line shares more similarities with MIN^+ cell lines, and this line will be referred to as MIN positive only (Knutsen et al., 2010). Two aneuploid colorectal cell lines with more complex karyotypes, the HT-29 and SW480 lines were also analysed in this study. The HT-29 and SW480 lines have high levels of chromosomal aberrations and are CIN^+.

1.5.2 p21 and p53 knock-out CRC Lines

As previous research on colorectal cancer cell lines in this lab has highlighted the activation of proteins in the DNA damage response network following curcumin treatment, the role of the downstream effectors of this network, p21 and p53, in maintaining G_2/M arrest was studied using the HCT116 $p21^{-/-}$, and HCT116 $p53^{-/-}$ knock-out lines (Bunz et al., 1998).

The p53 protein plays a key role in regulating G_2/M arrest, and can also mediate G_1/S phase arrest following DNA damage. While p21 is known to be central in the G_1/S checkpoint, previous studies on the HCT116 cell line have illustrated the importance of both p21 and p53 in sustaining G_2/M arrest following DNA damage (Bunz et al., 1998). It has been shown in human cancer cells that those lacking either the p21 or p53 proteins can arrest in G_2 following DNA damage, and it has been postulated that this is due to the integrity of the Chk1-CDC25C-CDK1 pathway (Bunz et al., 1998).

1.6 Aims and Objectives

1.6.1 Characterisation of Curcumin-Induced G_2/M Arrest

While many studies have been performed investigating the levels of G_2/M arrest, or the effects of curcumin on the mitotic spindle, comprehensive studies investigating both G_2/M boundary and M phase arrest in colorectal cancer have not been performed. The goal of this first part of the study was to establish levels of both G_2/M boundary arrest and M phase arrest using a combination of FACS cell cycle analysis and fluorescence microscopy in the five CRC lines. The levels of cell cycle arrest were investigated over a range of 12-72 hours, so that the treatment duration that resulted in the highest levels of curcumin-induced G_2/M boundary and M phase arrest in these lines could be ascertained. The panel of CRC cell lines was selected to facilitate comparisons regarding the importance of mismatch repair function, genomic stability, and p21 and p53 status on curcumin-induced G_2/M and M cell cycle arrest.

1.6.2 Characterisation of Curcumin-Induced Mitotic Arrest

The goals of this chapter were to further characterise curcumin-induced mitotic arrest in the panel of CRC cell lines. Previous studies have documented the effects of curcumin on the mitotic spindle, but these have failed to provide quantification and statistical analysis of the effects of curcumin on mitotic spindle formation, centrosomal separation, and chromosomal bridge formation. To elucidate whether curcumin arrested cells at a specific stage of mitosis quantification of the numbers of cells in each stage was performed using fluorescence microscopy. The effects of curcumin on chromosome alignment in the early stages of mitosis were investigated using immunofluorescence techniques. To discover whether curcumin treatment

affected the localisation of the mitotic kinase Aurora B, image analysis of control and curcumin-treated cells was carried out.

1.6.3 Mechanisms of Curcumin-Induced Cell Cycle Arrest

To elucidate whether CRC cells undergoing M phase arrest had DNA damage they were stained with pH3 and pH2A.X antibodies and analysed using immunofluorescence microscopy. To find out whether inhibitors of DNA damage signalling such as caffeine or debromohymenialdisine (DBH) affected levels of curcumin-induced G_2/M boundary and M phase arrest both FACS cell cycle analysis and fluorescence microscopy experiments were carried out. To investigate whether curcumin treatment affected the total protein levels of the CDC25C phophatase Western blotting experiments were performed. Levels of activating phosphorylation of the Chk1 kinase were also investigated using this technique. These experiments were also repeated in the presence of the DNA damage signalling inhibitor caffeine.

1.6.4 The Effects of Curcumin on NKG2DL Expression

Curcumin has previously been shown to activate DNA damage signalling and the heat shock response in cancer cells. To investigate whether curcumin treatment could alter the expression of the NKG2DL via heat-shock and DNA damage signalling-dependent mechanisms in these CRC lines, FACS analysis experiments were carried out with a range of positive controls including hydroxyurea, etoposide and nocodazole. The cell surface expression of NKG2DL studied included MICA/MICB, ULBP1, ULBP2, and ULBP3.

Chapter 2 Materials and Methods

2.1 Antibodies

2.1.1 Primary Antibody Details

Table 6 List of Primary Antibodies

Antibody Target	Antibody Codes	Company	Source
α-Tubulin (T-02)	Sc-8035	Santa Cruz	Mouse mAb
α-Tubulin	T5168	Sigma-Aldrich	Mouse mAb
Actin (1-19)-R	Sc-1616-R	Santa Cruz	Rabbit pAb
Aurora B	Ab2254	Abcam	Rabbit pAb
CDC25C (H6)	Sc-13138	Santa Cruz	Mouse mAb
Centrin	Sc-27793-R	Santa Cruz	Rabbit pAb
HSP70/HSC70	SPA-820	Stressgen	Mouse mAb (IgG_1)
Isotype Control	M5284	Sigma-Aldrich	Mouse mAb (IgG1)
Isotype Control	M5409	Sigma-Aldrich	Mouse mAb (IgG2a)
MICA/MICB	BAMO1	BAMOMAB	Mouse mAb (IgG1)
pChk1 (345)	2341	Cell Signaling	Rabbit mAb
pH3 (Ser10)	9706	Cell Signaling	Mouse mAb (IgG1)
pH3(Ser10) (D2C8)	3377	Cell Signaling	Rabbit mAb
pH2A.X (Ser139)	05-636	Millipore	Mouse mAb
ULBP1	AUMO2	BAMOMAB	Mouse IgG2a
ULBP2	BUMO1	BAMOMAB	Mouse IgG1
ULBP3	CUMO3	BAMOMAB	Mouse IgG1

List of polyclonal (pAb) and monoclonal (mAb) antibodies used in all experiments with product code, company name, the animal they were raised in. Details of antibody isotype are included for those used in FACS analysis experiments

2.1.2 Secondary Antibody Details

Table 7 List of Secondary Antibodies

Antibody Target	Antibody Codes	Company	Source
Anti-rabbit IRDye 680 CW (Red)	926-32221	LI-COR Biosciences	Goat
Anti-mouse IRDye 800 CW (Green)	926-32210	LI-COR Biosciences	Goat
Anti-mouse Alexa Fluor 488	A11001	Invitrogen	Goat
Anti-mouse Alexa Fluor 680	A10038	Invitrogen	Donkey
Anti-rabbit Alexa Fluor 594	A11012	Invitrogen	Goat
Anti-mouse fluorescein isothiocyanate (FITC) conjugated	F0257	Sigma-Aldrich	Goat

List of secondary antibodies used in Western blotting, FACS, and immunofluorescence experiments.

2.2 Dyes and Molecular Weight Markers

2.2.1 On-Cell Western Stains

The Sapphire700™ stain was used as a nuclear/cytoplasmic dye in the OnCell Western assay (LI-COR Biosciences).

2.2.2 Western Blotting

The molecular weight marker used in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels was the PageRuler™ (SM0671) Prestained Protein Ladder (Fermentas).

2.2.3 FACS Analysis

PI (PI), and diamidino-2-phenylindole (DAPI) dilactate (both Sigma-Aldrich) were used as nuclear dyes in FACS experiments.

2.2.4 Immunofluorescence Microscopy

DAPI dilactate or Hoescht 33342 (Sigma-Aldrich) were used to stain DNA in immunofluorescence experiments.

2.3 Buffers, Solutions and Other Reagents

2.3.1 Buffers for Western Blotting

1M Tris at pH 6.8 was prepared in a final volume of 500ml in distilled H_2O by dissolving 72.64g of Trizma base and adjusting pH with HCl. 1.5M Tris at pH 8.8 was prepared in a final volume of 500ml in distilled H_2O by dissolving 108.96g of Tris and adjusting pH with HCl. To prepare a 10X PBS-Tween 20 solution, 50 stock tablets (Sigma-Aldrich) to prepare phosphate buffered saline (PBS) were added to 990ml of distilled H_2O and 10ml of Tween 20. A 10% $^{w}/_{v}$ ammonium persulfate solution (APS) was made up by adding 2g of APS powder to distilled H_2O, adjusting the final volume to 20ml. Running buffer was prepared by adding 500ml of 10X Tris/Glycine/SDS buffer (National Diagnostics, Geneflow) to 4500ml dH_2O. Transfer buffer was prepared by adding 500ml of 10X Tris/Glycine buffer (National Diagnostics, Geneflow) and 1000ml methanol to 3500ml dH_2O.

Sample buffer (4X) was prepared by adding 2.51ml 1M Tris to 0.8g SDS performed in fume hood), 0.004g bromophenol blue, 0.3085g dithiothreitol (DTT) (fume hood), and 4ml of glycerol and made up to 10ml with dH_2O. (all reagents used from Sigma-Aldrich).

2.3.2 Gels and Nitrocellulose Paper for Western Blotting

The Dodeca kit was used for Western blotting analysis if six or more gels were being run (Biorad). Resolving gels (either 8% or 10% $^{w}/_{v}$ SDS) were used and were comprised of dH_2O, 30% $^{w}/_{v}$ acrylamide (Protogel, Geneflow), Tris 1.5M, SDS, APS (10% $^{w}/_{v}$ solution) and tetramethylethylenediamine (TEMED, Sigma-Aldrich). When preparing gels, the TEMED was added last (performed in the fume hood). Stacking gels were prepared and were composed of dH_2O, 30% $^{w}/_{v}$ acrylamide, 1.0M Tris, SDS, APS (10% $^{w}/_{v}$ solution), APS and TEMED. Protran nitrocellulose paper was used to transfer proteins during Western blotting analysis (Whatman, Schleicher and Schuell).

2.3.4 Lysis Buffer to Prepare Lysates for Western Blotting

Lysis buffer was composed of 20mM Tris (pH 7.5), 150mM NaCl, 1mM ethylenediaminetetraacetic acid (EDTA), 1mM ethylene glycol tetraacetic acid (EGTA), 1% $^{v}/_{v}$ Triton X-100, and 2.5mM Sodium Pyrophosphate, 1mM Na_3VO_3, and 1 x protease inhibitor cocktail (Sigma-Aldrich) was added immediately prior to use .

2.3.5 Pre-Extraction Brinkley Buffer used to Prepare Cells for Microscopy

Brinkley 1980 buffer (80mM 1,4-Piperazinediethanesulfonic acid, PIPES, pH6.9, 1mM EDTA, 1mM $MgCl_2$, 80mM KOH) with 0.5% $^{v}/_{v}$ Triton X-100 was prepared to use as a pre-extraction buffer when staining cells with α-tubulin to help visualise microtubules. All the reagents used to prepare this buffer were from Sigma-Aldrich. This buffer was stored at 4°C.

2.4 Cell Lines Used in this Study

2.4.1 Genetic Profiles of the CRC Panel

A panel of five CRC lines was selected to investigate curcumin-induced G_2/M arrest and their genetic differences are summarised in Table 6.

Table 8 Profile of CRC Cell Lines

Cell Line	p21 status	p53 status	CIN/MIN	MMR status
HCT116 Wt/HCT116 $p53^{+/+}$	Wild type	Wild type	MIN	MMR deficient (MLH1 negative)
HCT116 $p53^{-/-}$	Wild type	Knocked out	MIN	MMR deficient (MLH1 negative)
HCT116 $p21^{-/-}$	Knocked out	Wild type	MIN	MMR deficient (MLH1 negative)
HT-29	Wild-type	Mutated	CIN	MMR proficient
SW480	Wild-type	Mutated	CIN	MMR proficient

The HCT116 $p53^{+/+}$, HCT116 $p53^{-/-}$, HCT116 $p21^{-/-}$, HT-29 and SW480 lines were selected to investigate whether checkpoint proteins, the CIN/MIN phenotype or MMR status affect the way CRC cells respond to curcumin-induced cell cycle arrest. The genotype control HCT116 $p53^{+/+}$ cells are the exact HCT116 Wt strain used in the Vogelstein lab to construct the HCT116 $p53^{-/-}$ cell line via targeted homologous recombination of the p53 gene (Bunz et al., 1998).

2.4.2 Routine Cell Culture

The HCT-116 Wt (ATCC CCL-247), and the HCT116 $p53^{+/+}$, p21-/- and p53-/- (gift to Dr Lynne Howells from Dr Bert Vogelstein) lines were cultured in McCoy's medium (36600, McCoy's 5A) with 10% $^{v}/_{v}$ foetal calf serum (FCS, Sigma-Aldrich) and 1% $^{v}/_{v}$ Glutamax (Gibco). All cells were incubated at 37°C with 5% CO_2 in air. Cells were split at a ratio of 1/3-1/8 by removing medium from flasks (600ml Falcon, USA), washing with PBS, and adding 1 X trypsin (Sigma-Aldrich). Cells were incubated briefly, the trypsin neutralised using complete medium, and cells centrifuged for 5 minutes at 1400rpm (Beckman Coulter Allegra™ 6KR). The supernatant was removed and the cell pellet resuspended in fresh medium. This cell suspension was then counted and distributed proportionately to new flasks containing fresh medium (small flask Nunclon, NUNC, medium flask 75cm^2 Nunclon, NUNC, large flask, 600ml Falcon). HT-29 and HBL100 cells were cultured in DMEM medium (Sigma-Aldrich) with 10% $^{v}/_{v}$ FCS. SW480 and MDA-MB 468 cells were cultured in RPMI medium (Sigma-Aldrich) with 10% $^{v}/_{v}$ FCS. All cells lines tested negative for mycoplasma infection and were cultured without antibiotics. Cells were maintained in a Heraeus incubator at 37°C.

2.4.3 Cell Storage and Resurrection

To resurrect cells, cryotubes were removed from liquid nitrogen and placed briefly in a water bath at 37°C until defrosted. Cells were then resuspended in approximately 9ml of fresh medium and spun down. Following centrifugation, cells are resuspended in 10ml of medium and seeded in a small flask.

To prepare for storage in liquid nitrogen, after removal from a large flask and centrifugation, cells were then resuspended in FCS with 10% $^{v}/_{v}$ DMSO to prevent ice-crystals forming. Approximately 1ml of 1 x 10^{6} cells was then aliquoted per cryotube.

2.5 Treatments Used in this Study

2.5.1 Rationale for Treatment Concentrations

A drawback in the experimental design of many studies investigating curcumin using colorectal cancer cell lines is the use of unrealistically high concentrations. The concentrations of curcumin were chosen to reflect the bioavailability in the human gut following the oral ingestion of curcumin tablets. Phase I clinical studies have shown that concentrations up to 8µM have been found in colorectal tissue of patients taking 0.45-3.6g curcumin a day for a week prior to surgery (Hsu and Cheng, 2007, Cheng et al., 2001, Garcea et al., 2005). A concentration range of 1-10µM of curcumin was used in the majority of these studies.

2.5.2 Preparation of Curcumin Stocks

Curcumin was prepared with limited exposure to light, by dissolving curcumin powder (Sigma-Aldrich) in the solvent DMSO (Sigma-Aldrich) to make up a stock concentration of 100mM. Curcumin stock was stored in black eppendorfs at -20°C. Curcumin was mixed with the relevant proportion of medium prior to treatment of cells. A DMSO control was included in each experiment and final DMSO levels in cell media did not exceed 0.01% $^{v}/_{v}$. Fresh stocks of curcumin were prepared at least every two months.

2.5.3 Preparation of Nocodazole, Caffeine, DBH, HU and Etoposide Stocks

Nocodazole stocks were prepared by dissolving nocodazole powder (Sigma-Aldrich) in DMSO at a concentration of 1mg/ml or 3.3mM, and stored frozen at -20°C in 0.5ml eppendorfs. Caffeine and hydroxyurea (HU) stocks were prepared by dissolving in filtered sterile distilled water at a concentration of 200mM and 100mM respectively, and kept frozen at -20°C in 1.5ml eppendorfs until required (both reagents from Sigma-Aldrich). Debromohymenialdisine (DBH, Santa Cruz) and etoposide (Sigma-Aldrich) stocks were prepared by dissolving in DMSO at a concentration of 100mM and were kept frozen -20°C in 1.5ml black eppendorfs until required.

2.6 Western Blotting Protocol

2.6.1 Preparation of Cell Extracts for Western Blotting Analysis

Cells were plated (Nunclon™ Surface, NUNC™, Denmark) in 9cm^2 plates approximately 24 hours prior to treatment, at densities relative to the incubation period following treatment. Plates with treatment durations of 12, 24 and 48 hours were seeded at a density of 1.0 x 10^6. Cells were treated with 1-20µM of curcumin, DMSO alone as a vehicle control, or medium alone as a negative control. In experiments investigating the effects of caffeine on curcumin treatment cells were pre-treated for one hour with 10mM caffeine then treated with 10µM curcumin. Following incubation the medium was removed and spun down to retain floating cells. Adherent cells on the plate were gently washed in 5ml of ice-cold PBS twice. Then, 600µl of chilled lysis buffer was added and the plates incubated on ice for 10 minutes. Cells were removed from the

plate by scraping (Cell lifter TRP®, Switzerland) and the solution added to the small pellet of detached cells and transferred to eppendorfs. All samples were stored at -20°C. To determine the levels of protein in each sample the Bradford assay was performed with a Bio-Rad (Bio-Rad Laboratories) protein quantification kit.

2.6.2 Materials for Protein Separation with SDS Page Gels

Tris buffer (1.25M) was adjusted to pH 8.8 using HCl. PBS-Tween (PBS-T) was made up using phosphate buffer saline tablets (Sigma-Aldrich) and addition of 0.1% $^{v}/_{v}$ TWEEN® 20 (Sigma-Aldrich). Lysis buffer (10X) contained 20mM Tris, 150mM NaCl, 1mM EDTA, 1% $^{v}/_{v}$ Triton X-100, 2.5mM sodium pyrophosphate, 1mM sodium orthovanadate and protease inhibitor cocktail (Sigma-Aldrich). Lysis buffer (1X) was used to harvest cells. Running buffer for Western blots (Ultrapure 10X Tris/Glycine/SDS, Geneflow) was made following the instructions of the manufacturer, as was transfer buffer (Ultrapure 10X Tris/Glycine, Geneflow) used in protein transfer from gels to membranes.

2.6.3 Western Blotting Analysis

Samples in loading buffer containing 30µg of protein were loaded alongside Pageruler™ Prestained Protein Ladder (Fermentas Life Sciences) in wells of 10% ^{w}/v or 15% ^{w}/v SDS-polyacrylamide gels in a Western blotting tank (BIORAD Mini PROTEAN® 3) and run at 100V (BIORAD Power Pac 200). Separated proteins were then transferred (using BIORAD Trans-Blot® kit) to nitrocellulose membranes at 100V for 60 minutes. Following transfer Odyssey blocking buffer (LI-COR® Biosciences) was used to

block membranes for approximately 1 hour. Following blocking, membranes were washed three times for five minutes with PBS-T. Primary antibodies were generally diluted to 1:2000 in Odyssey Blocking Buffer diluted 1:1 in PBS-T. Loading control antibodies against actin or α-tubulin were diluted to 1:4000. Membranes were incubated with the primary antibodies overnight. Following incubation, membranes were washed three times for five minutes with PBS-T. Secondary antibodies were prepared at a dilution factor of 1:15,000 in Odyssey blocking buffer. A goat anti-rabbit antibody (IRDye 680 conjugated goat anti-rabbit IgG, LI-COR Biosciences) was used to detect rabbit primary antibodies. A goat anti-mouse antibody (IRDye 800 CW conjugated goat anti-mouse IgG, LI-COR Biosciences) was used to detect mouse primary antibodies. The membranes were incubated with the secondary antibodies for approximately 1 hour. During this period, the boxes containing the membranes were wrapped in aluminium foil to prevent degradation of the signal from the secondary antibodies. Following incubation with the secondary antibodies the membranes were washed three times with PBS-T. They were then scanned using the Odyssey Infrared Imaging System and analysed using Odyssey software. The parameter used to normalise the primary antibodies to the controls was the integrated intensity of the antibody signals. The field integrated intensity found in the feature reports following analysis of each blot is independent of the resolution of the image and is proportional to the quantity of dye-labelled antibodies on the membrane.

2.7 FACS Analysis Protocols

2.7.1 Preparation of Samples for Intracellular Staining with PI

Cells were plated (Nunclon™ Surface, NUNC™, Denmark) in 6-well plates approximately 24 hours prior to treatment. Cells were plated at densities relative to the incubation period following treatment. Wells with treatment durations of 12 hours were seeded at a density of $1x10^5$, 24 hours at 2×10^5 cells, 48 hours at 1.5×10^5, and 72 hours at 1.0×10^5. Prior to treatment all medium was removed from wells, and 4mls of fresh medium was added to each well. Cells were treated in duplicate with 1ml of medium alone, 1ml of medium with DMSO, or medium with curcumin to give final concentrations of 1-20µM per well. At harvest times the medium was removed and wells were washed with 2mls of PBS. After washing, 1ml of 1 x trypsin was added per well and plates were returned to the incubator for a further 5 minutes. After this step 1ml of medium was then added per well to neutralise the trypsin. Samples were removed from the wells and added to the media removed previously in 15ml Falcon tubes. Samples were then centrifuged for 5 minutes at 1,400rpm using a Beckman Coulter Allegra 6KR centrifuge. Supernatants were discarded and the cell pellets resuspended in 2mls of PBS. Samples were centrifuged again for 5 minutes at 1,400rpm. Following washing with PBS, the excess solution was poured off, and cells were fixed with 2mls of 70% $^{v}/_{v}$ ethanol (EtOH) using a vortex mixer. The samples were then stored in the cold room at 4°C overnight to allow membranes to permeabilise. To prepare the samples for DNA cell cycle analysis, they were spun at 1,400rpm for 5 minutes. The EtOH was discarded and the cell pellets were resuspended in 1ml of PBS containing ribonuclease A (1mg/ml, Sigma-Aldrich) and PI (50µg/ml, Sigma-Aldrich).

The samples were incubated overnight at 4°C to facilitate DNA staining. Prior to analysis cells were transferred to 5ml FACS tubes.

In addition, cell cycle analysis samples were prepared following 12 hours treatment with medium alone, DMSO, 10mM caffeine, 10µM curcumin, 10mM caffeine + 10µM curcumin, or 0.33µM of nocodazole. Cells were seeded at 1 x 10^6 in 9cm dishes and treated the following evening. Cells were harvested the following morning and prepared for analysis as above.

2.7.2 Preparation of Samples for Intracellular Staining with pH3 Antibodies and DAPI

Cells were seeded at 1 x 10^6 in 9cm dishes and treated the following evening with DMSO, 10mM caffeine, 10µM DBH, 10µM curcumin, 10mM caffeine + 10µM curcumin, 10µM DBH + 10µM curcumin or 0.33µM of nocodazole. Cells were harvested the following morning. All medium was removed from the dishes and cells were washed with 2mls of PBS. After washing, 1ml of 1 x trypsin was added per well and plates were returned to the incubator for a further 5 minutes. After this step 1ml of medium was then added per well to neutralise the trypsin. Samples were removed from the wells and transferred to 15ml Falcon tubes with respective media and floating cells and centrifuged for 5 minutes at 1,400rpm using a Beckman Coulter Allegra 6KR centrifuge. Cells were then resuspended in 1ml 2% $^{v}/_{v}$ formaldehyde, 1% $^{w}/_{v}$ bovine serum albumin (BSA) solution for 10-15 minutes. Cells were centrifuged again and all remaining supernatant removed. Cells were resuspended in ice cold methanol at -20°C for 10-15 minutes. Following this step, cells were centrifuged and washed with 1% $^{w}/_{v}$ BSA twice. Samples were incubated with primary mouse phospho-

histone H3 Ab (dilution factor 1:50) or mouse IgG isotype control (Sigma-Aldrich) diluted in 1% $^{w}/_{v}$ BSA for 1 hour at room temperature. After incubation with the primary antibody, the cells were centrifuged and washed with 1% $^{w}/_{v}$ BSA twice. The samples were then incubated with the anti-mouse Alexa 688 conjugated antibodies diluted (1:50) in 1% $^{w}/_{v}$ BSA for one hour at room temperature. Following incubation with the secondary antibodies cells were centrifuged, the supernatant discarded, and resuspended in PBS two times. The cells were spun again and resuspended in 1ml DAPI solution (0.1% $^{v}/_{v}$ Triton-X 100, 250µg/ml DAPI dilactate in PBS). Cells were syringed using a 23G needle to remove clumps and transferred to FACS tubes immediately prior to analysis.

2.7.3 Preparation of Cell Samples for Cell Surface Staining with anti-NKG2DL Antibodies

Cells were seeded at 1 x 10^6 in 9cm dishes and treated for 12 or 24 hours with DMSO, 10µM curcumin, 0.33µM nocodazole, 5mM hydroxyurea, or 50µM etoposide. All medium was removed from the dishes, transferred to 15ml Falcon tubes, and adherent cells were washed with 2mls of PBS. After washing, 1ml of 1 x trypsin was added per well and plates were returned to the incubator for a further 5 minutes. After this step 1ml of medium was then added per well to neutralise the trypsin. Samples were removed from the wells and transferred to the 15ml Falcon tubes containing respective media and floating cells and centrifuged for 5 minutes at 1,400rpm using a Beckman Coulter Allegra 6KR centrifuge. The supernatant was discarded and cells were resuspended in ice-cold PBS, centrifuged again and the

supernatant discarded. Cells were then resusupended in 1ml ice-cold PBS containing 10% $^{v}/_{v}$ FCS and 250µl of cell solution was transferred to FACS tubes. Primary antibodies were then added to the cell samples (1:50) and incubated for 30-60 minutes at 4°C, except for isotype control samples (incubated with mouse IgG control, Sigma-Aldrich) and secondary only samples which contained no primary antibody or isotype control antibodies). Cells were then washed twice with ice cold PBS and resuspended in PBS containing 3% $^{w}/_{v}$ BSA. Secondary antibodies were then added to the cell samples and incubated for 30 minutes at 4°C, and protected from light using aluminium foil (anti-mouse antibodies were diluted as follows, 1:20 FITC-conjugated, 1:50-Alexa 688 conjugated). Following incubation with the secondary antibodies cells were washed and resuspended in Hank's buffered saline solution (Gibco). Samples were placed on ice and 2 minutes prior to analysis either PI or DAPI was added to the samples to facilitate assessment of cell viability (except for unstained controls).

2.7.4 FACS Data Capture and Analysis (Cell Cycle using PI)

Cells that were stained with propium iodide to assess DNA content were analysed using the Becton Dickinson FACScan Flow Cytometer. Data were captured using a DNA analysis CellQuest program. Gating was used to exclude doublets and debris. At least 5000 counts per sample were analysed. The Modfit analysis software was used to give the relative cell cycle distributions of each sample. Each FACS analysis experiment was repeated at least three times. The Students T test was used to compare the means of data sets.

2.7.5 FACS Analysis Data Analysis of Sub-G_1 Content

Cells that were stained with propium iodide to assess DNA content were analysed using the Becton Dickinson FACScan Flow Cytometer. Data were captured using a DNA analysis CellQuest program. Gating was used to exclude doublets and debris. At least 5000 counts per sample were analysed. The CellQuest analysis software was used measure the apoptotic population as a percentage of the total cell population but assessing the sub-G_1 content of each sample. The marker M1 was used to gate the sub-G_1 content and the marker M2 was used to gate G_1, S and G_2/M cells. Each FACS analysis experiment was repeated at least three times. The Students T test was used to compare the means of data sets.

2.7.6 FACS Data Capture and Analysis (Cell Cycle and Mitotic Index using DAPI and anti-pH3 Antibodies)

Cells that were stained with DAPI to assess DNA content and primary antibodies to phospho-histone H3 and Alexa-conjugated 688 secondary antibodies to evaluate the mitotic index were analysed using the Becton Dickinson FACS Aria II. Data was captured using the FACS Diva 6 programme. At least 10,000 counts per sample were analysed. Gating was used to exclude doublets and debris. The Modfit analysis software was used to give the relative cell cycle distributions of each sample. The WinMDI program was used to calculate mitotic populations. Each FACS analysis experiment was repeated at least three times. The Students T test was used to compare the means of data sets.

2.7.7 FACS Data Capture and Analysis (Cell Surface Expression of NKG2DL, FITC-Conjugated Secondary, PI for Viability)

Cells that were stained with primary antibodies to NKG2DL coupled with anti-FITC secondary antibodies, and propium iodide to assess cell viability were analysed using the Becton Dickinson FACScan Flow Cytometer and data captured using the CellQuest program. At least 5000 counts per sample were analysed. Doublets, debris, and non-viable cells were excluded using gating and the cell surface expression of NG2DL on live cells was analysed using Cell Quest Software.

2.7.8 FACS Data Capture and Analysis (Cell Surface Expression of NKG2DL, Alexa 800-Conjugated-Secondary, DAPI for Viability)

Cells that were stained with primary antibodies to NKG2DL and Alexa 800 conjugated secondary antibodies were analysed using the Becton Dickinson FACS Aria II and data captured using the FACS Diva 6 programme. DAPI was used to assess cell viability. At least 10,000 counts per sample were analysed. Doublets, debris, and non-viable cells were excluded using gating and the cell surface expression of NG2DL on live cells. The WinMDI program was used to analyse data captured.

2.8 Immunocytochemistry

2.8.1 Treatments

Cells were seeded in plates at a density of $1x10^6$. Approximately 28-30 hours later cells were treated with either DMSO alone, 1-10µM curcumin, 10mM caffeine, 10µDBH,

10mM caffeine + 10µM curcumin, 10µM DBH + 10µM curcumin, or 0.33µM nocodazole as a positive control. Coverslips were coated with a 10% $^{v}/_{v}$ poly-L-lysine solution (final concentration 1mg/ml) (Sigma-Aldrich), incubated at room temperature for 10 minutes, then rinsed with de-ionised triple distilled water and left to dry overnight in the incubator at 37°C. After treatment for 12 hours, mitotic cells were harvested via mitotic shake off and transferred to 15ml Falcon tubes. Adherent cells were gently washed with PBS and 2mls of trypsin was added, and plates returned to the incubator until all cells had detached. The detached cells were added to the mitotic shake-off cells and the Falcon tube inverted to mix the populations. Approximately 500µl of the combined cell suspension was dropped onto pre-coated coverslips in a 6-well plate using a P1000 Gilson pipette, and plates were incubated until cells had settled and attached.

2.8.2 Immunofluorescence Staining

All medium was then removed from the cover slips and wells were rinsed twice with PBS. The cells were then fixed and permeabilised by the addition of ice-cold methanol and were incubated at -20°C for 30 minutes. When preparing cells for staining with α-tubulin antibodies, pre-extraction buffer was added to the wells for 30 seconds, then removed, prior to the addition of methanol. After this step all methanol was removed and cells were rinsed with PBS three times. Cells were then blocked for 30 minutes at room temperature with 3% $^{w}/_{v}$ milk in PBS. Upon removal of the milk solution, all cells except those in the antibody control wells were incubated with primary antibodies at a dilution factor of 1:1000 for one hour in 3% $^{w}/_{v}$ milk in PBS. All primary antibody

solution was removed and cells were gently rinsed with PBS three times. A secondary antibody solution containing secondary fluorescent antibodies and Hoescht/DAPI in 3% $^{w}/_{v}$ milk was added to all wells. Plates were immediately wrapped in aluminium foil to protect the fluorescent antibodies and dye from light and were incubated at room temperature for one hour. The solution was then removed and cells carefully washed three times with PBS. Cover slips were dipped in deionised triple-distilled water prior to mounting to remove salt residues. Cover slips were mounted onto slides using SlowFade reagent to prevent photo-bleaching of the fluorescent antibodies (Invitrogen). Filter paper was used to absorb excess reagent. Slides were left to cure for at least 24 hours, and then sealed using clear nail varnish.

2.8.3 Mitotic Index Analysis

Cells were analysed using fluorescence microscopy to evaluate the mitotic index. At least 100 cells were scored per sample and results are representative of at least three independent experiments. The Axioskop 2 plus (Carl Zeiss) fluorescent microscope with a 40X objective lense was used to view mitotic figures. The mitotic index was evaluated using phospho-Histone 3 staining and Hoescht. The difference in means of the mitotic index between DMSO control and treated populations was compared using the Student's T test statistical analysis.

2.8.4 Analysis of Stage of Mitosis

Slides with DMSO and curcumin treated cells were prepared and stained with antibodies to α-tubulin and centrin, and with DAPI to stain DNA. Prophase/prometaphase cells were defined by the appearance of condensed chromosomes. Metaphase cells were defined as having a bipolar mitotic spindle with chromosomes clearly aligned on the metaphase plate. In anaphase, sister chromatid separation could be visualised and in telophase this separation has progressed and contraction of the spindle-midbody could be seen. At least fifty cells were counted per slide, and this experiment was repeated three times.

2.8.5 Analysis of Chromosomal Bridges

To examine whether recently divided cells were linked by chromosomal bridges cells were stained with pH3 and Aurora B antibodies and DAPI. The numbers of chromosomal bridges between recently divided cells were counted on slides containing DMSO or curcumin-treated cells. At least 50 cells were counted per slide. This experiment was repeated three times.

2.8.6 Analysis of Metaphase Spindle Formation

Cells were prepared for immunofluorescence analysis and stained with antibodies to α-tubulin and centrin and chromatin was stained with DAPI. Normal metaphase spindles were defined as bi-polar with chromosomes aligned correctly on the

metaphase plate. At least 50 cells were counted per slide, and this experiment was repeated at least three times.

2.8.7 Analysis of Centrosomal Separation

Antibodies to centrin and α-tubulin were used to stain microtubules and centrioles. DNA was stained with DAPI. Centrosomes were defined as separated if they were more than 2 microns apart. Images were calibrated on the Openlab software and the 'ruler' tool was used to measure the distance between centrosomes. At least 50 cells were counted per slide and data were captured from three independent experiments.

2.8.8 Image Capture and Analysis

Images were captured from the Nikon microscope with an Xcite illumination system using a Hamamatsu ORCA-R^2 digital camera (pixel size 6.5µM x 6.5µM) connected to a Mac system running Openlab software (Improvision). Figures were compiled using Adobe Photoshop Creative Suite software.

Chapter 3 Defining Curcumin-Induced G_2/M Cell Cycle Arrest in CRC Lines

3.1 Introduction

The effects of curcumin treatment in preclinical *in vitro* models of colorectal cancer have shown that this agent generally induces cell cycle arrest at the G_2/M phase of the cell cycle (Chen et al., 1999, Hanif et al., 1997, Howells et al., 2007, Jaiswal et al., 2002).

In the Lovo colorectal cancer cell line research has shown that treatment resulted in arrest in both the S and G_2/M phases of the cell cycle (Chen et al., 1999). The authors noted that this arrest prevented cells from re-entering the cell cycle, contributing to the anti-proliferative effects of treatment in this line.

A previous study from this lab analysing curcumin-induced cell cycle arrest in the HCT116 colorectal cell line found that concentrations of 10-20μM induced significant G_2/M arrest at 24 and 48 hour time points (Howells et al., 2007). This study also showed that 20μM induced G_2/M arrest in the normal colon-derived HCEC line, but not in the HT-29 cell line under the same conditions.

Research on the importance of the cell cycle proteins p21 and p53 in mediating curcumin-induced cell cycle arrest found that in the HCT116 $p53^{+/+}$, HCT116 $p21^{-/-}$, and HCT116 $p53^{-/-}$ cell lines, treatment with 20μM curcumin resulted in G_2/M arrest in all cell lines following 15 and 30 hours treatment (Jaiswal et al., 2002).

Investigations into the anti-proliferative effects of curcumin on the HT-29 and HCT-15 colorectal cancer cell lines over a 12 to 48 hour period found that curcumin treatment

induced the highest levels of G_2/M arrest in these lines after 12 hours treatment (Hanif et al., 1997).

In these previous studies characterisation of cell cycle arrest was performed by FACS cell cycle analysis alone, by measuring the total DNA content in each cell line to elucidate the stage of the cell cycle. Analysis of DNA content alone does not differentiate between cells undergoing cell cycle arrest at the G_2/M boundary, or within M phase. Interestingly, research has indicated that curcumin treatment impaired mitotic spindle structure in the HT-29 colorectal cancer cell line and induced M phase arrest in the MCF-7 breast cancer cell line (Gupta et al., 2006, Holy, 2002).

To characterise the nature of G_2/M arrest in a panel of colorectal cancer cell lines, FACS analysis experiments were carried out to elucidate combined levels of G_2/M and M checkpoint arrest, then mitotic index experiments were performed to specifically identify levels of M phase arrest. FACS analysis was performed over 12-72 hours to find when the greatest G_2/M arrest occurred and to further compare the differences in arrest between the HCT116 wild-type, HCT116 $p21^{-/-}$, and HCT116 $p53^{-/-}$ lines. Cell cycle analysis of the HT-29 and SW480 CRC lines was also carried out at the 12 hour time point.

Previous investigation of the effects of curcumin on the mitotic index of the MCF-7 human breast cancer cell line showed that treatment with 10μM of curcumin increased the mitotic index by approximately 15% compared to the control levels (Holy, 2002). However, no statistical analysis was included to show whether this finding was significant. In this present study, pilot mitotic experiments were performed with two breast cancer cell lines, the HBL100 and MDA-MB-468 lines.

To determine whether curcumin induced M phase arrest in the panel of colorectal cancer cell lines, a range of mitotic index experiments using immunofluorescence microscopy was performed. A time course experiment was carried out to determine the peak levels of mitotic arrest over a 36 hour period in the HCT116 lines. Since the highest levels of M phase arrest were found after 12 hours treatment in the HCT116 $p53^{+/+}$ line, this treatment duration was then used for all further mitotic index experiments.

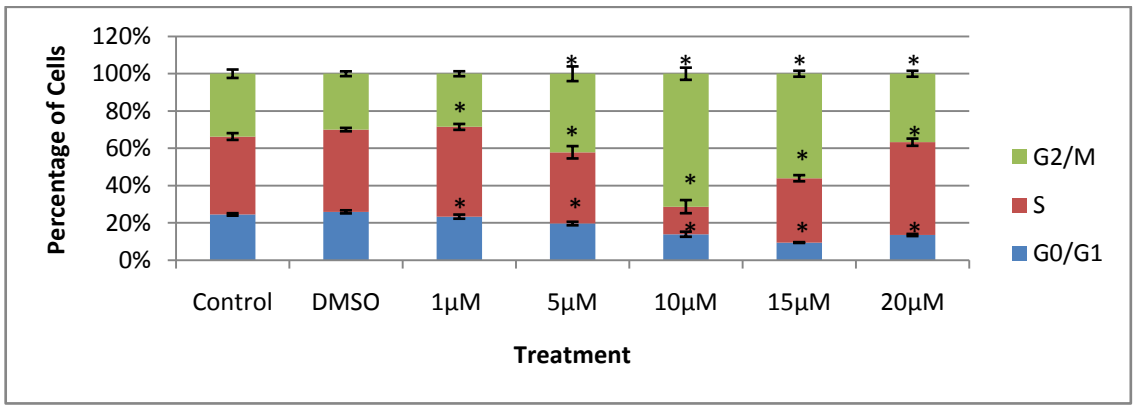
Conclusions regarding the nature of curcumin-induced cell cycle arrest in these colorectal cancer lines could then be made by comparing overall levels of G_2/M and M checkpoint arrest found using FACS cell cycle analysis and specific M phase arrest identified using fluorescence microscopy.

3.2 Results

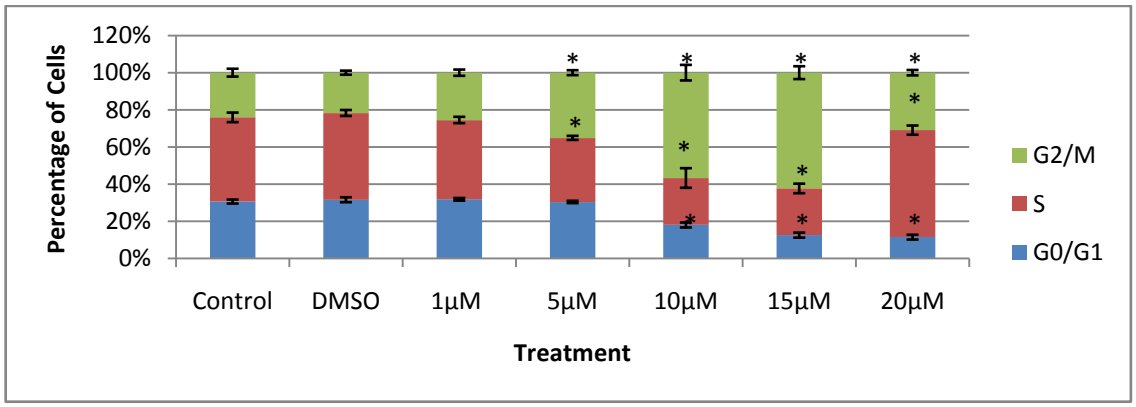
3.2.1 FACS Cell Cycle Analysis Confirms Curcumin Induces G_2/M Arrest in HCT116 Lines

Initial studies focussed on examining the effects of curcumin treatment on the HCT116 Wt, HCT116 $p53^{-/-}$, and HCT116 $p21^{-/-}$ lines for treatment durations of 24, 48 and 72 hours using a concentration range of 5-20μM curcumin. FACS cell cycle studies were carried out by labelling the total DNA content of cells using PI to determine the stage of the cell cycle.

(A)



(B)



(C)

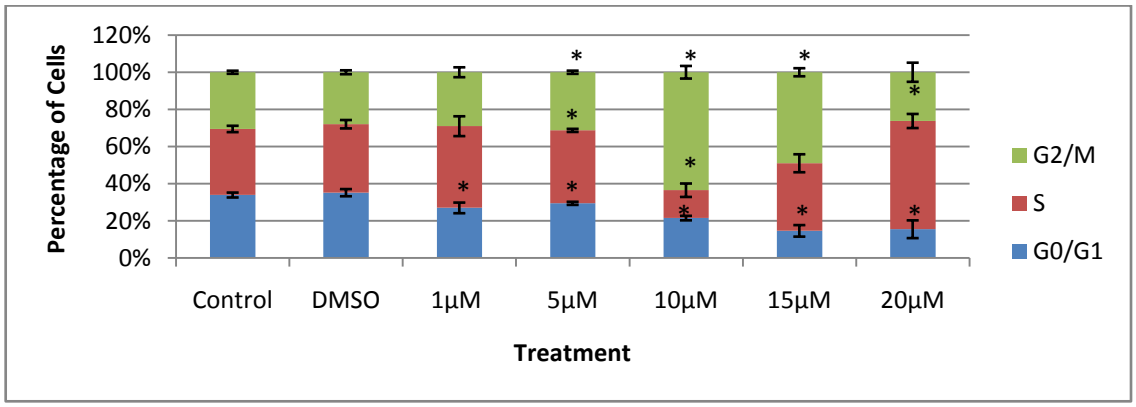


Figure 6 Curcumin Induces G_2/M Cell Cycle Arrest in HCT116 Wt Cells After 24-72 Hours

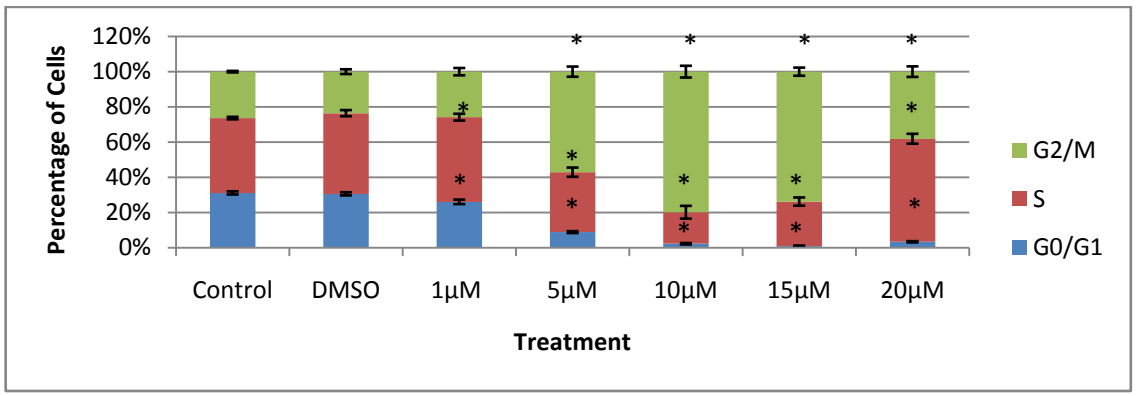
HCT116 Wt cells were treated with medium alone, DMSO, or curcumin for (A) 24 hours, (B) 48 hours, (C) 72 hours and analysed using FACS cell cycle analysis. Error bars show the standard error of the mean. * indicates a significant difference ($p<0.05$) in the mean number of cells in each phase following curcumin treatment compared to the DMSO control, following a two-tailed T test, assuming equal variance.

Treatment with 5-20µM of curcumin for 24 hours induced significant ($p<0.05$) G_2/M arrest in the HCT116 Wt line (Figure 6 A). The highest levels of G_2/M arrest occurred following treatment with 10µM of curcumin, with an average of 72% of cells in this phase compared to an average of 30% in the DMSO control. There were corresponding significant decreases in the number of cells in G_0/G_1 and S phases following treatment with 5-20µM. The lowest average levels of cells found in G_0/G_1 occur after treatment with 10µM of curcumin with 10% of cells compared to an average of 26% in the DMSO control. The most significant decrease in cells in S phase occurred after treatment with 10µM of curcumin. Analysis showed that this treatment resulted in 16% less cells in S phase compared to the DMSO control.

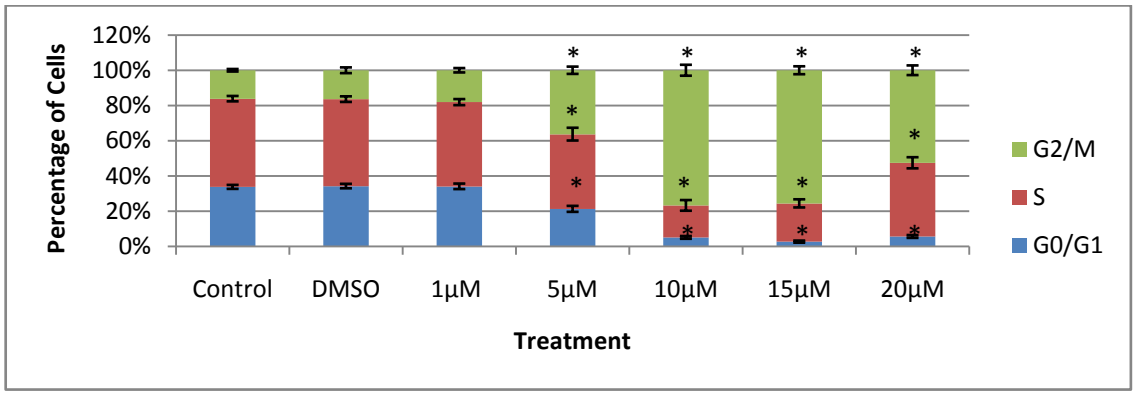
Treatment for 48 hours resulted in significant G_2/M arrest in the HCT116 Wt cell line (Figure 6 B). Following treatment with 15µM of curcumin, an average of 62% of cells arrested in G_2/M compared to 22% of cells in the DMSO control. Treatment with concentrations of curcumin, 10µM and above resulted in a significant decrease in cells in the $G_{0/}G_1$ phase of the cell cycle. For example, cells treated with 20µM of curcumin had an average of 11% of cells in this phase whereas the DMSO control had an average of 32% of cells in G_0/G_1. Cells treated with 20µM of curcumin also had a significant increase in cells in S phase, with an average of 58% cells in contrast to 47% in the DMSO control. This is in contrast to cells treated with 5-15µM of curcumin that displayed a significant decrease in the average number of cells in S phase. This was most pronounced after treatment with 15µM of curcumin, where there was an average of 25% of cells in S phase compared to 47% in the DMSO control.

Figure 6 C shows that G_2/M arrest was maintained following curcumin treatment for 72 hours. The highest levels of G_2/M arrest were found following treatment with 10µM of curcumin, with an average of 63% compared to 28% in the DMSO control. Treatments with 10-20µM of curcumin resulted in a significant decrease in cells in the G_0/G_1 phase of the cell cycle, with an average of 15% of cells in this phase after treatment with 15µM of curcumin compared to 35% in the DMSO control. Figure 6 C also shows that a significant number of cells arrest in the S phase of the cell cycle following treatment with 20µM of curcumin for 72 hours, where there was an average of 58% of cells in S phase, compared to 37% in the DMSO control.

(A)



(B)



(C)

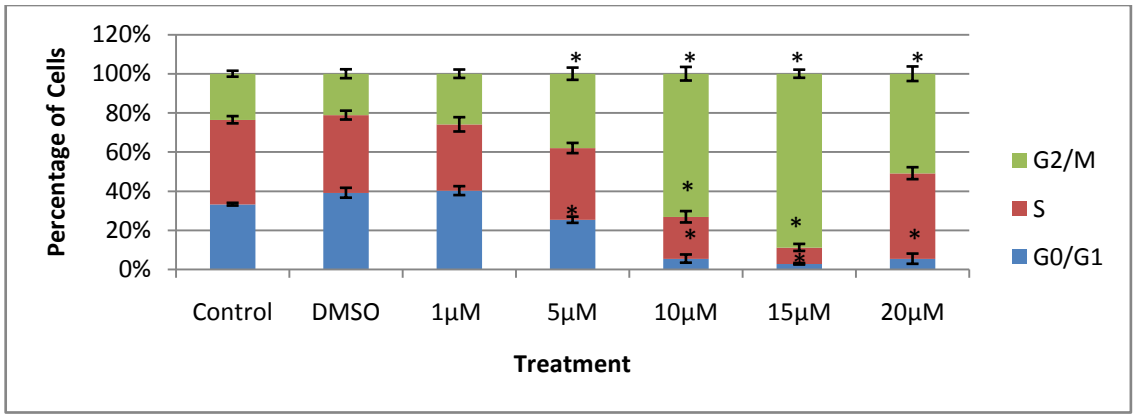


Figure 7 Curcumin Induces G_2/M Cell Cycle Arrest in HCT116 $p53^{-/-}$ Cells After 24-72 Hours

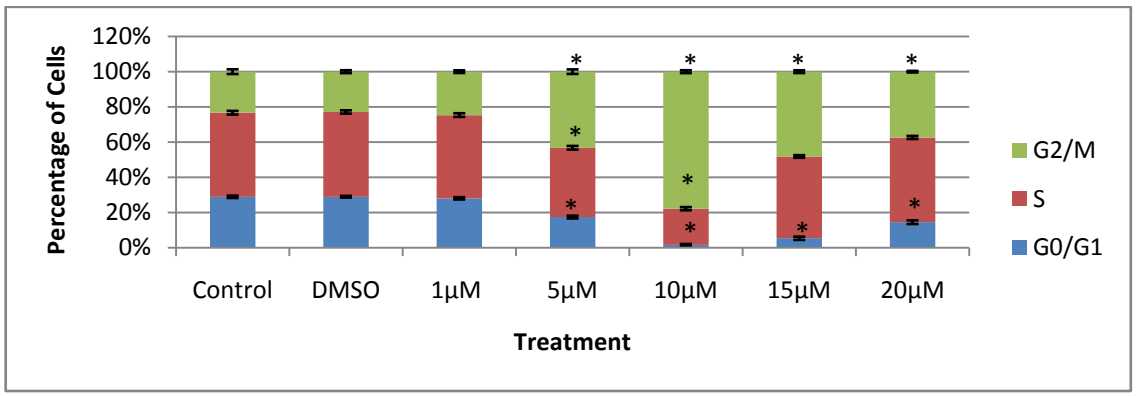
HCT116 $p53^{-/-}$ cells were treated with medium alone, DMSO, or curcumin for (A) 24 hours, (B) 48 hours, (C) 72 hours and analysed using FACS cell cycle analysis. Error bars show the standard error of the mean. * indicates a significant difference ($p<0.05$) in the mean number of cells in each phase following curcumin treatment compared to the DMSO control, following a two-tailed T test, assuming equal variance.

Curcumin treatment also resulted in G_2/M arrest in the HCT116 $p53^{-/-}$ line, with peak levels of arrest at 24 hours following treatment with 10µM (Figure 7 A). Cells treated with 5-20µM of curcumin had a significantly less cells in G_0/G_1, just 1%, following treatment with 15µM of curcumin, in contrast to an average of 29% in the DMSO control. Cells treated with 5-15µM also showed a decrease in the number of cells in S phase. In contrast, cells treated with 20µM showed a significant increase in cells in S phase, with an average of 59% compared to 44% in the DMSO control.

HCT116 $p53^{-/-}$ cells treated with curcumin for 48 hours underwent significant G_2/M arrest, with the highest levels of arrest at 77% following treatment with 10µM (Figure 7 B). Treatments with 10 and 15µM of curcumin resulted in the highest levels of G_2/M arrest, and corresponding significant decreases in S and G_0/G_1 phases. Cells treated with 5-20µM of curcumin also had significantly lower levels of cells in G_0/G_1 phase, with the lowest levels found following treatment with 15µM with an average of 3% of cells compared to 34% in the DMSO control.

Treatment for 72 hours resulted in significant G_2/M arrest in the HCT116 $p53^{-/-}$ cell line (Figure 7 C). The highest levels of arrest occurred after treatment with 15µM curcumin, resulting in an average of 89% of cells in G_2/M compared to 21% in the DMSO control. HCT116 $p53^{-/-}$ cells treated with 5-20µM of curcumin also had a significant decrease in the percentage of cells in the G_0/G_1 phase of the cell cycle. Treatments that resulted in the highest levels of G_2/M arrest, 10µM and 15µM, had corresponding significant decreases in the percentage of cells in S phase. The HCT116 $p53^{-/-}$ cells treated with 15µM of curcumin for 72hours had an average of 8% cells in S phase in contrast to an average of 40% in the DMSO control.

(A)



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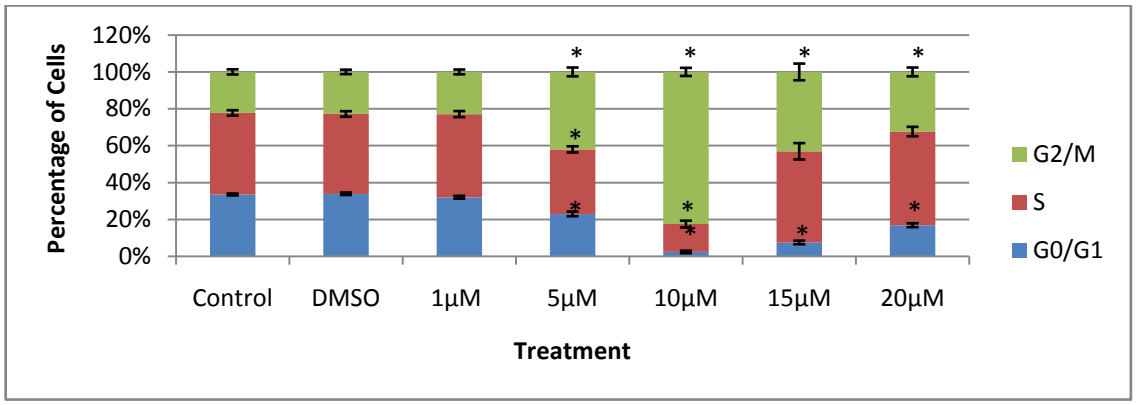


(C)

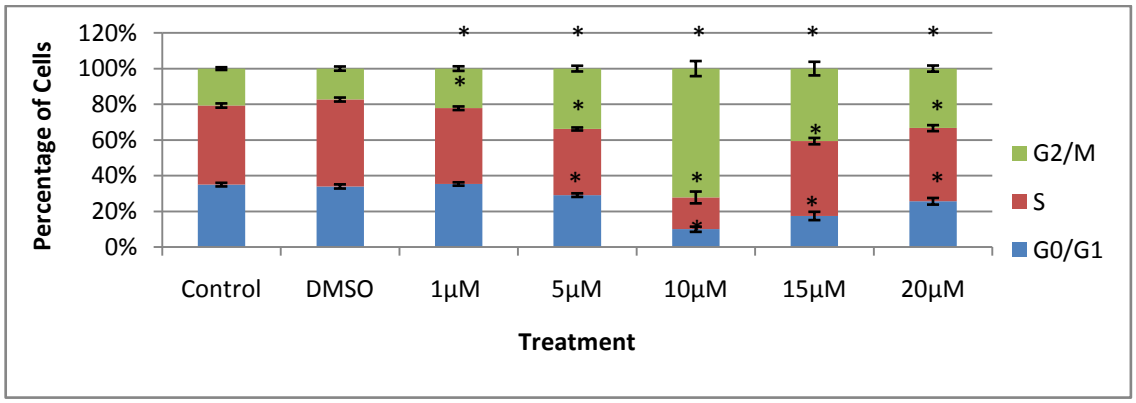


Figure 8 Curcumin Induces G_2/M Cell Cycle Arrest in HCT116 p21$^{-/-}$ Cells

HCT116 p21$^{-/-}$ cells were treated with medium alone, DMSO, or curcumin for (A) 24 hours, (B) 48 hours, (C) 72 hours and analysed using FACS cell cycle analysis. Error bars show the standard error of the mean. * indicates a significant difference ($p<0.05$) in the mean number of cells in each phase following curcumin treatment compared to the DMSO control, following a two-tailed T test, assuming equal variance.

Treatment with curcumin resulted in a significant G_2/M arrest in HCT116 $p21^{-/-}$ cells at all time points. The highest levels of arrest took place after treatment with 10µM with an average of 78% in G_2/M compared to 23% in the DMSO control, and these levels were arrest were similar to those found at the later timepoints (Figure 8 A). Treatments with 10 and 15µM of curcumin resulted in a significant decrease in cells in S phase, 20% compared to 48% in the DMSO control. Similar decreases in the average percentage of cells in G_0/G_1 were also observed in the HCT116 $p21^{-/-}$ line following treatment with 5-20µM for 24 hours.

Following treatment for 48 hours, 5-20µM of curcumin resulted in a significant decrease in the number of cells in G_0/G_1 phase of the cell cycle with an average of 2% of cells in this phase after treatment with 10µM of curcumin in contrast to 34% in the DMSO control. In addition, cells treated with 5µM and 10µM of curcumin also had significantly lower levels of cells in S phase compared to the DMSO control (Figure 8 B).

Figure 8 C illustrates that treatment with 1-20µM of curcumin for 72 hours resulted in significant G_2/M arrest in HCT116 $p21^{-/-}$ cells. Significantly lower levels of cells in both G_0/G_1 and S phases were found following treatment with 5-20µM of curcumin, and this was most pronounced with the 10µM treatment concentration. This concentration resulted in an average of 10% of cells in G_0/G_1 compared to 40% in the DMSO control, and an average of 18% of cells in S phase compared to 49% in the DMSO control.

In summary, these results show that the highest levels of G_2/M arrest in the HCT116 Wt line were found following 24 hours treatment with 10μM of curcumin, with an average of 72% of cells in G_2/M. In the HCT116 $p53^{-/-}$ line the highest levels were achieved with 89% of cells in G_2/M following treatment for 72 hours with 15μM of curcumin. In contrast the HCT116 $p21^{-/-}$ line had the highest levels of arrest following 48 hours treatment with 10μM of curcumin with an average of 83% of cells in G_2/M.

Treatment with the lowest concentration of curcumin, 1μM, induced significant G_2/M arrest in the HCT116 $p21^{-/-}$ line following 72 hours treatment. Treatment with the highest concentration (20μM) in these experiments did not result in the highest levels of G_2/M arrest. Instead, treatment with this dose resulted in a significant increase in S phase arrest in all cell lines at various time points.

3.2.2 Curcumin Treatment Induces Significant G_2/M Arrest After 12 Hours in CIN and MIN CRC Lines

After investigating of the effects of curcumin treatment on the HCT116 lines over a 24-72 hour period, further cell cycle studies were carried out to examine the effects of treatment for 12 hours. As 10μM curcumin was the most effective at inducing G_2/M arrest at the 24 hour time point, this was selected to be the maximum concentration for the 12 hour time point. A physiologically relevant concentration range of 1-10μM was used for these experiments. In addition to the MIN HCT116$^{+/+}$ HCT116 $p21^{-/-}$ and HCT116 $p53^{-/-}$ cell lines, the CIN HT-29 and the SW480 CRC lines were also analysed.

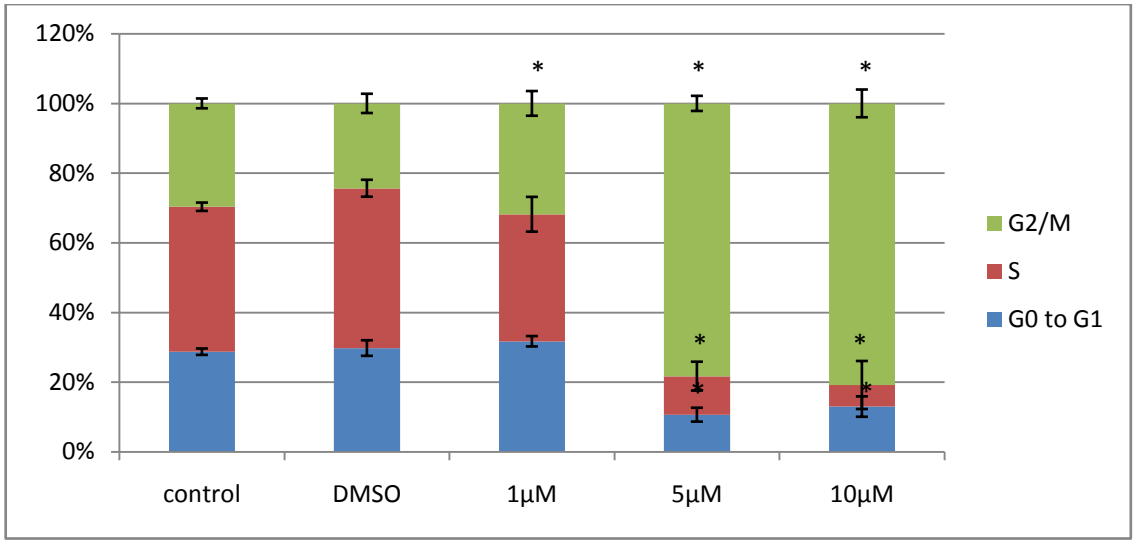


Figure 9 Curcumin Induces G_2/M Arrest in the HCT116 $p53^{+/+}$ Cell Line After 12 Hours

HCT116 $p53^{+/+}$ cells were treated with curcumin or DMSO alone for 12 hours then harvested for FACS cell cycle analysis. Error bars show the standard deviation. * indicates a significant difference ($p<0.05$) in the mean number of cells in each phase following curcumin treatment compared to the DMSO control, following a two-tailed T test, assuming equal variance.

Treatment with curcumin for 12 hours resulted in a significant G_2/M arrest in the HCT116 $p53^{+/+}$ cell line (Figure 9). Of note, treatment with 1µM of curcumin resulted in a significant increase in cells in G_2/M compared to the DMSO control, while 10µM of curcumin results in the highest number of cells in G_2/M arrest, with an average of 81% compared to 24% in the DMSO control. The average number of cells in both G_0/G_1 and S phases significantly decreased following treatment with both 5 and 10µM of curcumin. After treatment with 10µM of curcumin there were just 6% cells in S phase in comparison to 46% in the DMSO control.

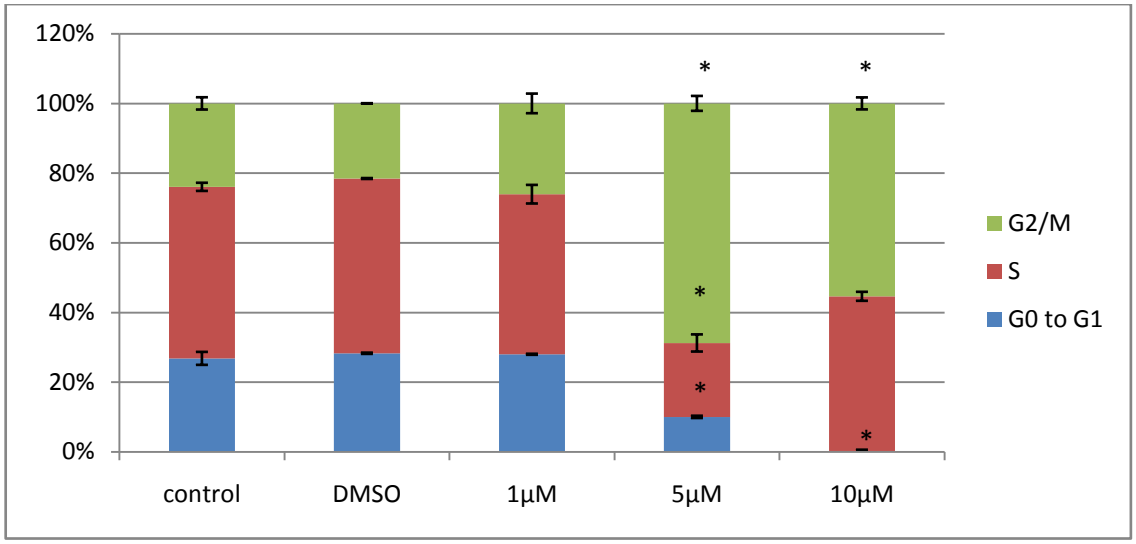


Figure 10 Curcumin Induces G_2/M Arrest in HCT116 $p53^{-/-}$ Cells After 12 Hours

HCT116 $p53^{-/-}$ cells were treated with curcumin or DMSO alone for 12 hours then harvested for FACS cell cycle analysis. Error bars show the standard deviation* indicates a significant difference ($p<0.05$) in the mean number of cells in each phase following curcumin treatment compared to the DMSO control, following a two-tailed T test, assuming equal variance.

Treatment with 5-10µM of curcumin for 12 hours results in a significant G_2/M arrest in the HCT116 $p53^{-/-}$ cell line. Figure 10 shows that treatment with 5µM results in the highest levels of G_2/M arrest with an average of 71% compared to 24% in the DMSO control. At this concentration there was a significant drop in the number of cells in both G_0/G_1 and S phases, with an average of 9% and 20%, compared to the DMSO controls (27% and 49% respectively). With 10µM there was a less pronounced G_2/M arrest, with an average of 58%. However, at this concentration the levels of cells in G_0/G_1 phase had decreased even further, with an average of less than 1%.

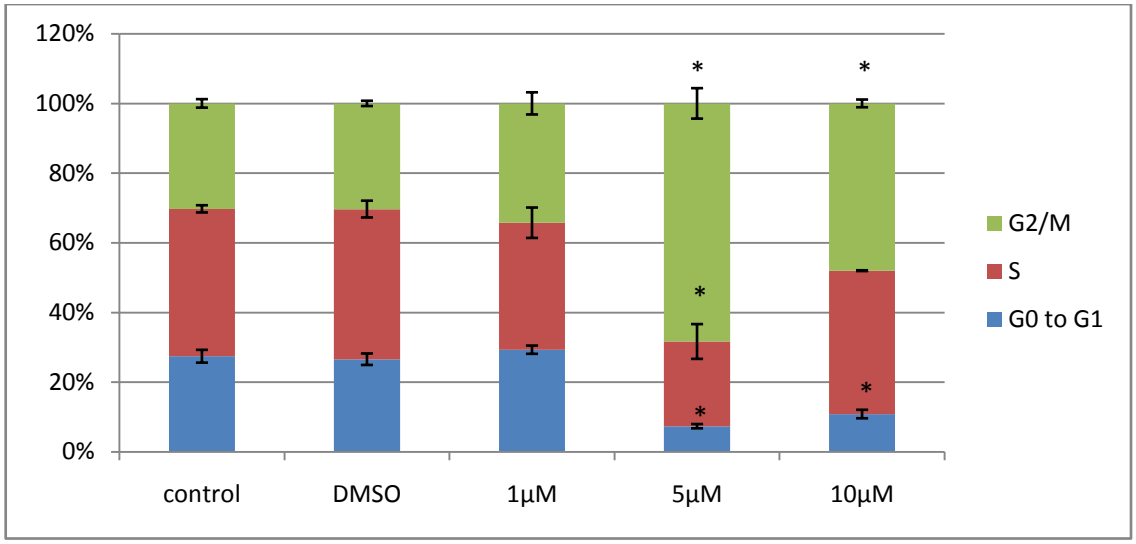


Figure 11 Curcumin Induces G_2/M Arrest in the HCT116 $21^{-/-}$ Line After 12 Hours

HCT116 $p21^{-/-}$ cells were treated with curcumin or DMSO alone for 12 hours then harvested for FACS cell cycle analysis. Error bars show the standard deviation. * indicates a significant difference ($p<0.05$) in the mean number of cells in each phase following curcumin treatment compared to the DMSO control, following a two-tailed T test, assuming equal variance.

Similar results were obtained in the HCT116 $p21^{-/-}$ line (Figure 11). The main difference was that the levels of G_2/M arrest were slightly lower, with an average of 64% compared to 71% in the HCT116 $p53^{-/-}$ line and 81% in the HCT116 $p53^{+/+}$ line.

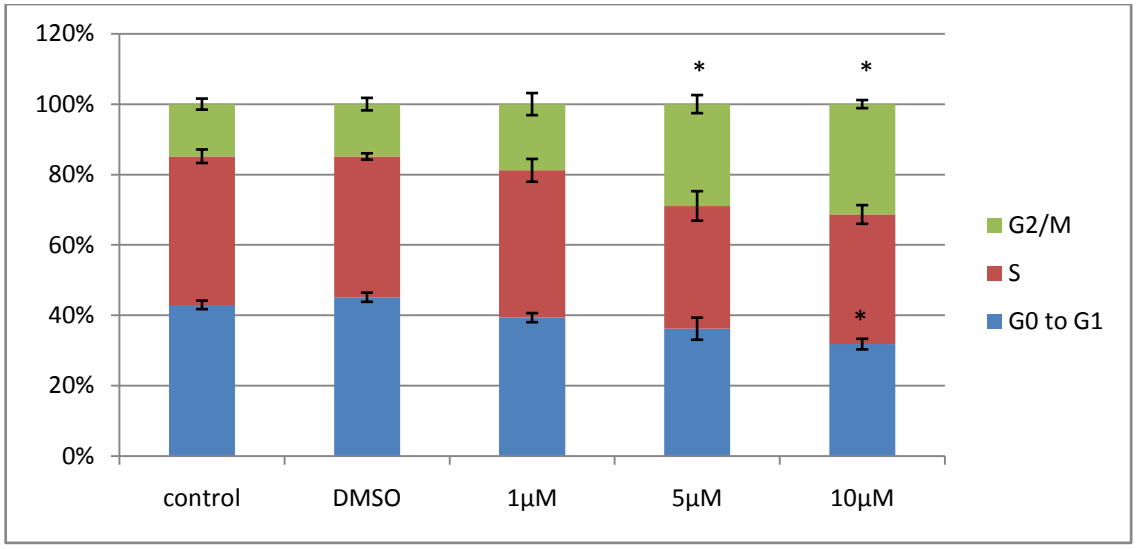


Figure 12 Curcumin Induces G_2/M arrest in HT-29 CRC CIN Line

HT-29 colorectal cancer cells were treated with curcumin or DMSO alone for 12 hours then harvested for FACS cell cycle analysis. Error bars show the standard deviation* indicates a significant difference ($p<0.05$) in the mean number of cells in each phase following curcumin treatment compared to the DMSO control, following a two-tailed T test, assuming equal variance.

Significant G_2/M cell cycle arrest was achieved in the HT-29 cell line following treatment with 5-10µM of curcumin (Figure 12). The highest levels of arrest occurred following 10µM curcumin treatment with an average of 31% of cells compared to an average of 15% in the DMSO control. The levels of cells in G_0/G_1 phase inversely correlated with the concentrations of curcumin, with an average of 39%, 36% and 32% of cells in S phase following treatments of 1, 5 and 10µM of curcumin.

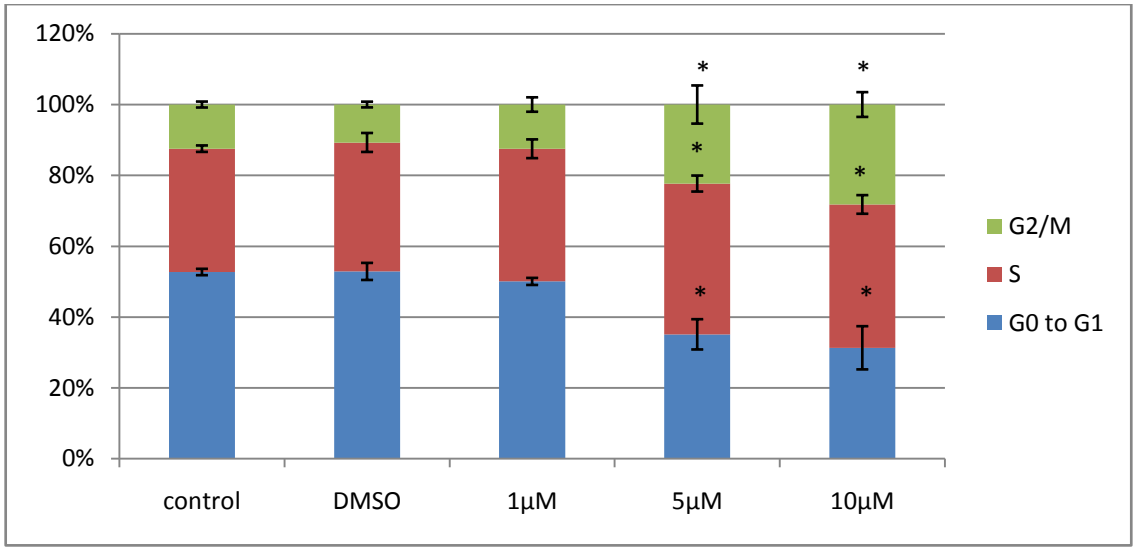


Figure 13 Curcumin Induces in G_2/M Arrest in the SW480 CRC CIN Line

SW480 colorectal cancer cells were treated with curcumin or DMSO alone for 12 hours then harvested for FACS cell cycle analysis. Error bars show the standard deviation. * indicates a significant difference ($p<0.05$) in the mean number of cells in each phase following curcumin treatment compared to the DMSO control, following a two-tailed T test, assuming equal variance.

SW480 cells treated with 5-10µM of curcumin for 12 hours underwent a small but significant G_2/M arrest (Figure 13). Treatment with 10µM was the most effective at inducing G_2/M arrest, with an average of 28% of cells in contrast to 11% in the DMSO control. There were correspondingly lower levels of cells in the G_0/G_1 phase after curcumin treatment.

The main findings of these FACS cell cycle analysis experiments are that G_2/M arrest is induced with lower concentrations of curcumin (1-10μM) following the shorter treatment duration (12 hours). Treatment with 10μM resulted in the highest levels of arrest in the HCT116 $p53^{+/+}$ line, with an average of 81% in G_2/M, which is approximately 9% higher than the average for the same treatment for 24 hours. In addition, 12 hours treatment with just 1μM of curcumin resulted in a significant G_2/M arrest in this line after 12 hours.

In the HCT116 $p53^{-/-}$ line treatment for 12 hours with a 5μM concentration of curcumin displayed the highest levels of arrest with 71% of cells in G_2/M. In contrast to the genotype control, these levels of G_2/M arrest are lower than those observed over longer treatment durations of 24, 48 and 72 hours in this line.

Treatment with 5μM of curcumin in the HCT116 $p21^{-/-}$ line resulted in the maximal levels of G_2/M arrest, with an average of 64% of cells in this phase. Again, these levels are much lower than those found over longer treatment times where levels of over 80% of the cell population in G_2/M were achieved.

While significant G_2/M arrest was achieved following curcumin treatment in the CIN lines, the average percentage of cells in this phase following curcumin treatment was generally much lower than in the HCT116 MIN lines.

3.2.3 Pilot Studies Confirm Curcumin Induces Significant Mitotic Arrest in the HBL100 and MDA468 Lines

The mitotic index is defined as the ratio of cells in mitosis to the total number of cells and can be assessed using fluorescence microscopy to analyse phospho-histone H3 (pH3) staining in combination with nuclear morphology. Histone H3 is phosphorylated by Aurora B at the onset of mitosis and this process can be visualised using anti-pH3 antibodies in conjunction with fluorescently labelled secondary antibodies. The spindle poison nocodazole was used as a positive control for all mitotic index experiments at a concentration of 0.33μM (0.1μg/ml^{-1}). As previously published data had indicated curcumin induced mitotic arrest in the MCF-7 breast cancer line (Holy, 2002), pilot experiments were carried out in the HBL100 and MDA MB 468 breast cancer cell lines with concentrations of 10-30μM for 24 hours.

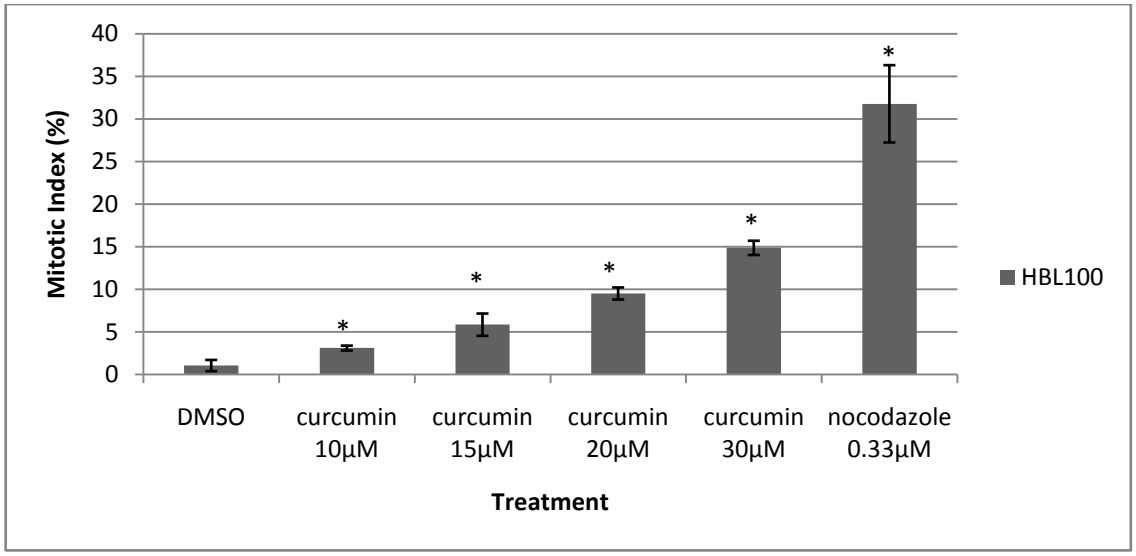


Figure 14 Curcumin Treatment Induces M Phase Arrest in Breast Cancer Line HBL100

HBL100 cells were treated with curcumin for 24 hours, harvested and prepared for immunofluorescence microscopy. Cells were stained with anti-pH3 antibodies to identify mitotic cells and Hoechst to stain DNA. DMSO and 0.33µM of nocodazole were used as negative and positive controls. Data shown are the mean of at least three independent experiments. Error bars show the standard deviation.* indicates a significant ($P<0.05$) difference in means compared to the DMSO control with a two-tailed T test assuming equal variance.

Treatment with curcumin for 24 hours in the HBL100 cell line resulted in a significant dose dependent increase in the average number of cells in M phase (Figure 14). Following treatment with 30µM, 15% of cells were in M phase compared to an average of 1% in the DMSO control. Following nocodazole treatment 32% of cells were in M phase.

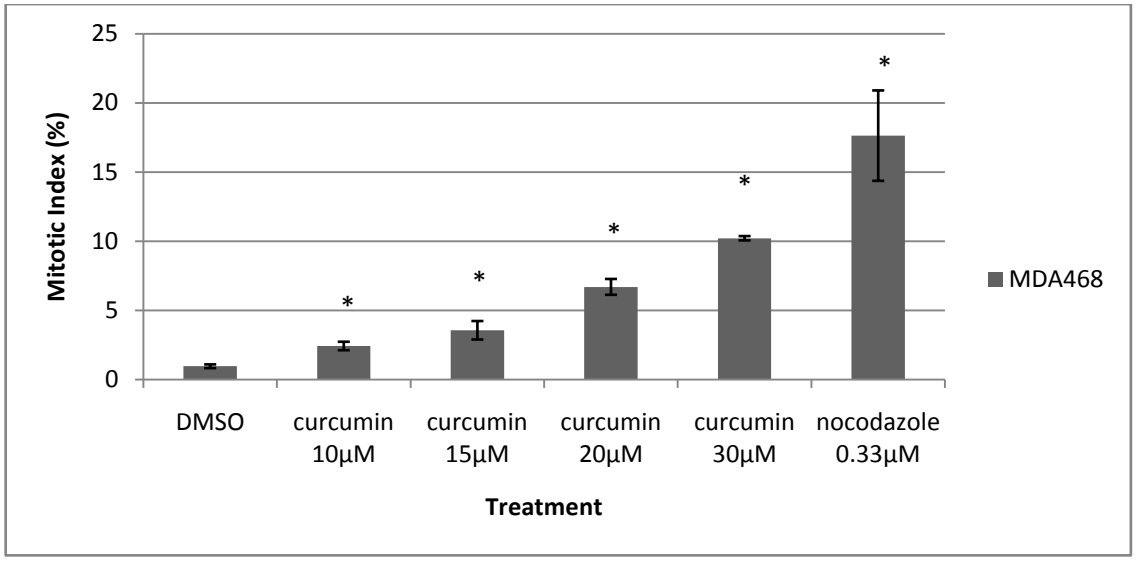


Figure 15 Curcumin Treatment Results in M Phase Arrest in the MDA-MB-668 Breast Cancer Line

MDA-MB-468 breast cancer cells were treated with curcumin for 24 hours and their mitotic index analysed using fluorescence microscopy after co-staining with pH3 antibodies and Hoechst. DMSO and 0.33µM of nocodazole were used as negative and positive controls. Data shown are the mean of at least three independent experiments. Error bars show the standard deviation.* indicates a significant ($P<0.05$) difference in means compared to the DMSO control with a two-tailed T test assuming equal variance.

Treatment with curcumin for 24 hours also induces significant dose-dependent M phase arrest in the MDA-MB-468 breast cancer cell line (Figure 15), with a maximum of 10% of cells in M phase compared to 1% in the DMSO control. Treatment with 0.33µM nocodazole resulted in an average of 18% of cells in M phase.

These mitotic index studies on the breast cancer cell lines showed that curcumin treatment resulted in higher levels of M phase arrest in the HBL100 cell line than in the MDA-MB-468 line. Lower levels of M phase arrest also occurred in the nocodazole control in the MDA-MB-468 breast cancer cell line with an average of approximately 18%, compared to the HBL100 which had an average of over 30%.

3.2.4 Curcumin Treatment Results in Peak Levels of Mitotic Arrest After 12 Hours in the HCT116 $p53^{+/+}$ and HCT116 $p21^{-/-}$ Lines

A time course experiment was then performed over 36 hours to ascertain the peak of curcumin-induced mitotic arrest in the HCT116 $p53^{+/+}$, HCT116 $p53^{-/-}$ and HCT116 $p21^{-/-}$ cell lines.

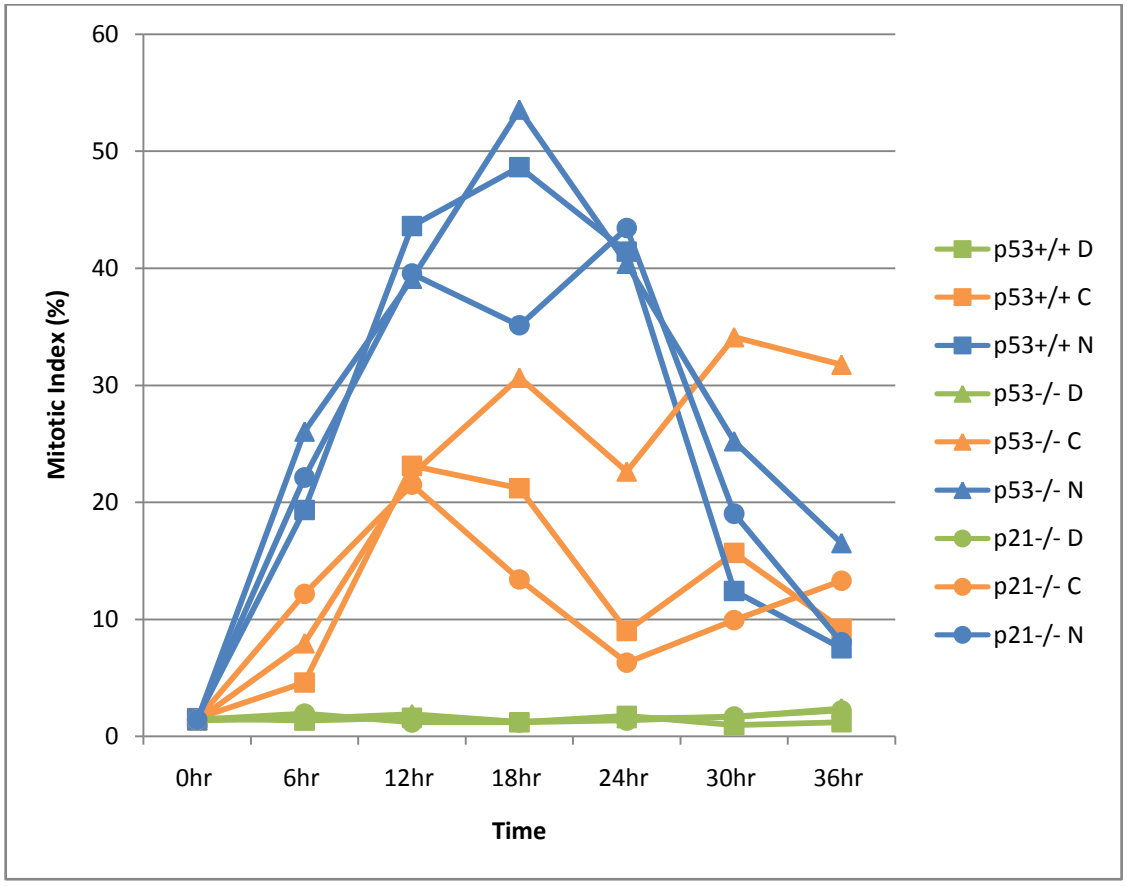


Figure 16 p21 and p53 Status Affects Peak Levels of Curcumin-induced Mitotic Arrest in the HCT116 Cell Lines

HCT116 $p53^{+/+}$, HCT116 $p53^{-/-}$, and HCT116 $p21^{-/-}$ lines were treated with 10µM of curcumin for 0-36 hours and then analysed using fluorescence microscopy to evaluate the mitotic index. DMSO and 0.33µM of nocodazole were used as negative and positive controls. For each data point 1000+ cells were analysed using anti-pH3 antibodies and Hoechst staining (n=1).

In the HCT116 $p53^{+/+}$ cell line the highest levels of curcumin-induced M phase arrest were found at the 12 hour time point, with an average of 23% (Figure 16). In contrast untreated cells had a mitotic index of around 1-2%. The peak mitotic index with 0.33µM nocodazole was 49% at 18 hours. Of the three HCT116 lines, the HCT116 $p53^{-/-}$ line displayed the highest levels of mitotic arrest following curcumin treatment, peaking at 34% at 30 hours. Treatment with nocodazole for 18 hours resulted in the highest levels of mitotic arrest with an average of 54% cells in the HCT116 $p53^{-/-}$ line.

The time-course showed that the highest levels of M phase arrest following curcumin treatment also occurred at the 12 hour time point in the HCT116 $p21^{-/-}$ line, with an average of 21% of cells in M phase. In this line the highest mitotic index after treatment with nocodazole was found at the 24 hour time point with an average of 43%.

With the exception of the HCT116 $p53^{-/-}$ line, the highest levels of mitotic arrest following curcumin treatment occurred at the 12 hour time point and subsequently dropped. The levels of mitotic arrest induced with nocodazole were generally highest at the 18 hour time point with the exception of the HCT116 $p21^{-/-}$ line. At the 30 hour time point, the levels of curcumin-induced mitotic arrest increased compared to levels at 24 hours in all cell lines. However, a similar effect was not observed at the 30 hour time point in the nocodazole control treated cells. As Figure 16 shows that curcumin treatment for 12 hours generally resulted in the highest levels of G_2/M arrest in the HCT116 lines, all further mitotic index experiments were carried out using this treatment duration.

3.2.5 Curcumin Treatment Results in Significant Mitotic Arrest After 12 Hours in the HCT116 Lines

In light of the time course data, mitotic arrest in the panel of five CRC lines was examined by evaluating the mitotic index following treatment for 12 hours with a range of 1-10µM of curcumin.

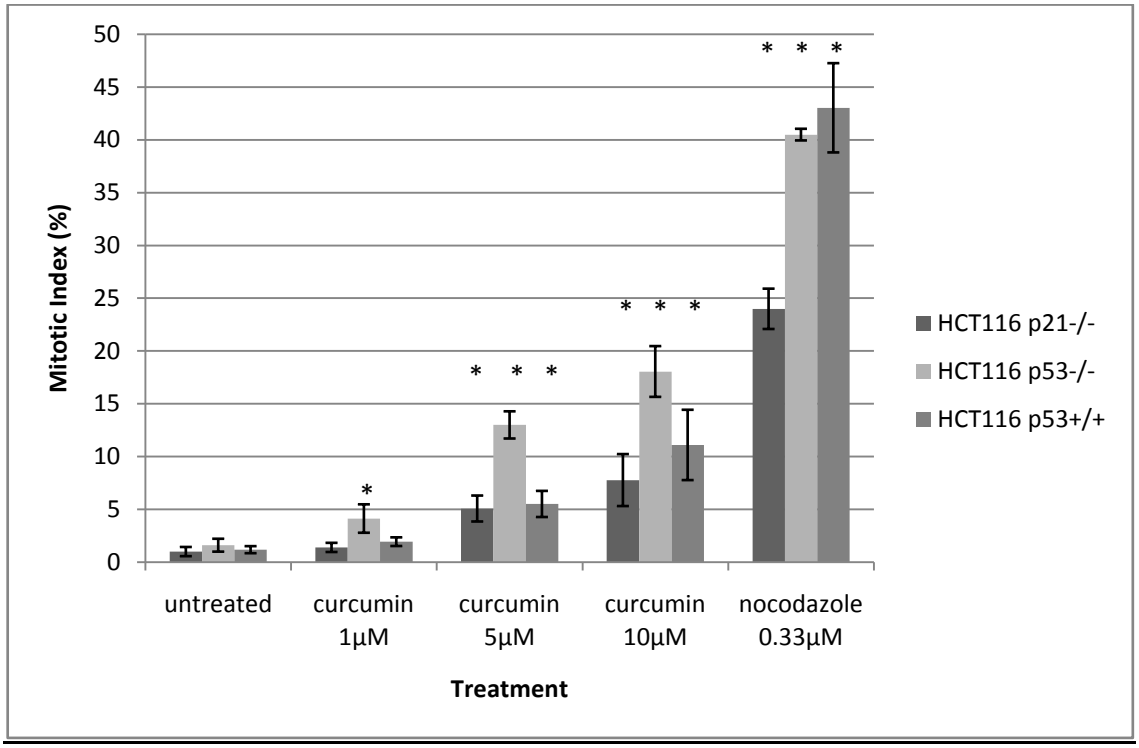


Figure 17 Curcumin Treatment Induces Mitotic Arrest in HCT116 $p53^{+/+}$, $p53^{-/-}$, $p21^{-/-}$ CRC Lines

HCT116 $p53^{+/+}$, HCT116 $p21^{-/-}$, and HCT116 $p53^{-/-}$ cells were treated with curcumin for 12 hours, co-stained with anti-pH3 antibodies and Hoechst and their mitotic index assessed using fluorescence microscopy. DMSO and 0.33µM of nocodazole were included as negative and positive controls. At least 500 cells were scored per data point. Data shown are the mean of at least three independent experiments. Error bars show the standard deviation.* indicates a significant ($P<0.05$) difference in means compared to the DMSO control with a two-tailed T test assuming equal variance.

HCT116 $p53^{+/+}$, $p53^{-/-}$, and $p21^{-/-}$ lines were analysed following curcumin treatment with immunofluorescence microscopy to evaluate the mitotic index (Figure 17). Results showed a significant ($P<0.05$) increase following 5 and 10μM curcumin treatment in all three cell lines. There was a significant increase in the mitotic index following treatment with just 1μM in the HCT116 $p53^{-/-}$ line, with an average of 4% cells in M phase compared to an average of 2% in the DMSO control. The highest levels of curcumin-induced M phase arrest were found in the HCT116 $p53^{-/-}$ line, with an average of 18% cells in M phase following treatment with 10μM. These results were similar to findings from the time course experiment, where the average number of cells in M phase after 12 hours treatment in the HCT116 $p53^{-/-}$ line was 23% (Figure 16). In contrast, both the HCT116 $p53^{+/+}$ and HCT116 $p21^{-/-}$ cell lines had means of 11% and 8% respectively. Figure 17 also shows that the HCT116 $p21^{-/-}$ cell line has the lowest average number of cells in M phase following nocodazole treatment. Data from Figure 17 shows that the mean mitotic index of the HCT116 $p21^{-/-}$ line following treatment with 0.33μM nocodazole for 12 hours is 24% in contrast to 40% in Figure 16. Figure 17 shows that of the three HCT116 lines, the HCT116 $p53^{-/-}$ line is consistently the most sensitive to curcumin-induced M phase arrest.

3.2.6 Curcumin Treatment Fails to Induce Mitotic Arrest After 12 Hours in the CIN HT-29 Line

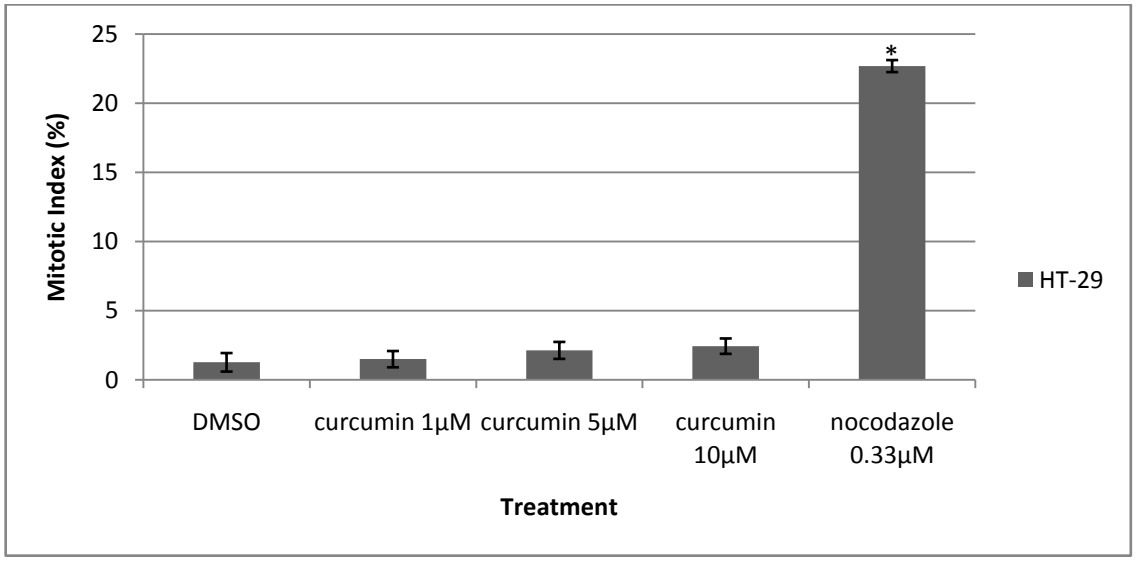


Figure 18 Curcumin Treatment Fails to Induce Mitotic Arrest in the CIN CRC Line HT-29

HT-29 cells were treated with curcumin for 12 hours and mitotic index assessed using fluorescence microscopy. Cells were co-stained with anti-pH3 antibodies and Hoechst, and DMSO and 0.33µM of nocodazole were used as negative and positive controls. Data shown are the mean of at least three independent experiments. Error bars show the standard deviation.* indicates there is a significant ($P<0.05$) difference in means compared to the DMSO control with a two-tailed T test assuming equal variance.

In the CIN HT-29 cell line curcumin treatment for 12 hours did not induce a significant M phase arrest (Figure 18). In contrast, treatment with 0.33µM of nocodazole resulted in an average of 23% of cells arresting in M phase. This level of nocodazole-induced M phase arrest is comparable to levels observed in the HCT116 $p21^{-/-}$ line under similar conditions.

3.2.7 Curcumin Treatment Induces Significant Mitotic Arrest After 12 Hours in the CIN SW480 Line

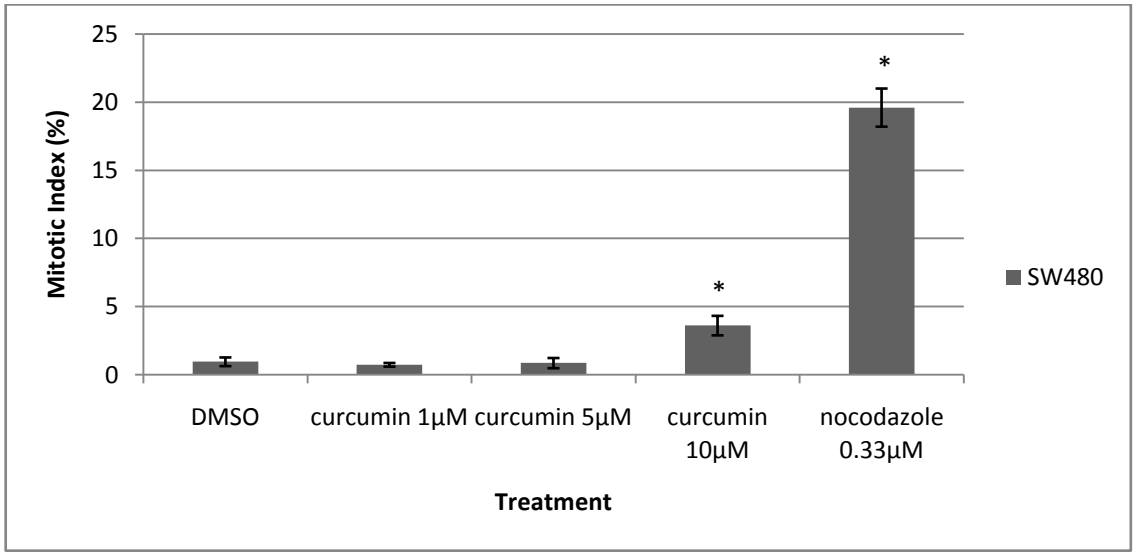


Figure 19 Curcumin Induces M Phase Arrest in the CIN SW480 Line

SW480 cells were treated with curcumin for 12 hours and mitotic index assessed using fluorescence microscopy. Cells were co-stained with pH3 antibodies and Hoechst. DMSO and 0.33µM of nocodazole were used as negative and positive controls. Data shown are the mean of at least three independent experiments. Error bars show the standard deviation. Bars marked with * indicate there is a significant ($P<0.05$) difference in means compared to the DMSO control with a two-tailed T test assuming equal variance.

Figure 19 shows that treatment with 10µM of curcumin for 12 hours resulted in a small, but significant increase in the number of SW480 cells in M phase (4%), compared to 1% in the DMSO control. As in the HT-29 line, treatment with nocodazole was more effective, resulting in an average mitotic index of 20%.

3.3 Discussion

The experiments described above analysed in depth the ability of curcumin to induce cell cycle arrest in a panel of colorectal cancer cell lines following treatment for various times and at different doses. Effects of curcumin treatment on G_2/M cell cycle arrest have been described previously, but it was important to re-establish the basic parameters of arrest. These data could then be used in conjunction with novel mitotic index data to draw new conclusions regarding the nature of this arrest, and to define whether arrest occurred at the G_2/M boundary, during mitosis, or at both checkpoints of the cell cycle.

3.3.1 Curcumin Induces G_2/M Cell Cycle Arrest in both MIN and CIN CRC Lines

FACS cell cycle analysis showed that treatment with 10μM of curcumin induced significant G_2/M arrest in all HCT116 lines over 72 hours (Howells et al., 2007, Jaiswal et al., 2002). These findings are in accordance with previous research, but are the first to characterise arrest at the 12 hour time point.

In general the HCT116 Wt/HCT116 $p53^{+/+}$ cell line displayed higher levels of G_2/M arrest at the 12 and 24 hour time points and levels dropped at later time points. In comparison, levels of arrest in the HCT116 $p53^{-/-}$ line consistently increased with time, with the highest levels found after 72 hours treatment. The HCT116 $p21^{-/-}$ line had increasing levels of G_2/M arrest between the 12 and 24 hour time points, with levels peaking at 48 hours, and dropping by the 72 hour time point.

The study carried out by Jaiswal et al. (2002) investigating the effects of curcumin on the HCT116 panel, revealed that G_2/M phase arrest was p53-independent in agreement with the results presented here. However, as they used 20µM of curcumin for 15 and 30 hours, direct comparisons cannot be made. Nevertheless, there are similarities in the response of these lines to curcumin treatment in the two studies. In the HCT116 $p53^{-/-}$ cell line, treatment with 20µM curcumin for 15 hours resulted in less than 1% of cells in G_0/G_1 phase. Figure 10 shows that in the same cell line, treatment with 10µM of curcumin also results in similar levels of cells in G_0/G_1 phase. This was also found in the same cell line at the 24 hour time point, and in the HCT116 $p21^{-/-}$ line at the 12 and 48 and 15 and 30 hour time points in response to curcumin. Previous research using the colorectal cancer Lovo cell line had noted that arrest in S and G_2/M phases of the cell cycle prevented progression of the cells through the cell cycle (Chen et al., 1999). In the HCT116 lines tested in this present study, high levels of G_2/M arrest were accompanied by corresponding low levels of cells in the G_0/G_1 phase of the cell cycle. Lower levels of cells progressing through the cell cycle following curcumin treatment could contribute to the anti-proliferative effects of curcumin found in the HCT116 colorectal cancer lines (Hanif et al., 1997, Reddy et al., 2006).

While the CIN lines did show a significant increase in cells in G_2/M phase following curcumin treatment for 12 hours, levels were much lower than those found in the HCT116 lines. The HT-29 line had an average of 31% of cells undergoing G_2/M arrest following 10µM of curcumin treatment compared to 81% in the HCT116 $p53^{+/+}$ line. The SW480 line had similar levels of arrest to the HT-29 line with an average of 28% cells in G_2/M after 10µM of curcumin treatment.

3.3.2 Curcumin Induces M Phase Arrest in Breast and Colorectal Cancer Cell Lines

Mitotic index experiments confirmed that curcumin induces significant mitotic arrest in the HBL100 and MDA468 breast cancer lines. These data confirm findings from a past study investigating the effects of curcumin on MCF7 cells (Holy, 2002). However, in the present study cells were co-stained with pH3 antibodies as a marker of mitosis, and this additional stain was used in combination with nuclear morphology to identify mitotic cells instead of nuclear morphology alone as described by Holy. Additionally, statistical analysis was performed in the present study to confirm significant changes in the mitotic index following curcumin treatment.

Observations over 36 hours in the three HCT116 lines highlighted key differences and similarities in the nature of curcumin-induced M phase arrest in each line (Figure 16). The peak mitotic index occurred at 12 hours in the HCT116 $p53^{+/+}$ and HCT116 $p21^{-/-}$ lines. In comparison, the peak in the HCT116 $p53^{-/-}$ line was found after 30 hours treatment with curcumin. The HCT116 $p53^{-/-}$ line showed the highest levels of mitotic arrest following curcumin treatment and this arrest was maintained over time. Further analysis of mitotic arrest after 12 hours treatment with curcumin in the HCT116 panel confirmed that the HCT116 $p53^{-/-}$ was the most sensitive to arrest, with a significant increase in mitotic index following treatment with just 1μM.

Compared to the MIN HCT116 lines, the CIN HT-29 and SW480 colorectal cancer lines were significantly less sensitive to curcumin treatment with regards to mitotic arrest. In SW480 cells there was a small but significant increase following 12 hours treatment with 10μM of curcumin. The average mitotic index following treatment with the nocodazole was also lower in the HT-29 and SW480 lines and these findings are in

agreement with a previous study investigating the effects of mitotic checkpoint genes on M phase arrest in human cancer cell lines (Cahill et al., 1998). This research showed that when MIN lines were transfected with a mutant version of the *hBUB1* gene, levels of M phase arrest were reduced and comparable to those found in CIN lines. These findings led the authors to hypothesise that the lower levels of M phase arrest found in the CIN lines were a result of a compromised spindle assembly checkpoint. Later research argued that while CIN lines achieved lower levels of mitotic arrest compared to MIN lines in response to agents that induce spindle damage, CIN lines do have a robust checkpoint (Tighe, 2001).They proposed that differences in mitotic arrest were linked to the function of the adenomatous polyposis coli gene (*APC*) which is frequently found to be mutated in CIN CRC lines (Morin et al., 1997). When the MIN HCT116 line was transfected with a mutant version of the *APC* gene, this resulted in a partial abrogation of M phase arrest, with levels similar to those of the CIN lines tested (Tighe, 2001). Of the panel of cell lines in this study, the HCT116 lines have a wild-type version of the *APC* gene, and the HT-29 and SW480 lines have a mutant version of this gene (Morin et al., 1997, Morin et al., 1996). While there are clear differences in the levels of curcumin-induced M phase arrest between the MIN and CIN lines, further analysis of the involvement of APC, or spindle assembly checkpoint proteins such as Bub1, BubR1 and Mad2 would be required to draw conclusions as to the importance of these proteins in curcumin-induced mitotic arrest.

3.3.3 Integrating FACS and Mitotic Index Data Regarding Curcumin-Induced G_2/M and M Checkpoint Arrest

In the HCT116 $p53^{+/+}$ line cell cycle analysis showed that a range of 1-10μM of curcumin treatment resulted in a significant G_2/M arrest after 12 hours. For the same time point concentrations of 5 and 10μM of curcumin resulted in a significant M phase arrest as shown by mitotic index studies. The increase in cells in G_2/M arrest as shown by FACS is greater than the increased percentage of cells in M phase as shown by mitotic index analysis. This indicates that following curcumin treatment the HCT116 $p53^{+/+}$ cells undergo a combination of G_2/M boundary and M phase arrest. Similar results were obtained for the HCT116 $p53^{-/-}$ and HCT116 $p21^{-/-}$ lines. FACS cell cycle studies of the HCT116 genotype control and knock-out lines showed that treatment with 5μM of curcumin for 12 hours consistently induced the highest levels of G_2/M arrest. These data show that curcumin can induce high levels of cell cycle arrest at physiologically relevant concentrations in colorectal cancer cell lines regardless of p53 status. The mitotic index studies of these lines revealed that 10μM curcumin resulted in the highest levels of M phase arrest following 12 hours treatment in the HCT116 $p53^{+/+}$ and HCT116 $p21^{-/-}$ lines. As the levels of G_2/M arrest assessed by FACS analysis decrease with 10μM of curcumin at this time point the ratio of cells undergoing M phase arrest to those undergoing G_2 arrest alone increases with this higher concentration. Interestingly, the HCT116 $p53^{-/-}$ line was the most sensitive to M phase arrest and this may be due to the reduced capacity to maintain G_2/M checkpoint arrest in cells lacking the p53 protein (Bunz et al., 1998). Cells lacking p53 may be unable to arrest as effectively at the G_2/M boundary, so these cells continue to enter into mitosis and arrest at the mitotic checkpoint.

FACS analysis of the HT-29 line following treatment with 5 or 10µM of curcumin for 12 hours showed significant G_2/M arrest. The levels of arrest found after treatment with 10µM were comparable to those found in an earlier study (Hanif et al., 1997). However this line did not undergo M phase arrest when analysed using the mitotic index assay. It can therefore be concluded that these cells are arresting predominantly at the G_2/M checkpoint in response to curcumin.

In the SW480 cell line FACS analysis showed that 5-10µM curcumin treatment for 12 hours resulted in a significant G_2/M arrest. At this time point 10µM also resulted in a significant M phase arrest in this cell line. This suggests that 5µM of curcumin treatment predominantly results in G_2/M arrest, and that 10µM of curcumin treatment for 12 hours results in both G_2 and M phase arrest.

In summary, curcumin treatment resulted in G_2/M arrest in all cell lines tested, and specific M phase arrest in four out of the five lines. Of note, the HCT116 $p53^{-/-}$cell line was the most sensitive to curcumin treatment with just 1µM resulting in a significant increase in the mitotic index. The CIN HT-29 and SW480 lines were the least sensitive to curcumin treatment, and had the lowest levels of both G_2/M and M phase arrest.

Chapter 4 Characterisation of Curcumin-Induced Mitotic Arrest

4.1 Introduction

Recent research has highlighted the potential for curcumin to be used as a novel anti-mitotic agent in the treatment of cancer. It was shown to act by disrupting the microtubule network in dividing cancer cells via inhibition of microtubule assembly (Gupta et al., 2006). Following the discovery that treatment stained tubulin polymers yellow, curcumin was found to be incorporated into microtubule polymers in a concentration-dependent fashion. This binding resulted in conformational changes in tubulin and reduced GTPase activity of microtubules resulting in altered microtubule assembly dynamics.

Earlier studies using the MCF-7 breast cancer cell line indicated curcumin disrupted mitotic spindle structure and image analysis revealed the accumulation of mitotic cells with monopolar spindles (Holy, 2002). This study also showed abnormal distribution of the microtubule-bundling protein NuMA (nuclear mitotic apparatus protein) in curcumin-treated mitotic cells.

An increase in the mitotic index in mouse progenitor cells transfected with the Bcr-Abl oncoprotein was observed alongside mitotic spindle disorganisation and failed chromosome segregation following curcumin treatment (Wolanin, 2006). This was accompanied by mislocalisation of Aurora B, and a downregulation of survivin mRNA levels. The authors proposed that these were linked as similar patterns of Aurora B staining were observed following survivin knock-down with siRNA.

Research on the effects of curcumin on the human promyelocytic leukemia HL-60 line showed that treatment resulted in aberrant mitosis followed by cell death. Total protein levels of the chromosomal passenger complex member, survivin, were also shown to be decreased following curcumin treatment. This study also observed tubulin staining by curcumin, indicating incorporation of this compound into microtubule polymers, and images showed disruption of the cytoskeleton in mitotic cells (Magalska et al., 2006).

To investigate the effects of curcumin on mitosis in CRC cells, images of control and curcumin-treated cells were captured. Chromosomal bridge formation, centrosomal separation, the stage of mitosis and levels of mitotic spindle abnormalities were quantified to further characterise curcumin-induced mitotic arrest. Additionally, the localisation of Aurora B was studied in curcumin-treated CRC cells during mitosis.

4.2 Results

4.2.1 The Effects of Curcumin on Mitotic Progression

To elucidate whether curcumin induced arrest at a specific stage of mitosis, control and curcumin-treated mitotic cells were examined using fluorescence microscopy.

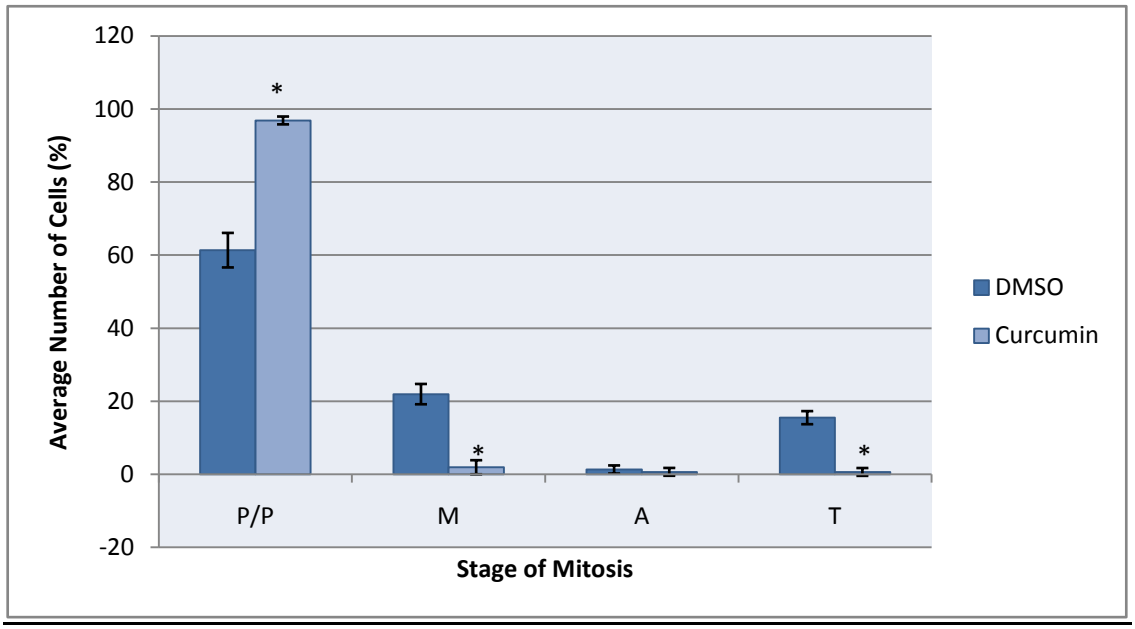


Figure 20 Curcumin Induces M Phase Arrest at the Prophase/Prometaphase Stage of Mitosis

HCT116 $p53^{+/+}$ cells were treated with either DMSO alone or 10µM of curcumin for 12 hours and prepared for image analysis using fluorescent microscopy. Cells were stained with antibodies against α-tubulin and centrin. DNA was stained with DAPI. Prophase/prometaphase cells (P/P) were defined by the appearance of condensed chromosomes. Metaphase cells (M) were defined as having a bipolar mitotic spindle with chromosomes clearly aligned on the metaphase plate. In anaphase (A) sister chromatid separation could be visualised, and in telophase (T) this separation had progressed and contraction of the spindle mid-body could be seen. Fifty mitotic cells were counted per slide, and results are representative of three separate experiments. Error bars show the standard deviation and * indicates a significant ($p<0.05$) difference in means compared to the DMSO control following a two-tailed t-test assuming equal variance.

Curcumin specifically induces M phase arrest at prophase/prometaphase in the HCT116 $p53^{+/+}$ cell line, with an average increase of 36% of cells at this stage in mitosis (Figure 20). This is accompanied by a significant decrease in cells found in metaphase and telophase, later phases of mitosis, when compared to the DMSO controls.

Asynchronous populations of cells were treated with DMSO and curcumin to prepare these slides and representative images of interphase cells are included as a control in all figures. To visualise the effects of curcumin on the mitotic spindle and spindle poles at each stage of mitosis, cells were stained with anti-α-tubulin and anti-centrin antibodies. DNA was also stained with DAPI to examine the effects of curcumin on chromatin.

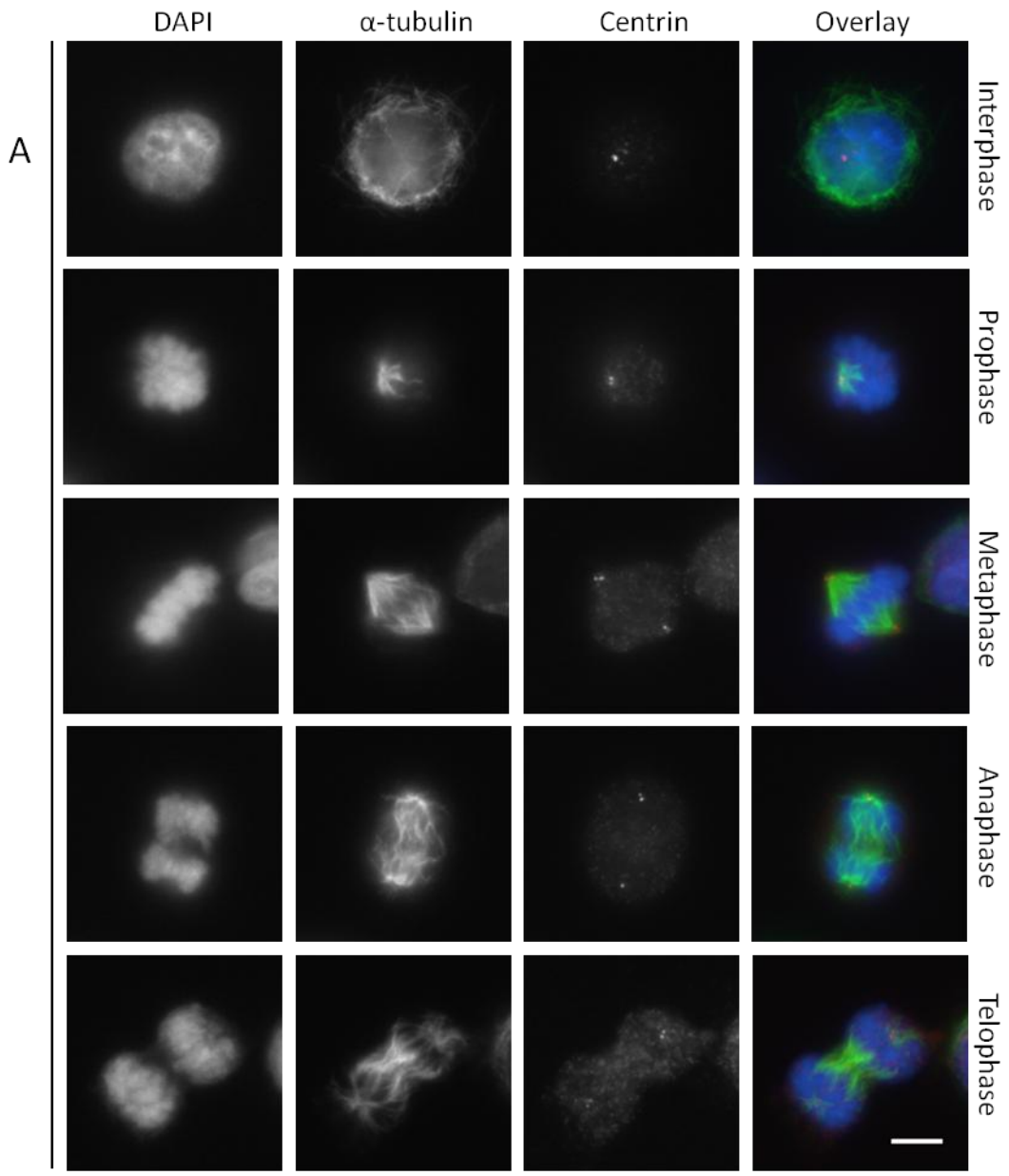


Figure 21 Curcumin Treatment Impairs Normal Mitotic Progression

HCT116 $p53^{+/+}$ cells were treated with DMSO alone (A) or 10μM curcumin (B) for 12 hours and prepared for analysis using fluorescence microscopy. Cells were stained with antibodies against α-tubulin (green) to show the microtubule network and against centrin (red) to show the centrioles. DNA was stained with DAPI (blue). Representative images of interphase, metaphase, anaphase, and telophase cells are shown for each treatment. In the majority of cells a cut phenotype was observed, however in a small percentage of cells chromosomes separated correctly. Images are representative of at least three different experiments. Scale bars 5μm.

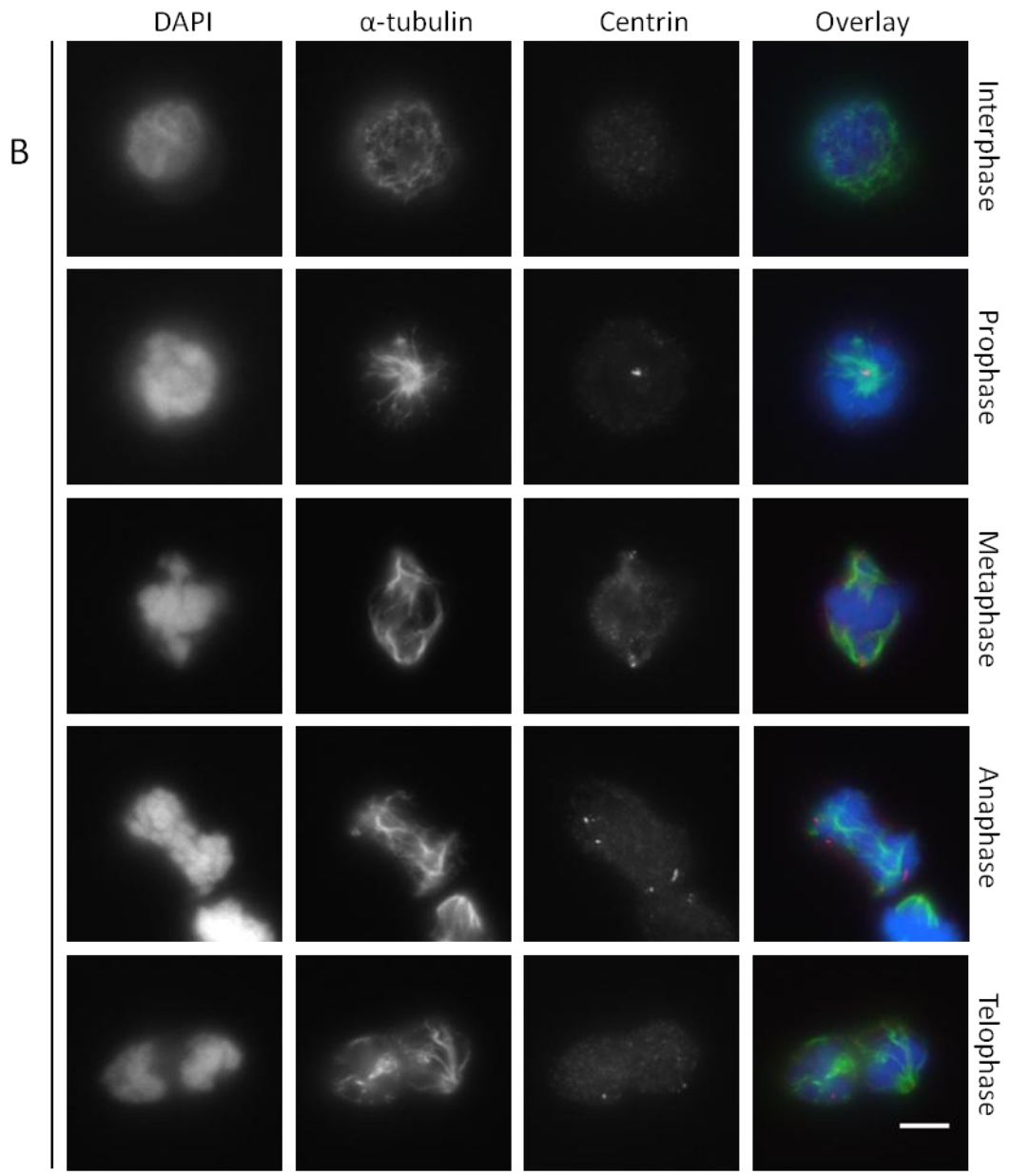


Figure 21 Continued

Figure 21 shows representative images of the microtubule network in control and curcumin-treated cells respectively at various stages of the cell cycle. In control cells progression through each stage of mitosis can be seen. Condensation of chromosomes and the start of mitotic spindle formation can be seen in both the curcumin and DMSO-treated representative prophase cells.

A metaphase cell with bipolar mitotic spindle formation and alignment of chromosomes on the metaphase plate is shown in Figure 21 A. In contrast, Figure 21 B shows a metaphase cell with mitotic spindle abnormalities. While spindle pole separation has occurred, chromosomes appear entangled in the mitotic spindle. The chromosomes are not aligned on the spindle equator, and the spindle itself is not as symmetrical as that seen in the DMSO control.

A control anaphase cell with sister chromatid separation, exhibits two sets of chromosomes which have moved from the spindle equator to towards the spindle poles (Figure 21 A). However, in the curcumin-treated 'anaphase' cell chromatid disjunction has not occurred, and the sister chromosomes are seen as one large asymmetrical mass instead of two sets of independent chromosomes. Sister chromatids have not separated properly, and as a consequence some un-separated chromosomes may be trapped in the mid-body during contraction. Curcumin treated anaphase cells are similar to the *cut* (chromosomes untimely torn) phenotype observed in fission yeast cells expressing a non-degradable version of Cut2, and human cells expressing a non-degradable version of securin (Funabiki et al., 1996, Hagting et al., 2002, Hirano et al., 1986). In the representative image of the curcumin-

treated cell in late telophase, the spindle mid-body cannot be visualised clearly, whereas in the DMSO treated cell contraction of the spindle midbody can be seen.

4.2.2 Characterising the Effects of Curcumin on Chromosomal Bridges

As curcumin treatment resulted in the appearance of a cut phenotype, the number of chromosomal bridges in control and treated cells were compared, to further examine the effects of curcumin on chromosome separation.

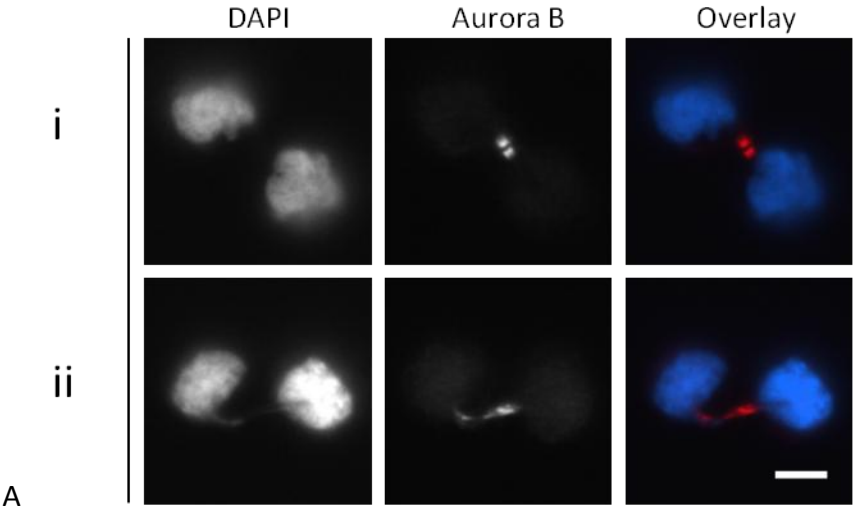


Figure 22 A Curcumin Treatment Does Not Significantly Alter Chromosomal Bridge Formation in HCT116 p53$^{+/+}$ Cells

(A) Representative images of HCT116 p53$^{+/+}$ cells stained for chromatin (DAPI, blue) and Aurora B (red) show (i) two normal nuclei in late cytokinesis with no chromosomal bridge (ii) a chromosomal bridge formed between two nuclei in cytokinesis.

(B) HCT116 p53$^{+/+}$ cells were treated for 12 hours with either DMSO alone or 10µM of curcumin and prepared for immunofluorescent microscopy. At least 50 cells were counted per slide. Results are a mean of at least three separate experiments and error bars show the standard deviation. The difference in means was compared with a two-tailed T test assuming equal variance and $P > 0.05$.

B

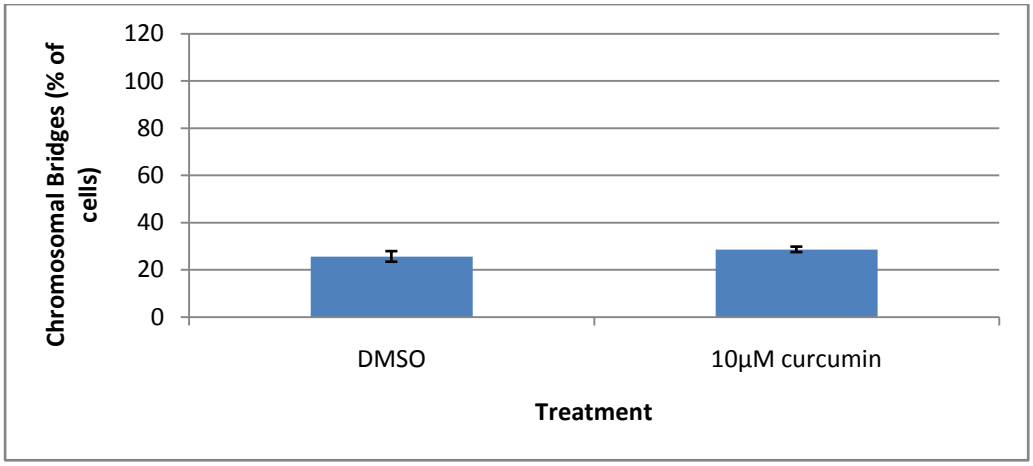


Figure 23 B Curcumin Treatment Does Not Significantly Alter Chromosomal Bridge Formation in HCT116 p53$^{+/+}$ Cells

Curcumin treatment does not significantly alter the levels of chromosomal bridge formation in HCT116 p53$^{+/+}$ cells (Figure 22 B). Control and curcumin-treated samples had an average of approximately 26% and 29% of cells with chromosomal bridges respectively.

4.2.3 Curcumin Treatment Disrupts Early Mitotic Events in CRC Lines

Following these preliminary experiments on the HCT116 p53$^{+/+}$ cells, especially those indicating that curcumin induces arrest in prophase, the effects on early mitotic events in CRC cells were characterised in further detail. Cells were stained for pH3 to identify mitotic cells, centrin to visualise spindle poles, and DNA to visualise chromosomes. Representative images of control and curcumin-treated prophase and metaphase cells were captured.

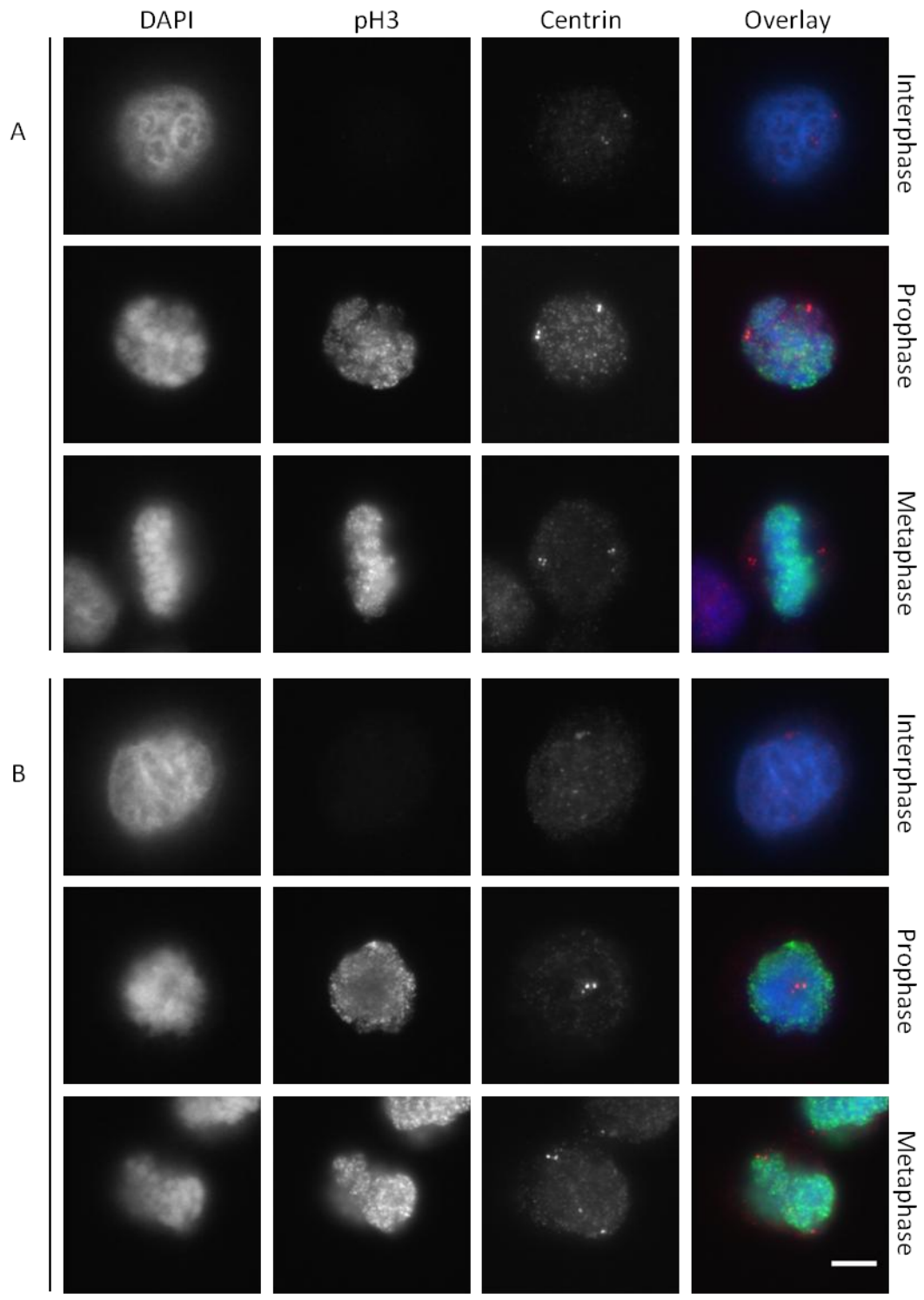


Figure 24 Curcumin Treatment Inhibits Chromosomal Alignment During Early Mitosis in the HCT116 p53$^{+/+}$ line

HCT116 p53$^{+/+}$ cells were treated with DMSO alone (A) or 10µM of curcumin (B) for 12 hours then prepared for immunofluorescence microscopy. Cells were stained with anti-pH3 (green) and anti-centrin antibodies (red). DNA was stained with DAPI (blue). Representative control and curcumin-treated cells are shown in interphase, prophase, and metaphase (n=3). Scale bar 5µm.

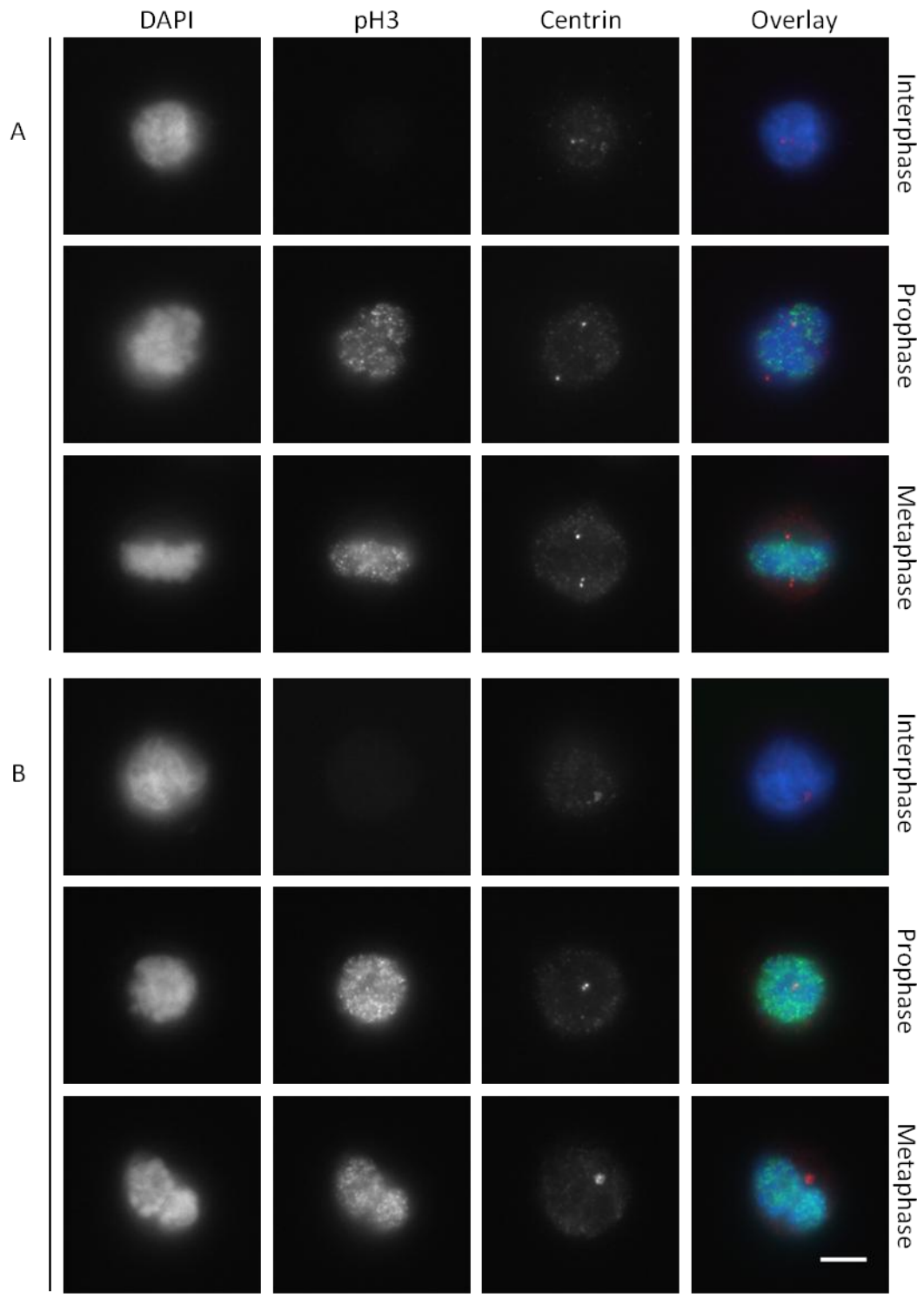


Figure 25 Curcumin Treatment Disrupts Chromosomal Arrangement in Early Mitotic HCT116 p53$^{-/-}$ Cells

HCT116 p53$^{-/-}$ cells were treated with DMSO alone (A) or 10μM of curcumin (B) for 12 hours then stained with anti-pH3 (green) and anti-centrin antibodies (red). DNA was stained with DAPI (blue). Representative cells from each treatment are shown in interphase, prophase, and metaphase (n=3). Scale bar 5μm.

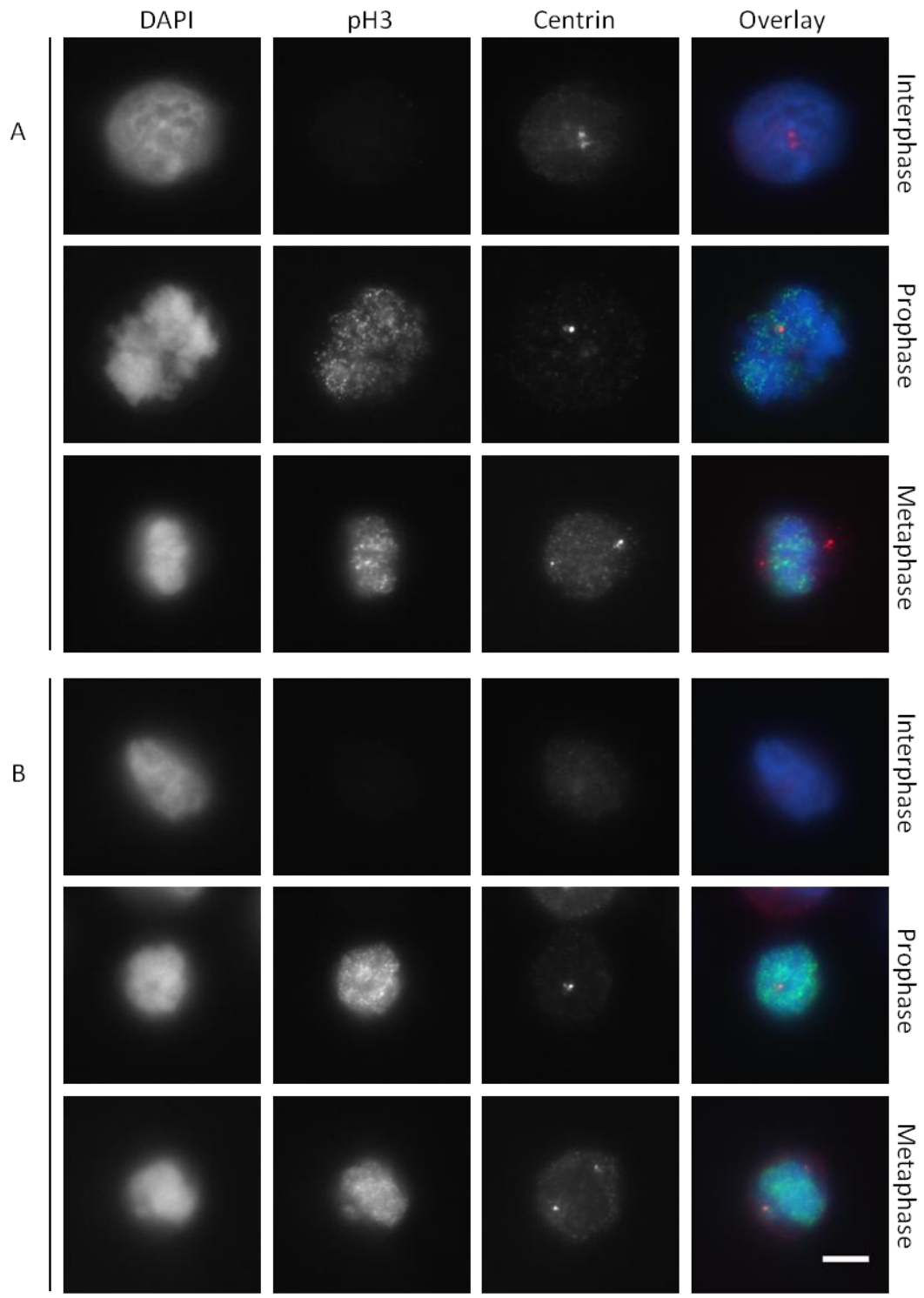


Figure 26 Curcumin Treatment Inhibits Chromosomal Organisation in Early Mitotic HCT116 p21$^{-/-}$ Cells

HCT116 p21$^{-/-}$ cells were treated with either DMSO alone (A) or 10μM of curcumin (B) for 12 hours. Cells were then prepared for immunofluorescence microscopy and stained with anti-pH3 antibodies (green) and anti-centrin antibodies (red). DNA was stained with DAPI (blue). Representative cells from each treatment are shown in interphase, prophase, and metaphase (n=3). Scale bar 5μm.

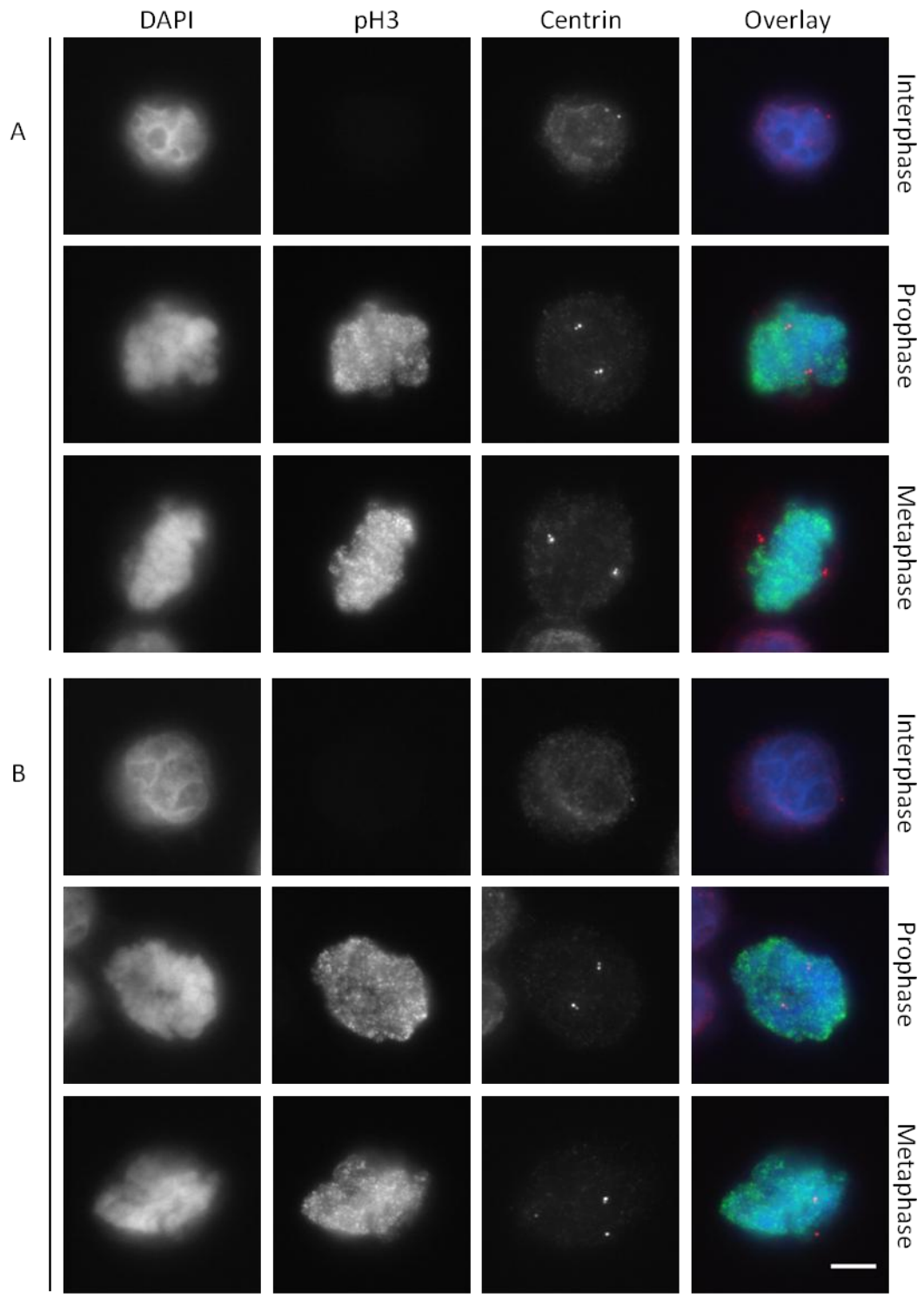


Figure 27 Curcumin Treatment Results in Chromosomal Disarray in Early Mitosis in the HT-29 Line

HT-29 cells were treated with either DMSO alone (A) or 10µM of curcumin (B) for 12 hours. Cells were then prepared for immunofluorescence microscopy and stained with antibodies against pH3 (green) and centrin (red). DNA was stained with DAPI (blue). Representative cells from each treatment are shown in interphase, prophase, and metaphase (n=3). Scale bar 5µm.

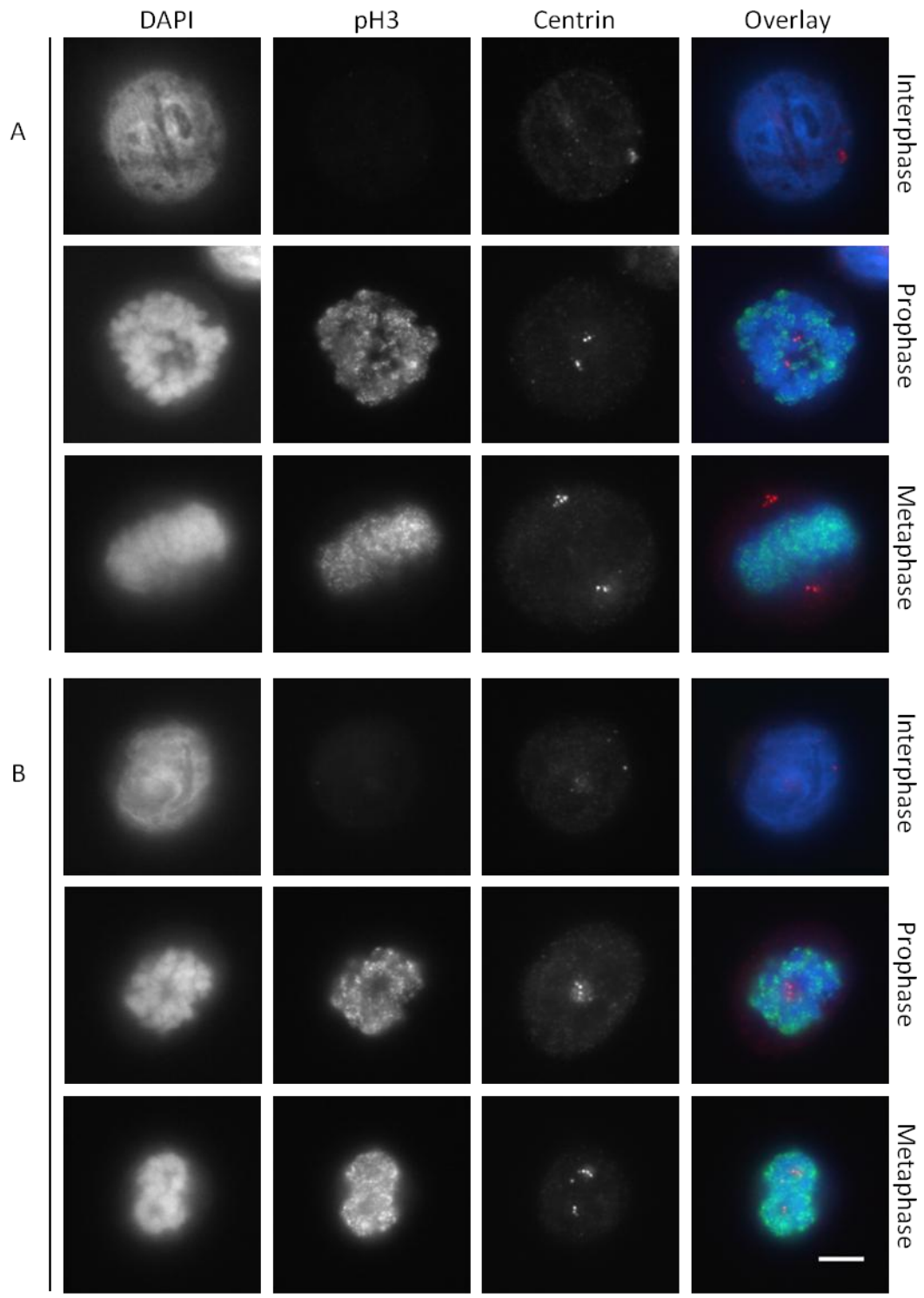


Figure 28 Curcumin Treatment Impairs Chromosomal Organisation in Early Mitotic SW480 Cells

SW480 cells were treated with either DMSO alone (A) or 10µM of curcumin (B) for 12 hours. Cells were then prepared for immunofluorescence microscopy and stained with antibodies against pH3 (green) and centrin (red). DNA was stained with DAPI (blue). Representative interphase, prophase, and metaphase cells are shown for each treatment (n=3). Scale bar 5µm.

Figures 23-27 show representative images of early mitotic cells treated with curcumin from the HCT116 $p53^{+/+}$, HCT116 $p53^{-/-}$, HCT116 $p21^{-/-}$, HT-29, and SW480 colorectal cancer cell lines.

Curcumin-treated prophase cells differed in appearance from DMSO controls in all cell lines. Chromosomes generally appeared more condensed in curcumin-treated cells. In some cells there appeared to be an area in the centre of the chromosomes with lower levels of pH3 staining. In these cells, unseparated centrosomes were usually located in this area, as shown by centrin staining (Figures 23, 24, 25 and 27).

In all lines, curcumin-treated cells were impaired in their ability to align chromosomes on the metaphase plate. In comparison to the DMSO controls, treated cells generally failed to exhibit bi-orientated chromosomes between the spindle poles. In some metaphase cells, as in Figure 24, spindle-pole separation had failed.

These images indicate that curcumin treatment results in impaired chromosomal organisation and alignment in early mitotic cells in conjunction with spindle-pole abnormalities. To investigate the effects of curcumin on mitotic spindle formation in CRC cells a further series of experiments was carried out using fluorescence microscopy.

4.2.4 Characterising the Effects of Curcumin on the Mitotic Spindles of CRC Cells

Curcumin treatment has previously been shown to disrupt mitotic spindle formation in dividing breast, cervical, colorectal, and promyelotic leukemia human cancer cells, and mouse progenitor cells transfected with the Bcr-Abl oncoprotein (Gupta et al., 2006, Holy, 2002, Magalska et al., 2006, Wolanin, 2006). To investigate such an effect in these CRC lines, immunofluorescence microscopy was performed using antibodies to α-tubulin and centrin to stain the mitotic spindle and spindle poles respectively and DAPI was used to stain chromatin. The number of normal metaphase mitotic spindles was quantified for each cell line using immunofluorescence microscopy.

Since the previous set of images indicated that curcumin treatment impaired spindle pole separation, additional experiments were performed to determine whether curcumin treatment significantly affects levels of centrosomal separation in the HCT116 $p53^{+/+}$ line.

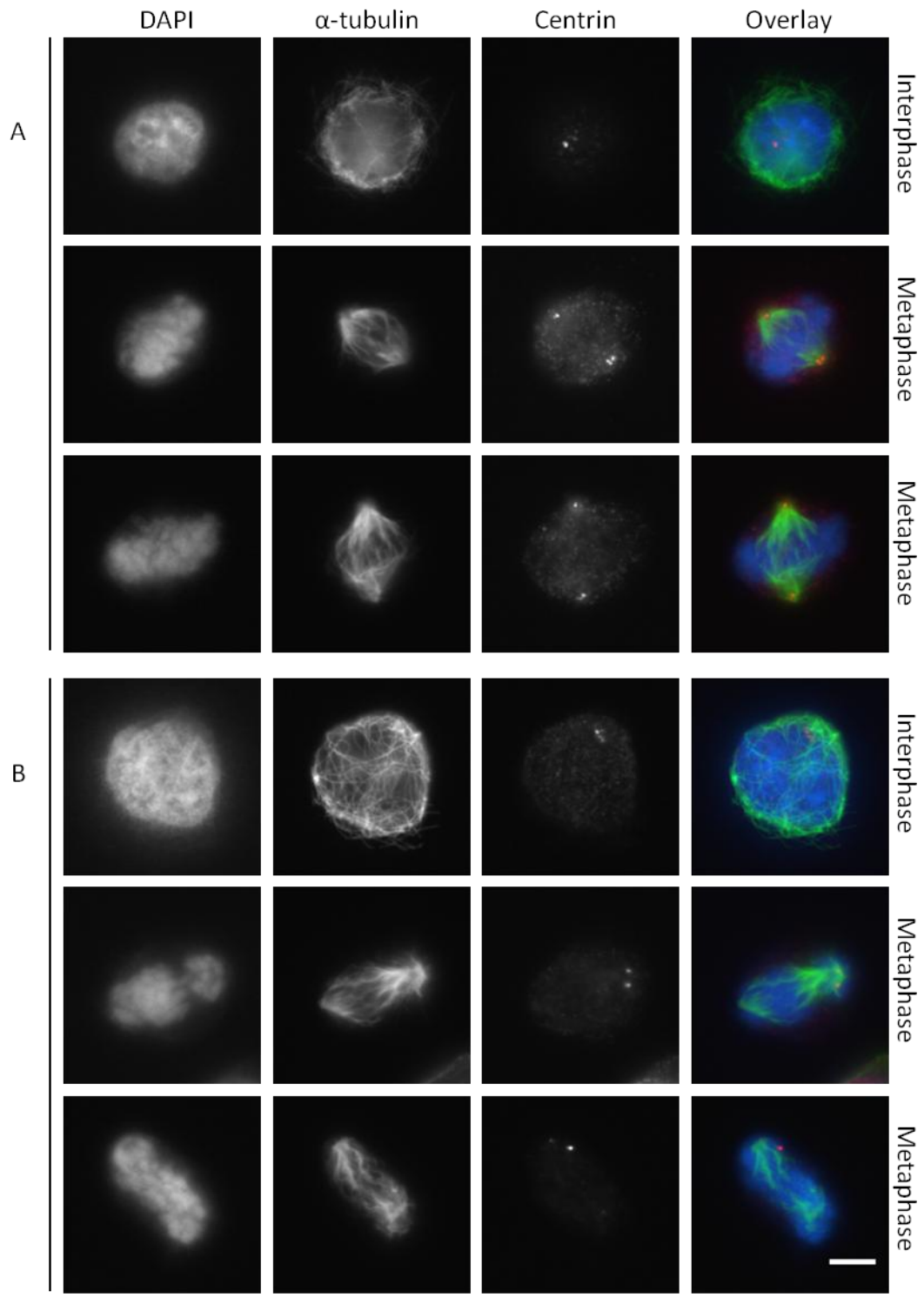


Figure 29 Curcumin Treatment Inhibits Mitotic Spindle Formation in HCT116 p53$^{+/+}$ Cells

HCT116 p53$^{+/+}$ cells were treated for 12 hours with either DMSO alone (A) or 10μM of curcumin (B). Cells were stained with antibodies against α-tubulin (green) to visualise the mitotic spindle and centrin (red) to visualise centrosomes. Control interphase and two representative metaphase cells are shown for each treatment (n=3). Scale bar 5μm.

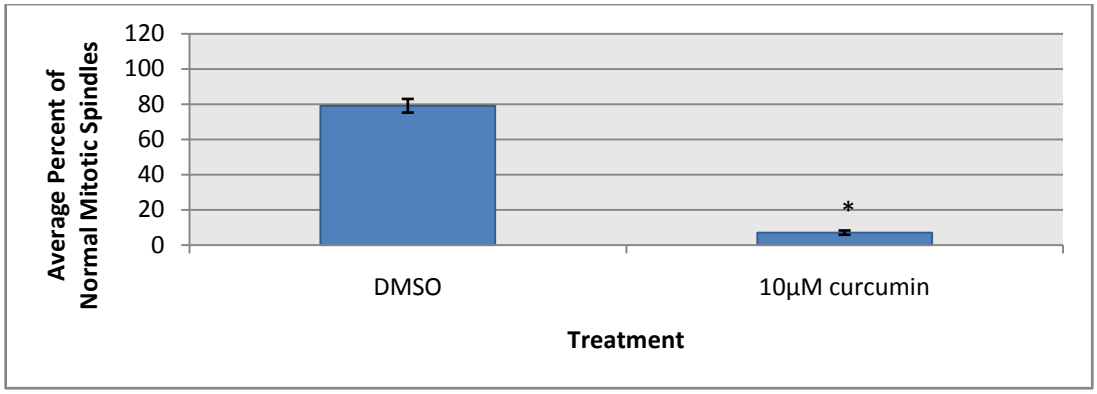


Figure 30 Curcumin Treatment Significantly Inhibits Metaphase Mitotic Spindle Formation in HCT116 p53$^{+/+}$ Cells

HCT116 p53$^{+/+}$ cells were treated for 12 hours, harvested and analysed using fluorescence microscopy. Normal metaphase mitotic spindles were defined as bi-polar with chromosomes aligned on the metaphase plate. At least 50 cells were counted per slide and data shown are the average of at least three separate experiments. Error bars show the standard deviation and * indicates a significant (P<0.05) difference compared to the DMSO control following a two-tailed t-test assuming equal variance.

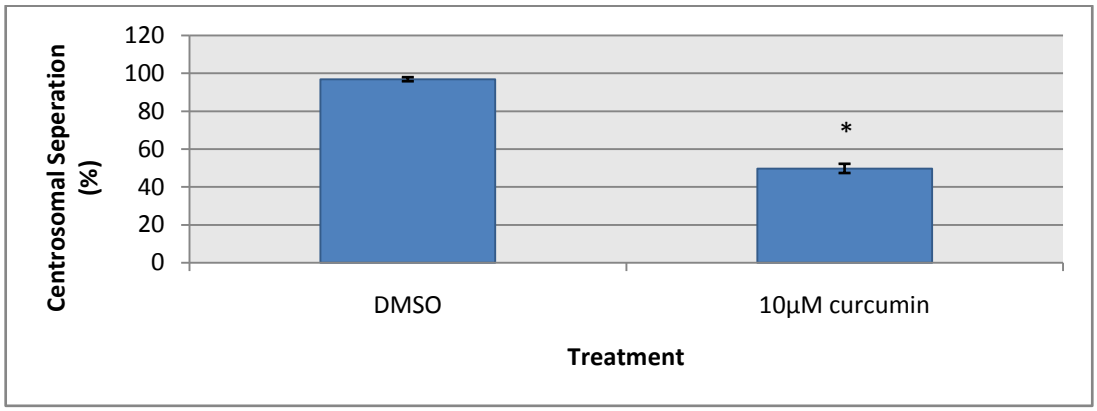


Figure 31 Curcumin Treatment Significantly Inhibits Centrosomal Separation During Mitosis in HCT116 p53$^{+/+}$ Cells

HCT116 p53$^{+/+}$ cells were treated for 12 hours, harvested and analysed using fluorescent microscopy. Centrosomes were defined as separated if they were more than 2 microns apart. At least 50 cells were counted per slide. Data shown are the average of at least three separate experiments. Error bars show the standard deviation. * indicates a significant (P<0.05) difference compared to the DMSO control following a two-tailed t-test assuming equal variance.

Curcumin treatment results in mitotic spindle abnormalities in metaphase cells in the HCT116 $p53^{+/+}$ line (Figure 28). In part A of this figure control metaphase cells with bipolar mitotic spindles and chromosomes neatly aligned perpendicular to the spindle can be seen. In comparison, curcumin-treated cells in part B feature a tangled mass of chromosomes in parallel with the mitotic spindle. The spindle structure of curcumin-treated cells is impaired; with the microtubule network appearing distorted and shaped by the chromosomes within.

Quantification of these spindle abnormalities in metaphase control and curcumin-treated cells reveals that there are significantly higher levels of abnormalities in curcumin-treated cells (Figure 29). Around 79% of control cells had normal mitotic spindles in metaphase, whereas only 7% are normal in curcumin-treated HCT116 $p53^{+/+}$ cells.

Following curcumin treatment mitotic spindle abnormalities increase as the levels of centrosomal separation decrease (Figure 30). Control HCT116 $p53^{+/+}$ cells had an average of 97% of cells featuring separated centrosomes during mitosis, whereas curcumin-treated cells had an average of 50% with separated centrosomes.

The effects of curcumin on the mitotic spindles of the remaining CRC lines were then investigated.

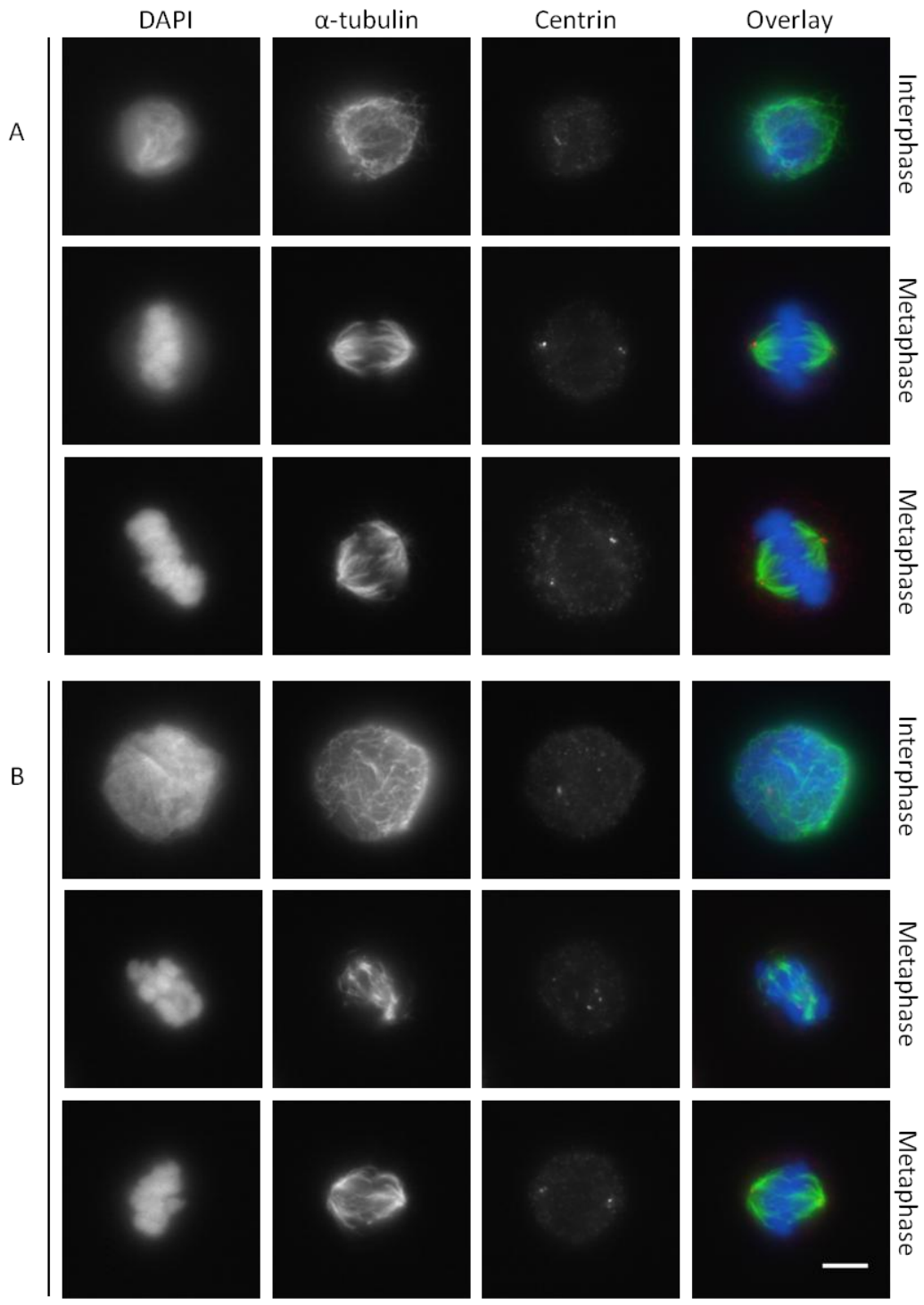


Figure 32 Curcumin Treatment Impairs Mitotic Spindle Formation in the HCT116 p53$^{-/-}$ Cell Line

HCT116 p53$^{-/-}$ cells were treated for 12 hours with either DMSO alone (A) or 10μM of curcumin (B). Cells were stained with antibodies against α-tubulin (green) to visualise the mitotic spindle and centrin (red) to visualise centrosomes. Control interphase and two representative metaphase cells are shown for each treatment (n=3). Scale bar 5μm.

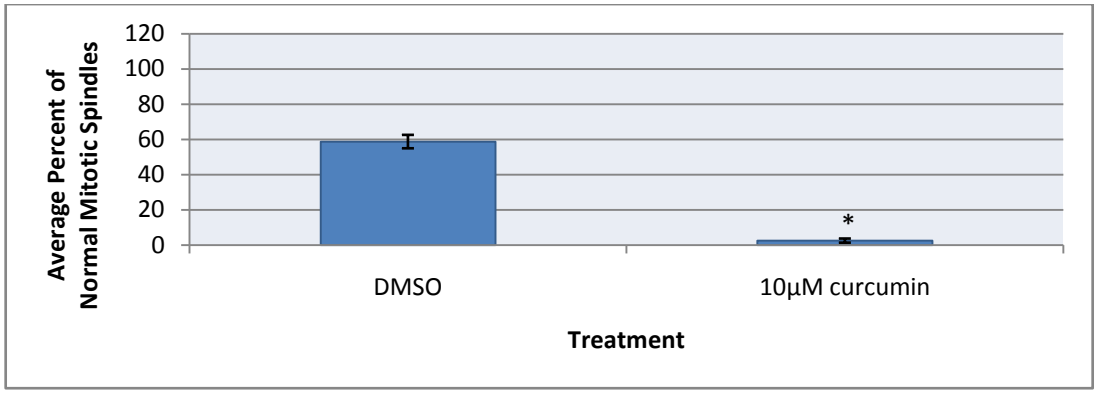


Figure 33 Curcumin Significantly Inhibits Metaphase Mitotic Spindle Formation in HCT116 p53$^{-/-}$ Cells

HCT116 p53$^{-/-}$ cells were treated for 12 hours, harvested and analysed using fluorescence microscopy. At least 50 cells were counted per slide and data shown are the average of at least three separate experiments. Error bars show the standard deviation and * indicates a significant ($P<0.05$) difference compared to the DMSO control following a two-tailed t-test assuming equal variance.

As with the HCT116 p53$^{+/+}$ line, the microtubule network in resting curcumin-treated HCT116 p53$^{-/-}$ cells appears more tightly wrapped around the nucleus than in the DMSO control (Figure 31). The mitotic spindle is severely distorted and the chromosomes appear more disordered than those shown in the control metaphase cells.

Curcumin treatment results in a significant increase in mitotic spindle abnormalities in the HCT116 p53$^{-/-}$ line (Figure 32). An average of 59% of all control cells exhibit normal metaphase mitotic spindle formation compared to an average of 2% in the curcumin-treated cells. However, higher baseline levels mitotic spindle abnormalities were found in the HCT116 p53$^{-/-}$ control cells compared to controls in the HCT116 p53$^{+/+}$ line. (Only 59% in the HCT116 p53$^{-/-}$ line were normal in comparison to 79% in the HCT116 p53$^{+/+}$ line).

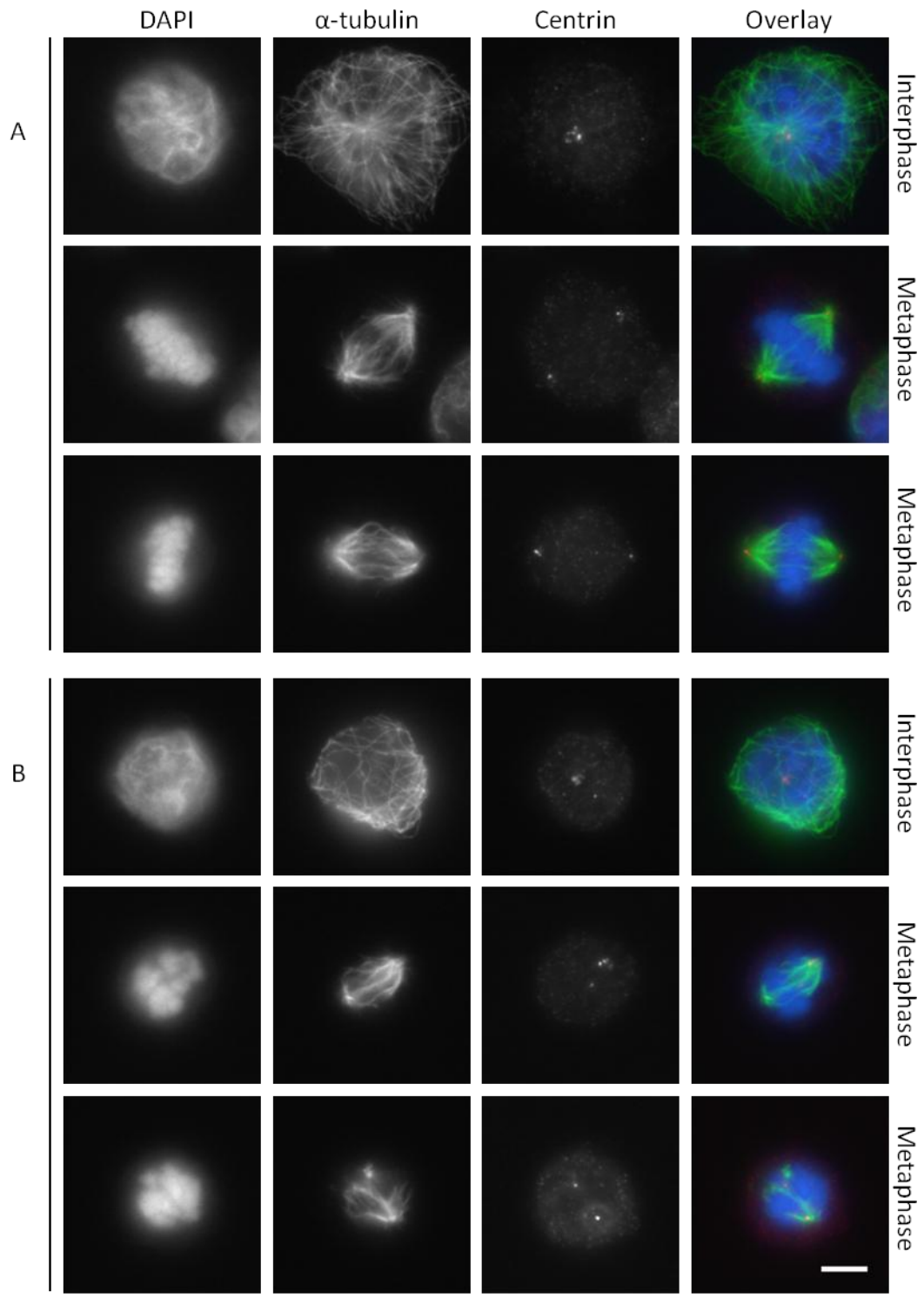


Figure 34 Curcumin Treatment Results in Abnormal Mitotic Spindle Formation in the HCT116 p21$^{-/-}$ Line

HCT116 p21$^{-/-}$ cells were treated with either DMSO alone (A) or 10μM of curcumin (B) for 12 hours. Cells were stained with antibodies against α-tubulin (green) to visualise the mitotic spindle and centrin (red) to visualise centrosomes. Control interphase and two representative metaphase cells are shown for each treatment (n=3). Scale bar 5μm.

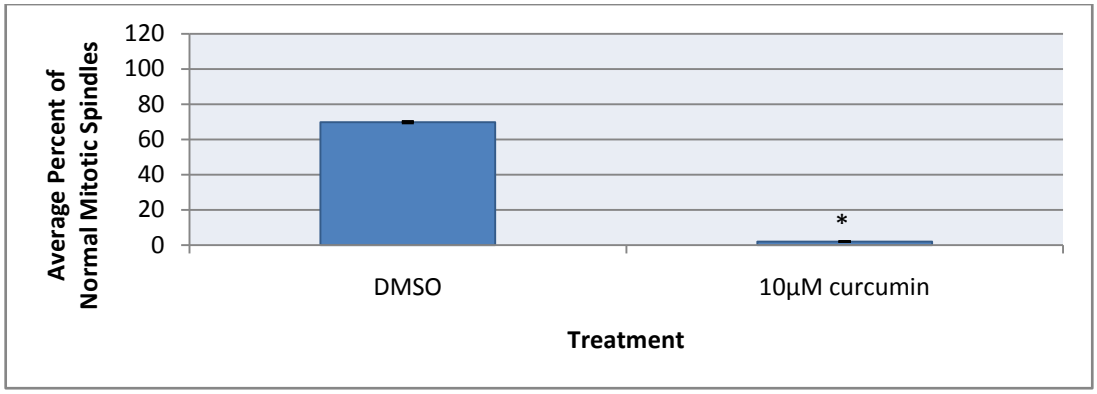


Figure 35 Curcumin Significantly Inhibits Normal Metaphase Mitotic Spindle Formation in the HCT116 p21$^{-/-}$ Line

HCT116 p21$^{-/-}$ cells were treated with DMSO or 10µM curcumin for 12 hours, harvested and analysed using fluorescence microscopy. At least 50 cells were counted per slide and data shown are the average of at least three separate experiments. Error bars show the standard deviation and * indicates a significant ($P<0.05$) difference compared to the DMSO control following a two-tailed t-test assuming equal variance.

Figures 33 and 34 reveal the effects of curcumin on the mitotic spindle of HCT116 p21$^{-/-}$ cells. Instead of chromosomes aligned neatly on the mitotic spindle equator as seen in Figure 33 A, those of curcumin-treated cells (Figure 33 B), were found in a spherical mass in the centre of the spindle. Curcumin-treated metaphase cells also lacked symmetrical arrays of microtubules between spindle poles.

Curcumin treatment significantly reduces the formation of normal mitotic spindles during metaphase in HCT116 p21$^{-/-}$ cells, with approximately 70% less cells found with normal spindle structure compared to the DMSO control (Figure 34). The baseline levels of normal mitotic spindle formation in the HCT116 p21$^{-/-}$ cell line are approximately 10% higher than those in the HCT116 p53$^{-/-}$ line but 10% lower than those found in HCT116 p53$^{+/+}$ cells.

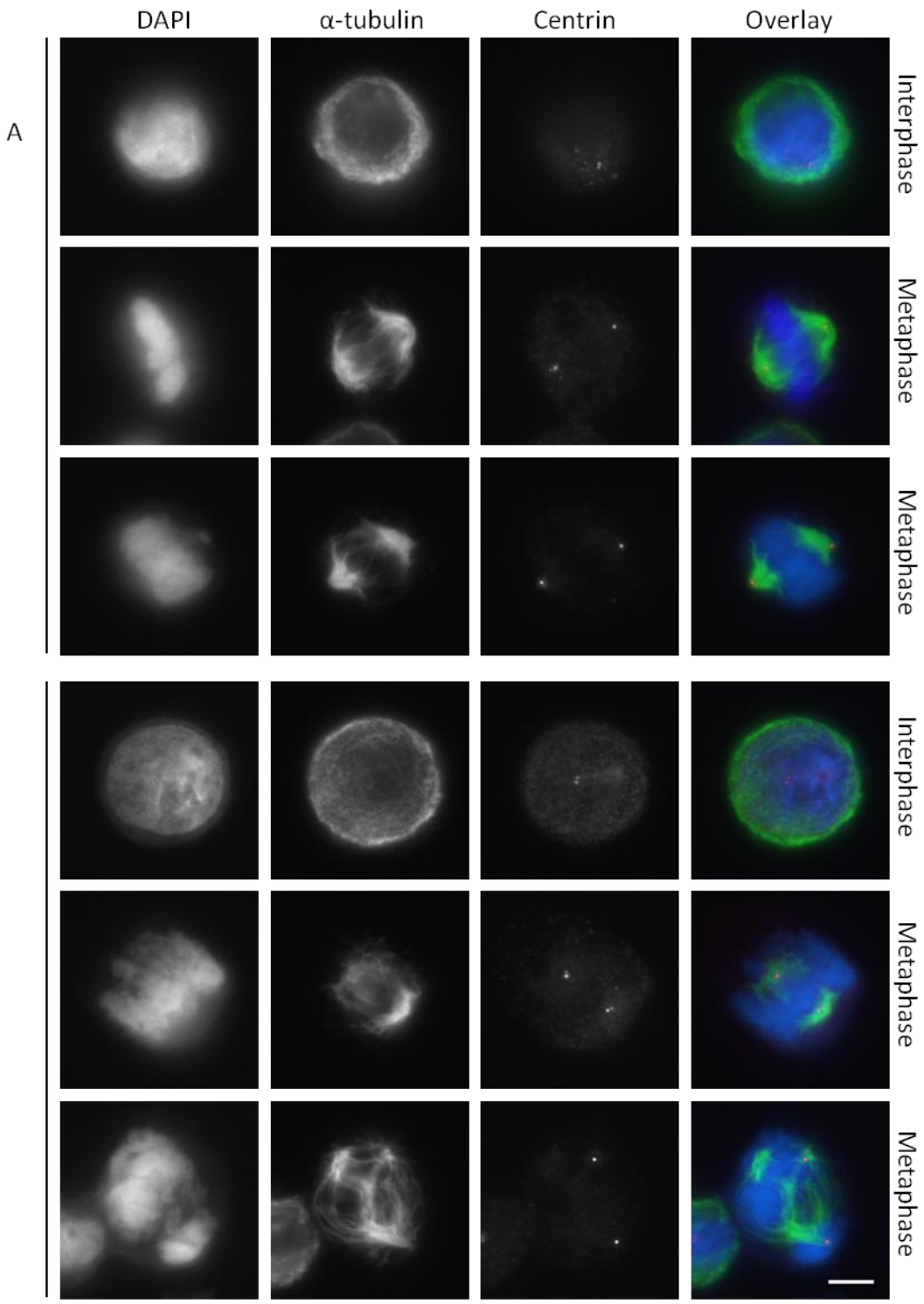


Figure 36 Curcumin Treatment Inhibits Mitotic Spindle Formation in the HT-29 Cell Line

HT-29 cells were treated with either DMSO alone (A) or 10μM of curcumin (B) for 12 hours. Cells were stained with antibodies against α-tubulin (green) to visualise the mitotic spindle and centrin (red) to visualise centrosomes. For each treatment one interphase control and two representative metaphase cells are shown (n=3). Scale bar 5μm.

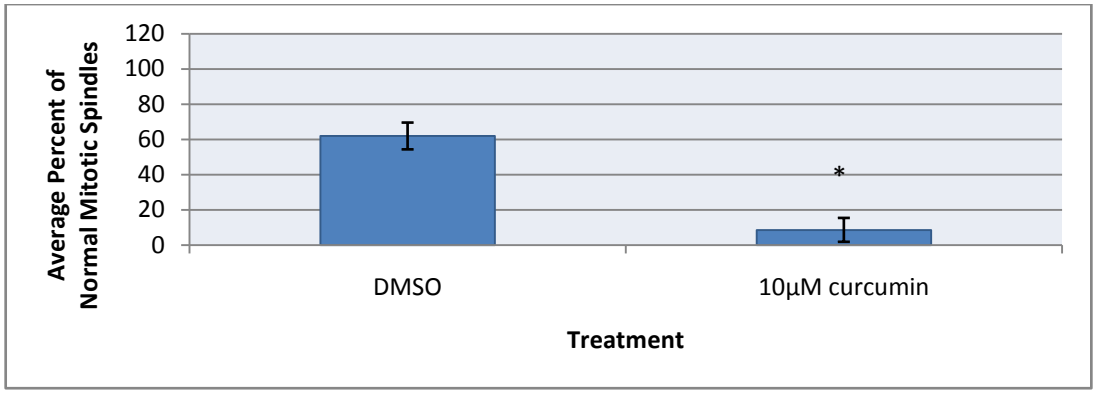


Figure 37 Metaphase Mitotic Spindle Formation is Significantly Impaired Following Curcumin Treatment in the HT-29 Cell Line

HT-29 cells were treated for 12 hours, harvested and analysed using fluorescence microscopy. Normal metaphase mitotic spindles were defined as being bi-polar with chromosomes aligned on the metaphase plate. At least 50 cells were counted per slide and data shown are the average of at least three separate experiments. Error bars show the standard deviation and * indicates a significant ($P<0.05$) difference compared to the DMSO control following a two-tailed t-test assuming equal variance.

Curcumin treatment significantly impairs mitotic spindle formation in the HT-29 line (Figures 35 and 36). As in the HCT116 lines, the curcumin-treated interphase cell appears to have a more compact microtubule network. This network appears disorganised in both curcumin-treated mitotic cells, and chromosomes appear entangled. Curcumin-treated HT-29 metaphase cells failed to form normal arrays of microtubules from the spindle poles.

The average number of normal mitotic spindles in control HT-29 cell populations (approximately 62%) is similar to those found in the HCT116 $p53^{-/-}$ line (59%). Following curcumin treatment the average number of cells with normal mitotic spindles is reduced to 9%.

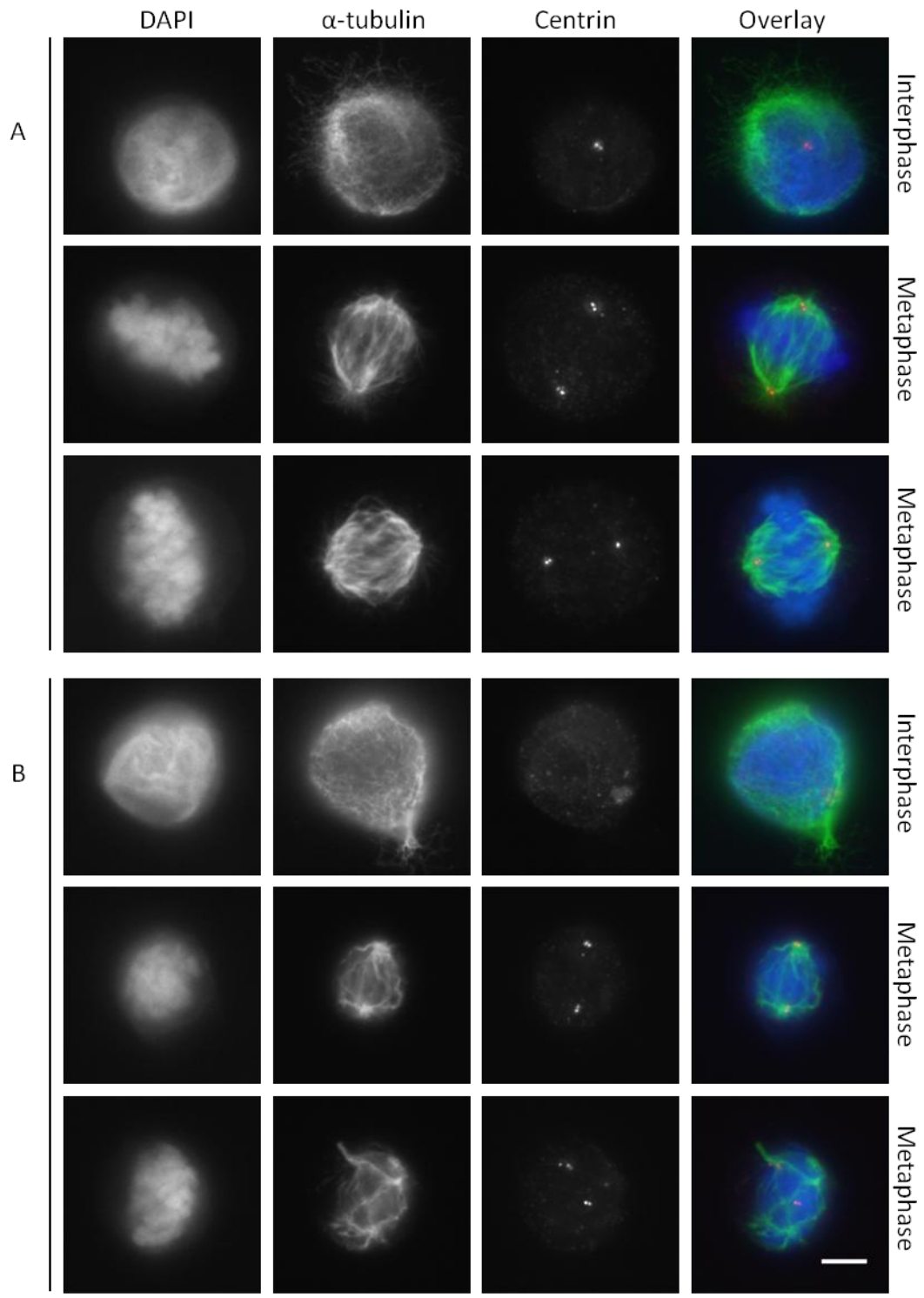


Figure 38 Curcumin Treatment Results in Abnormal Mitotic Spindle Formation in the SW480 Cell Line

SW480 cells were treated with either DMSO alone (A) or 10μM of curcumin (B) for 12 hours and then prepared for immunofluorescence microscopy. Cells were stained with anti-α-tubulin antibodies (green) to visualise the mitotic spindle and anti-centrin antibodies (red) to visualise centrosomes. Control interphase and two representative metaphase cells are shown for each treatment (n=3). Scale bar 5μm.

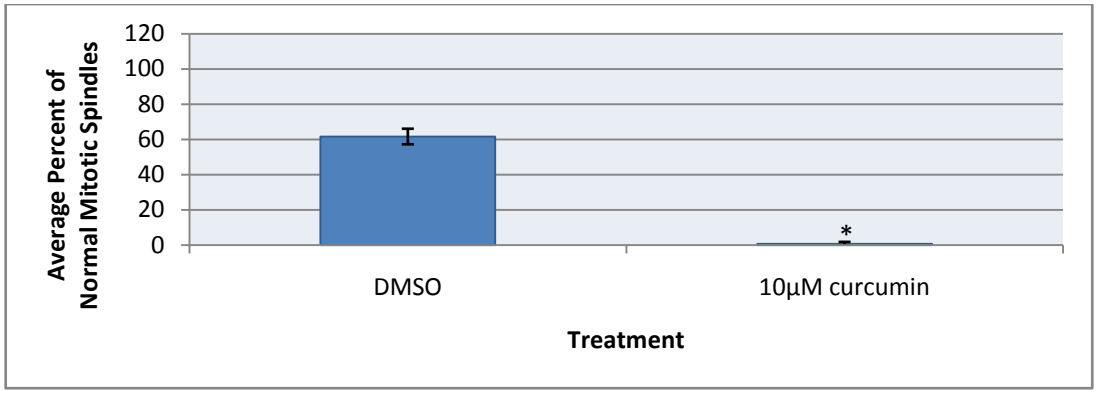


Figure 39 Curcumin Treatment Significantly Reduces Metaphase Mitotic Spindle Formation in SW480 Cells

SW480 cells were treated for 12 hours, harvested and analysed using fluorescence microscopy. At least 50 cells were counted per slide and data shown are the average of at least three separate experiments. Error bars show the standard deviation. * indicates a significant ($P<0.05$) difference compared to the DMSO control following a two-tailed t-test assuming equal variance.

Images of SW480 cells in Figure 37 show that curcumin treatment results in a failure to align chromosomes correctly on the spindle equator. The microtubule network in both interphase and mitotic curcumin-treated cells appears more compressed than in the representative control images. Figure 38 shows curcumin treatment significantly impairs mitotic spindle formation in SW480 metaphase cells after 12 hours.

The levels of normal mitotic spindle formation in control cells (62%) are comparable to those found in the HCT116 $p53^{-/-}$ (59%) and HT-29 lines (62%). Following curcumin treatment an average of just 1% of cells were found with normal mitotic spindles in the SW480 cell line.

4.2.5 Curcumin Treatment Results in Aurora B Mislocalisation in Dividing CRC Cells

Previous research has shown that curcumin treatment results in mislocalisation of the mitotic kinase Aurora B during mitosis in mouse progenitor cells transfected with the human Bcr-Abl oncoprotein (Wolanin, 2006). To investigate whether this occurred in the HCT116 $p53^{+/+}$ CRC line, images of control and curcumin treated cells were captured at representative stages of mitosis. Fluorescence microscopy experiments were performed using antibodies to Aurora B and pH3 to identify mitotic cells.

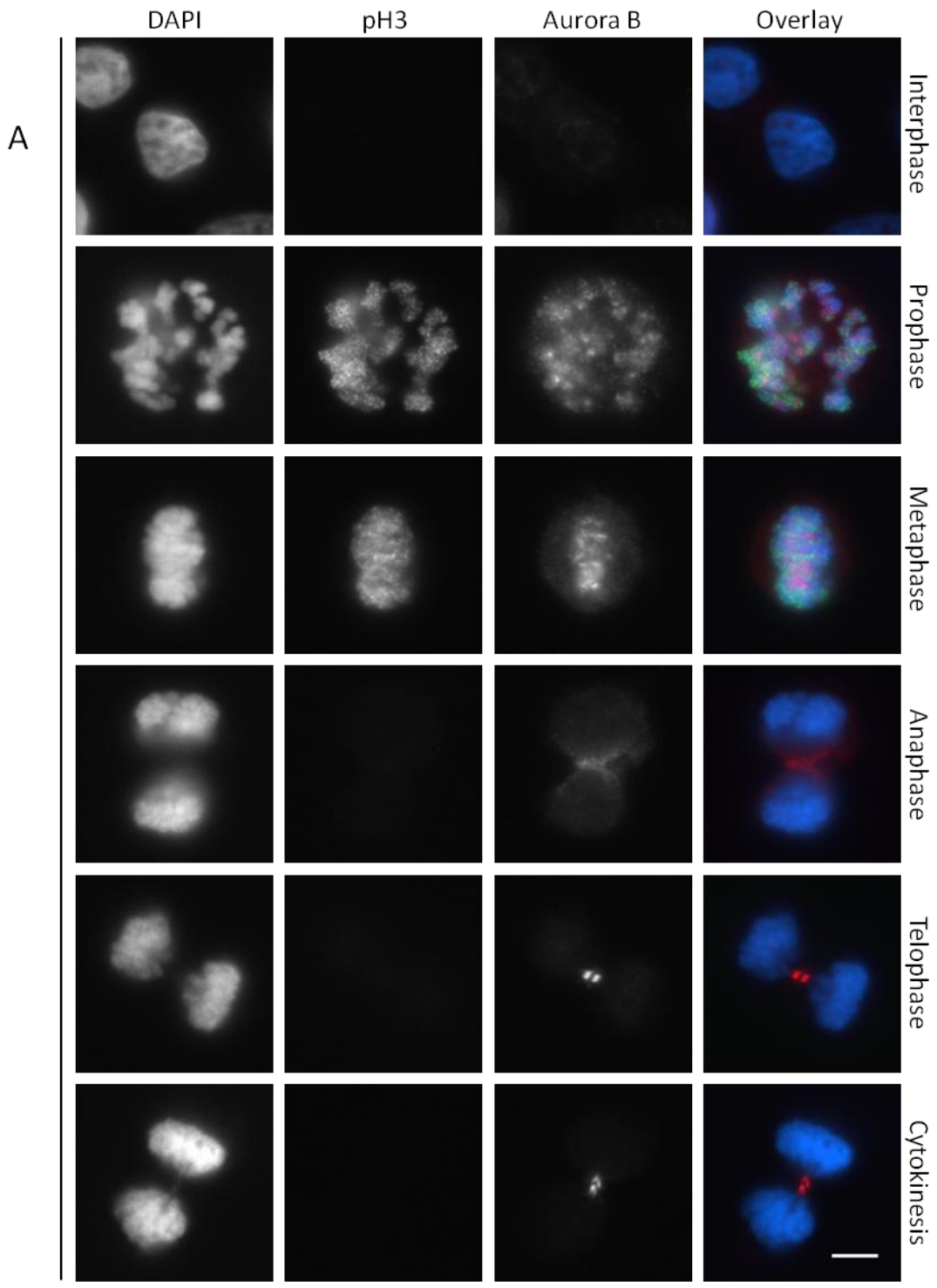


Figure 40 Aurora B is Mislocalised Following Curcumin Treatment in HCT116 p53$^{+/+}$ Cells

HCT116 p53$^{+/+}$ cells were treated for 12 hours with either DMSO alone (A) or 10µM of curcumin (B). Cells were prepared for immunofluorescence microscopy and stained with antibodies against phospho-histone H3 (pH3, green) and Aurora B (red). DNA was stained with DAPI (blue). Representative images of cells are shown in interphase and each stage of mitosis (prophase, metaphase, anaphase, telophase and cytokinesis). Images are representative of at least three separate experiments.

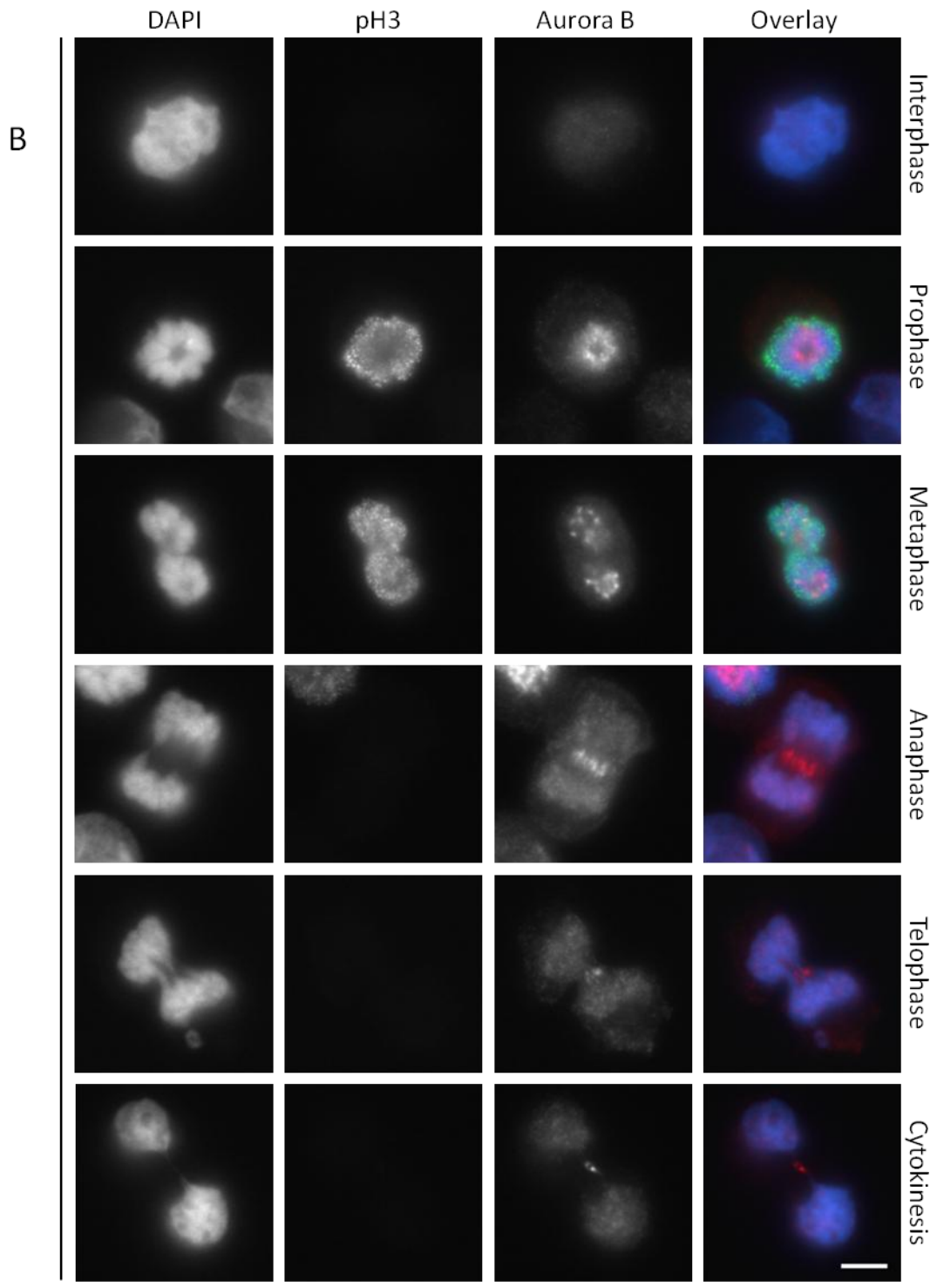


Figure 39 Continued

In control HCT116 $p53^{+/+}$ cells Aurora B staining is indicative of localisation at the centromeres and chromosome arms of prophase/prometaphase cells and this pattern can be seen in Figure 39 A. However, the pattern of localisation is different in Figure 39 B, with Aurora B staining a ring at the centre of the condensed chromosomes.

In metaphase cells treated with DMSO, Aurora B staining is indicative of localisation at the centromeres of aligned chromosomes on the metaphase plate. In curcumin-treated cells Aurora B staining is less even, with foci of brighter staining, instead of the even staining seen in the control cells in A. Control anaphase cells generally feature Aurora B staining at the midzone and cortex (Figure 39 A). Aurora B staining at the spindle midzone is also seen in curcumin-treated anaphase cells, however there is still Aurora B present on chromatin.

The panel featured in Figure 39 A shows a late telophase cell with Aurora B staining only at the cleavage furrow. In contrast, the curcumin-treated telophase cell appears to have higher levels of midbody and chromatin Aurora B staining. However, in curcumin-treated cells, some Aurora B staining can still be seen on chromatin. In the curcumin-treated telophase cell shown, there is still chromatin present in the spindle midzone and this appears to be in the process of being compressed by the cleavage apparatus. In cells undergoing the final stages of cytokinesis, Aurora B staining appears only at the cleavage furrow between the nuclei of the control cells.

The images of curcumin-treated cells stained with Aurora B and pH3 are comparable to cells with a *cut* phenotype, especially the image of the cell in telophase where sister chromatids have failed to separate properly and chromatin is still present in the spindle midzone.

4.3 Discussion

4.3.1 Curcumin Treatment Disrupts Formation of the Mitotic Spindle

In order to clarify the stage of mitotic arrest following curcumin treatment, quantification of the average number of cells at each stage of mitosis was performed using fluorescence microscopy. These data show that curcumin treatment results in a significant increase in cells in prophase/prometaphase, with a corresponding decrease in cells in the later stages of mitosis, anaphase and telophase. Previous studies indicated that curcumin can be incorporated into tubulin complexes resulting in a conformational change in tubulin, lowered GTPase activity of microtubules, and impaired microtubule assembly (Gupta et al., 2006). In addition, other research has identified that NuMA localisation has been disturbed by curcumin-treatment, which may be linked to mitotic spindle abnormalities (Holy, 2002). The impairment of microtubule dynamics and the disruption of NuMA localisation may contribute to the inability to form a bipolar mitotic spindle with symmetrical astral arrays of microtubules in curcumin-treated cells (Gaglio et al., 1995). A recent study has shown that curcumin perturbed the localisation of the kinesin protein Eg5, and prevented mitotic spindle reassembly following release from nocodazole (Banerjee et al., 2010). Kinesins are required for bi-polar mitotic spindle formation, and inhibition can result in the formation of cells with monopolar spindles (Blangy et al., 1995) similar to those seen following curcumin treatment.

The appearance of cells with monopolar spindles has been noted in various investigations (Banerjee et al., 2010, Basile et al., 2009, Holy, 2002) and in the present study experiments to quantify levels of centrosomal separation were designed to

investigate this phenomenon. It was found that treatment with curcumin resulted in a significant decrease in levels of centrosomal separation in the HCT116 $p53^{+/+}$ cell line, a factor that would contribute to an increased number of cells with monopolar spindles. Curcumin treatment has been shown to down-regulate Plk1 gene expression in the HT-29 colorectal cancer line (van Erk, 2004), and Eg5 in the MCF-7 breast line and it is likely that curcumin treatment may affect centrosomal regulators in the HCT116 line also. Future studies looking at the effects of curcumin on centrosomal regulators such as Plk1, Eg5, Aurora A and Nek 2 could be carried out to further investigate the inhibition of centrosomal separation in CRC lines.

Baseline levels of mitotic spindle abnormalities were higher in the CIN HT-29 and SW480 cell lines than in the MIN HCT116 $p53^{+/+}$ line, in accordance with recently published data (Silkworth et al., 2009). Interestingly, levels of mitotic spindle abnormalities in control HCT116 $p53^{-/-}$ cells were comparable to those found in the CIN lines. Curcumin treatment resulted in a significant increase in the formation of mitotic spindle abnormalities in all lines tested. Microtubule capture of chromosomes may be impaired by inhibition of microtubule dynamics, and this would reduce successful sister chromatid bi-orientation on the mitotic spindle. Studies have shown that the mitotic process takes longer in CIN cell lines compared to MIN lines, and it is proposed that this may be a result of an increased length of time taken to form secure microtubule attachments to kinetochores in these cells due to an increased chromosomal content relative to diploid lines (Silkworth et al., 2009). If curcumin inhibits the microtubule capture of chromosomes via perturbed microtubule dynamics, it is possible that the CIN lines may be more vulnerable to treatment during

this process. Data presented here show that the CIN SW480 was highly sensitive to the effects of curcumin, with mitotic spindle abnormalities in 99% of cells following treatment.

4.3.2 Activation of the Spindle Assembly Checkpoint by Curcumin

A new study has found that curcumin treatment increased the accumulation of checkpoint proteins Mad2 and BubR1 at the kinetochores of curcumin-treated (36µM, 24 hours) MCF-7 cells, indicating activation of the spindle assembly checkpoint (Banerjee et al., 2010). To investigate whether activation of the spindle checkpoint is responsible for curcumin-induced cell cycle arrest in CRC lines, experiments using siRNA to knock-out Mad2 or BubR1 could be carried out. If these components of the spindle checkpoint were integral to curcumin-induced mitotic arrest, it is likely that knock-down of these proteins using siRNA would result in abrogation of M phase arrest.

Curcumin has been shown to inhibit the activity of the proteasome in HCT116 and SW480 colorectal cancer cells (Milacic et al., 2008), reducing activity by 32% and 42% respectively at the same concentration used in experiments reported here, 10µM. The destruction of securin at the onset of anaphase is crucial for sister chromatid separation during anaphase (CohenFix et al., 1996, Funabiki et al., 1996). Impairment of proteasomal activity may reduce the capacity of the APC/C to degrade securin and result in decreased levels of sister chromatid separation during anaphase in curcumin-treated cells. This should be taken into consideration if future experiments using siRNA to knock-out components of the spindle checkpoint were to be conducted. If

curcumin is inhibiting the activity of the APC/C, then knock-down of Mad2 or BubR1 may not result in a clean abrogation of mitotic arrest.

4.3.3 Impairment of the DNA Decatenation Process by Curcumin

Another factor that may contribute to ineffective bi-orientation of sister chromatids on the mitotic spindle is impairment of the DNA decatenation process. Previous analysis of the effects of curcumin on mitotic cells has included images of cells with unusual chromatin staining (Magalska et al., 2006, Wolanin, 2006). Condensed chromosomes appear compacted in a ring, with a gap in the middle. To further investigate the effects of curcumin on the chromosomal organisation of early mitotic cells a series of image analysis experiments was carried out. Prophase cells from all five lines examined appear to have similar chromosomal disorganisation, where unseparated centrosomes can be visualised. The unusual appearance of chromosomes may be in part due to abnormal spindle pole localisation and spindle formation. However, inhibition of DNA decatenation may also contribute to the tangled appearance of chromosomes. Curcumin has been shown to act as a topoisomerase II poison, and these enzymes are essential for the decatenation of chromosomes prior to and during mitosis (Luo et al., 2009, Martin-Cordero et al., 2003). In addition, inhibition of DNA decatenation by topoisomerase II poisoning may also contribute to chromosomal disjunction during anaphase, due to a failure to separate entangled chromosomes correctly (Downes et al., 1991). Partial inhibition of topoisomerase II following curcumin treatment may result in sister chromatid segregation abnormalities.

4.3.4 Curcumin Treatment Results in Failed Sister Chromatid Disjunction, Comparable to the *Cut* Phenotype

The *cut* phenotype was first observed in fission yeast in cells expressing mutant versions of the *cut* gene (Hirano et al., 1986). It was shown that although mitotic chromosomes formed in these cells, they failed to separate properly and cytokinesis continued. It was later shown that fission yeast cells expressing a non-degradable version of the cut2 protein presented the same phenotype (Funabiki et al., 1996). This type of abnormal mitosis with failed sister chromatid disjunction was also observed in fission yeast cells with a mutant version of the gene that encodes the topo II enzyme, *top2* (Uemura and Yanagida, 1986).

In human cells, expression of a non-degradable version of the cut2 homologue, securin, results in a *cut* phenotype (Hagting et al., 2002). Securin is required to inhibit the protease separase until the metaphase anaphase transition. At the onset of anaphase securin is degraded, releasing separase to cleaves cohesin in a timely fashion facilitating sister chromatid disjunction (Jallepalli et al., 2001). Furthermore, inhibitors of DNA topo II similarly prevent chromatid separation but do not prevent mitotic exit in mammalian cells (Downes et al., 1991). The topo II enzyme is required for correct chromosome segregation during mitosis, and may decatenate specific regions of mitotic chromosomes such as kinetochores in a precise temporal programme (Barthelmes et al., 2000, Bhat et al., 1996, Christensen et al., 2002, Coelho et al., 2003, Nitiss, 2009, Rattner et al., 1996). Pharmacological inhibition of the topo II protein or knock-down via siRNA can also lead to a failure of chromosomes to attach

correctly to the mitotic spindle, and failed centromeric disjunction during mitosis (Coelho et al., 2008, Porter and Farr, 2004).

It would be difficult to determine without further investigations whether curcumin is affecting separase, securin, or topo II, as inhibition of the activity of these proteins present a similar phenotype to those observed in this present study.

4.3.5 Effects of Curcumin on the Chromosomal Passenger Complex Protein Aurora B

Curcumin treatment has been shown to affect Aurora B localisation in a mouse model of myeloid leukemia (Wolanin, 2006). The present results further characterise the effects of curcumin treatment on Aurora B localisation at various stages of mitosis in colorectal cancer cells. The effects in prophase/prometaphase and metaphase could be a result of impaired chromosomal alignment. Aurora B may still be correctly localised at the kinetochores of these cells, however, as microtubule capture of chromosomes may be impaired by curcumin treatment, normal separation and alignment of chromosomes may be inhibited, resulting in altered kinetochore localisation. In telophase and cytokinesis, Aurora B staining cannot be seen on the separated chromosomes of control cells, instead it is focussed on the cleavage furrow. However, in curcumin-treated cells, residual Aurora B staining can still be seen on the chromatin. Most Aurora B is degraded during late telophase by the APC complex, but this protein can still be seen at the cleavage furrow. It is possible that APC/C inhibition by curcumin treatment may also contribute to impaired degradation of Aurora B at the end of mitosis. Aurora B is involved in regulating the timing of abscission by monitoring the presence of chromatin in the spindle midzone during cytokinesis

(Mendoza et al., 2009, Norden et al., 2006, Steigemann et al., 2009). Curcumin treatment may impair this regulatory process, resulting in abscission in the presence of chromatin as seen in Figure 39 B.

Wolanin et al. (2006) proposed that curcumin-induced mislocalisation of Aurora B staining is a result of reduced survivin levels in a mouse model of myeloid leukemia, and further investigations into the effects of curcumin treatment total survivin protein levels in this line could be perfomed using Western blotting analysis. The authors also suggest that this survivin down-regulation may contribute to mitotic arrest, and that this arrest is p53 mediated. Previous studies have shown that survivin downregulation contributed to a mitotic block, but that this arrest in mitosis was abrogated in the HCT116 $p53^{-/-}$ line (Beltrami et al., 2004). It is possible that if survivin were downregulated in this line that this could contribute to mislocalisation of Aurora B. However, it is unlikely that loss of survivin contributes to mitotic arrest in a similar p53-dependent mechanism after curcumin treatment, as results from the previous chapter show that the highest levels of mitotic arrest with this agent were found in the HCT116 $p53^{-/-}$ line.

Knock-down of the topo II enzyme using siRNA can result in a reduction of Aurora B kinase activity and mislocalisation of this protein during mitosis (Coelho et al., 2008). This study also found that reduced Aurora B kinase activity was dependent on the spindle checkpoint protein BubR1 (Coelho et al., 2008). It is possible that the mislocalisation of Aurora B in curcumin-treated cells is a result of the inhibition of the topo II enzyme.

Chapter 5 Mechanisms of Curcumin-Induced G_2/M and M Phase Cell Cycle Arrest

5.1 Introduction

Previous studies have suggested that microtubule depolymerisation may be linked to curcumin-induced M phase arrest (Holy, 2002, Singh et al., 1996). While curcumin-mediated G_2/M boundary arrest is thought to involve DNA damage network checkpoint signalling (Park et al., 2006, Tomita et al., 2006), the mechanisms of mitotic arrest have not been elucidated. Furthermore, the exact nature of DNA damage is still unclear. Treatment with curcumin has been associated with the production of reactive oxygen species (ROS), topoisomerase poisoning, and chromosomal alterations (Giri et al., 1990, Jiang et al., 2010, Lopez-Lazaro et al., 2007b). However, newly published research indicates that the status of DNA repair genes is a critical factor in the induction of curcumin-mediated cell cycle arrest (Jiang et al., 2010).

In a study investigating the genotoxicity of curcumin in the K562 leukemia cell line, it was found that curcumin acted as a topo I and topo II poison, and that curcumin-induced DNA damage could be inhibited when cells were treated in combination with ROS inhibitors (Lopez-Lazaro et al., 2007b). In tests on the K562 cell line, treatment with a relatively high range of curcumin concentrations (35-70µM) resulted in a significant increase in topo I and topo II DNA complexes. Pre-treatment with the ROS inhibitor NAC protected cells from both topo I and topo II DNA complex formation. Curcumin treatment (50µM) was also found to significantly increase the levels of phospho histone H2A.X (pH2A.X) staining in a different model of leukemia, in the two HL-60 lines after 12 hours (Mosieniak et al., 2006).

Mitotic arrest induced by nocodazole, paclitaxel or monastrol has been previously shown to induce pH2A.X foci and to increase levels of ATM phosphorylation in the HCT116 cell line (Dalton et al., 2007). A significant increase in pH2A.X staining was found in prometaphase cells following treatment with nocodazole and CENP-E siRNA indicating the presence of single or double strand DNA breaks. These authors also found a significant increase in pH2A.X staining in untreated control prometaphase cells with spontaneous spindle defects. Cells that had divided following release from nocodazole-mediated mitotic arrest were found to have chromosomal aberrations, and the authors concluded that the DNA damage incurred during mitotic arrest could increase the occurrence of chromosomal alterations in daughter cells.

Research in this lab investigating the type of DNA damage following curcumin treatment in the HCT116 line found no significant increase in levels of oxidative DNA damage, when levels of 8-oxo-deoxyguanosine or 8-oxo-deoxyadenosine (8-oxo-dG, 8-oxo-dA) adducts were measured by mass spectrometry (Personal communication, Dr R. Cordell, Univ. of Leicester). However, a study using human hepatoma HepG2 cells found that curcumin treatment (~7-110µM) results in a significant increase in 8-oxo-dG formation in mitochondrial and nuclear DNA, and a significant increase in comet tail DNA (Cao and et al., 2006). Curcumin was shown to generate reactive oxygen species (ROS) in medium when addition of 5-100µM for 30 minutes resulted in a proportional increase in levels of hydrogen peroxide (H_2O_2) equivalents, as shown by the ferrous oxidation–xylenol orange FOX assay (Kelly et al., 2001). Curcumin treatment (5-50µM) induced DNA damage as measured by the comet assay in human lymphocytes and healthy gastric mucosa cells (Blasiak et al., 1999, Kelly et al., 2001).

While there is still controversy regarding the nature of DNA damage induced by curcumin, and whether this can occur at physiologically relevant concentrations, research indicates that curcumin activates the G_2/M DNA damage signalling checkpoint in a range of cancer cell lines (Park et al., 2006, Tomita et al., 2006).

Research investigating the effects of curcumin on non-epithelial cancer cell lines has shown that curcumin can activate G_2/M DNA damage checkpoint signalling. In primary adult T cell leukemia (ATL) lines, and HTLV-1 (Human T-lymphotropic virus type I) infected T cell lines treatment (50μM) reduced the total protein levels of cyclin D1, CDC25C, CDK1 and survivin, resulting in the induction of G_2/M and G_1 cell cycle arrest (Tomita et al., 2006).

However, the effects of curcumin on components of the DNA damage signalling network appear to be cell type specific. In a study using human bladder cancer cells, curcumin treatment (5-12.5μM) had no effect on total protein levels or the phosphorylation status of cell cycle proteins CDC25C, CDK1, or Wee1 (Park et al., 2006). However, this study found that curcumin significantly upregulated p21 RNA and total protein levels in cells undergoing G_2/M checkpoint arrest.

In the past year, there have been significant advances in the understanding of mechanisms of curcumin-induced G_2/M arrest. A recent study using pancreatic cells found components of the DNA damage signalling network, ATM and Chk1, were involved in curcumin-induced cell cycle arrest, and activation resulted in inhibition of the CDC25C phosphatase and loss of CDK1-cyclin B. (Sahu et al., 2009). Phosphorylation of histone H2A.X, a marker of DNA strand breaks was observed following curcumin treatment in the BxPC-3, but not the HPDE-6 line.

Other research published this year has highlighted how the status of DNA mismatch repair genes (MMR) can influence the response to curcumin treatment in HCT116 colorectal cancer cells (Jiang et al., 2010). In MMR-proficient cells, curcumin treatment (30μM) resulted in the formation of pH2A.X foci and Chk1-mediated cell cycle arrest. In contrast, there was significantly less pH2A.X formation and cell cycle arrest following curcumin treatment in MMR-deficient cells. The authors proposed that the source of DNA damage in this study was DSBs as a result of ROS and they showed that the phosphorylation levels of H2A.X, ATM and Chk1, and overall levels of cell cycle arrest were reduced when cells were treated with curcumin in combination with the ROS inhibitor N-acetyl-cysteine (NAC).

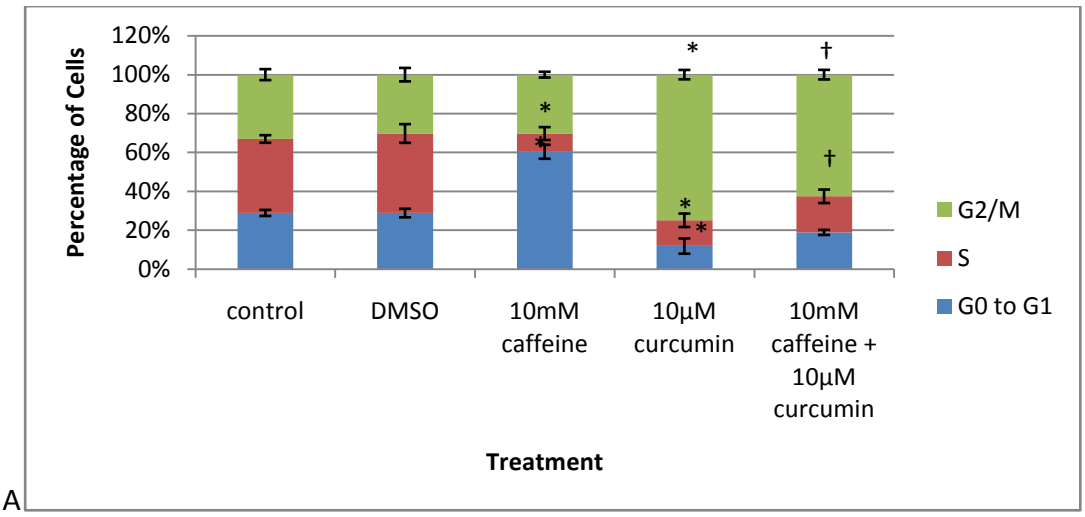
A comprehensive study investigating the effects of curcumin and its derivatives in the HCT116 lines, proposed that the increased levels of pH2A.X staining were a result of a prolonged mitotic block induced by curcumin (Basile et al., 2009). They showed that curcumin treatment (10μM) resulted in an increase in phosphorylation levels of histone H3, H2A.X, and ATM and that this corresponded to a significant increase in levels of G_2/M arrest.

In order to further examine the involvement of the DNA damage signalling network in curcumin-mediated G_2/M and M cell cycle arrest in colorectal cancer cell lines, experiments were carried out to investigate the effects of checkpoint inhibitors on levels of overall G_2/M and M phase arrest, the effects of treatment on key cell cycle proteins, and the levels of pH2A.X staining in mitotic cells.

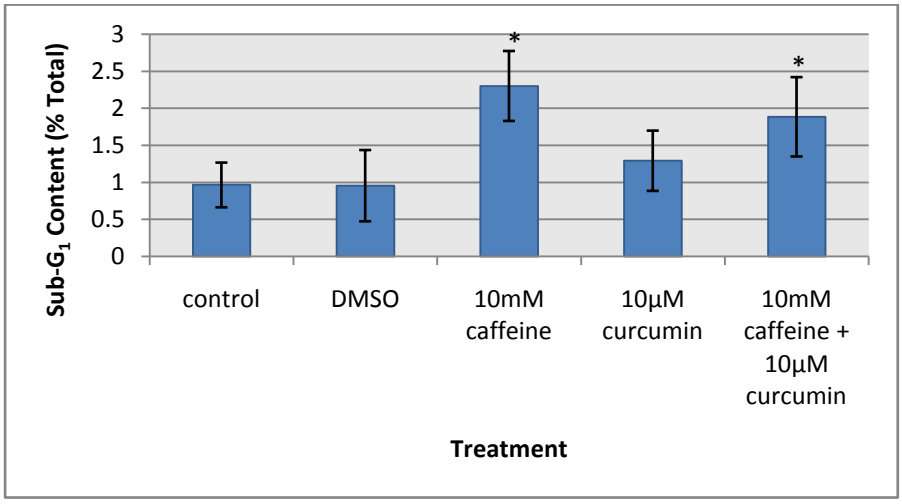
5.2 Results

5.2.1 Caffeine significantly reduces curcumin-induced G_2/M arrest in the MIN HCT116 Lines

Caffeine has been shown to inhibit the activity of the DNA damage network signalling kinases, ATM, ATR, DNA-PK and Chk1 at IC_{50} values of 0.2mM, 1.1mM, 10mM and 5mM respectively (Sarkaria et al., 1999). These kinases are involved in relaying the DNA damage signal at the G_2/M boundary that ultimately results in deactivation of CDK1-cyclinB1 complexes and prevents entry into mitosis. A concentration of 10mM caffeine was selected, as previous work investigating the effects of the chemopreventive agent resveratrol on the DNA damage signalling pathway had successfully used this concentration (Tyagi et al., 2005). To investigate whether caffeine pre-treatment could alter levels of G_2/M cell cycle arrest, FACS cell cycle analysis following treatment with caffeine and curcumin or curcumin alone was carried out in the MIN and CIN colorectal cancer cell lines. Levels of arrest were analysed after 12 hours treatment, as the highest levels of G_2/M and M phase arrest were shown to occur at this time point. Additional analysis of the sub-G_1 content of these lines was performed to investigate whether pre-treatment with caffeine altered the levels of apoptosis in each cell line.



A



B

Figure 41 Caffeine Reduces Levels of G_2/M Arrest in HCT116 $p53^{+/+}$ Cells Treated with Curcumin

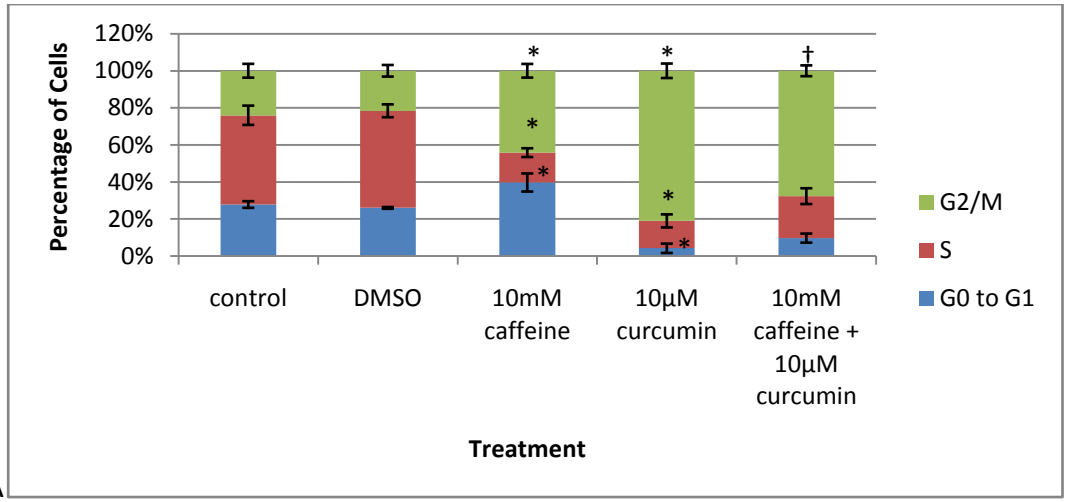
(A) HCT116 $p53^{+/+}$ cells were pre-treated with caffeine for 1 hour then treated with curcumin for 12 hours and evaluated using FACS cell cycle analysis. Control cells were treated with medium alone. Data shown are the mean (±SD) (n=3). * Shows a significant difference ($p<0.05$) in the mean number of cells in each phase following curcumin treatment compared to the DMSO control, and † indicates a difference between cells treated with caffeine alone and cells treated with a combination of caffeine and curcumin. P values were calculated using a 2-tailed T-Test assuming equal variance.

(B) The apoptotic population of these HCT116 $p53^{+/+}$ cells was measured as a percentage of the total cell populations with a sub-G_1 content. Data shown are the mean (±SD) (n=3). * Shows a significant difference ($p<0.05$) in the mean number of cells in each phase following treatment compared to the DMSO control. P values were calculated using a 2-tailed T-Test assuming equal variance.

In HCT116 $p53^{+/+}$ cells that were pre-treated with caffeine prior to curcumin treatment there was a 12% reduction in the number of cells in G_2/M (Figure 40 A). Caffeine treatment alone resulted in a significant increase in the number of cells in G_0/G_1, and a decrease in the number of cells in S phase in this cell line.

There is a small but significant increase in the sub-G_1 content of cells treated with caffeine alone or a combination of curcumin and caffeine (Figure 40 B).

A



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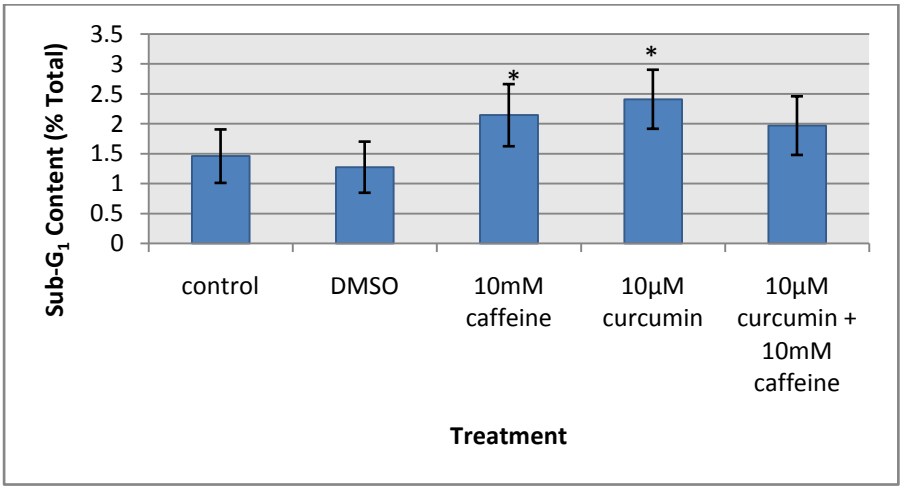


Figure 42 Pre-treatment with Caffeine Results in Reduced Levels of G_2/M Arrest in the HCT116 $p53^{-/-}$ Line

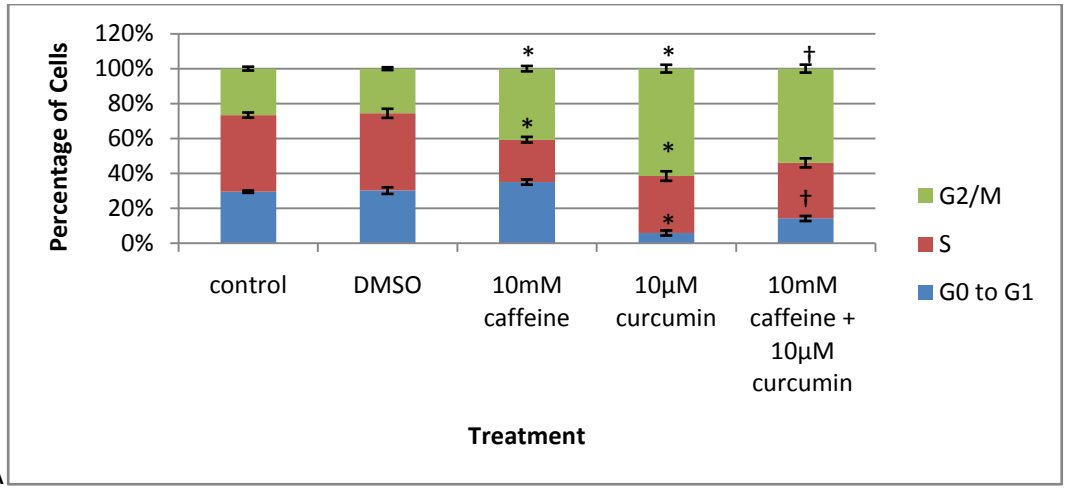
(A) HCT116 $p53^{-/-}$ cells were pre-treated with caffeine for 1 hour then treated with curcumin for 12 hours and evaluated using FACS cell cycle analysis. Control cells were treated with medium alone. Data shown are the mean (±SD) of at least 3 independent experiments. * Shows a significant difference ($p<0.05$) in the mean number of cells in each phase following curcumin treatment compared to the DMSO control, and † indicates a difference between cells treated with caffeine alone and cells treated with a combination of caffeine and curcumin. P values were calculated using a 2-tailed T-Test assuming equal variance.

(B) The apoptotic population of these HCT116 $p53^{-/-}$ cells was measured as a percentage of the total cell populations with a sub-G_1 content. Data shown are the mean (±SD) (n=3). * Shows a significant difference ($p<0.05$) in the mean sub-G_1 content following treatment compared to the DMSO control. P values were calculated using a 2-tailed T-Test assuming equal variance.

Pre-treatment with caffeine prior to curcumin treatment for 12 hours resulted in 13% less cells in G_2/M in the HCT116 $p53^{-/-}$ cell line (Figure 41 A). Interestingly, in this p53-deficient line caffeine treatment alone increased the number of cells in G_0/G_1 and G_2/M compared to the DMSO control.

Analysis of the sub-G_1 content of these cells shows that treatment with caffeine and curcumin alone results in a significant increase in apoptotic cells compared to the DMSO control (Figure 41 B).

A



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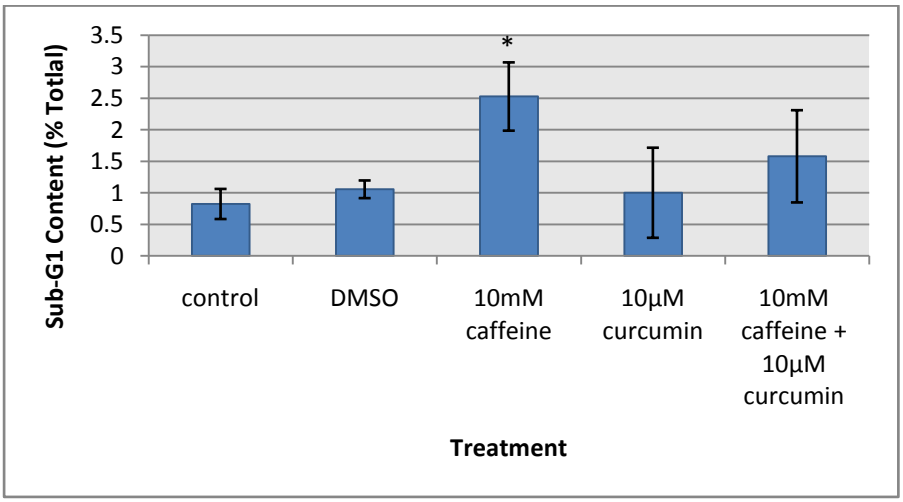


Figure 43 Lower Levels of Curcumin-Induced G_2/M Arrest in HCT116 $p21^{-/-}$ Cells Pre-Treated with Caffeine

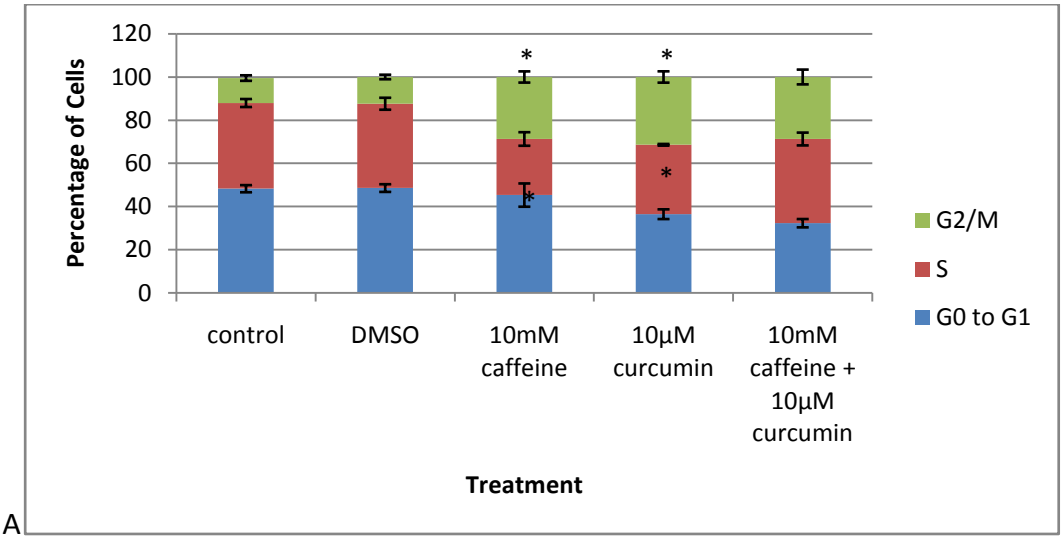
(A) HCT116 $p21^{-/-}$ cells were pre-treated with caffeine for 1 hour then treated with curcumin for 12 hours and evaluated using FACS cell cycle analysis. Control cells were treated with medium alone. Data shown are the mean (±SD) of at least 3 independent experiments. * Shows a significant difference ($p<0.05$) in the mean number of cells in each phase following curcumin treatment compared to the DMSO control, and † indicates a difference between cells treated with caffeine alone and cells treated with a combination of caffeine and curcumin. P values were calculated using a 2-tailed T-Test assuming equal variance.

(B) The apoptotic population of these HCT116 $p21^{-/-}$ cells was measured as a percentage of the total cell populations with a sub-G_1 content. Data shown are the mean (±SD) (n=3). * Shows a significant difference ($p<0.05$) in the mean sub-G_1 content following treatment compared to the DMSO control. P values were calculated using a 2-tailed T-Test assuming equal variance.

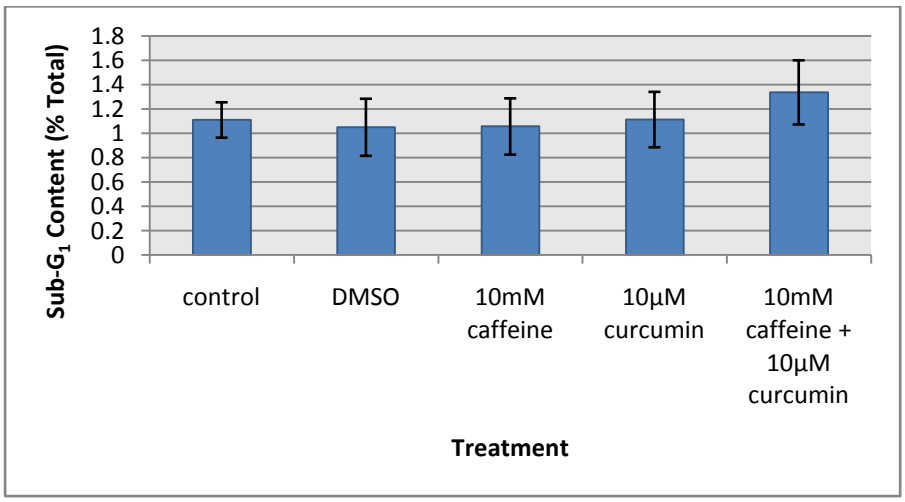
Treatment with the inhibitor caffeine before curcumin treatment resulted in an 8% reduction in the number of cells in G_2/M arrest in the HCT116 $p21^{-/-}$ line (Figure 42 A). Treatment with caffeine alone resulted in a significant increase in cells in G_2/M, and a decrease in the number of cells in S phase.

Analysis of the apoptotic population shows that there is a significant increase in the sub-G_1 content of cells treated with caffeine alone compared to the DMSO control (Figure 42 B).

5.2.2 Caffeine Does Not Reduce Curcumin-Mediated G_2/M Arrest in the CIN lines



A



B

Figure 44 Pre-Treatment with Caffeine Does Not Alter Levels of Curcumin Induced G_2/M Arrest in HT-29 Cells

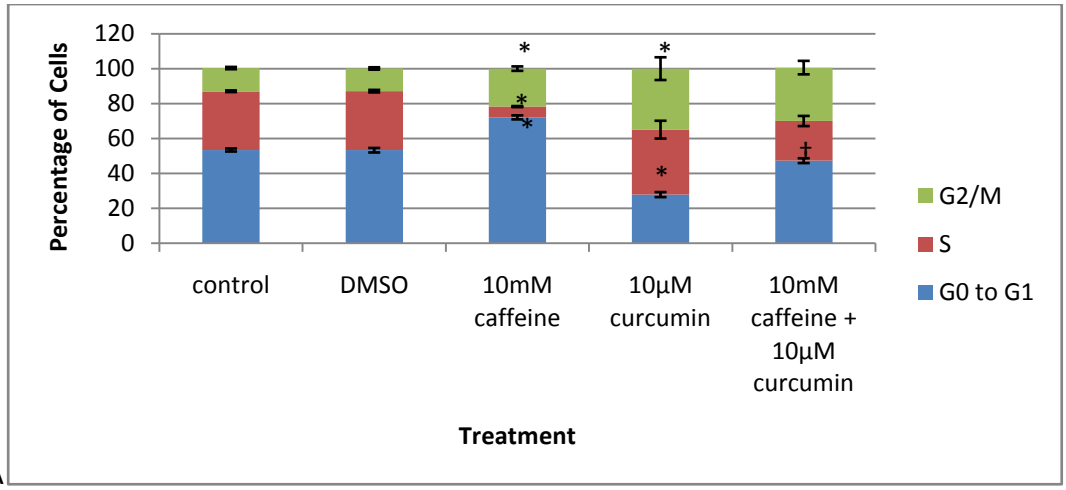
(A) HT-29 cells were pre-treated with caffeine for 1 hour then treated with 10µM of curcumin for 12 hours and evaluated using FACS cell cycle analysis. Control cells were treated with medium alone. Data shown are the mean (±SD) of at least 3 independent experiments. * Shows a significant difference ($p<0.05$) in cells in G_2/M compared to the DMSO control, and † indicates a difference between cells treated with caffeine alone and cells treated with a combination of caffeine and curcumin. P values were calculated using a 2-tailed T-Test assuming equal variance.

(B) The apoptotic population of these HT-29 cells was measured as a percentage of the total cell populations with a sub-G_1 content. Data shown are the mean (±SD) (n=3). * Shows a significant difference ($p<0.05$) in the mean sub-G_1 content following treatment compared to the DMSO control. P values were calculated using a 2-tailed T-Test assuming equal variance.

Pre-treatment with caffeine prior to curcumin did not result in a significant decrease in the number of cells in G_2/M in the CIN HT-29 cell line (Figure 43 A). Treatment with caffeine alone resulted in a significant increase in the number of cells in G_2/M and a decrease in the number of cells in S phase.

Analysis of the sub-G_1 content of these HT-29 cells showed no significant changes in the number of cells undergoing apoptosis following treatment with caffeine, curcumin or a combination of caffeine and curcumin (Figure 43 B).

A



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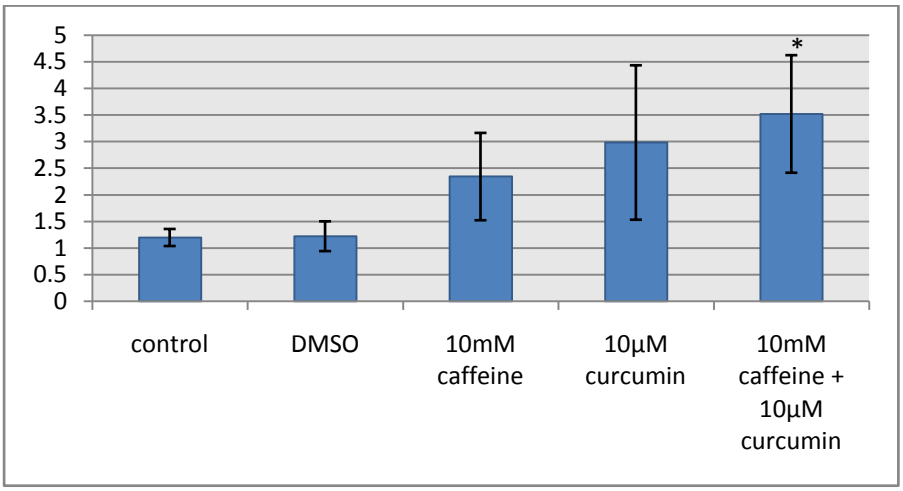


Figure 45 Caffeine Does Not Affect Curcumin-Induced G_2/M Arrest in the SW480 Line

(A) SW480 cells were pre-treated with caffeine for 1 hour then treated with 10µM of curcumin for 12 hours and evaluated using FACS cell cycle analysis. Control cells were treated with medium alone. Data shown are the mean (±SD) (n=3). * Shows a significant difference ($p<0.05$) in the mean number of cells in each phase following curcumin treatment compared to the DMSO control, and † indicates a difference between cells treated with caffeine alone and cells treated with a combination of caffeine and curcumin. P values were calculated using a 2-tailed T-Test assuming equal variance.

(B) The apoptotic population of these SW480 cells was measured as a percentage of the total cell populations with a sub-G_1 content. Data shown are the mean (±SD) (n=3). * Shows a significant difference ($p<0.05$) in the mean number of cells in each phase following treatment compared to the DMSO control. P values were calculated using a 2-tailed T-Test assuming equal variance.

Caffeine pre-treatment did not significantly affect the levels of curcumin-induced G_2/M arrest in the CIN SW480 line, but it did increase the number of cells in G_0/G_1 (Figure 44 A). Caffeine treatment alone altered the cell cycle profile, and there was a significant increase in the number of cells in G_0/G_1 and G_2/M.

Pre-treatment with caffeine followed by curcumin treatment significantly increased the sub-G_1 content of these SW480 cells compared to the DMSO control. There was an average increase in the percentage of sub-G_1 cells following treatment with caffeine and curcumin alone compared to the DMSO control but these changes were not found to be significant.

To summarise, FACS cell cycle analysis shows that caffeine significantly reduces but does not abrogate curcumin-mediated G_2/M arrest in the MIN HCT116 lines. In contrast, caffeine pre-treatment does not significantly alter levels of curcumin-induced G_2/M arrest in the CIN HT-29 and SW480 lines.

Treatment with caffeine alone was found to significantly increase the sub-G_1 content of the MIN HCT116 lines indicating higher levels of apoptosis. Treatment with curcumin alone significantly increased the sub-G_1 content in the HCT116 $p53^{-/-}$ line. Pre-treatment with caffeine followed by curcumin treatment significantly increased levels of apoptosis in the HCT116 $p53^{+/+}$ and SW480 lines.

5.2.3 Caffeine Inhibits Curcumin-Induced Mitotic Arrest in MIN HCT116 Lines

While caffeine pre-treatment was initially expected to directly affect levels of G_2/M boundary arrest, mitotic index experiments were performed to investigate the effects of caffeine on curcumin-induced M phase arrest.

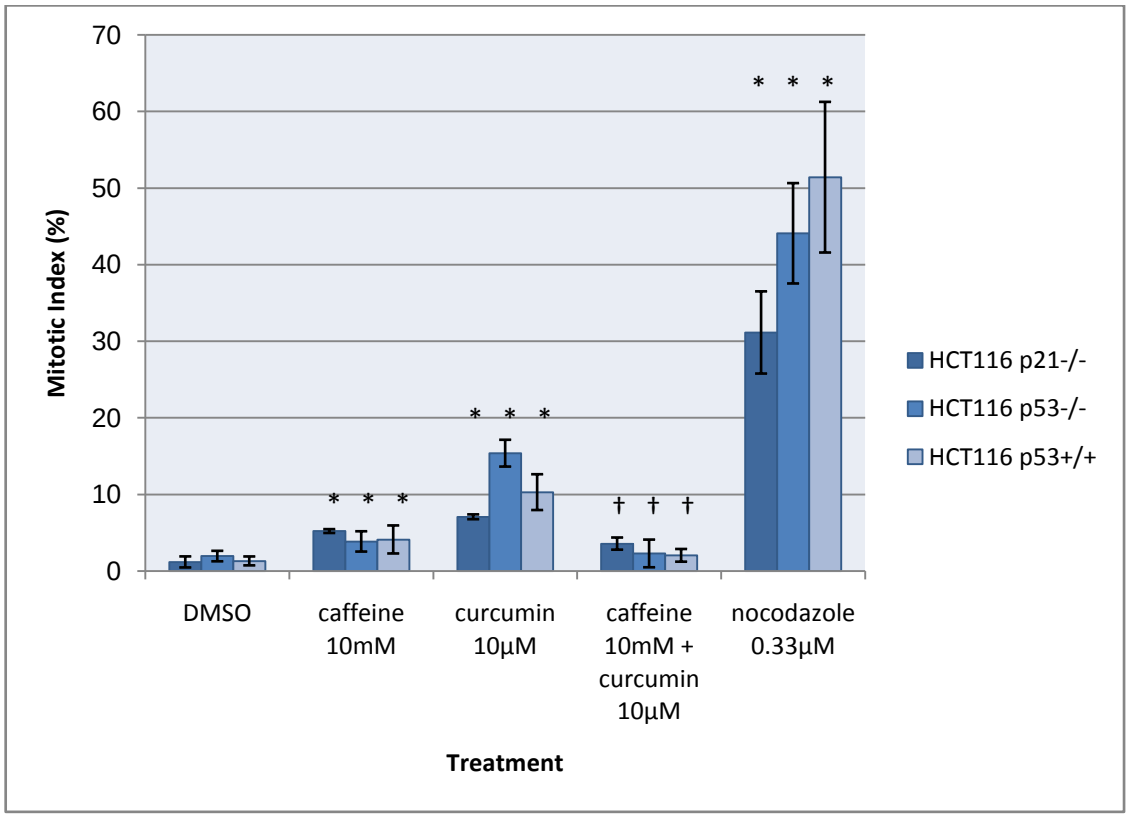


Figure 46 Caffeine Pre-Treatment Abrogates M Phase Arrest in HCT116 Lines

HCT116 Wt, HCT116 p21-/-, and HCT116 p53-/- cells were pre-treated with caffeine for 1 hour, treated with 10µM curcumin for 12 hours, and their mitotic index assessed using fluorescence microscopy. Cells were co-stained with pH3 antibodies and DAPI. DMSO and nocodazole were used as negative and positive controls. At least 500 cells were scored per data point. Data shown are the mean of at least three independent experiments. * indicates a significant difference from the DMSO control, and † indicates a significant ($P<0.05$) difference in means of the mitotic index of the HCT116 cells pre-treated with caffeine prior to curcumin treatment compared to cells treated with curcumin alone.

Pre-treatment with caffeine prior to curcumin treatment results in a significant reduction of the mitotic index in all HCT116 lines (Figure 45). Levels of mitotic arrest following curcumin treatment in the HCT116 $p53^{+/+}$ line were 10%, and this dropped to 2% in cells pre-treated with caffeine. There was a smaller reduction, from 7% to 4% in the HCT116 $p21^{-/-}$ cell line. The greatest loss of mitotic arrest was found in the HCT116 $p53^{-/-}$ line, where levels fell from 15% to 2%. Caffeine alone resulted in a significant increase in mitotic index by between 2-4% compared to the DMSO control.

5.2.4 Caffeine Inhibits Curcumin-Induced Mitotic Arrest in the CIN Lines

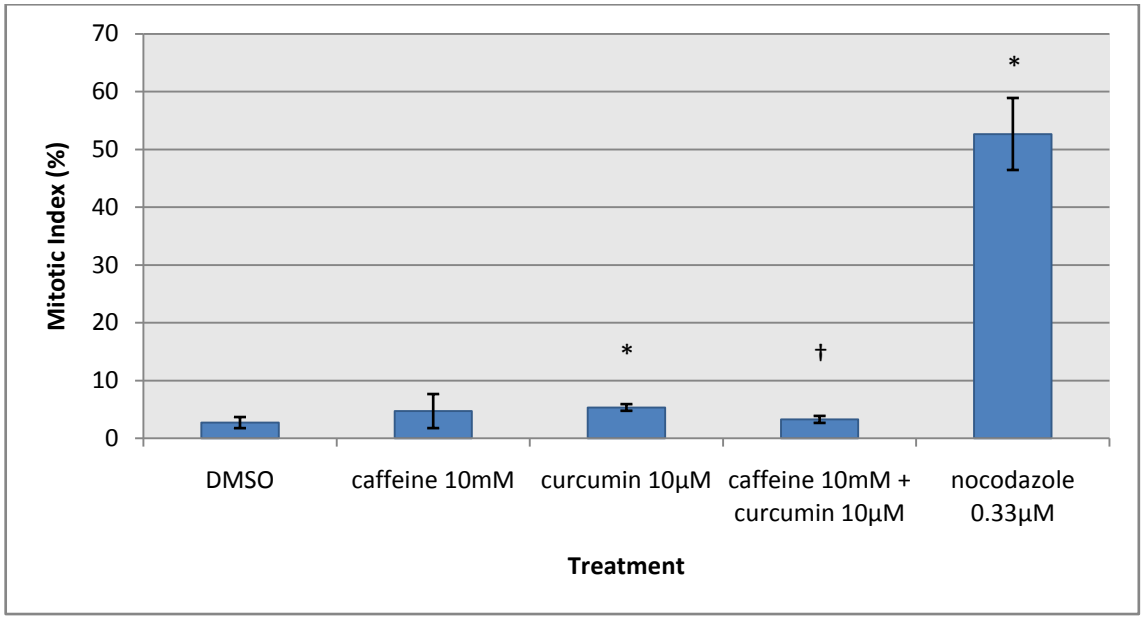


Figure 47 Caffeine Pre-Treatment Significantly Reduces the Mitotic Index in Curcumin-Treated Cells

HT-29 cells were pre-treated with caffeine for 1 hour, treated with curcumin for 12 hours, and their mitotic index assessed using fluorescence microscopy. Cells were co-stained with pH3 antibodies and DAPI. DMSO and nocodazole were used as negative and positive controls. At least 100 cells were scored per data point. Data shown are the mean (±SD) of at least three independent experiments. * indicates a significant ($P<0.05$) difference compared to the DMSO control. † indicates a difference in means between the curcumin-treated population and the caffeine and curcumin-treated cells.

There was a significant reduction in the mitotic index of caffeine and curcumin-treated HT-29 cells compared to those treated with curcumin alone (Figure 46). Following treatment with curcumin for 12 hours the mitotic index increased by 2%. The mitotic index returned to control levels when cells were pre-treated with caffeine. While curcumin was not found to significantly induce mitotic arrest in a previous experiment (Figure 18), this discrepancy may be due to the subtle changes in the mitotic index following treatment in this line.

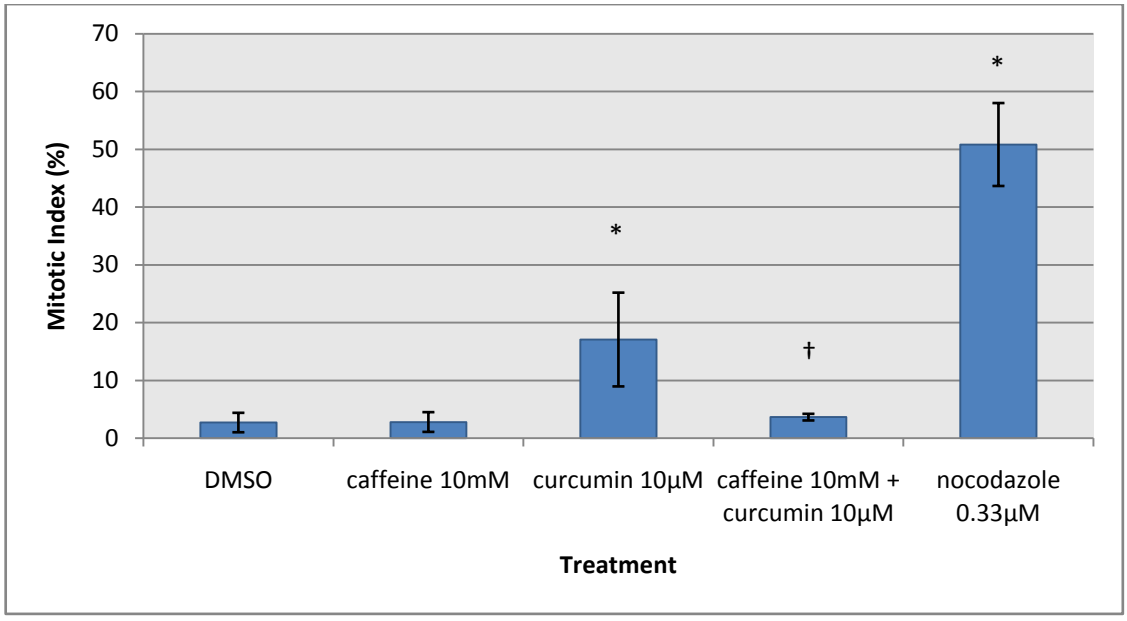


Figure 48 Caffeine Inhibits Curcumin-Induced M Phase Arrest in Curcumin Treated SW480 Cells

SW480 cells were pre-treated with caffeine for 1 hour, treated with curcumin for 12 hours, and their mitotic index assessed using fluorescence microscopy. Cells were co-stained with pH3 antibodies and DAPI. DMSO and nocodazole were used as negative and positive controls. At least 100 cells were scored per data point. Data shown are the mean (±SD) of at least three independent experiments. Bars marked with * indicate there is a significant ($P<0.05$) difference from DMSO control. † indicates there is a difference in means between the cells treated with curcumin alone and those treated with caffeine and curcumin.

Pre-treatment with the inhibitor caffeine significantly attenuates curcumin-induced mitotic arrest in the SW480 cell line (Figure 47). The mitotic index is reduced by an average of 13% in cells pre-treated with caffeine prior to curcumin treatment, compared to those treated with curcumin alone.

To summarise, caffeine pre-treatment was much more effective in inhibiting curcumin-induced mitotic arrest than G_2/M arrest in all of the CRC lines tested.

5.2.5 DBH Reduces Curcumin-Mediated Mitotic Arrest in the HCT116 p53$^{+/+}$ line

As treatment with caffeine implicated DNA damage signalling in curcumin-mediated M phase arrest, further experiments were carried out using the Chk1/Chk2 inhibitor debromohymenialdisine (DBH). DBH was discovered during a cell-based screen of marine invertebrate extracts (Curman et al., 2001). It was isolated from samples of the marine sponge *Stylissa flabeliformis* collected near Matapore Island in Papua New Guinea and was found to be a potent Chk1 and Chk2 inhibitor. This checkpoint kinase inhibitor has an IC_{50} of 3µM for Chk1 and 3.5µM for Chk2. DBH does not inhibit the activity of ATM, ATR or DNA-PK, having a narrower range of inhibition than caffeine.

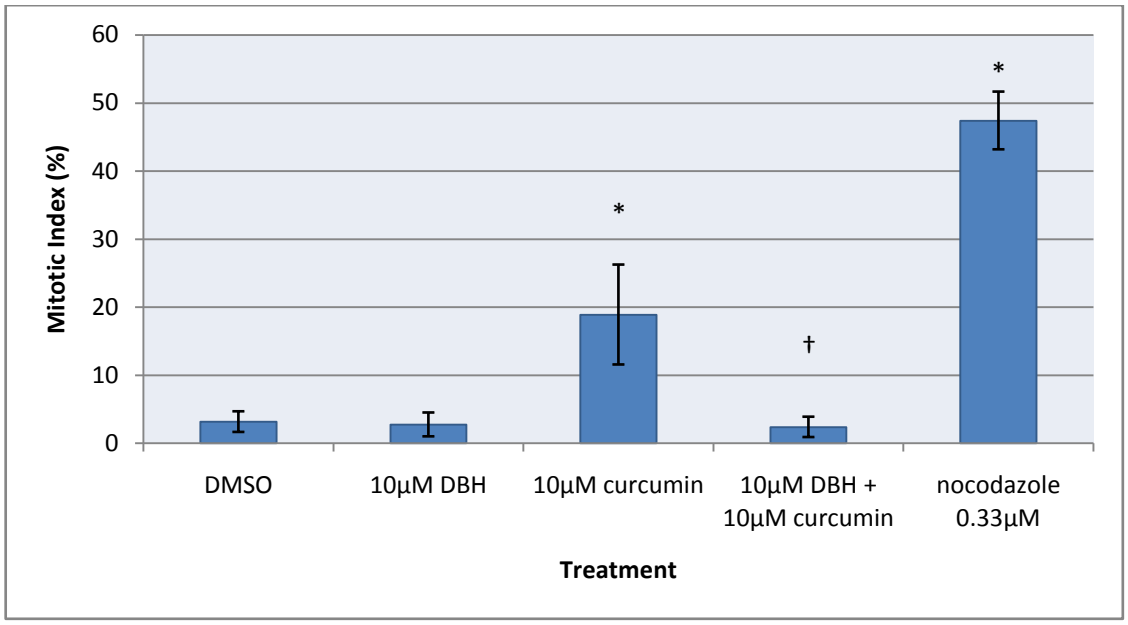


Figure 49 DBH Inhibits Curcumin-Induced Mitotic Arrest in HCT116 p53$^{+/+}$ Cells

HCT116 p53$^{+/+}$ cells were pre-treated with DBH for 1 hour, treated with curcumin for 12 hours, and their mitotic index assessed using fluorescence microscopy. Cells were co-stained with pH3 antibodies and DAPI. DMSO and nocodazole were used as negative and positive controls. At least 100 cells were scored per data point. Data shown are the mean (±SD) of at least three independent experiments. * indicates a significant ($P<0.05$) difference compared to the DMSO control. † indicates a difference between the curcumin-treated population and the DBH and curcumin treated cells.

Pre-treatment with the inhibitor DBH inhibits curcumin-induced M phase arrest in the HCT116 $p53^{+/+}$ cell line (Figure 48). In cells pre-treated with DBH prior to curcumin treatment the mitotic index was just 2% in comparison to 19% in those treated with curcumin alone.

5.2.6 Confirmation That Checkpoint Inhibitors Block Curcumin-Induced Mitotic Arrest

To confirm the studies described above with respect to mitotic index data, simultaneous analysis of DNA content and pH3 staining was performed by further FACS analysis. The total DNA content of cells was quantified by staining with DAPI and analysis with the UV/450/50-laser. Total pH3 (Ser10) staining was analysed by using a primary antibody coupled to long range red secondary antibody (Alexa 680) and excitation using the R/670/14-A laser. Curcumin fluoresces with an emission spectrum at 460-640nm peaking at approximately 520nm (exciting wavelength 440nm) (Kunwar et al., 2006, Stockert et al., 1989). When analysing curcumin-treated cells this natural fluorescence can directly overlap with the emission spectra of fluorescein isothiocyanate (FITC: emission peak 521nm) in the FL-2 channel, and to a lesser extent with PI in the FL-1 channel (PI: emission peak 620nm). To prevent overlaps with the emission spectra of curcumin, long-range red secondary antibodies and DAPI were selected to stain curcumin-treated cells. Tests using curcumin-treated cells and samples containing curcumin alone have confirmed that there is no spill-over of the natural fluorescence of this agent into spectra to be generated with the UV/450/50 and R/680/14-A lasers.

Simultaneous analysis of the cell cycle profile and pH3 staining of cells was carried out following treatment with curcumin and pre-treatment with checkpoint inhibitors caffeine and DBH. Nocodazole was used as a positive control for pH3 staining.

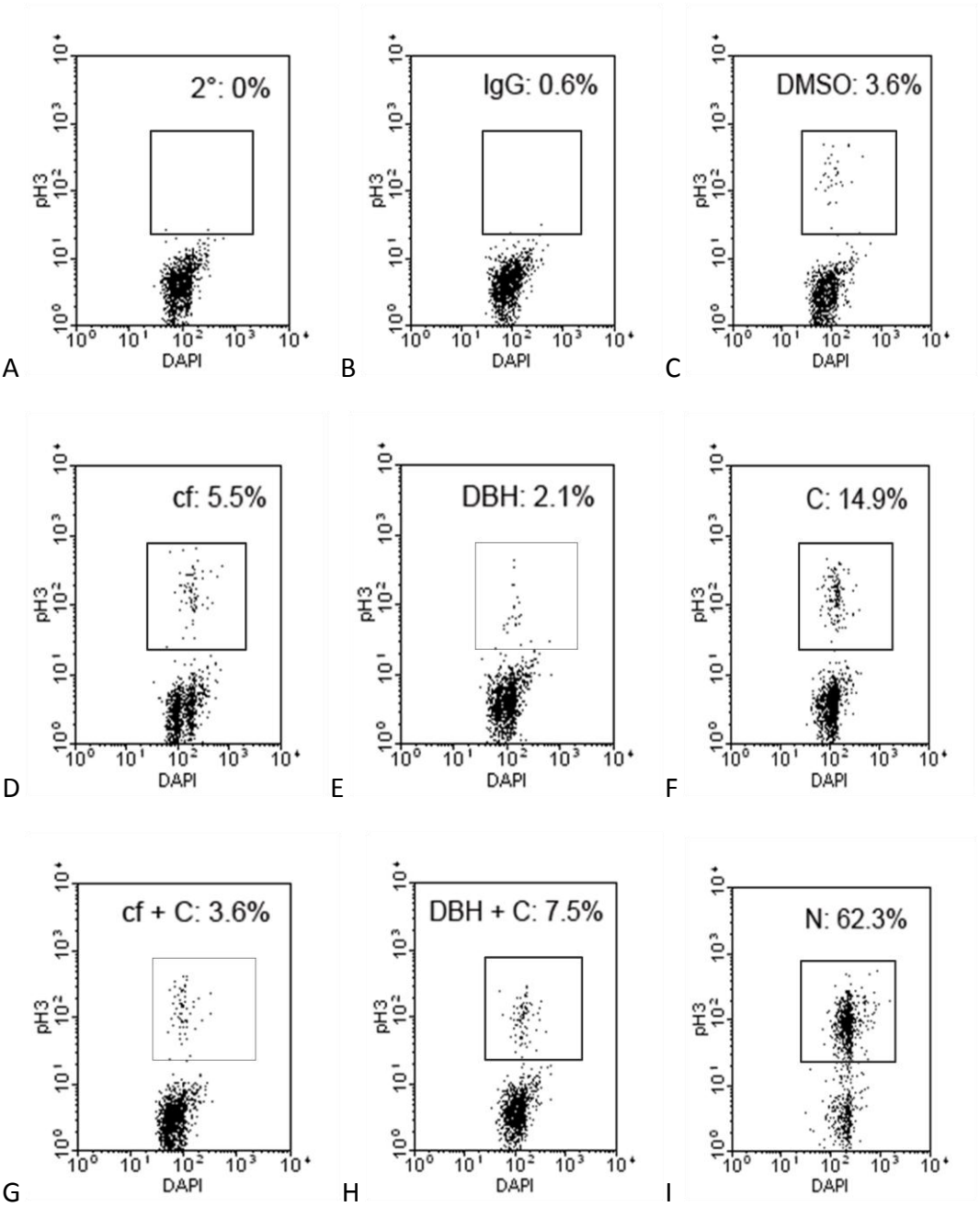
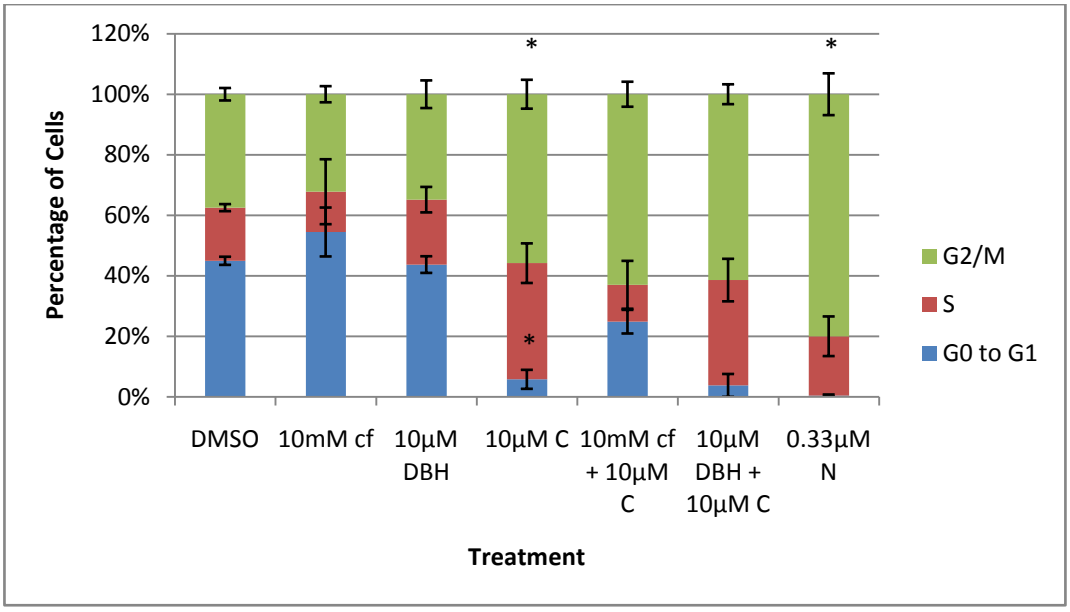


Figure 50 Checkpoint Inhibitors Reduce Curcumin-Mediated Mitotic Arrest

HCT116 $p53^{+/+}$ cells were stained with a primary antibody to pH3 to quantify the mitotic index, and the DNA stain DAPI to evaluate the phase of the cell cycle. A long range red fluorescent secondary antibody (Alexa 680) was used to label phospho-H3 positive cells. Data are presented in a dot plot with DAPI vs. pH3 staining and gating of the mitotic population: (A) Secondary only control with DAPI (B) IgG_1 Isotype Control (C) DMSO (D) 10mM caffeine (E) 10µM DBH (F) 10µM curcumin (G) 10mM caffeine + 10µM curcumin (H) 10µM DBH + 10µM curcumin (I) 0.33µM nocodazole Data are from one experiment to illustrate changes in the mitotic index.



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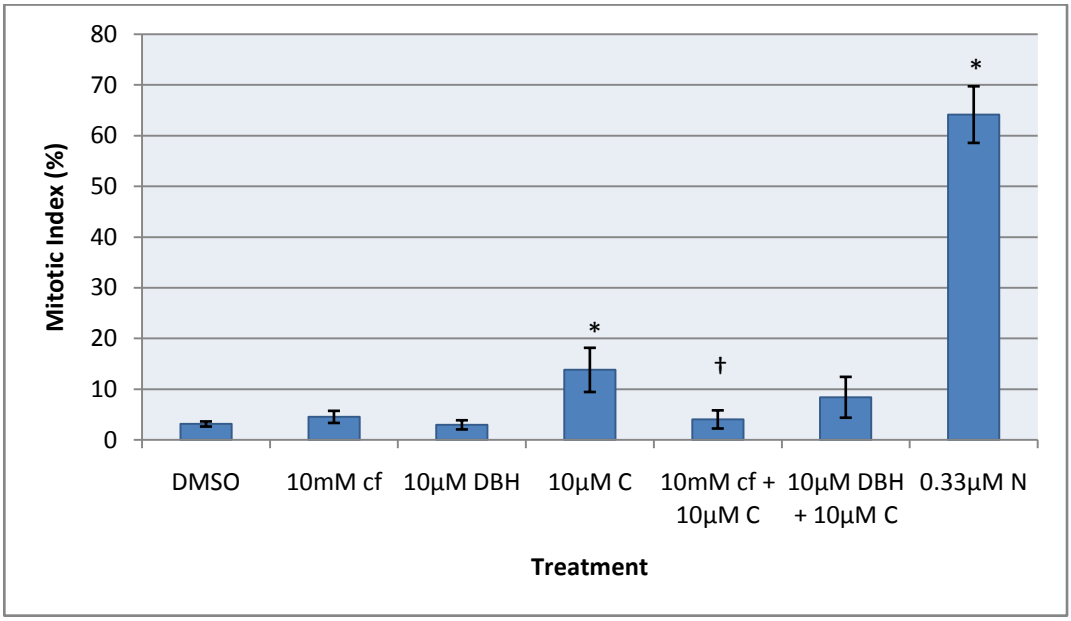


B

Figure 51 Pre-Treatment with Caffeine Reduces Curcumin-Mediated Mitotic Arrest

HCT116 $p53^{+/+}$ cells were pre-treated with caffeine or DBH for 1 hour then curcumin for 12 hours and processed with FACS analysis to measure both DNA content (DAPI) and pH3 staining. Data were then analysed to evaluate the stage of the cell cycle (A) or mitotic index (B). Nocodazole was used as a positive control for M phase arrest. Data shown are the mean (±SEM) of 3 independent experiments. Bars marked with * show a significant difference ($p<0.05$) from the average percentage of cells compared to the DMSO control. † indicates a difference between the curcumin-treated population and the inhibitor and curcumin treated cells P values were calculated using a 2-tailed T-Test assuming equal variance. At least 10,000 cells were analysed per data point.

An alternative analysis of the cell cycle profile using pH3 and DAPI stains confirms that curcumin treatment results in G_2/M and M phase arrest (Figures 49 and 50). Figure 49 highlights the changes in both DNA content and pH3 staining following treatment with curcumin between the DMSO control (C) and curcumin-treated cells (F). The nocodazole control (I) has the greatest increase in the number of pH3 positive 4N cells.

FACS cell cycle analysis using the DNA stain DAPI shows that curcumin treatment results in a significant increase in the number of cells undergoing G_2/M arrest, with an average of 56% of cells in this phase compared to 38% in the DMSO control (Figure 50 A). These levels are different to those found using PI as a DNA stain in Figure 40 that showed that the average number of cells in G_2/M following treatment with 10µM of curcumin for 12 hours was 75% in contrast to 30% in the DMSO control. No significant differences in the levels of G_2/M arrest were found following pre-treatment with the inhibitors caffeine or DBH prior to curcumin treatment (Figure 50 A). Again, this is in contrast to findings in Figure 40 that showed that there was a small but significant reduction in the levels of G_2/M arrest following pre-treatment with caffeine before curcumin treatment. However, the standard deviation was much higher in the samples stained with DAPI and this may account for the differences in significance between the two experiments.

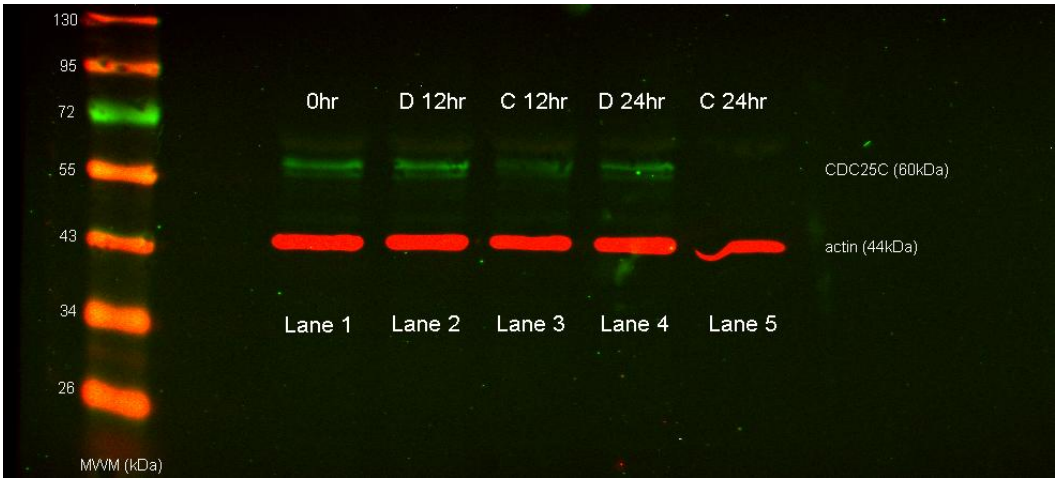
FACS analysis of the number of pH3 positive cells confirms that 12 hours curcumin treatment results in a significant increase in the mitotic index in the HCT116 $p53^{+/+}$ line (Figure 50 B). Pre-treatment with 10mM of caffeine results in an abrogation of mitotic arrest and this confirms previous findings using fluorescence microscopy to evaluate

the mitotic index (Figure 45). However, Figure 50 B shows that despite a decrease in the average number of mitotic cells following pre-treatment with the Chk1/Chk2 inhibitor DBH, this is not significant. These findings differ to those in Figure 48, where fluorescence microscopy was used to calculate the mitotic index.

5.2.7 Curcumin Treatment Down-regulates Total CDC25C Protein Levels

The CDC25C phosphatase removes inhibitory phosphate residues from CDK1-cyclin B complexes, facilitating the transition from G_2 to M phase. Nuclear exclusion of this phosphatase or destruction by proteasomal degradation can result in G_2/M cell cycle arrest. Curcumin treatment has previously been shown to result in a total reduction of CDC25C protein levels in T cell lines (Tomita et al., 2006). In order to investigate whether loss of CDC25C contributed to curcumin-induced G_2/M arrest in the HCT116 $p53^{+/+}$ or HCT116 $p53^{-/-}$ lines, Western blotting experiments were carried out and analysed using the Odyssey imaging system. Total CDC25C integrated intensity levels have been normalised to those of the loading control and are presented as a percentage of levels found in the 0 hour untreated lysates.

A



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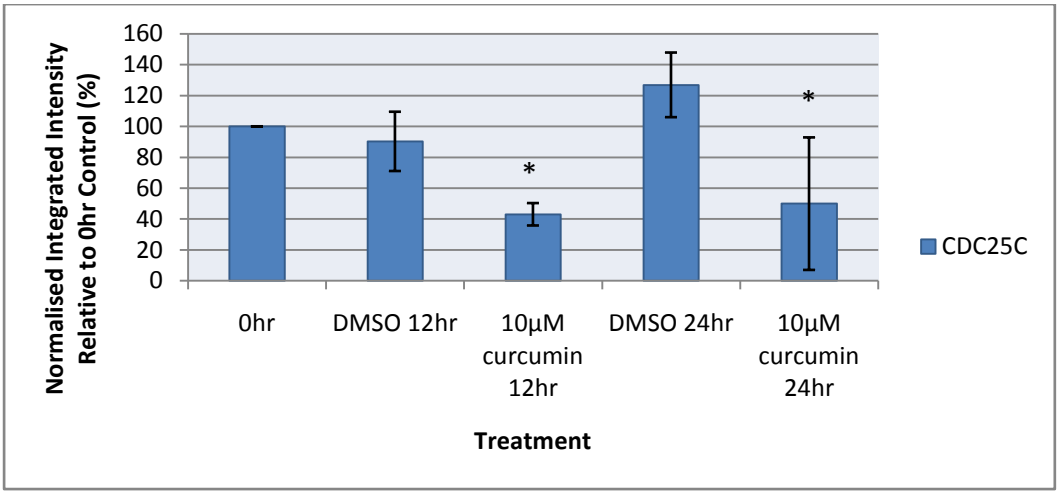


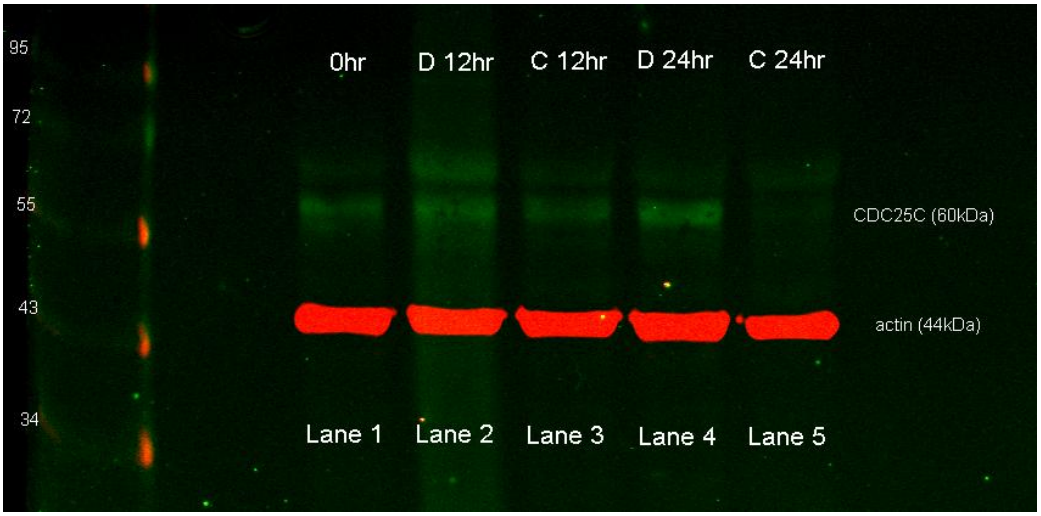
Figure 52 Curcumin Treatment Reduces Total CDC25C Protein Levels in the HCT116 p53$^{+/+}$ Line

(A) HCT116 p53$^{+/+}$ cells were treated with medium alone (Lane 1 0hr), DMSO alone (Lane 2, 12hr, Lane 4 24hr) or 10 µM curcumin (Lane 3, 12hr, Lane 5, 24hr) for the times indicated, and prepared for Western blotting analysis. CDC25C (green) and actin (red) levels were captured using the Odyssey scanner.

(B) Integrated intensity levels of CDC25C were normalised to those of the actin loading control and are shown as a relative percentage compared to the 0hr control. Bars marked with * indicate there is a significant ($P<0.05$) difference between the means of DMSO control samples and those treated with curcumin for the time point indicated. Error bars show the standard deviation. Data shown are the mean of at least three independent experiments.

Curcumin treatment results in a significant reduction in CDC25C total protein levels at both the 12 and 24 hour time points in the HCT116 $p53^{+/+}$ line (Figure 51). Following 12 hours treatment with curcumin, levels of CDC25C were 43% of those found in the 0 hour control, compared to 90% in the DMSO control for that time point. After treatment for 24 hours levels of CDC25C were 50% of those found in the 0 hour control.

A



B

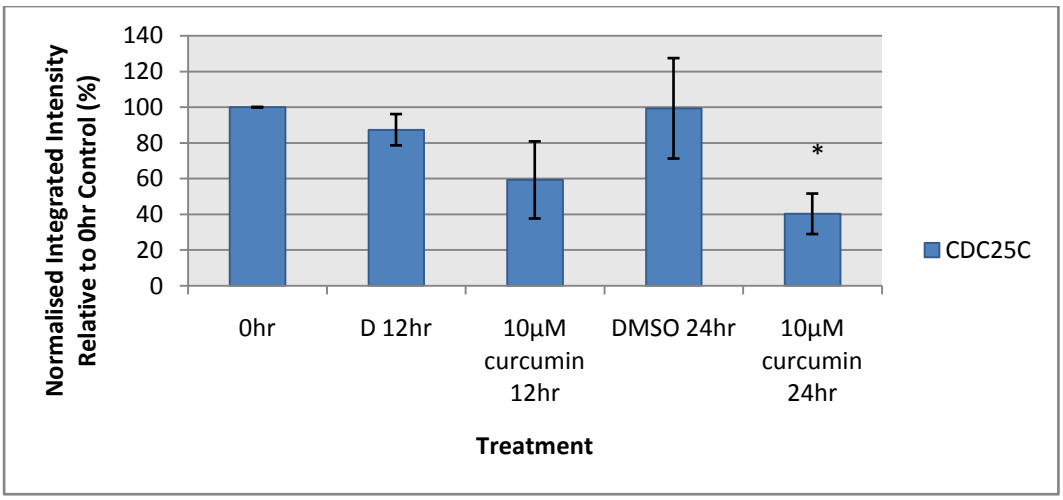


Figure 53 Curcumin Treatment Reduces Total CDC25C Levels in the HCT116 p53$^{-/-}$ Line

(A) HCT116 p53$^{-/-}$ cells were treated with medium alone (Lane 1 0hr), DMSO alone (Lane 2, 12hr, Lane 4 24hr) or 10 µM curcumin (Lane 3, 12hr, Lane 5, 24hr) for the times indicated, and prepared for Western blotting analysis. CDC25C (green) and actin (red) levels were captured using the Odyssey scanner.

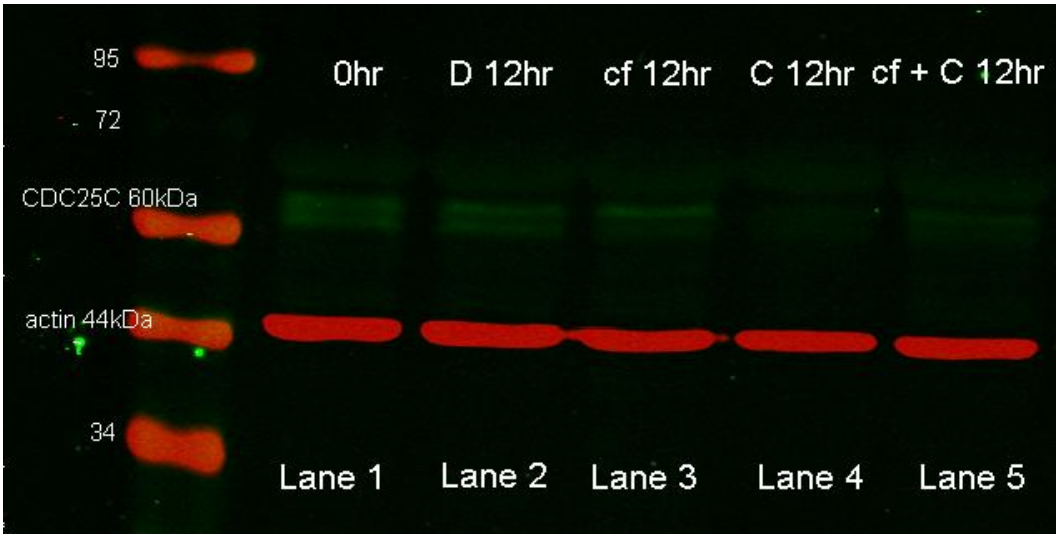
(B) Integrated intensity levels of CDC25C were normalised to those of the actin loading control and are shown as a relative percentage compared to the 0hr control. Bars marked with * indicate there is a significant ($P<0.05$) difference between the means of DMSO control samples and those treated with curcumin for the time point indicated. Error bars show the standard deviation. Data shown are the mean of at least three independent experiments.

Treatment with curcumin results in a significant decrease in total CDC25C levels after 24 hours with 40% compared to the DMSO control in the HCT116 $p53^{-/-}$ line (Figure 52). Due to the large error bars, in contrast to the HCT116 $p53^{+/+}$ line a significant difference in CDC25C levels at 12 hours was not apparent.

5.2.8 The Levels of CDC25C Following Caffeine and Curcumin Treatment

As caffeine pre-treatment was found to significantly reduce levels of G_2/M and M cell cycle arrest in the HCT116 $p53^{+/+}$ line, the effect of caffeine pre-treatment on key cell cycle proteins was investigated. Previous studies have shown that pre-treatment with caffeine could prevent inhibitory phosphorylation of the CDC25C phosphatase (Tyagi et al., 2005). To investigate whether caffeine pre-treatment could rescue CDC25C degradation, pre-treated HCT116 $p53^{+/+}$ cells were exposed to curcumin treatment for 12 hours, as the greatest loss of CDC25C was found at this time point.

A



B

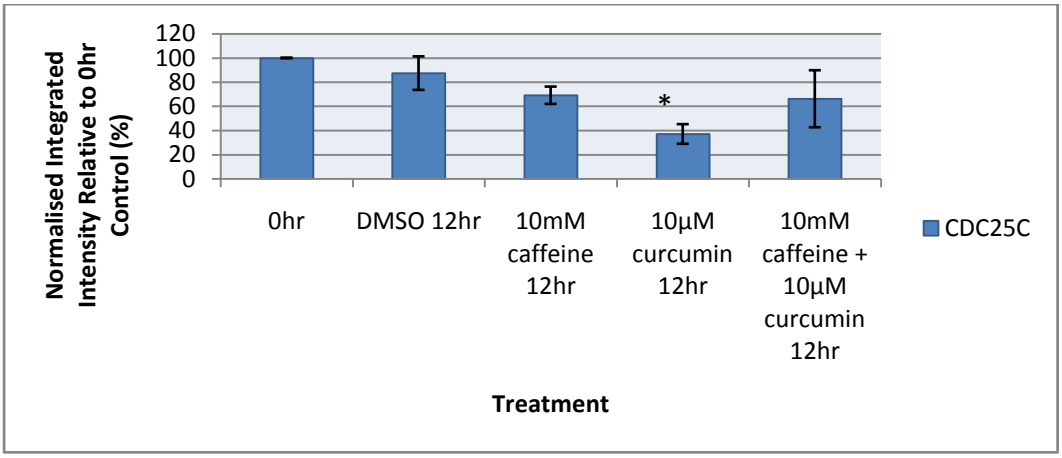


Figure 54 Caffeine Pre-Treatment Does Not Significantly Alter CDC25C Levels in Curcumin-Treated Cells

(A) HCT116 $p53^{+/+}$ cells were treated with medium alone (Lane 1, 0hr) DMSO (Lane 2) or 10µM curcumin (Lane 4) for 12 hours, or pre-treated with 10mM caffeine for 1 hour, followed by DMSO (Lane 3) or curcumin (Lane 5) for 12 hours. Cells were then harvested and analysed by Western blotting. Total CDC25C (green) and actin (red) levels were captured using the Odyssey scanner.

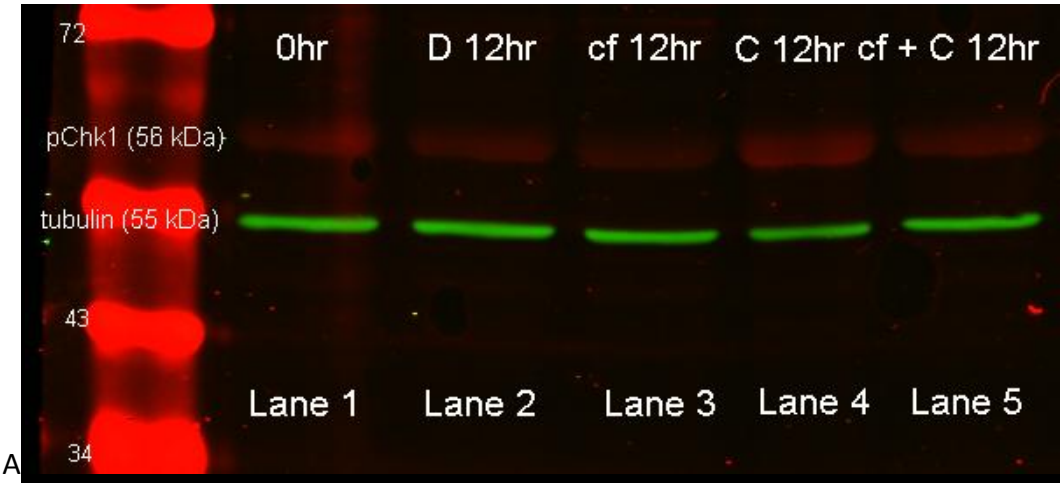
(B) Integrated intensity levels of CDC25C were normalised to those of the actin loading control and are shown as a relative percentage compared to the 0hr control. Bars marked with * indicate there is a significant ($P<0.05$) difference between the means of DMSO control samples and those treated with curcumin for the time point indicated. Error bars show the standard deviation. Data shown are the mean of at least three independent experiments.

Pre-treatment with caffeine did not prevent the reduction of CDC25C total protein levels following curcumin treatment in the HCT116 $p53^{+/+}$ line (Figure 53).

5.2.9 Curcumin-Mediated Chk1 Phosphorylation is Inhibited by Caffeine

Loss of CDC25C during checkpoint activation can be mediated by the Chk1 and Chk2 kinases. Pre-treatment with caffeine (10mM) has been shown to reverse resveratrol-mediated Chk1 phosphorylation (Tyagi et al., 2005). To investigate the effects of these kinases in curcumin-mediated G_2/M arrest in the HCT116 $p53^{+/+}$ line, cells were pre-treated with caffeine prior to curcumin treatment. Levels of Chk1 phosphorylation at Ser345 were then measured by Western blotting.

A



B

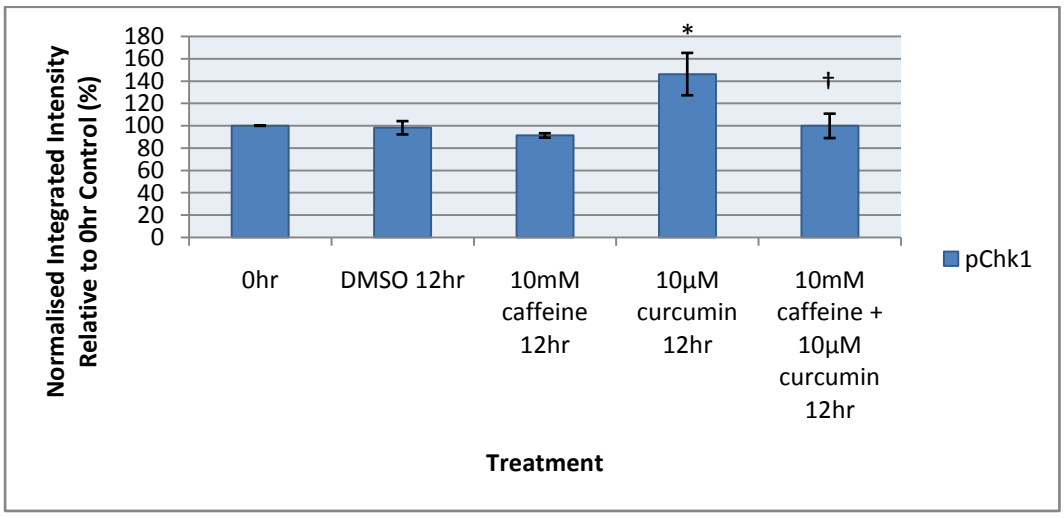


Figure 55 Caffeine Pre-Treatment Reduces Chk1 Phosphorylation in Curcumin-Treated HCT116 p53$^{+/+}$ Line

(A) HCT116 p53$^{+/+}$ cells were prepared as in Figure 53 Levels of pChk1 (Ser345, red) and tubulin (green) were captured in separate channels using the Odyssey scanner.

(B) Integrated intensity levels of pChk1 were normalised to those of the tubulin loading control and are shown as a relative percentage compared to the 0hr control Bar marked with * indicates a significant ($P<0.05$) difference from the DMSO control sample, † indicates a significant ($P<0.05$) difference between the cells treated with curcumin and those pre-treated with caffeine then treated with curcumin. Data shown are the mean (±SD) of at least three independent experiments.

Curcumin treatment for 12 hours significantly increases levels of Chk1 phosphorylation and this is abrogated by pre-treatment with caffeine (Figure 54). Levels of pChk1 rose by 66% compared to the DMSO control. In cells pre-treated with caffeine then treated with curcumin there was significantly less Chk1 phosphorylation (by 46%) compared to cells treated with curcumin alone.

5.2.10 Curcumin-Mediated Mitotic Arrest Results in an Increase in pH2A.X Foci

Previous work has shown that an increase in pH2A.X staining can be seen in HCT116 cells treated with spindle poisons such as nocodazole, paclitaxel or monastrol due to aberrant mitosis (Dalton et al., 2007). To examine whether curcumin treatment altered pH2A.X staining in mitotic HCT116 $p53^{+/+}$ cells, immunofluorescence experiments were carried out. Cells were stained with antibodies to pH3 (Ser10) to identify mitotic cells, and pH2A.X (Ser139) to identify cells with DNA single and double-strand breaks. Chromatin was stained with DAPI.

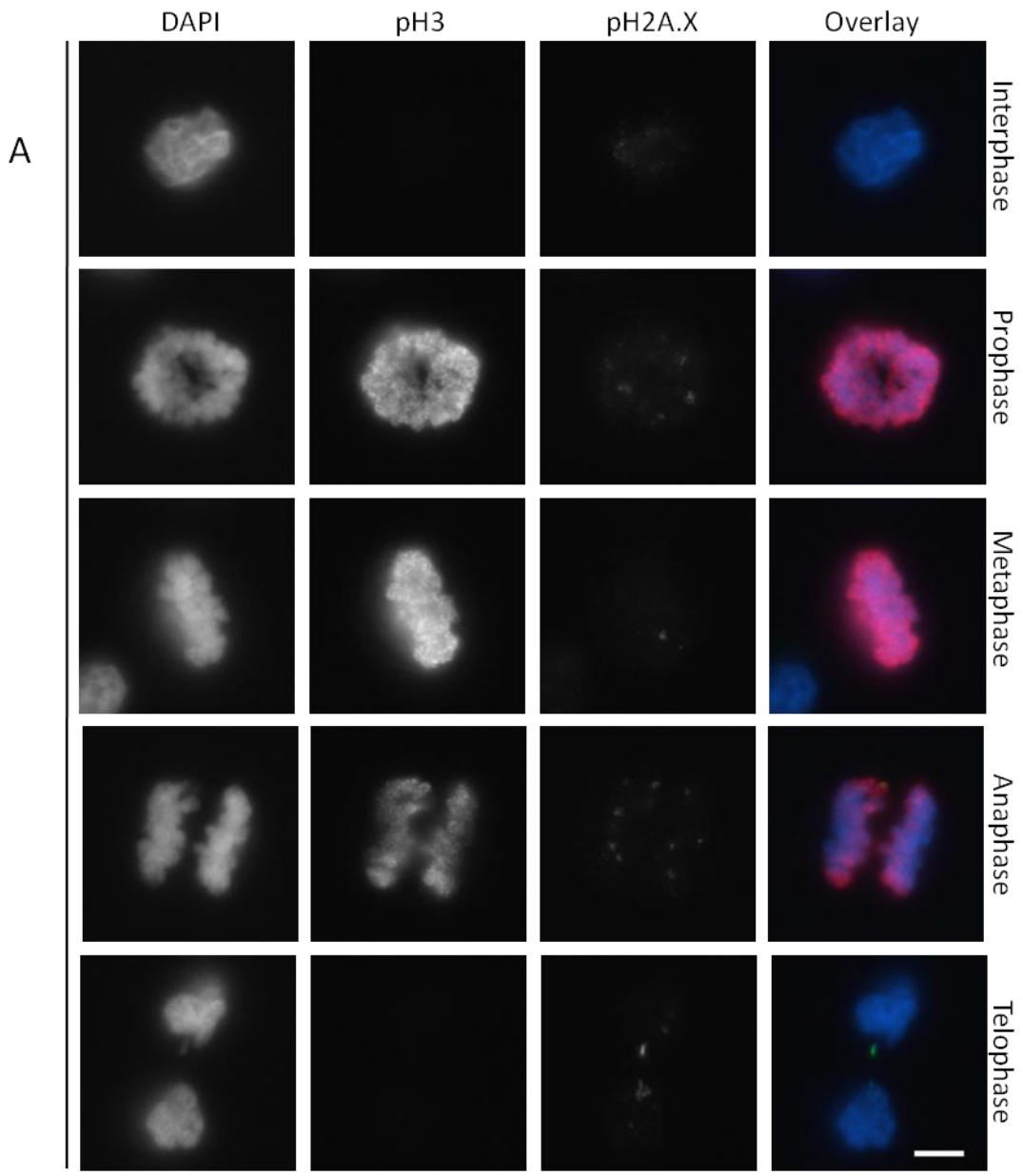


Figure 56 Curcumin Treatment Results in Increased pH2A.X Staining in Mitotic Cells

HCT116 $p53^{+/+}$ cells were treated for 12 hours with either DMSO alone (A) or 10µM curcumin (B). Cells were stained with antibodies against pH3 (red) and pH2A.X (green). DNA was stained with DAPI (blue). Representative images of cells are shown in interphase and each stage of mitosis (prophase, metaphase, anaphase, and telophase). Images are representative of at least three separate experiments. Exposure and gain were maintained and comparable for pH2A.X staining. Scale bar 5µm.

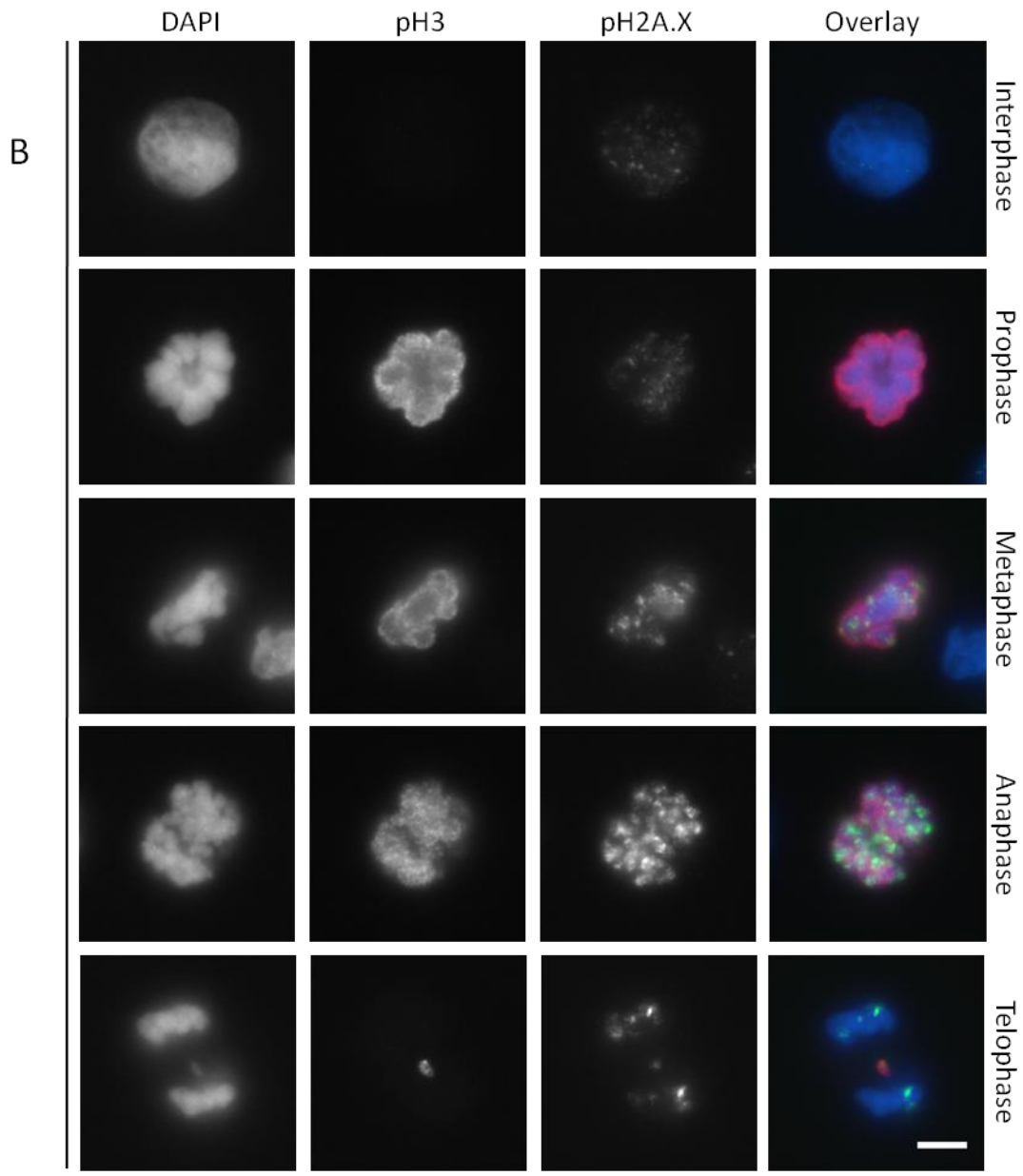


Figure 55 Continued

Treatment with curcumin results in increased pH2A.X staining in mitotic cells (Figure 55). In DMSO-treated cells low levels of pH2A.X staining can be seen throughout mitosis. In a cell in late telophase, a small amount of staining can be seen between the chromatin of the two nuclei.

In cells treated with curcumin, higher levels of pH2A.X staining can be seen, generally in cells in the later stages of mitosis. In the representative interphase and prophase cells, the levels of pH2A.X staining are relatively similar to those found in the DMSO control. However, levels of pH2A.X staining are higher during metaphase in curcumin-treated cells. The highest levels of pH2A.X staining in Figure 55 can be seen in the anaphase cell. This cell appears to have tangled chromosomes that have not clearly separated. Fewer areas of intense pH2A.X staining or foci can be seen in curcumin-treated cells undergoing telophase.

Cells in the later stages of mitosis resemble cells with a *cut* phenotype, where normal mitotic chromosome separation has failed, and this is most notable in curcumin-treated anaphase cells that have the highest levels of pH2A.X staining.

5.3 Discussion

5.3.1 Effect of Checkpoint Inhibitors on G_2/M and M Phase Arrest

Pre-treatment with caffeine significantly reduced levels of curcumin-induced G_2/M arrest to a limited extent in the HCT116 $p53^{+/+}$, HCT116 $p53^{-/-}$, HCT116 $p21^{-/-}$ lines, but not in the HT-29 or SW480 lines. However, it was discovered that such pre-treatment significantly inhibited M phase arrest in all 5 lines. The reduction in levels of M phase arrest generally correlated with the overall reduction in G_2/M arrest in the HCT116 lines. The reduction in mitotic arrest in the HT-29 line was just 2%, and this low percentage may explain why a significant difference in overall G_2/M and M arrest was not detected using FACS cell cycle analysis. Interestingly, the reduction in M phase arrest found using fluorescence microscopy was found to be 13% in the SW480 line, whereas no significant reduction in G_2/M arrest was found with FACS analysis following caffeine pre-treatment in this line. However, the average number of cells in G_2/M was shown to be reduced following caffeine pre-treatment, although this difference was not statistically significant.

Taken together, these results indicate that inhibition of the DNA damage network signalling via pre-treatment with caffeine may affects the levels of curcumin-induced mitotic arrest rather than arrest at the G_2/M boundary. This implies that there could be ATM/ATR independent mechanisms of G_2/M boundary cell cycle arrest following curcumin treatment. Curcumin may induce antephase arrest, which involves CHFR, p38, Plk1 and is independent of ATM signalling (Kang et al., 2002, Mikhailov et al., 2005, Scolnick and Halazonetis, 2000, Summers et al., 2005). There is contradictory evidence regarding curcumin-mediated p38 activation, but recent studies, including

those in this lab, have shown that this agent can activate p38 in leukemia, breast and oral cancer cells (Andreadi et al., 2006, Chang et al., 2010, Chen et al., 2010). Curcumin has also been shown to downregulate the transcription of Plk1 (van Erk, 2004). To investigate the role of p38 signalling in G_2/M arrest in these CRC lines, pre-treatment with a p38 inhibitor such as SB 203580 could be carried out prior to curcumin treatment (Matsusaka and Pines, 2004, Mikhailov et al., 2004). If antephase signalling is involved in curcumin-induced arrest, a significant reduction in the number of G_2/M cells would be expected and this could be measured using FACS cell cycle analysis.

Pre-treatment with the Chk1 and Chk2 inhibitor DBH significantly reduced curcumin-induced mitotic arrest in the HCT116 $p53^{+/+}$ line as measured by fluorescence microscopy, indicating that one or both of these kinases may contribute to the regulation of curcumin-induced M phase arrest.

FACS analysis that simultaneously measured DNA content and the mitotic index in the HCT116 $p53^{+/+}$ following treatment with curcumin alone or in combination with checkpoint inhibitors caffeine or DBH, essentially confirmed data found in mitotic index experiments carried out using microscopy. However, pre-treatment with DBH reduced the mitotic index to a lesser extent than the results from the fluorescence microscopy experiments indicated. Furthermore, no significant effect on levels of overall G_2/M arrest was found following caffeine pre-treatment when cells were stained with DAPI, whereas a significant reduction of G_2/M arrest was found in cells stained with PI. This may be due to variations in analysis as the error bars in experiments using PI were much lower, so small changes in the average number of cells in G_2/M would be more likely to be detected using this DNA stain.

The levels of apoptotic cells were analysed in the studies with curcumin and caffeine. The HCT116 $p53^{-/-}$ line was the most sensitive to curcumin treatment and was the only line to have a significant increase in sub-G_1 content following curcumin treatment for 12 hours. Pre-treatment with caffeine followed by curcumin treatment resulted in an increase in apoptotic cells in the HCT116 $p53^{+/+}$ line and the SW480 cell line. However in this study pre-treatment with caffeine alone resulted in an increase in the number of apoptotic cells in the MIN HCT116 lines.

Previous research investigating the effects of caffeine on overall levels of curcumin-induced G_2/M arrest in the HCT116 line found no significant effect on levels of arrest (Basile et al., 2009). The differences in those experiments compared to the present study are that they co-treated with caffeine instead of pre-treating, they used a lower concentration (1mM compared to 10mM (IC_{50}: 0.2mM ATM, 1.1mM ATR, 10mM DNA-PK, 5mM Chk1) and they measured levels of arrest after 24 hours instead of 12. The concentration of curcumin was the same (10μM). Their study additionally found that curcumin activated ATM via phosphorylation at Ser-1981. As they found there were no significant differences in the overall levels of G_2/M arrest, they concluded that curcumin-mediated M phase arrest did not involve ATM. While caffeine resulted in a significant reduction in levels of curcumin-mediated G_2/M arrest in this present study, this decrease was modest and related to the reduction of cells in mitotic arrest. It is possible that Basile et al. did not find significant differences in levels of G_2/M arrest as the lower concentration of caffeine used may not have had the same effect on reducing the number of cells in mitosis. Data presented here suggest that DNA

damage signalling may be involved in curcumin-mediated mitotic arrest but further experiments would be required to confirm this.

5.3.3 Effect of Curcumin Treatment on Cell Cycle Proteins

Curcumin treatment was found to significantly decrease the total protein levels of the CDC25C phosphatase. This is in accordance with previous research that has shown that curcumin has a negative effect on the activity of this phosphatase via inhibitory phosphorylation or reduction of total protein levels (Sahu et al., 2009, Tomita et al., 2006). While curcumin has been shown to impair the activity of the proteasome (Milacic et al., 2008), loss of CDC25C still occurs in curcumin-treated cells. Other studies have found that in certain circumstances proteasomal inhibitors do not rescue CDC25C degradation and have proven that CDC25C may be degraded via a calpain-dependent pathway (Garcia-Morales, 2007). Loss of CDC25C during checkpoint activation can be mediated by the Chk1 and Chk2 kinases. Previous research that investigated the mechanisms of curcumin-mediated G_2/M arrest has shown that this is ATM/Chk1 mediated (Sahu et al., 2009). Knock-out of either of these kinases using siRNA results in an abrogation of G_2/M arrest. In this present study, pre-treatment with caffeine prior to curcumin treatment resulted in higher total protein levels of CDC25C, but differences compared to cells treated with curcumin alone were not significant. At the concentrations used in this study, caffeine treatment (10mM) may inhibit the activity of Chk1 and Chk2, thus impairing their ability to target CDC25C for proteasomal degradation (Sarkaria et al., 1999).

While curcumin activated the DNA damage signalling network, leading to activatory phosphorylation of the Chk1 protein, this was significantly reduced by caffeine pre-treatment. As caffeine has been shown to inhibit the activity of the ATM/ATR kinases at this concentration, this suggests that these kinases may be involved in the activation of Chk1 following curcumin-mediated DNA damage during mitotic arrest and further experiments would be required to confirm this hypothesis. This is in line with recent research that has shown that the activity of the ATM kinase is essential for Chk1 activation following curcumin treatment in pancreatic cancer cells (Sahu et al., 2009).

5.3.4 The Importance of MMR Genes in Curcumin-Induced G_2/M Arrest

Research investigating the importance of the MMR genes in curcumin-induced G_2/M arrest found that in HCT116 cells with engineered MMR deficiency (either Msh2 or Mlh1), there was a partial reduction in the levels of checkpoint kinase activation and G_2/M arrest, compared to MMR proficient control cells (Jiang et al., 2010). These authors found that the status of the Chk1 kinase was more critical than the status of Msh2 or Mlh1 in the induction of G_2/M arrest, as Chk1 knock-down via siRNA, or pharmacological inhibition of this kinase with UCN-01 (0.3µM), prevented curcumin-induced arrest, with numbers of cells in G_2/M comparable to those for untreated cells. Knock-down of mismatch repair genes Msh2 or Mlh1 reduced levels of curcumin-induced G_2/M arrest, but to a lesser extent.

Of the cell lines in this present study, the HCT116 lines are MMR deficient (Mlh1 negative), and the HT-29 and SW480 lines are MMR proficient. The highest levels of

G_2/M arrest were found in the MMR deficient HCT116 lines. However, it is possible that greater levels of curcumin-mediated G_2/M cell cycle arrest could be achieved in the HCT116 lines used in this study by transfecting cells with a functional copy of Mlh1 or Msh2. Furthermore, the research done by Jiang et al. (2010) did not include other MMR competent or deficient lines for comparison when investigating the importance of MMR gene status in curcumin-mediated G_2/M arrest.

5.3.5 The Importance of the Spindle Assembly Checkpoint in Curcumin-Induced M Phase Arrest

Recent research in yeast has found that homologues of ATM and ATR kinases are crucial for DNA-damage mediated activation of the spindle-assembly checkpoint in budding yeast cells (Kim and Burke, 2008). Activation of the DNA damage signalling network during mitosis prevents the destruction of Pds1, a homologue of securin. The activity of Chk1 is required for regulation of spindle checkpoint function in humans, and knock-down of this protein with siRNA resulted in abrogation of taxol-mediated mitotic arrest (Zachos et al., 2007). Interestingly, knock-down of Chk1 failed to prevent nocodazole-mediated mitotic arrest, implying that there is an alternative Chk1-independent pathway that maintains arrest in these cells. Curcumin has recently been shown to activate the spindle assembly checkpoint in MCF-7 breast cancer cells (Jiang et al., 2010). Treatment with 36µM of curcumin for 24 hours resulted in the accumulation of spindle assembly checkpoint proteins Mad2 and BubR1 at kinetochores, and these experiments could be repeated in CRC lines using the

concentrations used in this study (10μM), in the presence of pharmacological inhibitors of Chk1 or after establishing knock-down of Chk1 using siRNA.

The highest levels of curcumin-mediated mitotic arrest were found in the MIN HCT116 lines with lower levels in the CIN HT-29 and SW480 lines. It has been proposed that CIN lines have a less robust spindle checkpoint, and are therefore less sensitive to agents that induce mitotic arrest (Cahill et al., 1998). If mitotic arrest is a result of DNA damage network activation of the spindle assembly checkpoint, then lines with a less robust spindle checkpoint such as the CIN HT-29 and SW480 lines would be expected to have a lower mitotic index following treatment with curcumin. However, mitotic arrest in both CIN and MIN lines would be abrogated by inhibition of the DNA damage signalling network. Results from this study indicate that mitotic arrest following curcumin treatment is a result of DNA damage activation of the spindle assembly checkpoint in CRC lines.

5.3.6 Decatenation Failure Following Curcumin Treatment Results in DNA Strand Breaks in Mitotic Cells

The presence of single or double strand breaks in DNA can be measured using anti-pH2A.X antibodies. DNA breaks, especially DSBs, result in the phosphorylation of histone H2A.X (Rogakou et al., 1998). Histone H2A.X is phosphorylated on Ser-139 by the checkpoint kinases ATM, ATR or DNA-PK. Each strand break is visualised by a focus of pH2A.X staining, and levels of intensity correlate to levels of DNA damage. Curcumin treatment increased the levels of pH2A.X staining in HCT116 $p53^{+/+}$ cells, and this is in accordance with other recent research in colorectal and pancreatic

cancer cells (Basile et al., 2009, Sahu et al., 2009). Jiang et al. (2010) proposed that the DNA damage was caused by repair of 8-oxo-dG adducts generated by reactive-oxygen species formation following curcumin treatment. They found that after treatment with curcumin (30μM) for 60minutes, levels of pH2A.X in lysates analysed by Western blotting, were lower in MMR deficient cells. However, no statistical analysis was performed to provide evidence for an increase in oxidative damage in this study, and levels of both 8-oxo-dG adducts and ROS species following curcumin treatment were comparable to controls.

In contrast Basile et al. (2009) suggested that the increase in histone H2A.X phosphorylation was caused by a prolonged curcumin-mediated mitotic arrest. Other studies have found that curcumin acts as a topo II poison, and single and double strand breaks as a result of the formation of permanent topo I-DNA and topo II-DNA complexes are the source of DNA damage (Lopez-Lazaro et al., 2007b, Martin-Cordero et al., 2003, Roth et al., 1998, Snyder and Arnone, 2002).

As discussed in the introduction, there are two types of DNA topoisomerase inhibitors: the topoisomerase poisons and the catalytic inhibitors of topoisomerases. Topoisomerase poisons stabilise the usually transient strand breaks generated by topoisomerase activity. These strand breaks can be lethal or nonlethal depending on conditions. In contrast, the catalytic inhibitors of the topoisomerases act by blocking the enzymatic activity of the topoisomerases, thus preventing the formation of any strand breaks. It should be noted that if these two types of topoisomerase inhibitor are used in combination, then the activity and toxicity of the topoisomerase poisons is

reduced, as their efficacy is dependent on the catalytic activity of the topoisomerase enzymes.

In two separate studies that investigated the source of DNA damage induced by curcumin it was found that pre-treatment with the ROS inhibitor N-acetylcysteine (NAC) prevented the formation of topo I-DNA and topo II-DNA complexes, reduced pH2A.X and pATM levels, and prevented G_2/M arrest (Jiang et al., 2010, Lopez-Lazaro et al., 2007b). Both studies concluded that curcumin-mediated DNA damage was a result of reactive oxygen species generation, as the free radical scavenger NAC protected cells from the effects of DNA damage induced by curcumin. However, both authors failed to note that, at the concentrations used in their studies (5-10mM), NAC would reduce the catalytic activity of topo II α by over 75% (Grdina et al., 1998). While ROS generated by curcumin treatment may have been responsible for the DNA damage manifested in both studies, the possibility that NAC acted as a topoisomerase inhibitor, thus preventing the damage generated by curcumin-mediated topo II poisoning, cannot be ruled out. By preventing the catalytic activity of topo II, the generation of single or double strand breaks by topo II-DNA complexes would be greatly reduced, and this would explain why levels of topo II-DNA complexes were relative to those found in untreated cells, and why there was reduced pH2A.X and pATM, and lower levels of G_2/M arrest found in both studies.

Intriguingly, research has shown that caffeine can also act as a catalytic inhibitor of topo I and topo II, and co-treatment with caffeine can protect against DNA damage induced by topo II poisons (Russo et al., 1991, Shin et al., 1990). Further investigations are required to elucidate whether the abrogation of curcumin-mediated mitotic arrest

via caffeine pre-treatment is a result of caffeine's ability to inhibit the catalytic activity of the ATM/ATR kinases, or the catalytic activity of topo II.

Data found in this present study indicate that the DNA damage induced by curcumin in colorectal cancer cells is a result of aberrant mitosis rather than reactive-oxygen species generation and this is supported by the lack of pH2A.X foci in curcumin-treated interphase cells. Positive controls for ROS generation such as hydrogen peroxide could be included in future immunofluorescence experiments to confirm the differences in pH2A.X staining in curcumin and hydrogen peroxide-treated interphase cells. Furthermore, the aberrant mitosis and pH2A.X staining induced by curcumin treatment may be a result of topoisomerase poisoning. Both NAC and caffeine may inhibit the catalytic activity of the topoisomerase enzymes in addition to ROS generation or DNA damage signalling following curcumin treatment, and further investigations are required to confirm this hypothesis.

5.3.7 Mitotic Spindle Abnormalities Could Contribute to Curcumin-Induced DNA Damage

Image analysis revealed that mitotic rather than interphase cells had higher levels of pH2A.X staining, implying that aberrant mitosis following curcumin treatment resulted in DNA damage. Recent work has shown that nocodazole treatment increased the levels of pH2A.X staining specifically in mitotic cells in 6 out of 8 colorectal cancer lines tested (Dalton et al., 2007). The highest levels of pH2A.X staining were generally found in prometaphase cells, and the authors proposed that this was linked to abnormalities in spindle structure caused by treatment, as a study of untreated control cells

revealed high levels of pH2A.X staining in cells with spontaneous spindle defects. However, the mechanism for the generation of DNA damage in these mitotic cells was unknown. Data in the previous chapter highlighted that curcumin treatment causes prophase/prometaphase arrest in the HCT116 $p53^{+/+}$ line with very few cells progressing to the later stages of mitosis. Another study investigating the effects of topoisomerase poisons found that HCT116 $p53^{+/+}$ cells undergoing mitosis in the presence of the topoisomerase inhibitor adriamycin (750nM) arrested in prometaphase, and that cell cycle arrest was dependent on Mad2 (Vogel et al., 2005).

Additional DNA strand breaks may be generated by spindle forces acting upon the kinetochores of nondecatenated chromosomes. If curcumin was acting as a topoisomerase poison in the HCT116 $p53^{+/+}$ line in this study, then this would explain the tangled appearance of chromosomes. Spindle forces acting to align and separate sister chromatids during mitosis could explain the high levels of pH2A.X staining visualised in metaphase and anaphase cells in Figure 55. The levels of pH2A.X staining appear to be highest in cells that resemble the *cut* phenotype, seen previously in human cells treated with topoisomerase inhibitors, or that express mutant versions of the securin gene that fail to separate mitotic chromosomes correctly (Gimenez-Abian and Clarke, 2009, Hagting et al., 2002).

In summary, image analysis suggests that curcumin may be acting as a topoisomerase poison in this study, and additional breaks may be acquired during the mitotic process due to spindle abnormalities, and failures in the sister chromatid decatenation and disjunction processes. However, further research using physiologically relevant

concentrations of curcumin in colorectal cancer lines is required, to provide conclusive evidence regarding the source of DNA damage in this panel of colorectal cancer lines.

5.3.8 Similarities to Other Chemopreventive Agents in Mechanisms of Cell-Cycle Arrest

One of the mechanisms of curcumin induced G_2/M boundary arrest was destruction of the CDC25C phosphatase protein. Negative regulation of the CDC25C phosphatase via inhibitory phosphorylation or proteosomal destruction is one of the central mechanism of G_2/M arrest induced by other cell cycle-interactive chemopreventive agents such as 3,3'-diindolylmethane (DIM), diallyl trisulphide (DATS), gallic acid and resveratrol (Agarwal et al., 2006, Herman-Antosiewicz and Singh, 2005, Kandala and Srivastava, 2006, Tyagi et al., 2005, Xiao et al., 2005). This present study found that curcumin treatment significantly reduced the total protein levels of CDC25C in the HCT116 lines in a p53-independent mechanism. Additionally, gallic acid and DATS-mediated mitotic arrest was shown to be dependent on the Chk1 kinase (Herman-Antosiewicz and Singh, 2005, Xiao et al., 2009). The present study has shown that DNA damage signalling is involved in curcumin-mediated mitotic arrest.

DIM is a topo II catalytic inhibitor, and treatment with this agent resulted in phosphorylation of histone H2A.X and Chk2-mediated G_2/M arrest (Gong et al., 2006, Kandala and Srivastava, 2006). DIM treatment also blocked sister chromatid separation during mitosis, a hallmark of decatenation failure following topoisomerase inhibition (Downes et al., 1991, Gong et al., 2006).

The chemopreventive agents EGCG (epigallocatechin gallate), luteolin, and genistein act as topo II poisons and significantly increase the formation of topo II-DNA complexes (Bandele and Osheroff, 2008, Lopez-Lazaro et al., 2007a). Resveratrol has recently been shown to act as a catalytic inhibitor of topo II, but high levels of pH2A.X led the authors to propose that this agent could also act as a topo II poison in human glioblastoma cells (Leone et al., 2010). Curcumin has previously been shown to act as a topoisomerase poison in Chinese hamster lung cells, and human leukemia K562 cells (Lopez-Lazaro et al., 2007b, Snyder and Arnone, 2002) and data from the present study suggest that this may be the main source of DNA damage in these colorectal cancer cells.

Chapter 6 Curcumin and the NKG2D Ligands

6.1 Introduction

Research has shown that curcumin treatment transactivates heatshock factor 1 (HSF-1) by dimer- or trimerisation, leading to increased binding to the heat shock element (HSE) of the *HSP70* gene, and increased total protein levels of the heat shock 70 (HSP70) protein in COLO205, HT-29 and SW620 colorectal cancer cell lines (Chen et al., 2001, Khar et al., 2001). The effects of curcumin treatment on the expression of NKG2DL such as MICA, MICB, ULBP1-3 have not been studied. However, the *MICA/MICB* genes also contain a HSE in their promoter region and are inducible by heat shock, it is plausible that curcumin treatment may result in increased transcription of these genes via HSF1 activation and DNA damage dependent signalling (Eagle et al., 2006, Gasser et al., 2005, Kim et al., 2006, Soriani et al., 2009). Furthermore, curcumin has been shown to act as a proteasomal inhibitor, and proteasomal inhibitors such as MG132 have been shown to increase levels of ULBP2 in Jurkat T cells (Milacic et al., 2008, Vales-Gomez, 2008). A study using head and neck squamous cell carcinoma (HNSCC) showed that treatment with proteasomal inhibitors, such as bortezomib, selectively induces ULBP1 mRNA expression and results in increased cell surface expression of ULBP1 in HNSCC cells (Butler et al., 2009). Bortezomib treatment has also been shown to result in increased MICA/MICB mRNA levels, and increased cell surface expression has been observed following treatment in hepatoma cells (Armeanu et al., 2008). Curcumin treatment may regulate the transcription and cell surface expression of NKG2DL such as MICA/MICB and ULBP1-2 via multiple mechanisms.

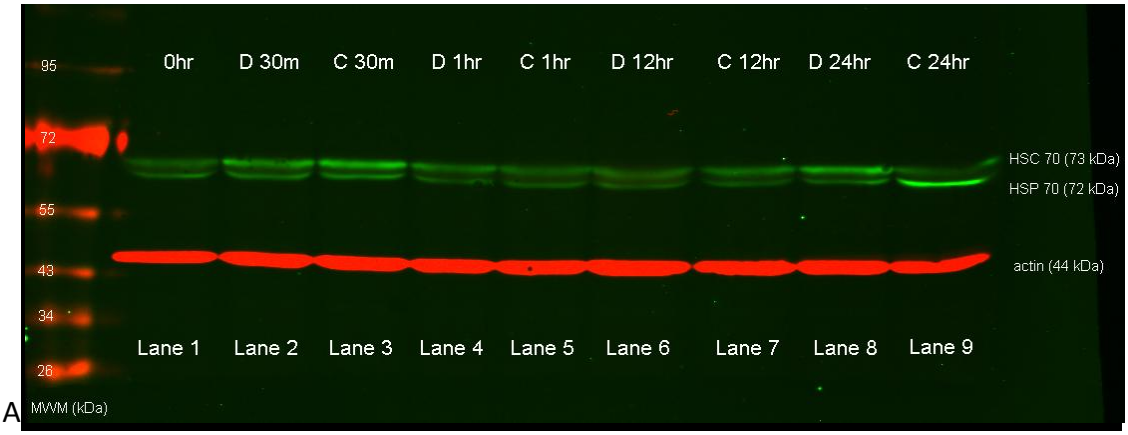
Previous studies have found that treatment with curcumin can prevent metastasis of tumour cells injected into immunodeficient nude mice (Bachmeier et al., 2007). These mice are impaired in their ability to generate mature αβ T cells and have an inhibited adaptive immune system. However, they may have a higher proportion of functional γδ T cells, which are capable of recognising NKG2DL, and the activation of the innate immune system by curcumin in these mice, may have contributed to the reduction of lung metastasis found in that study (Lake et al., 1991, Nonaka et al., 2005). In the Min mouse model lacking functional APC, curcumin treatment was shown to result in an increase in mucosal immune cells, indicating that curcumin is capable of modulating immune system functions (Churchill et al., 2000).

6.2 Results

6.2.1 Curcumin Treatment Upregulates Total Levels of HSP70 in the HCT116 Lines

Levels of the inducible HSP70 (72kDa) protein rise following genotoxic and proteotoxic stresses. HSP70 can act as a chaperone, facilitating the proteosomal destruction of damaged proteins (Kiang and Tsokos, 1998). Curcumin treatment has been shown to upregulate levels of HSP70 in COLO-205, HT-29, SW480 and SW620 colorectal cancer cells (Chen et al., 2001, Khar et al., 2001, Rashmi et al., 2003). To investigate whether curcumin treatment increased total levels of HSP70 in the HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cell lines, lysates were analysed using Western blotting. An antibody to both HSC70 and HSP70 proteins was used and the expression of HSC70 protein acted as an additional internal control, as in contrast to HSP70, the total protein levels of HSC70 do not increase during the stress response.

A



B

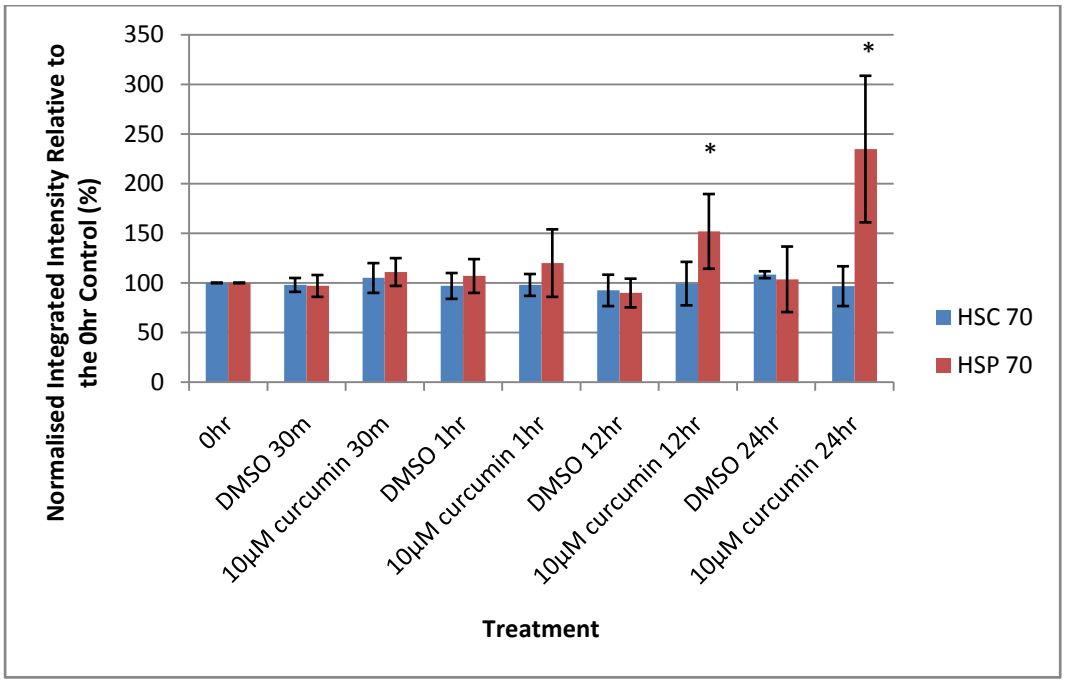


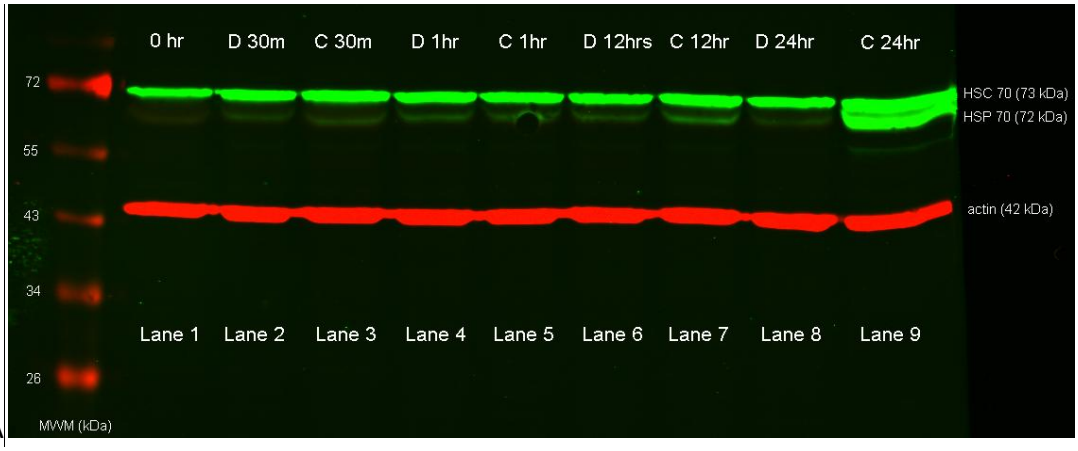
Figure 57 Curcumin Treatment Increases Total HSP70 Protein Levels in the HCT116 p53$^{+/+}$ Line

(A) HCT116 p53$^{+/+}$ cells were treated with media alone (Lane 1, 0hr), DMSO, (Lane 2, Lane 4, Lane 6, Lane 8), or 10μM curcumin (Lane 3, Lane 5, Lane 7, Lane 9) for 30m, 1hr, 12 or 24 hours, and then harvested to prepare lysates for Western blotting analysis. Total levels of HSC70/HSP70 (green) and actin (red) were captured using the Odyssey scanner.

(B) The HSC70/HSP70 levels acquired using the Odyssey imaging system were normalised to the actin loading control. Normalised integrated intensity levels of the HSC70/HSP70 proteins are shown relative to the 0hr control. Bars marked with * indicate there is a significant ($P<0.05$) difference between the means of DMSO control samples and those treated with curcumin for the time point indicated. Error bars show the standard deviation. Data shown are the mean of at least three independent experiments.

Treatment with curcumin resulted in a significant increase in HSP70 total protein levels at both time points in the HCT116 $p53^{+/+}$ line (Figure 56). Normalised HSP70 protein levels in curcumin-treated cells were 152% compared to 90% in the DMSO control following 12 hours treatment and rose to 235%, compared to 104% in the DMSO control after 24 hours. Levels of the HSC70 protein remained relatively constant following treatment with curcumin.

A



B

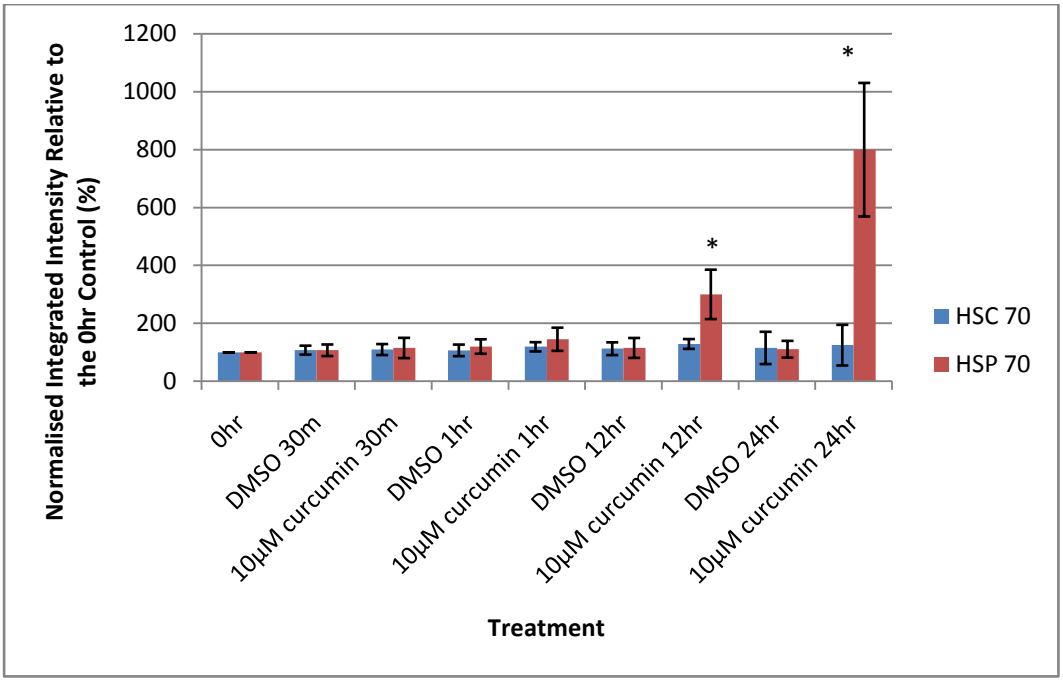


Figure 58 Curcumin Treatment Results in an Increase in Total HSP70 Levels in the HCT116 p53$^{-/-}$ Line

(A) HCT116 p53$^{-/-}$ cells were treated with media alone (Lane 1, 0hr), DMSO, (Lane 2, Lane 4, Lane 6, Lane 8), or 10μM curcumin (Lane 3, Lane 5, Lane 7, Lane 9) for 30m, 1hr, 12 or 24 hours, and then harvested to prepare lysates for Western blotting analysis. Total levels of HSC70/HSP70 (green) and actin (red) were captured using the Odyssey scanner.

(B) The HSC70/HSP70 levels acquired using the Odyssey imaging system were normalised to the actin loading control. Normalised integrated intensity levels of the HSC70/HSP70 proteins are shown relative to the 0hr control. Bars marked with * indicate there is a significant ($P<0.05$) difference between the means of DMSO control samples and those treated with curcumin for the time point indicated. Error bars show the standard deviation. Data shown are the mean of at least three independent experiments.

Treatment with curcumin also resulted in a significant increase in total HSP70 levels in HCT116 $p53^{-/-}$ cells (Figure 57). Following treatment for 12 hours, normalised protein levels of HSP70 rose to 300% compared to 115% found in the DMSO control. After 24 hours treatment with curcumin levels of HSP70 were 800%, compared to 111% in the DMSO control. Levels of HSC70 remained relatively constant throughout.

6.2.3 Curcumin May Increase Total Levels of MICA/MICB

The *MICA/MICB* genes contain HSE in their promoter region similar to the *HSP70* gene. To investigate whether curcumin treatment could result in increased protein levels of MICA/MICB via HSF1 activation and DNA damage dependent signalling, HCT116 cells were treated with curcumin and analysed using Western blotting.

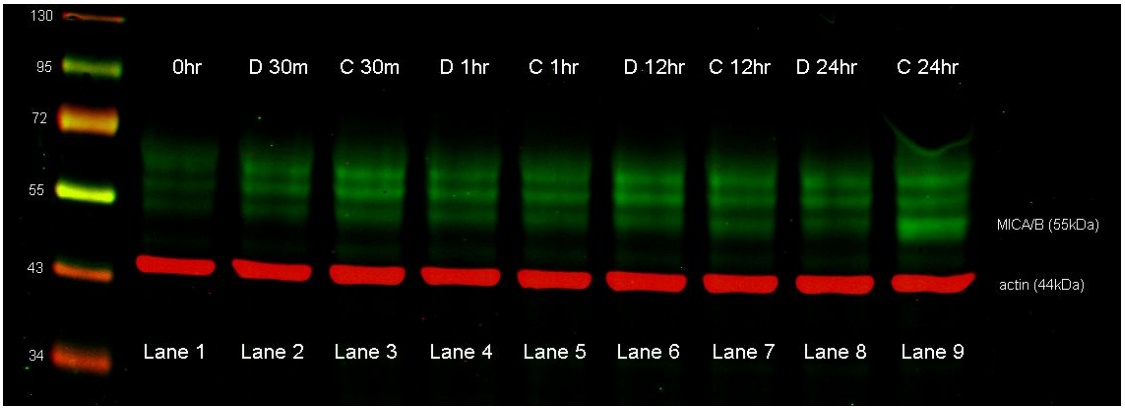


Figure 59 Curcumin Treatment May Upregulate Total Protein Levels of MICA/MICB

HCT116 $p53^{+/+}$ cells were treated with media alone (Lane 1, 0hr), DMSO, (Lane 2, Lane 4, Lane 6, Lane 8), or 10µM curcumin (Lane 3, Lane 5, Lane 7, Lane 9) for 30m, 1hr, 12 or 24 hours, and then harvested to prepare lysates for Western blotting analysis. Total levels of MICA/MICB (green) and actin (red) were captured using the Odyssey scanner. This is a representative image (n=2).

Figure 58 shows that the total protein levels of MICA/MICB may increase following curcumin treatment, most notably after 24 hours treatment. Further experiments would be required to confirm this preliminary data.

6.2.3 Analysis of NKG2DL Surface Expression following Curcumin Treatment

Following curcumin treatment, preliminary analysis of cell surface expression of ULBP1 using primary antibodies coupled to secondary FITC-conjugated antibodies showed high levels of fluorescence crossover into both the FL-1 and FL-2 channels from curcumin. To circumvent crossover of emission spectra in the presence of curcumin in the FL-1 and FL-2 channels, test samples were run using DAPI as a viability marker, and an Alexa 800-conjugated secondary antibody. DMSO was used as a negative control, and isotype controls, secondary only controls, and samples without DAPI staining were included in all experiments.

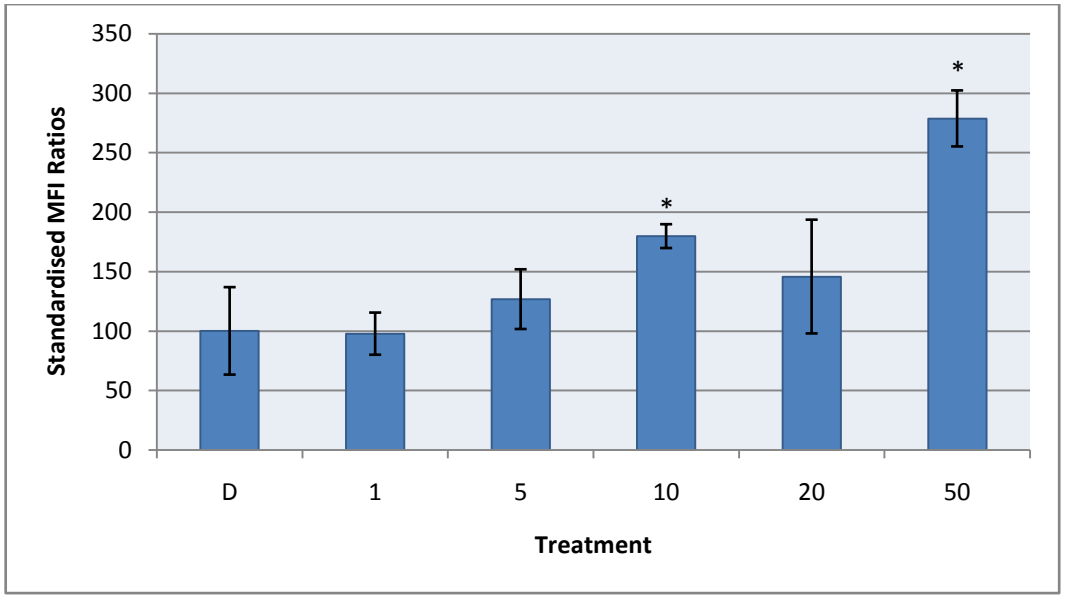


Figure 60 Curcumin Treatment Upregulates Cell Surface Expression of ULBP1

HCT116 $p53^{+/+}$ cells were treated with DMSO or 1-50µM curcumin for 12 hours, harvested and prepared for FACS analysis using anti-ULBP1 primary antibodies and anti-mouse Alexa-conjugated secondary antibodies. DAPI was used to assess cell viability. Data shown are the mean (±SD) of at least three independent experiments. Bars marked with * indicate there is a significant difference from the DMSO control. Gating was used to exclude doublets, debris and dead cells.

Figure 59 shows that treatment with 10µM and 50µM curcumin upregulates the cell surface expression of ULBP1 in the HCT116 $p53^{+/+}$ line. Preliminary experiments using an ULBP1 cell surface stain followed by fixing and permeabilisation and an internal pH3 and DAPI stain indicated that the cells with the highest levels of ULBP1 were in the G_2/M stage of the cell cycle, and stained positive for pH3 (data not shown). Further experiments are required to confirm whether cells undergoing mitotic arrest following curcumin treatment exhibit high levels of ULBP1 on the cell surface.

6.3 Discussion

These data show that curcumin treatment significantly upregulates the total protein levels of HSP70, a chaperone that can contribute to the degradation of damaged proteins during proteotoxic or genotoxic stress. High levels of HSP70 have been found in exosomes released from tumour cells, and it is possible that increased levels of HSP70 are responsible for the immunomodulatory properties of exosomes derived from curcumin-treated breast tumour cells (Mambula and Calderwood, 2006, Zhang et al., 2007a). Curcumin treatment has previously been shown to lead to transactivation of the heat shock factor 1 HSF1 (Chen et al., 2001). Transcription of both the *HSP70* genes and the *MICA/MICB* genes occur via in HSF-1-dependent manner, and this provides further evidence to suggest that treatment with curcumin may result in transcriptional activation of the *MICA/MICB* genes.

Treatment with curcumin increased the cell surface expression of ULBP1 (Figure 59). It is possible that curcumin may regulate ULBP1 transcription via inhibition of the proteasome in a similar manner to bortezomib (Butler et al., 2009, Milacic et al., 2008).

The preliminary results investigating expression of the MICA/MICB proteins suggest curcumin may affect levels of intracellular protein expression, but without statistical analysis, these results are inconclusive. Further method development is required to investigate the effects of curcumin on the cell surface expression of the NKG2DL on HCT116 cells, and factors such as antibody concentrations, antibody incubation times, and fixing reagents could all be optimised in future experiments. Treatments with both an extended time course and a range of concentrations are necessary to find the

peak expression of these cell surface ligands following curcumin treatment. To investigate whether curcumin treatment results in increased transcription of the *MICA/MICB* and *ULBP1* genes, studies using reporter plasmids encoding the promoter regions of the NKG2DL genes could be carried out (Lopez-Soto et al., 2009). These could be confirmed by real time reverse transcription polymerase chain reaction (qRT-PCR) experiments using primers specific for the NKG2DL (Kim et al., 2006, Vales-Gomez, 2008).

Recent studies have shown that levels of MICB may be higher in multiple myeloma cells undergoing G_2/M arrest following treatment with doxorubicin and melphalan, and it is possible that MICA/MICB cell surface expression in curcumin-treated cells may be linked to G_2/M boundary or M phase arrest (Soriani et al., 2009). Preliminary findings suggested that the cell surface levels of ULBP1 were highest in mitotic cells, but further experiments are required to confirm this data.

Increased expression of NKG2DL has been linked to tumour cell lysis and promotion of an anti-tumour immune response (Armeanu et al., 2008, Ebert and Groh, 2008, Groh et al., 1998, Kato et al., 2007, Lopez-Soto et al., 2009). While this avenue of research is still in the preliminary stages, it suggests a novel mechanism by which curcumin may act as a chemopreventive and chemotherapeutic agent in the treatment of CRC.

Chapter 7 General Discussion

7.1 The Effects of Curcumin on G_2/M Progression

7.1.1 Curcumin Treatment Results in Checkpoint Activation

These studies show that physiologically relevant concentrations of curcumin induce significant G_2/M arrest in the MIN HCT116 lines and in the CIN HT-29 and SW480 lines but that the CIN lines were less sensitive to mitotic arrest. These results are in line with other studies showing that CIN lines have a reduced capacity to undergo M phase arrest due to impaired mitotic checkpoint function (Cahill et al., 1998). FACS cell cycle analysis over a range of 12-72 hours, and a mitotic index time course experiment over 12-36 found that the highest levels of G_2/M and M phase arrest were generally found after 12 hours treatment in the HCT116 lines. This provides new evidence that curcumin exerts checkpoint activation at this shorter time point.

7.1.2 Mechanims of Curcumin-Induced Cell Cycle Arrest

Curcumin treatment reduced the total protein levels of the CDC25C protein phosphatase, and increased the activatory phosphorylation of the Chk1 kinase. Pre-treatment with caffeine failed to prevent curcumin mediated CDC25C degradation, however it did inhibit Chk1 phosphorylation. It is possible that the effects of caffeine pre-treatment on components of the DNA damage signalling network were more profound on the effector proteins directly affected by ATM/ATR signalling. Furthermore, at the concentrations used, caffeine treatment may have impaired the activity of the Chk1 and Chk2 kinases, thus inhibiting their ability to target CDC25C for

proteasomal degradation (Sarkaria et al., 1999). Studies using more specific inhibitors of the ATM/ATR and Chk kinases are required to investigate this phenomenon.

While treatment with caffeine abrogated mitotic arrest in all five cell lines, it did not significantly reduce the levels of G_2/M boundary arrest, implying that this may be independent of this particular DNA damage signalling pathway. Curcumin has previously been shown to activate the p38 MAPK, and it is possible that curcumin treatment triggers antephase arrest, independently of G_2/M DNA damage checkpoint signalling (Andreadi et al., 2006, Chen et al., 2010, Damelin and Bestor, 2007, Mikhailov et al., 2005). This checkpoint has been linked to treatment with spindle poisons such as colchicine, colcemid, and nocodazole, and topoisomerase inhibitors such as adriamycin, ICRF-193 and merbarone and it is plausible that curcumin treatment may trigger cell cycle arrest at the G_2/M boundary via this alternative pathway (Mikhailov et al., 2004, Rieder and Cole, 2000).

7.1.3 Curcumin Impairs Mitotic Spindle Formation

Data in this present study showed that curcumin treatment impairs the normal mitotic progression of cells which fail to arrest in late G_2. It has been suggested that there are three requirements that must be met during prometaphase for normal mitosis to occur: the establishment of a bipolar spindle axis, sister chromatid bi-orientation between the spindle poles, and alignment of chromosomes on the spindle equator (Sluder, 2004). Impairment of microtubule dynamics following curcumin treatment reduces the likelihood that these requirements will be met. Curcumin treatment significantly impaired correct mitotic spindle formation in all 5 CRC lines tested, and

inhibited centrosomal separation in the HCT116 $p53^{+/+}$ line. These data support previous studies on the effects of curcumin on the mitotic spindle, the more recent studies investigating spindle abnormalities and the activation of the spindle assembly checkpoint in curcumin-treated cells (Banerjee et al., 2010, Bielak-Zmijewska et al., 2010, Gupta et al., 2006). They also quantify the effects of treatment on centrosomal separation, and the formation of a bipolar mitotic spindle for the first time.

7.1.4 Impairment of Sister Chromatid Disjunction

Data presented here also suggest that curcumin may inhibit the activity of topoisomerase II, which is involved in decatenation of sister chromatids during mitosis, and required for ensuring amphitelic kinetochore attachment to the mitotic spindle (Barthelmes et al., 2000, Coelho et al., 2008, Coelho et al., 2003, Rattner et al., 1996). Curcumin treatment impaired mitotic progression in the HCT116 $p53^{+/+}$ line and resulted in abnormalities in prophase/prometaphase and metaphase in all five CRC lines tested. Analysis of data regarding the number of cells at the respective stages of mitosis in the presence of curcumin showed a decreased number of cells at anaphase and telophase. Image analysis revealed that curcumin treatment resulted in chromatin staining that was similar to that seen in fission yeast cells with a *cut* phenotype, human cells expressing a non-degradable version of securin, or those treated with a topo II poison (Gimenez-Abian and Clarke, 2009, Hagting et al., 2002, Hirano et al., 1986). Further research is required to determine whether sister chromatid disjunction failure following curcumin treatment is a result of inhibition of decatenation, or a

failure to cleave cohesin in a timely fashion as result of securin or separase inhibition (Downes et al., 1991, Hagting et al., 2002, Wang et al., 2010).

7.1.5 Deregulation of the Abscission Checkpoint

The localisation of the CPC member Aurora B was also affected following curcumin treatment, and this may contribute to aberrant mitosis. Recent studies in budding yeast and human cervical cells have highlighted the role of Aurora B in preventing cytokinesis while chromatin is present in the spindle midzone (Mendoza et al., 2009, Norden et al., 2006, Steigemann et al., 2009). The NoCut or abscission checkpoint ensures genomic integrity by monitoring chromosome segregation and prevents damage to unseparated chromosomes during cytokinesis. It is possible that the *cut* phenotype observed in late mitotic curcumin-treated cells, where chromatin is torn by the cleavage apparatus, may be linked to the deregulation of spatiotemporal mitotic events controlled by Aurora B, such as the NoCut checkpoint. Aurora B may also play a role in sister chromatid decatenation during metaphase by targeting topo II, but the exact mechanisms of regulation are yet to be defined (Morrison et al., 2002).

7.1.6 Aberrant Mitosis Following Curcumin Treatment Results in DNA Damage

A previous study has shown that treatment of HCT116 cells with spindle poisons such as nocodazole, paclitaxel and monastrol results in DNA damage as measured by pH2A.X foci. Similarly, curcumin treatment resulted in increased pH2A.X foci in mitotic, but not interphase, cells. This increased pH2A.X staining may be due to

damage generated by spindle forces acting upon non-disjoined chromosomes, permanent topo II-DNA complexes as a result of topo II poisoning, or a combination of both. Deregulation of the abscission checkpoint by curcumin treatment may result in nondisjoined chromosomes becoming trapped in the spindle midzone during cytokinesis leading to further DNA damage.

Treatment with another dietary polyphenol, resveratrol, and resveratrol analogues has been shown to induce mitotic arrest, alter Aurora B levels, activate spindle checkpoint proteins, inhibit topo II activity, and increase levels of pH2A.X foci (Bin Hong et al., 2009, Leone et al., 2010). It is possible that treatment with curcumin and resveratrol may induce DNA damage in mitotic cells through similar mechanisms.

7.1.7 Curcumin Treatment May Upregulate Colorectal Cancer Cell Surface Expression of NKG2DL

Treatment with curcumin significantly increased the total protein levels of HSP70 in both the HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ lines, and this may be a result of induced HSF-1 binding to the HSE in the promoter region of the HSP70 gene (Chen et al., 2001). The promoter region of the *MICA/MICB* genes also contains a HSE, and their transcription is also regulated in a DNA damage signalling-dependent manner involving ATM and Chk1 (Eagle et al., 2006, Gasser et al., 2005). Interestingly, this data suggests that nocodazole may increase the cell surface expression of MICA/MICB. The effect of spindle poisons on the regulation of MICA/MICB cell surface expression has not been studied, but it is possible that the DNA damage induced by mitotic arrest may lead to increased transcription of the *MICA/MICB* genes (Dalton et al. 2007).

The transcription of ULBP1, and cell surface expression of this protein is increased following proteasomal inhibition, and it is plausible that curcumin treatment may result in similar effects (Butler et al., 2009, Milacic et al., 2008). While this present study does not provide conclusive evidence that curcumin modulates NKG2DL cell surface expression, it provides a platform for future studies.

7.2 Future work

7.2.1 Further Characterisation of Curcumin-Mediated Checkpoint Signalling

To test the hypothesis that curcumin treatment results in antephase arrest via a p38-dependent pathway, pre-treatment using an inhibitor of p38 MAPK, prior to curcumin treatment could be carried out (Mikhailov et al., 2004). Investigations into the mechanisms of DNA damage during aberrant mitosis could confirm whether in the HCT116 $p53^{+/+}$ cell line, curcumin increases the levels of pH2A.X foci in a ROS-independent manner. This could be combined with other assays measuring DNA damage such as the comet assay, and assays investigating the generation of reactive oxygen species. ROS generation in cell culture media containing curcumin has been measured previously using the FOX assay (Kelly et al., 2001), ROS convert the colourless solution to orange, which is measured against a standard curve using known concentrations of H_20_2 as a control. The absorbance is generally measured around 560-595nm, and this study measured the absorbance at 560nm. As the peak emission of curcumin is 520nm (exciting wavelength 440nm) (Kunwar et al., 2006, Stockert et al., 1989), it is possible that the natural fluorescence of curcumin may contribute to absorbance levels. To investigate this, this experiment could be repeated

using curcumin only controls, without the addition of reagents containing ferrous ions or xylene orange dye. Furthermore fluorophotometric quantitation of oxidative stress following curcumin treatment using the dye 2',7'-dichlorofluorescein (DCFH-DA) may also be challenging as the emission peak of this dye is 520nm (excitation 490nm). Curcumin treatment significantly increased the levels of oxidative stress as measured by DCFH-DA in the lung cancer A-549 cell line (Lin et al., 2008). The natural fluorescence of curcumin may have affected these measurements. Far red dyes such naphthofluorescein, diaminocyanins, and MitoSOX have been used to investigate the general levels of ROS production, nitric oxide production, and to specifically monitor superoxide generation in mitochondria repectively (Mukhopadhyay et al., 2007, Sasaki et al., 2005, Xu et al., 2005). Using a far red dye to investigate ROS generation following curcumin treatment would prevent any crossover in emission spectra.

Knock-down of checkpoint signalling proteins ATM and Chk1 via siRNA abrogated curcumin-mediated cell cycle arrest in the BxP3 pancreatic cancer cell line (Sahu et al., 2009). Similar inhibition of these proteins could confirm they are involved in cell cycle arrest in colorectal cancer cell lines used here. Once knock down had been achieved, measurement of total DNA content and pH3 levels could be performed using FACS analysis. Furthermore, measurement of pH2A.X staining in knock-down cells would also indicate whether ATM contributes to phosphorylation of histone H2A.X at serine-139 following curcumin treatment. The role of Chk1 and ATM in the regulation of the SAC after treatment could also be investigated by determining whether proteins such as BubR1 and Mad2 correctly localise to kinetochores in cells lacking Chk1 or ATM. However, as Chk1 has been shown to contribute to the activity of the Aurora B kinase

and to SAC function, knock-down of these proteins alone may result in impaired SAC function (Zachos et al., 2007). Further experiments investigating the effects of curcumin on the mitotic spindle such as the microtubule regrowth assay, or kinesin ATP assays could be carried out in the presence of curcumin, in addition to investigations on the effects of curcumin on centrosomal regulators such as Aurora B, Nek2 and Plk1. This would help elucidate the mechanisms of spindle assembly checkpoint activation in curcumin treated cells.

7.2.2 Confirming that Curcumin Acts as a Topoisomerase Poison at Physiologically Relevant Concentrations

It would be important to analyse whether curcumin could induce the formation of topo II-DNA complexes in these cell lines at physiologically relevant concentrations, as previously such studies have generally used unrealistically high levels of this agent (Lopez-Lazaro et al., 2007b, Martin-Cordero et al., 2003, Roth et al., 1998, Snyder and Arnone, 2002). This could be carried out *in vitro* by measuring the cleavage of plasmid DNA, such as pRYG (Burden et al., 2001). Increased cleavage would indicate the formation of strand breaks following topo II-DNA complex formation as a result of topo II poisoning. To confirm whether caffeine inhibits the ability of curcumin to induce topo II-DNA complexes, thus reducing the levels of DNA damage resulting from curcumin treatment, samples containing a combination of caffeine and curcumin could also be tested. The ability of curcumin to act as a catalytic topo II inhibitor at these concentrations could also be tested by performing a series of decatenation reactions.

If curcumin were shown to act as a topo II inhibitor at these concentrations it would be interesting to investigate whether this had a link to Aurora B kinase activity in curcumin-treated cells, and this could be carried out using a kinase assay.

Further research investigating whether curcumin has the potential to induce nonlethal topo II-DNA strand breaks, or chromosomal instability as a result of the breakage of nondecatenated chromosomes during mitosis is required using physiologically relevant concentrations of curcumin.

7.2.3 Research on the Effects of Curcumin on NKG2DL Expression in CRC

While preliminary data suggest that curcumin treatment may upregulate the cell surface expression of NKG2D ligands such as MICA/MICB and ULBP1 in colorectal cancer cells further studies are required. Analysis of the effects of curcumin on NKG2DL at the transcriptional level could be carried out using qRT-PCR and transfection of reporter plasmids containing the promoter regions of the respective genes (Kim et al., 2006, Lopez-Soto et al., 2009, Vales-Gomez, 2008). The level of cell surface expression could be further quantified using flow cytometry, and more advanced staining techniques could be used in combination, to investigate links with the cell cycle and DNA damage. If the results from these studies were promising, experiments investigating NK cell-mediated lysis of curcumin-treated CRC cells could be carried out to determine the impact of treatment on the cytolytic activity of NK cells.

7.3 Clinical Implications

7.3.1 Curcumin as an Anti-Mitotic Agent

In various CRC lines, curcumin treatment contributes to a failure to meet necessary requirements in the early stages of mitosis, such as centrosomal separation, formation of a bipolar mitotic spindle, and sister chromatid disjunction, and may also impair events in the later stages of mitosis such as cytokinesis. These results were found using concentrations that were shown to have a significant effect on microtubule assembly in previous studies, and at physiologically relevant concentrations (Gupta et al., 2006, Sharma et al., 2005). These new data support the use of curcumin as an anti-mitotic agent in the treatment of CRC.

7.3.2 Targeting MSI^+ CRC with Combined Curcumin Chemotherapy

Previous research has indicated that CRC cells with MMR deficiency have an enhanced sensitivity to topoisomerase poisons (Fallik et al., 2003, Jacob et al., 2001). This may account for enhanced sensitivity to curcumin treatment as shown by increased levels of apoptosis in MMR deficient cells compared to MMR proficient cells in a recent study (Jiang et al., 2010). Research confirming the ability of curcumin to act as a topoisomerase poison, could validate the use of this agent in MSI^+ CRC chemotherapy. While cell death has not been investigated in this present study, it is likely that the aberrant mitosis found in curcumin-treated CRC cells would generally result in apoptosis or an apoptosis-independent form of cell death such as mitotic catastrophe under these conditions.

7.3.3 The Potential 'Dark Side' of Curcumin

A recent paper has advised caution regarding the mutagenic properties of curcumin and while most evidence shows that this agent exerts protective effects, the long term effects of higher doses of this agent, such as those used in clinical trials, are still unknown (Burgos-Moron et al., 2010). Research in this present study has highlighted the potential for curcumin to induce chromosomal aberrations as a result of decatenation failure and spindle abnormalities during mitosis. There are concerns regarding the incidence of secondary malignancies following treatment with topoisomerase inhibitors, as they have the potential to generate chromosomal translocations (Kudo et al., 1998, Pedersenbjergaard et al., 1993, Roulston et al., 1995). Research using genistein, resveratrol and curcumin has found that treatment with these compounds can lead to an increase in chromosomal aberrations in various normal and transformed mammalian cell lines (Giri et al., 1990, Leone et al., Lopez-Lazaro et al., 2007a). Genistein may increase the incidence of infant leukemia under certain conditions as a result of topo II poisoning (Strick et al., 2000). It is possible that some curcumin-treated cells may replicate with nonlethal levels of genomic damage acquired following aberrant mitosis. Further studies clarifying the source of mitotic DNA damage using physiologically relevant concentrations of curcumin in both normal and cancer cell lines are required to alleviate any concerns regarding the safety of this agent.

7.3.4 Curcumin Treatment Increases Danger Signals Sent from Colorectal Cancer Cells

While studies of the effects of curcumin on NKG2DL colorectal cancer cell surface expression are preliminary, it seems likely that the cells that manage to divide with chromosomal aberrations following treatment would be the most susceptible to immune cell-mediated lysis, due to increased cell surface expression of these damage associated-ligands (Gasser et al., 2005, Unni, 2008). Furthermore, curcumin treatment has been shown to induce apoptosis-independent cell death, such as mitotic catastrophe and necrosis, and this would contribute to danger signals such as HSP70 in the tissue microenvironment surrounding CRC cells, and an enhancement of the anti-tumour response (Dempe et al., 2008, Gallucci and Matzinger, 2001, Howells et al., 2007, Jiang et al., 2010, Magalska et al., 2006, Matzinger, 2002, Matzinger, 1998, O'Sullivan-Coyne et al., 2009, Wolanin, 2006).

Many conventional CRC chemotherapeutic agents activate anti-cancer immunity via the DNA damage or proteotoxic stress response, but impair systemic immune system function due to high toxicity. If the findings for curcumin were consolidated, it could be beneficial to combine it with these chemotherapeutic agents, to boost local anti-cancer immunity without further compromising immune system function.

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