INVESTIGATING THE ROLE OF ERK IN MICROTUBULE-INTERFERING AGENT-INDUCED CELL SURVIVAL AND DEATH

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PRITI GOPAR

Department of Biochemistry

University of Leicester

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Declaration

This thesis submitted for Masters of Philosophy entitled "Investigating the role of ERK in microtubule-interfering agent-induced cell survival and death" is based on the work conducted by the author in the Department of Biochemistry, University of Leicester, from January 2006 to July 2007. All of the work reported in this thesis is original unless otherwise stated. No part of this work has been submitted for another degree in this or any other institution.

Signed: Miss Priti Gopar

Date: June 2008

Abstract

Activation of the mitotic checkpoint by microtubule-interfering drugs (MIAs) such as Taxol causes mammalian cells to undergo apoptosis. Previous studies have shown that treatment with such drugs activate the extra-cellular regulated kinase (ERK) pathway, causing the cells to arrest in mitosis and then undergo apoptosis. ERK1/2 activation in response to MIAs has been implicated as having a role in mediating MIA-induced cell survival in certain cancer cell types such as lung cancers.

Using Hela cells, the results in this project confirm the activation of ERK1/2 upon introduction of MIAs such as Nocodazole, Taxol, Vinblastine and Vincristine. GFP-Erk1 expression showed a rapid cytoplasmic to nuclear translocation of ERK1/2 in response to MIAs, further suggesting a direct role for ERK1/2 in cell survival. Quantifying cell death in response to MIAs with or without the MEK/ERK inhibitor U0126 showed that ERK1/2 inhibition may increase cell death in comparison to MIA treatment alone. Subsequent data has shown, for the first time, that upon ERK1/2 inhibition by U0126, cell death occurs via the intrinsic apoptotic pathway, as seen by caspase-9 and caspase-3 cleavage after 12 hours of treatment with Taxol plus U0126. Furthermore, inhibiting ERK1/2 activation in Taxol-induced mitotic cells using the U0126 compound increases cell death suggesting that cells need to reach mitosis before the onset of apoptosis.

Preliminary data in this report also supports a role for active ERK1/2 in mediating cell survival via phosphorylation of the pro-apoptotic protein Bim and possibly caspase-9. Collectively, the results shown in this report provides potential new targets in the development of anti-cancer therapies.

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CONTENTS

Figures and Tables

Abbreviations

(A)	Attached (non-mitotic)
AP-1	Activating protein-1
ASK1	Apoptosis signal-regulating kinase 1
ATF-2	Activating transcription factor-2
Bim	BCL-2 –interacting mediator of cell death
BSA	Bovine serum albumin
CENP-E	Centromere-binding protein E
CR	Conserved regions
DD	Death domain
DED	Death effector domain
DKO	Double knockout
DMEM	Dulbecco's modified eagles medium
DNA	Deoxyribonucleic acid
DPC4	Deleted in pancreatic carcinoma-4
Cdk	Cyclin-dependent protein kinase
c-src	Cellular-src
ECL	Enhanced Chemiluminescence
EGF	Epidermal growth factor
Elk-1	Ets-like gene 1
ERK	Extracellular signal-regulated kinase
Ets1	v-ets erythroblastosis virus E26 oncogene homolog 1
FADD	Fas-associating protein with death domain
FBS	Foetal bovine serum
G_0	G Zero
$G_{1/2}$ phases	Gap phases 1 or 2
GAP	GTPase-activating protein
GCK	Germinal centre kinase
GDP	Guanine diphosphate
GEF	Guanine nucleotide exchange factor

GFP	Green fluorescent protein
GLK	GCK-like kinase
Gly	Glycine
GPCR	G-protein–coupled receptor
Grb2	Growth factor receptor bound protein 2
GTP	Guanaine triphosphate
Hela	Human cervical carcinoma cells
HPK1	Haematopoietic progenitor kinase 1
HSP	Heat-shock protein
JAK-STAT	Janus kinase-signal transducers and activators of transcription
JNK	c-Jun N-terminal kinase
LB	Lysogeny borth
(M)	Mitotic
MAP	Microtubule-associated proteins
MAPK	Mitogen-activated protein kinase
MIA	Microtubule-interfering agent
MKK	MAPK kinase
MKKK	MAPK kinase kinase
MKKKK	MAPK kinase kinase kinase
MKP3	MAPK phosphatase 3
MOS	Moloney sarcoma oncogene
MPF	Mitosis promoting factor
M-phase	Mitotic phase
MST	Mammalian STE20-like kinase
MTOC	Microtubule organising centre
MUK	MAPK upstream kinase
NFAT4	Nuclear factor of activated T-cells
NGF	Nerve growth factor
NIK	Nck-interacting kinase
NK	Natural killer
PAK	p21-activated kinase

PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PKA	c-AMP-dependent protein kinase
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PMA	Phorbol-myristate acetate
RIPA	Radioimmunoprecipitation
SAPK	Stress-activated protein kinase
SDS	Sodium dodecyl sulphate
SDS PAGE	Sodium dodecyl sulphate-polyacrylanide gel electrophoresis
SH	Src homology
SOC	Super optimized culture
SOS	Son of Sevenless
S-phase	DNA synthesis phase
SPRK	Src-homology-3 domain-contaning praline-rich kinase
TAK1	Transforming growth factor-β-activated kinase 1
TEMED	*N*,*N*,*N'*,*N'*-tetramethyl-ethane-1,2-diamine
tBid	Truncated Bid
TGF-β	Transforming growth factor β
Thr	Threonine
TNF	Tumour necrosis factor
TPl2	Tumour progression locus 2
Tyr	Tyrosine

CHAPTER 1
INTRODUCTION

1.1 The Cell Cycle

Cells in unicellular or multicellular organisms require a mechanism to reproduce by duplicating their contents and dividing into two. The cell division cycle is the fundamental mechanism by which all living things reproduce.

The fine details of the cell cycle may vary between organisms but certain requirements are universal. Firstly, to produce a pair of genetically identical daughter cells, the DNA must be faithfully replicated and the replicated chromosomes segregated into two separate cells. To achieve this, a complex set of cytoplasmic and nuclear processes need to be coordinated during the cell cycle. The complexity of the cell cycle became apparent when the cell cycle control system was identified (Matthews *et al.,* 1999).

1.1.1. Phases of the Cell Cycle

The cell cycle is divided into several distinct stages (Figure 1.1); **Interphase** – This is where the cell spends a significant amount of its time preparing to divide. During **S-phase**, the replication of DNA occurs, and the two gap phases; **G_1** and **G_2 phases** allow the cells time to grow and double their mass before dividing. During G_1, the cell monitors its environment and its size. The cell then makes the decision to commit to the DNA replication step, pause or exit the cell cycle (Johnson & Walker, 1999). G_2 is the second gap phase required for further cell growth and ensures that DNA replication is complete before entering M-phase (Alberts *et al.,* 1994). **M phase** is the mitotic phase and is further divided into five stages: **(i) Prophase -** where the chromatin condenses into chromosomes, the nuclear envelope breaks down and spindle forms and migrates to the opposite poles of the cell; **(ii) Prometaphase and metaphase** – where the chromosomes attach to the spindle and eventually align along the

centre of the cell; **(iii) Anaphase** – where the chromosomes separate to opposite ends of the cell; **(iv) Telophase –** where the nuclear envelope reforms and chromatin decondenses and **(v) Cytokinesis** - the formation of the actomyosin contractile ring and separation of the two identical daughter cells (Pines & Rieder, 2001). Cells can also leave the cell cycle by entering a specialised resting state, **G Zero (G_0)**. Cells can remain here for days and even up to years before resuming proliferation (Alberts *et al.*, 1994).

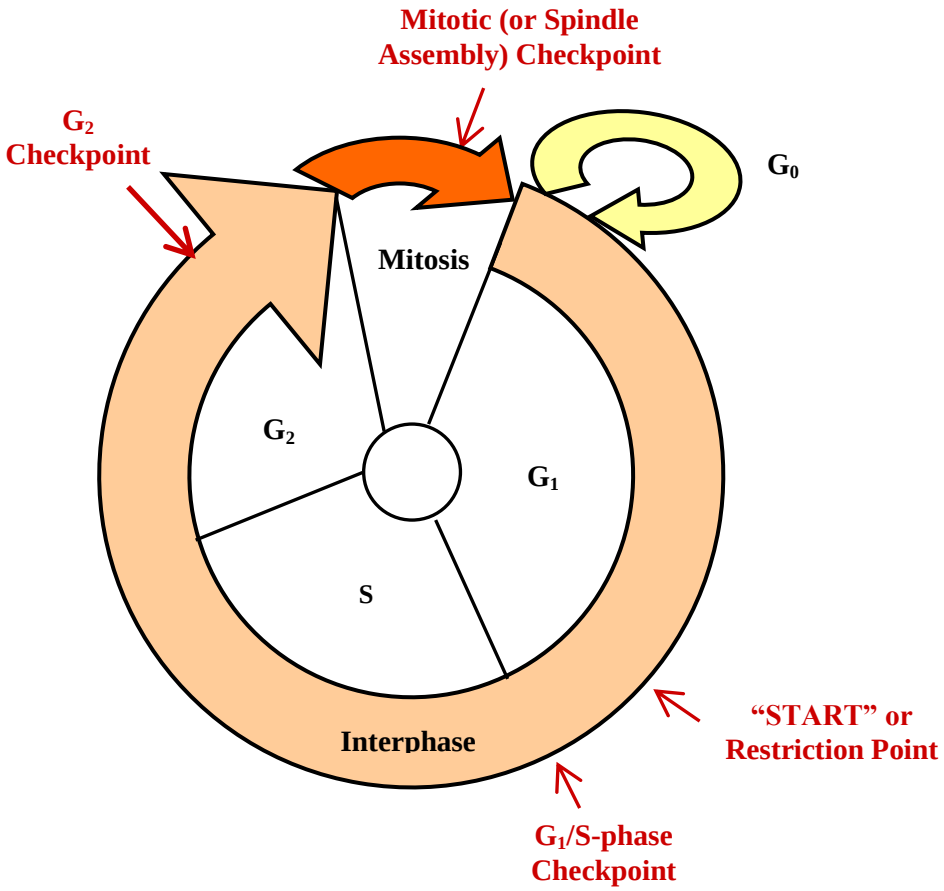


Figure 1.1: A summary of the phases of the cell cycle. The existence of specific checkpoints ensures the completion of the cycle in the correct order and without errors. The positions of the checkpoints are indicated by the arrows.

1.1.2. Cell Cycle Control in Eukaryotes

Eukaryotic cells have evolved a system of control to ensure that the cell cycle occurs in the correct sequence. The control system is made up of interacting proteins such as the cyclin-dependent protein kinases (Cdk's) and cyclins. Both Cdk's and cyclins act at critical points of the cycle using feedback signals from the processes that are being performed. Without such feedback, a delay or interruption in any of the processes within the cell division cycle can cause disaster such as uncontrolled proliferation (Alberts *et al.,* 1994). The regulation of Cdk's and cyclins is provided by specific checkpoints within the cell cycle (Figure 1.1) which assess the progress or completion of key events. Checkpoints provide a mechanism to monitor the cell's progress through the cell division cycle to ensure that one phase of the cell cycle has been executed correctly before allowing the cell to proceed to the next phase (Pines & Rieder, 2001). The cell cycle control system can also be regulated by signals from its environment and they generally act at three major checkpoints in the cell cycle (Figure 1.1); at late G_1 called "START" or the restriction point (Arellano & Moreno, 1997), just before entry into S-phase (G_1/S-phase transition), at G_2, before mitotic entry (Alberts *et al.,* 1994) and during mitosis, the mitotic (or spindle assembly) checkpoint (Rudner & Murray, 1996).

The Cdk's induce downstream processes by phosphorylating selected proteins on serine and threonine residues. The cyclins bind to Cdk molecules and control their ability to phosphorylate target proteins (Arellano & Moreno, 1997). It is the assembly and disassembly of the Cdk-cyclin complexes that drives the cell cycle (Figure 1.2).

There are two main classes of cyclins; mitotic cyclins**,** which bind to Cdk molecules during G_2 and are required for entry into mitosis, and G_1 cyclins**,** which bind to Cdk molecules during G_1 and are required for entry into S-phase (Alberts *et al.,* 1994). Yeast cells have played an important part in understanding the mechanism of the cell cycle. In yeast cells, the same

member of the Cdk family is required to regulate S-phase and mitosis (Lew & Reed, 1993), whereas in mammalian cells, different Cdk proteins are needed to regulate S-phase and mitosis (Johnson & Walker, 1999). Like yeast, mammalian cells also recruit specific cyclins to form complexes with Cdk's to complete discrete stages of the cell cycle (Johnson & Walker, 1999). For example, the Cdk1/Cyclin B complex (also called the Mitosis-Promoting Factor (MPF)) is required to initiate mitosis (Pines & Rieder, 2001). The Cdk2/cyclin E complex allows passage past the G_1 checkpoint (Johnson & Walker, 1999) and Cdk2/cyclin A is required subsequently to activate the DNA replication machinery (Matthews *et al.,* 1999).

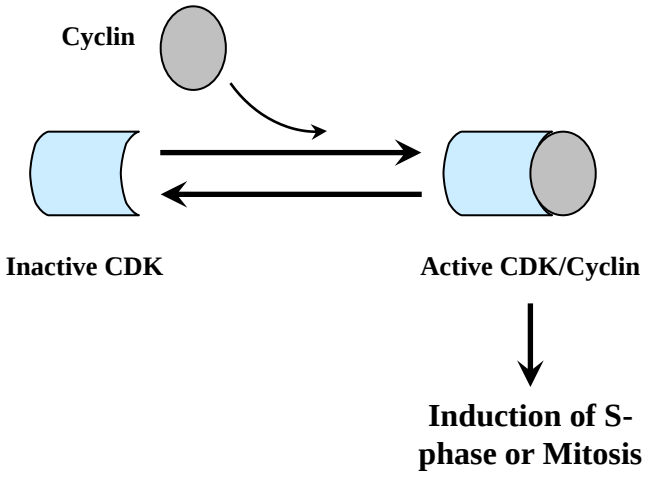


Figure 1.2: The cell cycle control system in eukaryotic cells involves CDK and cyclins. The assembly and disassembly of the cyclin-Cdk complex drives the cycle in one direction by allowing cells entry into each phase of the cell cycle.

1.2 Mitogen-Activated Protein Kinase (MAPK) Pathways

Growth factors play a significant role in regulating cell proliferation (Pardee, 1989). The behaviour of a cell is dependent on its environment and any change will be recognised by specific cell surface receptors which in turn activate intracellular signalling pathways to mount an appropriate response (Seger and Krebs, 1995).

There are many signalling pathways involved in producing an output in the cell. Examples include the Transforming growth factor β (TGF-β) signalling pathways (Miyazawa *et al.,* 2002), the Janus kinase-signal transducers and activators of transcription (JAK-STAT) signalling pathways (Aaronson & Horvath, 2002), the natural killer (NK) signalling pathways (Vivier *et al.,* 2004) and the apoptotic cell signalling pathways (Reed, 2000). In this project, however, I will only focus specifically on the Mitogen-Activated Protein Kinase (MAPK) family of pathways, which are important for the regulation of the cell cycle and as a target for chemotherapeutic drugs (Flaherty, 2006).

1.2.1 MAPK Activation Modules

A diverse array of stimuli activates MAPK pathways including growth factors, cytokines, irradiation, osmolarity and stress (Widman *et al.,* 1999). Figure 1.3 shows the basic assembly of the MAPK pathways that are conserved from yeast to humans.

In response to an external stimulus, the first kinase to be activated in this three-component module is the MAPK kinase kinase (MKKK) (Fanger *et al.,* 1997). Some MKKK's are activated by phosphorylation via an upstream MAPK kinase kinase kinase (MKKKK) or by interaction with a small GTP-binding protein of the Ras or Rho family (Widman *et al.,* 1999). MKKK's are serine/threonine protein kinases that phosphorylate and activate the next kinase in the module, a MAPK kinase (MKK). These kinases recognise a Threonine-X-Tyrosine (Thr-X-

Tyr) motif in the activation loop of MAPKs (Gartner *et al.*, 1992), which they phosphorylate, defining MKK's as dual-specificity kinases. MAPK, the final kinase in the module, phosphorylates substrates on their serine and threonine residues (Widman *et al*, 1999). The majority of defined substrates for MAPK are transcription factors (Yoon and Seger., 2006), but MAPK can also activate cytoplasmic substrates such as other protein kinases, phospholipases and cytoskeleton-associated proteins (Yoon and Seger., 2006).

MAPK's control fundamental cellular processes such as proliferation, differentiation, survival and apoptosis (Peyssonnaux and Eychene., 2001) through regulation of transcription, metabolism and cytoskeletal rearrangements.

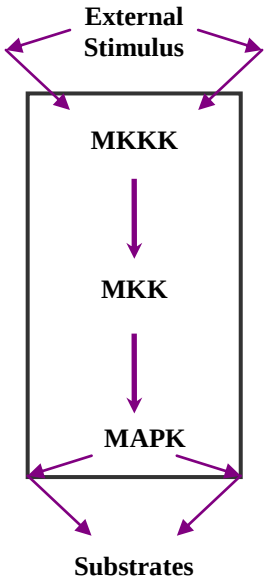


Figure 1.3: Basic MAPK module with the three kinases. Each is activated by its previous kinase or in the case of MKKK, upstream activators. The substrates are cytoplasmic and nuclear. Adapted from Widman *et al* (1999).

The existence of three tiers in this pathway is most likely essential for amplification and tight regulation of the transmitted signals. It also allows the pathway to target various substrates using different combinations of each component. MKKs recognise MAPKs by their tertiary structure, restricting the regulation of MAPK subtypes. In contrast, MKKKs are able to

recognise different MKK-MAPK combinations, extending the variety of target substrates even further (Widman *et al.*, 1999).

There are more known MKKKs than there are MAPKs in mammalian cells. There are even fewer MKKs as part of the module. The large number of MKKKs allows various stimuli to activate specific MAPK pathways.

To date, 14 MKKKs, 7 MKKs and 12 MAPKs have been identified in mammalian cells (Widman *et al.*, 1999) and each kinase belong to different subfamilies. Of the MKKKs, the Raf subfamily is the best characterised and encompasses B-RAF, A-RAF, and RAF1 (also known as C-RAF). The remaining subfamilies of the MKKKs are the MEK kinase subfamily, apoptosis signal-regulating kinase 1 (ASK1), tumour progression locus 2 (TPl2) and finally the more diverse subfamily comprising mammalian STE20-like kinase (MST), Src-homology-3 domain-containing proline-rich kinase (SPRK), MAPK upstream kinase (MUK), transforming growth factor-β-activated kinase 1 (TAK1) and Moloney sarcoma oncogene (MOS). In the MKK subfamily, MEK1 and MEK2 are closely related as are MKK3 and MKK6. The MAPKs are grouped into three main subfamilies; the extracellular signal-regulated kinases 1/2 (ERK1/2), p38 and c-jun NH2-terminal kinase (JNK) subfamilies, and correspond to the MAPK pathway that employs them (Figure 1.4). The first MAPK isoforms to be identified in mammals were ERK1 and ERK2 (formally known as p44 and p42 respectively – Seger and Krebs, 1995). A lesser characterised MAPK pathway, the ERK5 pathway (also known as BMK1, big mitogen-activated protein kinase 1) has also been found (Zhou *et al*., 1995) and an increasing amount of data for this pathway suggests that it plays a vital role in cell proliferation and survival (Girio *et* al., 2007, Kamakura *et al*., 1999, Nishimoto & Nishida, 2006, Raviv *et al*., 2004)

	JNK Pathway	p38 Pathway	ERK Pathway
MKKK:	MEKK1-4, TAK1, MUK, Tpl-2, SPRK, ASK1, MST	TAK1, ASK1, SPRK, PAK	Raf1, A-Raf, B-Raf, Mos, MEKK1, MEKK2, MEKK3, Tpl-2
MKK:	MKK4, MKK7	MKK3, MKK6	MEK1, MEK2
MAPK:	JNK1-3	p38α, p38β, p38γ, p38δ	ERK1, ERK2
	Substrates	Substrates	Substrates

Figure 1.4: Components of the JNK, p38 and ERK Pathways. The corresponding MKKs, MKKs, and MAPKs that are required for each cascade are shown in their respective columns. It is clear that a MKKK used in one pathway can also be used to activate another, e.g. MEKK1-2, Tpl-2, SPRK and ASK1. The information in this table has been taken from Widman *et al* (1999).

1.2.2 The JNK1, JNK2 and JNK3 MAPK Pathway

The c-Jun N-terminal kinase (JNK), also known as the stress-activated protein kinase pathway (SAPK, the rat homolog, Kyriakis *et al.*, 1994), is one of the several MAPK pathways identified in vertebrate cells (Johnson and Lapadat, 2002). Although this project will not be focussing on the JNK pathway or the p38 MAPK pathway, for completeness, a brief account of each is provided.

The JNK pathway is stimulated by a range of cellular stresses such as ultraviolet and ionising radiation, metabolic inhibitors, inflammatory cytokines and is involved in morphogenesis, inflammation, proliferation and apoptosis (Vlahopoulos & Zoumpourlis, 2004). In *Drosophila*

and other organisms, the JNK pathway is also required for embryonic development (Widman *et al.,* 1999).

The JNK MAPK's were discovered based on their activation in response to the inhibition of protein synthesis (Kyriakis *et al.,* 1994). They were then found to bind and phosphorylate the DNA-binding protein c-Jun and increase its transcriptional activity. c-Jun is a component of the activating protein-1 (AP-1) transcription complex, along with Fos (Derijard *et al*., 1994).

The components of the JNK pathway are listed in Figure 1.4. JNK1 and JNK2 are expressed ubiquitously, whereas the expression of JNK3 is limited to the brain (Yang *et al.*, 1997). JNKs can be activated through cell surface receptor families including the tumour necrosis factor (TNF) receptor family, G-protein–coupled receptors (GPCRs), tyrosine kinase receptors and cytokine receptors. JNK1/2 are activated by phosphorylation on threonine and tyrosine residues in the Thr-X-Tyr activation motif by one of the two dual specificity kinases; MKK4 and MKK7 (Sanchez *et al.,* 1994). These kinases are in turn activated by upstream MKKKs as shown in Figure 1.4.

Other kinases are also capable of activating JNKs when they are overexpressed in cells. Examples include p21-activated kinase (PAK), germinal centre kinase (GCK), Nck-interacting kinase (NIK), haematopoietic progenitor kinase 1 (HPK1), and GCK-like kinase (GLK). They regulate JNK activation by phosphorylating and activating specific MKKKs (Widman *et al.*, 1999).

The substrates of the JNK pathway are mostly transcription factors (Vlahopoulos & Zoumpourlis, 2004), in contrast to the ERK pathway that also has many cytoplasmic substrates. The known substrates for JNK include c-Jun, activating transcription factor-2 (ATF-2, Gupta *et al.,* 1995), Ets-like gene 1 (Elk-1, Zinck *et al.,* 1995), p53 (Hu *et al.,* 1997), deleted in

pancreatic carcinoma-4 (DPC4, Afti *et.,* 1997), nuclear factor of activated T-cells (NFAT4, Chow *et al.,* 1997) and ribonucleoprotein K (Habelhah *et al.,* 2001).

The MAPK cascades are activated when cells are under stress. The JNK pathway induces apoptosis in response to ultraviolet (Tournier *et al.*, 2000) and γ radiation (Chen *et al,* 1996), ceramide (Verheij *et al.,* 1996) and cytokines (Ichijo *et al.,* 1997). In neuronal PC-12 pheochromocytoma cells, removal of nerve growth factor induces the JNK and p38 pathways, but inhibits the ERK pathway, resulting in apoptosis. This demonstrated the importance in the balance between growth factor-activated ERK and stress-activated JNK/p38 pathways in determining whether a cell survives or undergoes apoptosis (Xia *et al.,* 1995).

1.2.3 The p38 MAPK Pathway

The p38 MAPK pathway is also involved in response to stress in eukaryotic and yeast cells and is activated by a diverse array of external stimuli including ultraviolet light, irradiation, heat shock, high osmotic stress, proinflammatory cytokines and certain mitogens (Widman *et al.*, 1999). It appears to respond to many stimuli that are responsible for activating the JNK pathway. Hence, not only does this prove that these two pathways are part of the same family of cascades; it also suggests a possible overlap between components (Figure 1.4) and the target substrates. p38 exists in different isoforms (α, β, γ, δ) where p38α has a 50% amino acid identity with ERK2 (Roux and Blenis, 2004).

Upon activation of the MKKKs in the p38 pathway, MKK3 and MKK6 are activated. They both show a high degree of specificity for p38 as they do not activate JNK or ERK1/2. MKK6 activates all p38 isoforms, whereas MKK3 is more selective by phosphorylating the α and β isoforms only (Enslen *et al.,* 2000). The p38 MAPK is sequentially activated by phosphorylation on a Thr-Gly-Tyr motif within its activation loop (Wilson *et al.,* 1996). Once

activated, it plays a major role in processes such as apoptosis, cytokine production, transcriptional regulation, and cytoskeletal reorganisation (Ono & Han, 2000). p38 has been implicated in HIV production and increased levels of p38 can be seen in Alzheimer's disease (Obata *et al.,* 2000).

p38 activity is critical for normal immune and inflammatory responses. It is activated in macrophages, neutrophils and T-cells (Ono and Han., 2000) and helps carry out their respective roles in mediating respiratory burst activity, chemotaxis, granular exocytosis and apoptosis (Ono and Han., 2000). In addition, the p38 pathway is also involved in apoptosis as briefly described in section 1.2.2 (page 11). In Rat-1 fibroblasts the outcome is very similar. In cells deprived of serum, blocking the activity of the p38 pathway using the inhibitor PD169316 (a pyridinyl imidazole compound) or administering insulin, decreases p38 activity and reduces the levels of apoptosis (Kummer *et al.,* 1997). Similarly in Hela cells, inhibiting p38 using p38 MAPK inhibitors SB203580 or SB202190 in Taxol-treated cells was found to suppress cell death (Deacon *et al.,* 2003), further supporting a role for p38 in apoptosis.

1.2.4 The ERK1/2 MAPK Pathway

The ERK1/2 pathway (Figure 1.5) is the first characterised and most studied of the MAPK pathways. Its components (Figure 1.4) indicate that this pathway has the most exclusive set of MKKKs to initiate the cascade, in comparison to the JNK and p38 pathways.

The two isoforms of ERK; ERK1 and ERK2, share an 81% amino acid identity and are expressed to various extents in all tissues (Chen *et al.,* 2001). They are activated by growth factors, phorbol esters, cytokines, microtubule disorganisation, and to a lesser extent, by ligands of the heterotrimeric GPCRs (Sugden and Clerk., 1997).

ERK1 and ERK2 are 44kDa and 42kDa proteins respectively (Widman *et al.*, 1999) and their activation leads to the subsequent activation of several transcription factors and other serine/threonine kinases contributing to cellular proliferation, differentiation, cell cycle regulation and cell survival (Johnson and Lapadat, 2002).

1.2.4i Ras and Raf Proteins

The first kinases to be activated are the MKKKs (Table 1.1) by the Ras oncoprotein. Ras is a membrane-anchored GTPase that acts as a biological switch relaying signals from ligand-stimulated receptors to the cytoplasmic ERK1/2 cascade (Peyssonnaux and Eychene., 2001). This was inferred from the observation that Ras activates Raf1 and that activated Raf1 is sufficient to stimulate the ERK1/2 pathway (Marais and Marshall., 1996). The RAS gene family comprises three functional genes, H-RAS, N-RAS and K-RAS, which encode 21kDa Ras proteins (Reuter *et al.*, 2000).

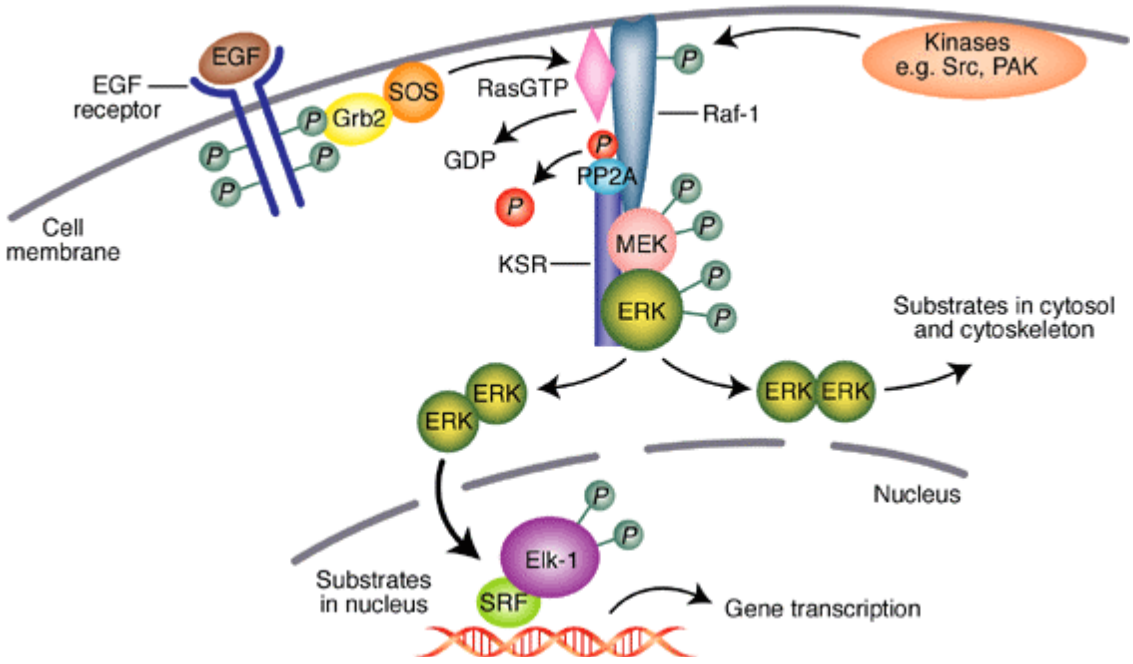


Figure 1.5: The organization and location of components making up the ERK1/2 pathway. The binding of a ligand to a cell surface receptor initiates a complex cascade through the cell into the nucleus. See text for further explanation of the cascade. Image taken from Kolch *et al* (2002).

Ras cycles between an active and an inactive conformation; Ras-GTP and Ras-GDP respectively. Regulatory proteins that control the GTP/GDP cycling of Ras include GTPase-activating proteins (GAPs), which work to hydrolyse GTP to GDP, and guanine nucleotide exchange factors (GEFs) such as Son of Sevenless (SOS) which induce the dissociation of GDP to allow GTP association (Reuter *et al.,* 2000). Active Ras transmits its signals to Ras effectors via direct activation, recruitment to the plasma membrane, and association with substrates (Vojtek & Der, 1998).

In 1994, Raf was found to be a multi-subunit protein complex with an apparent mass of 300-500kDa, whereas Raf1 alone is a 72kDa protein (Wartmann & Davis, 1994). Wartmann and Davis (1994) analysed the structure of Raf and demonstrated that it contains a single Raf protein kinase together with the molecular chaperones heat-shock protein 90 (hsp90) and p50 (Stancato *et al.,* 1993). Inactive, multi-subunit Raf is found in the cytoplasm. Growth factor binding to tyrosine kinase receptors causes an increase in the GTP-bound form of Ras via signalling that includes the Src homology 2 and Src homology 3 (SH2/SH3) growth factor receptor bound protein 2 (Grb2), and SOS (Egan *et al.,* 1993). Ras, in its GTP-bound form, binds to the N-terminal regulatory domain of Raf1-hsp90-p50 and localises the entire complex to the plasma membrane where it is activated. Raf1 then becomes phosphorylated on Tyr-340 and Tyr-341 by membrane bound tyrosine kinases including cellular-src (c-Src). To support the importance of tyrosine phosphorylation in Raf1 activation, Raf1 treatment with tyrosine phosphatases was shown to inactivate it (Dent *et al.,* 1995). However, there has been scepticism about tyrosine phosphorylation as mutations of tyrosines 340 and 341 on Raf1 only reduced its kinase activity in response to activated Ras, rather than eliminating it (Marais *et al.,* 1995). It was subsequently found that phosphorylation of Raf1 on serine 338 and to a lesser

extent on serine 339 (Diaz *et al.,* 1997), along with its tyrosine residues actually controls its kinase activity.

In addition to Ras, other proteins bind to and regulate Raf1 activity (Widman *et al.,* 1999). The 14-3-3 family of proteins for example are dimers that function as scaffolds or anchors to localise signalling proteins such as Raf1. They have been shown to be involved in cell cycle control in both yeast and mammalian cells (Hermeking *et al.,* 1997). The exact mechanism by which the 14-3-3 proteins carries out its function is not apparent but studies have suggested that it maintains Raf in its inactive state in the absence of GTP-bound Ras and then stabilises the active Raf produced during activation *in vivo* (Tzivion *et al.,* 1998).

Raf1 can also be phosphorylated by ERK1/2 and PKA (c-AMP-dependent protein kinase), which inhibit its Raf1 activity (Marais & Marshall, 1996). ERK1/2-mediated inhibition may be a feedback mechanism to limit the activation of the ERK1/2 pathway (Widman *et al.,* 1999). Inhibition by PKA provides a mechanism to negatively regulate the ERK1/2 pathway, allowing the control of these pathways by multiple inputs (Widman *et al.,* 1999).

The additional isoforms of Raf; A-Raf and B-Raf have a pattern of expression more restricted than Raf1 (Storm *et al.,* 1990). B-Raf is a 96kDa protein that is expressed in many neuronal and neuroendocrine cell types and also in the testis and spleen (Mercer & Pritchard, 2003). A-Raf, a smaller 68kDa Raf protein, is expressed in fibroblasts and other cell types. All three Rafs have homology in three conserved regions; CR1, CR2 and CR3, the rest being highly variable. CR1 and CR2 are present in the N-terminus regulatory domains, whereas CR3 is found in the C-terminus catalytic kinase domain (Morrison and Cutler., 1997).

Each isoform of Raf is able to induce ERK1/2 activation (Pritchard *et al.,* 1995) but they differ in their potency to activate the ERK1/2 pathway. It has been shown that B-Raf is more

effective at activating the ERK1/2 pathway than Raf1 and Raf1, in turn, is more effective than A-Raf at activating the ERK1/2 pathway (B-Raf >Raf1 >A-Raf) (Pritchard *et al.,* 1995).

The ERK pathway has been implicated in the suppression of apoptosis and most studies have suggested a functional redundancy among the Raf family, as all Raf kinases activate ERK1/2 through MEK1/2 (Pritchard *et al.,* 1995). In Raf knockouts, however, it seemed that mice lacking B-Raf, but not A-Raf or Raf1 showed disturbances in cell survival (Wojnowski *et al.,* 1997). It is possible that B-Raf possesses specific functions in cell death regulation or it may be due to B-Raf-specific activation by Rap-1, a GTP-binding protein, that is not connected to Ras (York *et al.,* 1998). In addition, Raf1 and A-Raf require tyrosine phosphorylation for maximal activation, whereas the corresponding tyrosine residues are missing from B-Raf (Fabian *et al.,* 1993). These data suggest a potential unique role for B-Raf in signalling cell survival by an unknown mechanism. To support this further, a study using Rat-1 fibroblast cell lines overexpressing B-Raf showed that it conferred resistance to apoptosis induced by serum deprivation as a result of constitutive activation of the ERK1/2 signalling pathway (Erhardt *et al.,* 1999). This apparent anti-apoptotic activity of B-Raf blocked caspase activation without interfering with the release of cytochrome c from mitochondria (Erhardt *et al.,* 1999). However, since then it has also been shown that Raf1 knockout in mice is essential for mouse development by increasing apoptosis of many tissues (Huser *et al.,* 2001). This highlights the importance of cell-type specificity which determines the funtion of the different Raf proteins.

During the cell cycle, levels of membrane-bound Raf1 and B-Raf proteins are constant throughout S-phase and G_2, but these levels decline during mitosis, and then increase again during G_1, whilst the levels of cytosolic Raf1 and B-Raf remain constant (Widman *et al.,* 1999). This suggests that the Raf proteins are not predominantly activated during mitosis and are required throughout S, G_1 and G_2.

A recent study investigated the role of Raf1 and A-Raf in G_1/S cell cycle progression and their effect on the cell cycle following knockout. Mice with inactivating mutations in both Raf1 and A-Raf were created and the embryos of double knockout (DKO) mice displayed severe defects that resulted in lethality at E10.5 (Mercer *et al.,* 2005). The cells in the DKO embryos showed no changes in apoptosis but displayed a generalised reduction in cell proliferation, which was apparent in their ability to delay entry into S phase. Results from additional tests indicated that both Raf1 and A-Raf have a combined role in regulating transient ERK1/2 activation and initiation of cell proliferation (Mercer *et al.,* 2005).

1.2.4ii MEK1 and MEK2

Raf activates MEK1 and MEK2. The primary amino acid sequence of MEK1 was elucidated from a complimentary-DNA (cDNA) sequence and the protein was found to be approximately 45kDa (Crews *et al.,* 1992). MEK1 and MEK2 are highly homologous in their primary amino acid sequences and function as dual threonine/tyrosine kinases (Widman *et al.,* 1999). Activated MEK1/2 phosphorylates and activates ERK1/2 on the Thr-X-Tyr motif in the activation loop of the ERK1/2 catalytic domain (Widman *et al.,* 1999). MEK1 is located outside the nucleus and in its inactive state it sequesters ERK1/2 in the cytoplasm (Yoon & Seger, 2006). Phosphorylation and activation of MEK1 result in the activation of ERK1/2 (Widman *et al.,* 1999). The activation of ERK1/2 induces a major conformational change and forces the detachment of MEK1 from ERK1/2 (Yoon & Seger, 2006). Activated ERK1/2 is then rapidly translocated to the nucleus (Brunet *et al.,* 1999) where it can regulate the activity of nuclear proteins including transcription factors, while MEK1 remains in the cytosol due to the presence of a nuclear export signal in its N-terminal region (Yoon & Seger, 2006).

Raf1 seems to activate both MEK1 and MEK2, but when recombinant Ras-GTP was used to bind cell lysates, a complex of Raf1 and MEK1 or B-Raf and MEK1 was found (Marais & Marshall, 1996). In addition, cells transformed by oncogenic forms of Ras showed increased MEK1 activity compared to MEK2, suggesting that Ras and Raf1 preferentially signal to ERK1/2 via MEK1 (Marais & Marshall, 1996). Collective research among many laboratories, each using different cell lines, found that MEK1 and MEK2 appeared to be differentially activated by Raf1, A-Raf, and B-Raf in different cell types and in response to various external stimuli (Bogoyevitch *et al.,* 1995, Lange-Carter & Johnson, 1994).

A study by Harding *et al* (2003) highlighted that in *Xenopus*, the MAPK cascade has well established roles in entry and exit from mitosis (i.e. during G_2/M progression), but relatively little is known about this pathway in mammalian cells. Harding *et al* (2003) found that membrane-bound MEK1 is strongly activated as cells enter mitosis, but that this activation is not coupled to ERK1/2, which is inactive in mitosis (Harding *et al.,* 2003). The uncoupling of MEK1 activation from ERK1/2 is mediated by direct modifications to MEK1 by the mitotic cell cycle machinery and requires active cyclin B-Cdc2 (Cdk1) (Harding *et al.,* 2003). This highlights an important functional consequence of the physical detachment seen upon ERK1/2 phosphorylation and activation by MEK1/2 which suggests that ERK1/2 may be inactive during mammalian cell mitosis.

1.2.4iii ERK1 and ERK2

ERK1 and ERK2 are 44- and 42-kDa isoforms, respectively (Widman *et al.,* 1999) displaying a high degree of similarity and are described as being functionally redundant, although they do

display differences in their substrate specificity (Seger & Krebs, 1995). As part of the MAPK module, they are activated last in response to a wide variety of growth factors and mitogens.

The full activation of ERK1/2 occurs as a result of phosphorylation of threonine and tyrosine residues in a Thr-X-Tyr motif (Yoon & Seger, 2006). Phosphorylation on both residues also seems to be the main requirement for translocation of ERK1/2 to the nucleus, as opposed to catalytic activity (Chen *et al.,* 1992). Monophosphorylation of either residue can cause some activation but may not be sufficient to exert their downstream effects under most conditions (Yoon & Seger, 2006). Upon phosphorylation of both residues, a conformational change occurs in ERK1/2, which changes the hydrogen bonds at the substrate binding site, and allows the side chain of the phospho-Tyr to face the surface of its substrates (Canagarajah *et al.,* 1997). However, this is not sufficient to provide high affinity and specificity. To allow proper substrate recognition, additional interaction domains (docking motifs) on ERK1/2 and their substrates are often involved in the phosphorylation process (Widman *et al.,* 1999), which help ERK1/2 activate the appropriate substrate required for a specific response.

ERK1 and 2 are proline-directed protein kinases, in that they phosphorylate serine or threonine residues nearby a proline residue. The main consensus sequence for recognition by ERK1/2 is Pro-Leu-(Ser or Thr)-Pro (Seger & Krebs, 1995). Several proteins can be phosphorylated by ERK1/2, including those in the cytoplasm and nucleus. About 160 substrates have already been identified and have been reviewed by Yoon & Seger (2006). Examples of ERK1/2 substrates include the S6 kinase p90 RSK (MAPK-activated protein kinase), cytosolic phosopholipase A_2, and the juxtamembrane region of the epidermal growth factor (EGF) receptor (Seger & Krebs, 1995). Many microtubule-associated proteins (MAPs) are also substrates for ERK1/2, including MAP-1, MAP-2, MAP-4, and Tau (Seger & Krebs, 1995). Examples of transcription factors in the nucleus that are also phosphorylated and activated by ERK1/2 are Elk1, v-ets

erythroblastosis virus E26 oncogene homolog 1 (Ets1), stress-activated protein kinase 1a (Sap1a), c-Myc, Tal, and Myb, whose activity is thought to be inhibited by ERK1/2 phosphorylation (Widman *et al.,* 1999).

ERK1/2 activity is predominantly regulated by the upstream components in the MAPK module (Table 1.1). However, ERK1/2 activity is also regulated by protein phosphatases, which are important determinants of the cellular responses regulated by the MAPK cascade (Widman *et al.,* 1999). ERK1/2 inactivation can be mediated by the removal of phosphate groups from either one or both of the regulatory residues. The best studied ERK1/2 phosphatase is the MAPK phosphatase 3 (MKP3) that is a product of an inducible gene, and is highly specific to ERK1/2 (Camps *et al.,* 1998). Another function of MKP3 is to act as a negative feedback, whereby its own phosphorylation leads to its degradation, which further facilitates ERK1/2 activity (Camps *et al.,* 1998).

1.2.5 The ERK5 Pathway

In comparison to the ERK1/2 pathway, the ERK5 pathway is less characterised. ERK5 was first identified from its direct and specific interaction with MEK5 (Zhou *et al*., 1995). It was classed as a novel signalling pathway as it harboured no interaction with the MEK1/ERK1 pathway (Zhou *et al*., 1995). The ERK5 signalling cascade acts through sequential activation of MEKK2/3, MEK5 and ERK5 and transmits signal to a variety of stress and mitogenic related targets (Raviv *et al*., 2004).

Unlike the MAPK pathways described above, who serve a single set of extracellular stimuli such as stress or mitogens, the ERK5 pathway serves both stress responses and proliferation (Raviv *et al*., 2004). ERK5 itself is twice the size of other MAPKs (Zhou *et al*., 1995) and its

substrates include myocyte enhancer factor 2 (MEF2 – Kato *et al*., 1997) and the ERK1/2 substrate c-Myc (Nishimoto & Nishida, 2006).

1.3 The ERK1/2 Pathway in the Cell Cycle

The activity of endogenous ERK1/2 has been shown to decrease as cells enter mitosis (Edelmann *et al.,* 1996). Because signalling pathways have more than one function, as demonstrated by their various substrates, it is difficult to establish a *bona fide* role for them. To investigate the ERK1/2 pathway, different cell lines have been used, which then raises the possibility that the response may be specific to the cell type.

Studies in *Xenopus* oocytes have provided information supporting the function of ERK1/2 in amphibian cell meiosis. Fan & Sun (2003) reviewed the function of ERK1/2 in mammalian cell meiosis where it plays a pivotal role in maintaining metaphase II arrest of the cycling oocyte until fertilisation. The ERK1/2 signalling pathway involved in *Xenopous* oocytes includes the serine/threonine protein kinase Mos (a MKKK) which is produced by the proto-oncogene c-mos that activates MEK1/2 which then activates ERK1/2 (Fan & Sun, 2003). Upon oocyte fertilisation, active ERK1/2 is important in reorganising the microtubule network and may assist in the transition from meiosis I to meiosis II (Fan and Sun., 2003). There is evidence showing partial association of ERK1/2 with the microtubule organising centres (MTOCs) present at the spindle poles as well as in the cytoplasm (Verlhac *et al.,* 1994), giving ERK1/2 an ideal location to carry out its function in meiosis.

The requirement for ERK1/2 in S-phase entry was demonstrated by a G_1 arrest in cells when ERK1/2 activity was blocked (Lavoie *et al.,* 1996). The mechanism by which ERK1/2 induces S-phase entry has been reviewed recently by Torii *et al* (2006). ERK1/2 activation is important

in activating the transcription factor Elk-1, which subsequently induces the expression of c-fos. The Fos and Jun families of nucleoproteins form homo or heterodimeric complexes that bind DNA and modulate expression of target genes required for S-phase entry, such as cyclin D. Cyclin D forms a complex with CDK4/6 (section 1.1) and eventually induces expression of cyclin E, along with other proteins required for S-phase entry (Torii *et al.*, 2006). Therefore, the activation of ERK1/2 triggers the induction of many genes important for S-phase entry.

In 1998, two independent groups published their findings that suggested a role for ERK1/2 in the mitotic phase of the cell cycle. Shapiro *et al* (1998) used a phospho-specific ERK1/2 antibody to demonstrate in NIH 3T3 cells, that active ERK1/2 and MEK1 become concentrated within the nucleus early in prophase before nuclear envelope breakdown. In late prophase, they became localised at spindle poles, but the intracellular distribution of ERK1/2 and MEK1 did not overlap, as active MEK1 was excluded from condensed chromosomes whereas active ERK1/2 associated with kinetochores and at the periphery of condensed chromosomes. Furthermore, Shapiro *et al* (1998) identified a link between ERK1/2 activity and the 3F3/2 phosphoantigen that regulate the metaphase-to-anaphase transition in mitosis, suggesting that 3F3/2 phosphoantigens are likely to be targets for ERK1/2 or ERK-related kinases. Shapiro *et al.,* (1998) proposed two roles for ERK1/2. One, that active ERK1/2 may act as a signal at kinetochores allowing cells to sense improper chromosome attachment to spindle microtubules via phosphorylation of the 3F3/2 phosphoantigen. Secondly, it was proposed that active ERK1/2 might regulate spindle assembly, possibly through regulation of the activity of motors or microtubule polymerisation.

Interestingly, Zecevic *et al* (1998), using a phospho-MAPK (ERK1/2) antibody, demonstrated that active ERK1/2 was localised at kinetochores, asters and at the midbody of dividing PtK1 cells. Active ERK1/2 was present on kinetochores in prophase and became undetectable by

mid-anaphase. They deduced that the intracellular localisation of active ERK1/2, consistent with its potential role in regulation of mitotic progression and the time course of its activation and inactivation, was consistent with a role in the regulation of chromosome movement. Zecevic *et al* (1998) co-immunoprecipitated active ERK1/2 with centromere-binding protein E (CENP-E, a kinetochore motor protein) during mitosis demonstrating that CENP-E can be phosphorylated by active ERK1/2 and may play a role in mediating interactions between chromosomes and microtubules.

In keeping with the idea that the ERK1/2 pathway is essential during mitotic progression, Hayne *et al* (2000) used Nocodazole (a microtubule depolymerising drug) to show that the ERK1/2 pathway is activated upon treatment with the drug, and that the ERK1/2 pathway is necessary for normal G_2/M progression, as inhibiting the pathway caused a block at G_2 (Hayne *et al.,* 2000). Not only does ERK1/2 seem important during mitosis, it has also been suggested that ERK1/2 has a role in regulating cell survival (Erhardt *et al.,* 1999). Erhardt *et al* (1999) described the B-Raf/MEK/ERK1/2 pathway as essential for cell survival in Rat-1 fibroblast cell lines since treatment with a MEK inhibitor or expression of a dominant inhibitory MEK mutant blocks the anti-apoptotic activity of B-Raf and that this pathway promotes apoptosis after growth factor deprivation (Erhardt *et al.,* 1999).

Thus, the role of the ERK1/2 pathway appears to be highly significant, not only during mitosis but also as a survival signal for the cell. Therefore there is no doubt that the accumulation of mutations in critical genes encoding proteins of the ERK1/2 MAPK signalling pathway may cause damaging effects such as altered programmes of cell proliferation, differentiation and death, resulting in cancer.

1.4 Ras and Raf Signalling in Cancer

The identification of the genes mutated in cancers suggests that they affect discrete pathways, each making distinct contributions to the malignant phenotype. Mutations in the Ras pathway (Figure 1.6) and in the retinoblastoma (Rb) protein, both important in driving cell division, affect cell cycle control and drive uncontrolled proliferation (McCormick, 1999).

Ras activates multiple pathways aside from the ERK pathway. These include the phosphatidylinositol 3-kinase (PI3K) pathway, the p120 Ras GAP pathway and the Ral GDS pathway (Vojtek & Der, 1998). Mutations in the Ras family of genes can be found in 20% to 30% of tumour types including adenocarcinomas of the pancreas, the colon, the lung, in thyroid tumours and in myeloid leukaemia (Bos, 1989). Constitutive activation of Ras results in constitutive activation of ERK1/2, hence rapid cell proliferation and cell survival, which is further enhanced by defects in other components of the Ras pathways such as Rb (McCormick, 1999). Mutations in the Rb protein inactivate Rb and disable the Rb checkpoint. This leads to up-regulation of cyclin D-CDK activity (brought about by active ERK1/2), allowing cells to escape senescence and differentiation (McCormick, 1999). Recently, mutations in Raf have also been discovered in cancers. In particular, the V600E mutation in B-Raf, where Valine at position 600 is substituted for Glutamic acid, is seen in approximately 70% of human malignant melanomas (Davies *et al.*, 2002).

The elucidation of key mutations within the ERK1/2 pathway has progressed and because Ras is central to many pathways and prominent in many tumours, it would be extremely useful to target the Ras signalling pathway to block cell survival effects, thereby increasing the efficiency of chemotherapy.

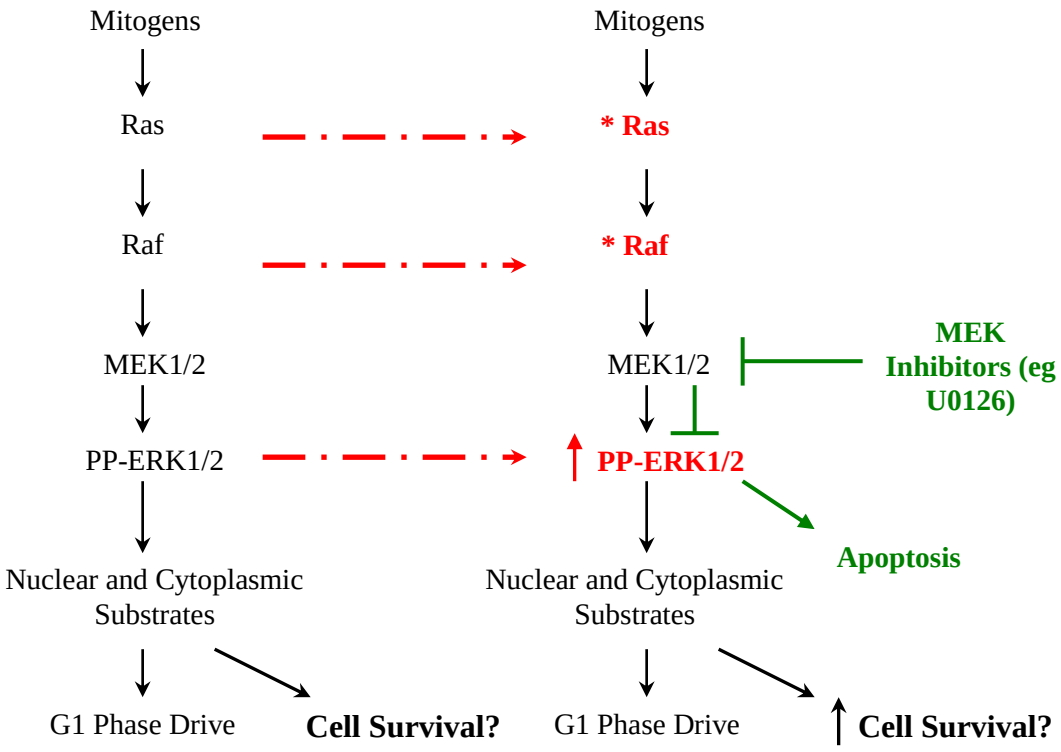


Figure 1.6: Key mutations within the ERK1/2 pathway. Mutations causing constitutive activation of either Ras (*Ras) or Raf (*Raf), result in increased activation of ERK1/2 (PP-ERK1/2). Inhibiting MEK1/2 activity (shown in green) decreases the downstream effects of mutated Ras or Raf providing a mechanism by which chemotherapeutic drugs can increase apoptosis of cancer cells.

It has been suggested that blocking the downstream affects of mutated components of the ERK1/2 pathway by inhibiting MEK1/2 (Figure 1.6) in cells treated with microtubule-interfering-agents (MIAs) may reduce potential survival effects of ERK1/2 and further induce apoptosis (McDaid and Horwitz., 2001, McDaid *et al.*, 2005, MacKeigan *et al.*, 2000). What remains is to find the mechanism by which ERK1/2 induces cell survival, which is currently unknown.

1.5 Cancer Chemotherapy and Microtubule-Interfering Agents (MIAs)

Anti-cancer drugs have been developed to target the destruction of cancer cells. The major groups of drugs used as chemotherapeutic agents include alkylating agents (Sanderson &

Shield, 1995), antimetabolites (Cole *et al*., 2005), topoisomerase I and topoisomerase II inhibitors (Rothenberg, 1997; Hande, 1998) and spindle inhibitors (Wood *et al.*, 2001). The alkylating agents consist of five major groups (Sanderson & Shield, 1995). These are the nitrogen mustards; the ethylenimines; the alkyl sulfonates; the nitrosoureas and the triazenes (Sanderson & Shield, 1995), which act directly on DNA, causing cross-linking of DNA strands, abnormal base-pairing or DNA strand breaks, thus preventing the cell from dividing (Sanderson & Shield, 1995). Antimetabolites are substances that closely resemble an essential nutrient that is required by the cell and therefore interferes with physiological reactions involving it (Cole *et al*., 2005). As a result antimetablites cause cell death by starvation, differentiation or alteration of cell function (Cole *et al*., 2005). Topoisomerase inhibitors stabilise the covalent link of topoisomerase enzymes to DNA creating permanent double-stranded breaks within the DNA strand, which then triggers apoptosis of the cell (Hande, 1998; Rothenberg, 1997). Finally, there are the spindle inhibitors (Wood *et al.*, 2001). These anti-tumour agents are usually derived from plants and so far are known to target β-tubulin (Wood *et al.*, 2005). Spindle inhibitors work by stabilising or destabilising microtubules, thereby blocking the ability of cells to divide (Wood *et al.*, 2001).

Chemotherapeutic microtubule-interfering agents or MIAs belong to the family of spindle inhibitors. MIAs are able to bind β-tubulin on microtubules and inhibit proliferation by acting on the mitotic spindle (section 1.5.1 & 1.5.2 (pages 27 to 29) - Mollinedo & Gajate, 2003). The biological consequences of interfering with microtubule dynamics in this way include M-phase arrest, inhibition of cell proliferation and apoptosis (Jordan & Wilson, 1998).

There are two types of MIAs that are currently used for cancer chemotherapy; firstly there are the vinca alkaloids (such as Vinblastine and Vincristine), which destabilise microtubules by inhibiting microtubule polymerisation (Mollinedo & Gajate, 2003). The second type are

taxanes (e.g. Taxol), which stabilise microtubules (Mollinedo & Gajate, 2003). Although each type of compound exerts opposite effects, they have the common property of suppressing microtubule dynamics and thereby microtubule function, leading to the disruption of mitotic spindle function and activation of the spindle-assembly checkpoint (Jordan & Wilson, 1998). Once the spindle checkpoint is activated, cells arrest at the metaphase to anaphase transition (Fang *et al.,* 1999).

However, the extent to which microtubules are involved in other cellular functions, including chemotaxis, intracellular transport and transmission of receptor signalling, renders non-malignant cells in interphase, vulnerable to the effects of these MIAs. This lack of selectivity towards tumour cells leads to a number of toxic side effects (Mollinedo & Gajate, 2003).

The way in which MIAs function to halt the cell cycle at a specific point has been the subject of research for many laboratories (Deacon *et al.,* 2003, Milross *et al.,* 1996, Weaver & Cleaveland, 2005). MIAs disrupt microtubule dynamics, activating the spindle-checkpoint, which initiates the ERK1/2 signalling pathway and blocking cells at M phase. Failure to proceed through the cell cycle is responsible for the activation of apoptosis (Deacon *et al.,* 2003). In addition, MIAs can promote a number of effects on the centrosomes, including abnormal centriole structure, centrosome fragmentation and inappropriate centrosome duplication (Wendell *et al.,* 1993).

1.5.1 Taxol

Taxol, also known as Paclitaxel, has been used for decades in the treatment of ovarian, breast and non-small cell lung cancers (Rao *et al.*, 1999). In 1971, Taxol was identified as the active constituent of the bark extract from the Pacific yew, *Taxus brevifolia* (Wani *et al.,* 1971). Mastropaolo *et al.*, (1995), determined the crystal structure of Taxol and found that it exists in

solution as a homodimer, interacting with solvent atoms through an extensive network of hydrogen bonds (Mastropaolo *et al.,* 1995).

The cellular targets of Taxol are the microtubules, specifically β-tubulin (Rao *et al.,* 1992), and its binding site is on the microtubule polymer. The addition of Taxol results in the reorganisation of the microtubule cytoskeleton, forming stable bundles of microtubules (Rao *et al.,* 1992). Normal microtubule dynamics are disrupted by Taxol and cells arrest in M-phase with inhibition of normal cell division (Schiff & Horwitz, 1980). The specific binding site for Taxol was determined and shown to occupy three distinct sites on β-tubulin; between residues 1-31, resides 217-233 and an arginine residue at position 282 (Rao *et al.*, 1999).

Clinically, Taxol is given as an infusion (drip) into the vein at a plasma concentration between 0.19 – 3.65 μg/ml and has many side effects including bruising, anaemia, hair loss, headaches (http//www.pakageinserts.bms.com/pi/pi_taxol.pdf).

1.5.2 Vinblastine/Vincristine

The vinca alkaloids are naturally occurring compounds that are found in the periwinkle plant *Vinca rosea Linn* (Mollinedo & Gajate, 2003). Vinblastine and Vincristine are two of the four compounds that belong to this group. The others are Vindesine and Vinorelbine, but for the purpose of this project I will not be talking in depth about them individually.

Although specific differences exist among the vinca alkaloids regarding their biological functions, the way they bind β-tubulin is similar. They bind to both high and low affinity sites on β-tubulin (Bhattacharyya & Wolff, 1976). The binding of vinca alkaloids to the high affinity sites located at the ends of microtubules results in disruption of microtubules. Low

concentrations of the drugs modify the dynamics at the ends of the microtubules, especially those involved in the mitotic spindle, which accelerates microtubule disassembly (Bhattacharyya & Wolff, 1976). This, however, does not result in gross microtubule disorganisation. At higher concentrations, the drugs are able to bind to low affinity binding sites as well as along the walls of microtubules leading to their disruption and disorganisation (Mollinedo & Gajate, 2003).

Clinically, Vinblastine and Vincristine are used for treatment of leukaemia, lymphoma, breast and lung cancers, and their side affects include bruising, anaemia, nausea, constipation and numbness/tingling in hands and feet. The plasma concentration for Vincristine varies from 0.59 to 213µg/ml (Krishna *et al.,* 2001) and for Vinblastine it ranges from 2.3 to 230µg/ml (Chao *et al.,* 1999).

Previous studies have investigated the effects of these MIAs on the activity of ERK1/2 (Hayne *et al.,* 2000, Boldt *et al.,* 2002, Deacon *et al.,* 2003) and found that ERK1/2 is activated independently of mitosis. In addition to activating ERK1/2, MIAs were also found to activate the spindle assembly checkpoint and induce a mitotic cell cycle arrest (Sorger *et al.,* 1997). As a result, it was suggested that MIA-mediated active ERK1/2 and therefore MIAs were responsible for favouring the survival of the cell (Boldt *et al.,* 2002). There has been a report of enhanced ERK1/2 activation in various human tumours (Kiyokawa *et al.,* 1994) which supports the idea that active ERK1/2 may mediate cell survival. However the mechanisms involved in ERK1/2 mediated cell survival requires the understanding of the apoptotic pathway.

1.6 MIAs and Apoptosis

Apoptosis, or cell suicide, is a form of cell death that is morphologically and biochemically distinct from necrosis (Budihardjo *et al.,* 1999). The three major components involved in apoptosis are the Bcl-2 family proteins; the caspases (Cysteine Aspartyl-specific Proteases), and the Apaf-1/CED-4 protein that relays the signal integrated by Bcl-2 family proteins to caspases (Budihardjo *et al.,* 1999). The activation of these proteins results in distinct changes that are characteristic of apoptosis. These include mitochondrial damage, nuclear membrane breakdown, DNA fragmentation, chromatin condensation, and the formation of apoptotic bodies (Thornberry & Lazebnik, 1998).

Apoptosis is triggered by two different pathways; the cell surface death receptor pathway, and the mitochondria-initiated pathway. Both pathways involve the activation of caspases, making them the key components in programmed cell death.

The first caspase to be discovered as a cytokine-processing enzyme was called interleukin-1 β-converting enzyme (ICE) (Budihardjo *et al.,* 1999). Since then, over 14 caspases have been cloned (Thornberry & Lazebnik, 1998) and numbered. Caspases 2, 3, 6, 7, 8, 9, and 10 are initiators and executioners of apoptosis, whereas Caspases 1 and caspase 11 function mainly in cytokine processing (Cohen, 1997). Caspases exist as inactive zymogens within the cell, termed procaspases. Upon an external signal, procaspases are proteolytically processed at conserved aspartic acid residues to assume an active state that consists of large and small catalytic subunits (Green & Reed, 1998).

1.6.1 Cell Death via Cell Surface Death Receptors

Cell surface death receptors are a family of transmembrane proteins belonging to the tumour necrosis factor (TNF) or nerve growth factor (NGF) receptor superfamily (Budihardjo *et al.*, 1999). Of this family, the Fas receptor is an important component in mediating this pathway. When the Fas receptor binds its ligand, Fas/CD95 ligand (FasL), it is recognised and translated into intracellular signals that lead to caspase activation by three distinct steps: ligand-induced receptor trimerisation; the recruitment of intracellular receptor-associated proteins, and the initiation of caspase activation (Figure 1.7, Budiharjo *et al.*, 1999). The cytoplasmic region of Fas contains a death domain (DD), which then recruits a DD-containing adaptor molecule, FADD (Fas-associating protein with death domain). The N terminus of FADD (called the death effecter domain, DED) is required to recruit upstream procaspases such as procaspase 8 and/or procaspase 10 that in turn brings about the final stages of cell death (Budiharjo *et al.*, 1999).

1.6.2 Mitochondrial-induced Cell Death

The Bcl-2 family of proteins, both pro- and anti-apoptotic forms, governs this pathway. The anti-apoptotic members of the family include Bcl-2, Bcl-X_l, Mcl-1, Bfl-1, Bcl-W and Boo, and the pro-apoptotic members include Bax, Bak, Bok, Bad, Bid, Bim, Bik, Hrk (Green & Reed, 1998). Many Bcl-2 proteins (usually the anti-apoptotic) are constitutively expressed in mitochondrial membranes whereas the pro-apoptotic members are localised to extra-mitochondrial compartments. Upon apoptosis induction, the pro-apoptotic proteins translocate to the mitochondria and induce mitochondrial membrane permeabilisation and the release of pro-apoptotic molecules such as cytochrome c into the cytosol (Green & Reed, 1998).

It is at this point where the two pathways of caspase activation merge. Activation of caspase 8 in the cell surface death receptor pathway provides the signal for cytochrome c release by

inducing Bid cleavage (Figure 1.7). Once released from the mitochondria, cytochrome c interacts with other cytosolic protein factors such as Apaf-1 and procaspase-9 (the apoptosome), to activate procaspase-3, known as an effector caspase that brings about the characteristics of apoptosis (Budihardjo *et al.*, 1999).

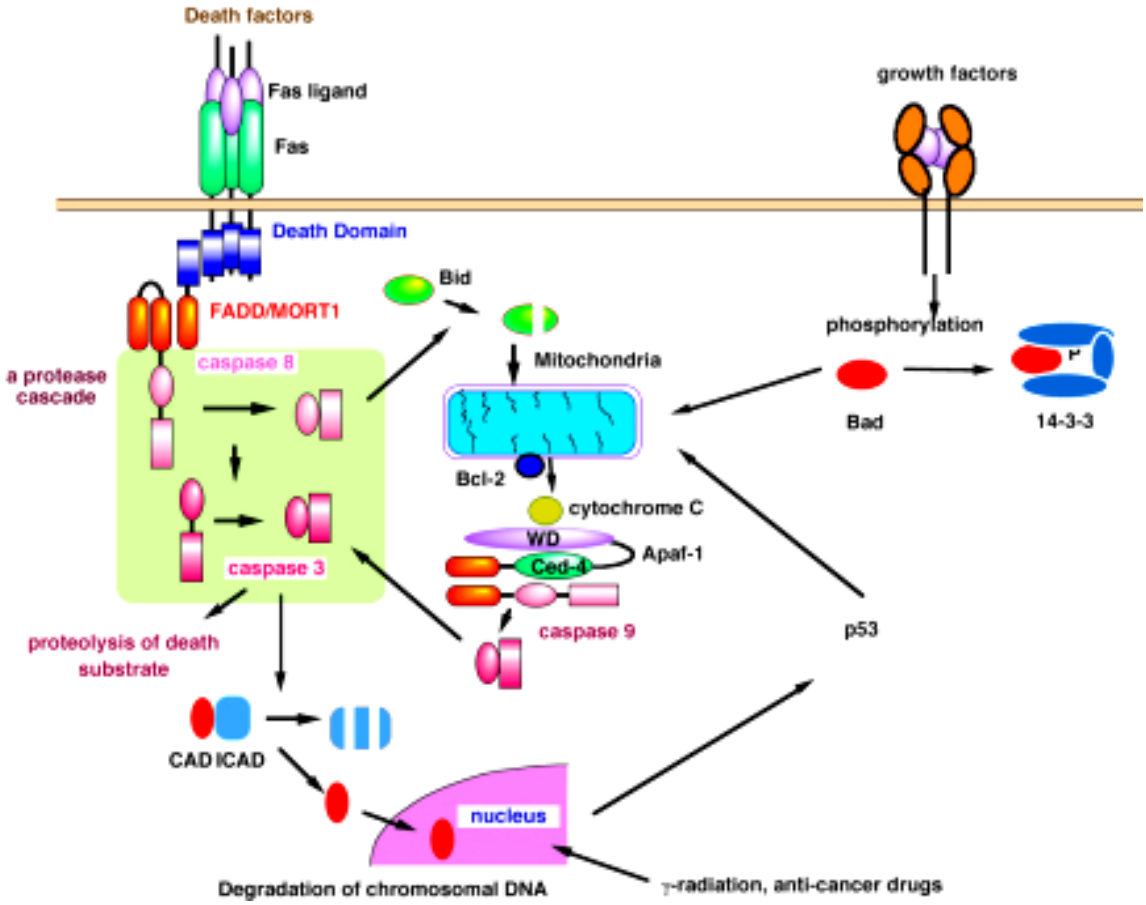


Figure 1.7: The Pathways Leading to Caspase Activation in Apoptosis. The text explains the cell surface death receptor-induced and mitochondrial-induced cell death. The alternative route to cell death includes p53 which is activated via damage to chromosomal DNA, and via Bad release from 14-3-3 proteins in growth factor deprivation. Image from the following website; http://claim.springer.de/EncRef/CancerResearch/samples/0003.htm

1.7 Background to the Project

Understanding how MIAs induce cell death will provide valuable information for improving anti-cancer therapy. Hence, previous studies in the laboratory have focussed on determining the mechanism of cell death following MIA-induced mitotic cell cycle arrest. Initial data in our lab (unpublished) suggested that mitotically-arrested cells undergo apoptosis by activation of the intrinsic apoptotic pathway. To determine this, Nocodazole (3μM) was added to a population of cells synchronised in G_1/S-phase by an aphidicolin-thymidine block. Nocodazole treatment for 12 hours or more induced the majority of cells in the population to arrest in mitosis (Figure 1.8). Apoptosis was assessed by immunocytochemistry to detect the presence of cleaved caspase-3 in Nocodazole-treated cells. Cells arrested in mitosis following treatment with Nocodazole displayed caspase-3 cleavage (Figure 1.9) suggesting that apoptosis only occurs in cells that have reached and arrested in mitosis. The data obtained for caspase-3 cleavage was quantified and it was found that apoptosis occurred in the mitotic population of cells only (Figure 1.10). There was a time-dependent increase in apoptosis, particularly between 12 to 24 hours post Nocodazole treatment (Figure 1.10).

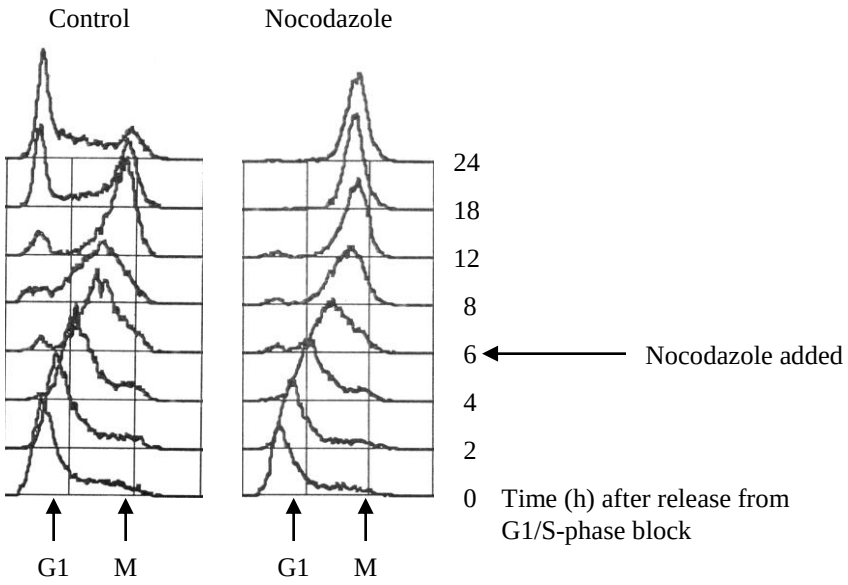


Figure 1.8: Nocodazole causes a mitotic block of synchronised Hela cells. 6 hours after a G1/S-phase block, cells were treated with Nocodazole (3µM) causing a shift in the DNA content by 24 hours as determined by flow cytometry (right image). This indicates mitotic arrest in response to Nocodazole treatment in comparison to the control (untreated cells, left image). G1=G1 phase, M=Mitotic phase.

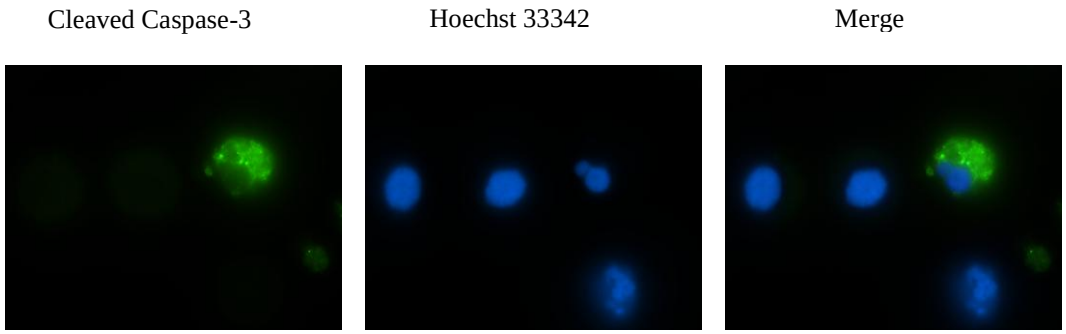


Figure 1.9: Caspase-3 is cleaved in Nocodazole-arrested mitotic cells. Hela cells were treated with Nocodazole (3µM) for 12 hours. Immunofluorescence was performed using an anti-cleaved caspase-3 (green) antibody and the DNA dye Hoechst 33342 (blue).

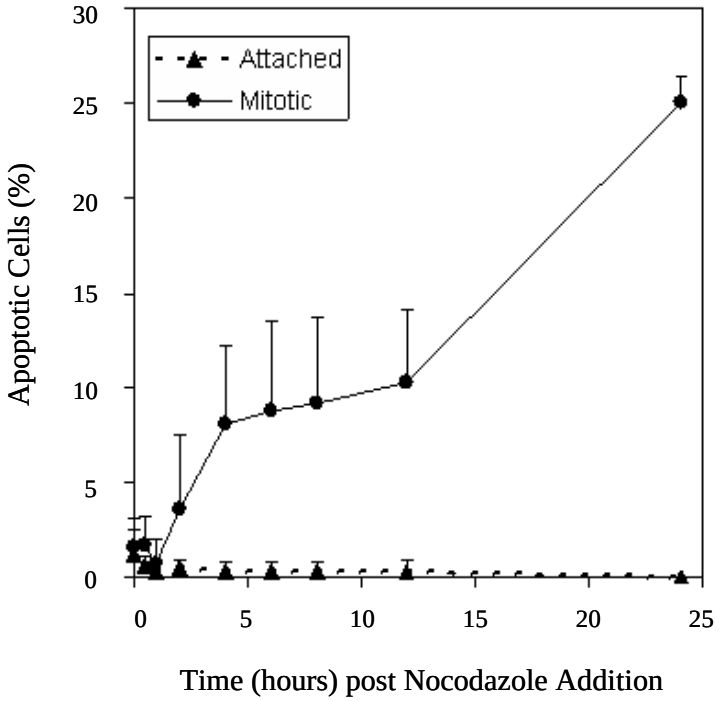


Figure 1.10: Apoptosis in Nocodaole-arrested mitotic cells. Hela cells were treated with Nocodazole (3μM) for the indicated periods and separated into a mitotic and non-mitotic (attached) population.. Immunocytochemistry was performed using an anti-cleaved caspase-3 antibody and the DNA dye Hoechst 33342. Approximately 50 – 100 cells were counted in random fields in 3 independent experiments and cells displaying cleaved caspase-3 were interpreted as apoptotic. Each time point indicates the mean (± SD).

Further evidence suggesting that mitotically arrested cells undergo apoptosis was the presence of cytoplasmic cytochrome c in mitotic cells after treatment with Nocodazole for 12, 18 and 24 hours (Figure 1.11a). Cytoplasmic cytochrome c was also detected in mitotic cells after 12 hour treatments with Taxol, Vinblastine and Vincristine (Figure 1.11b), indicating that these MIAs are responsible for mitotic arrest which then leads to the demise of the cell.

Evidence suggesting the involvement of the intrinsic pathway (caspase-9 and caspase-3) of apoptosis following Nocodazole treatment was obtained by both *in vitro* caspase activity assays (not shown) and by Western blotting (Figure 1.12). The Western blot shows that cells arrested in mitosis, following 12 and 24 hour treatment with Nocodazole, have increased activation of both caspase-9 and caspase-3 confirming a role for the intrinsic pathway.

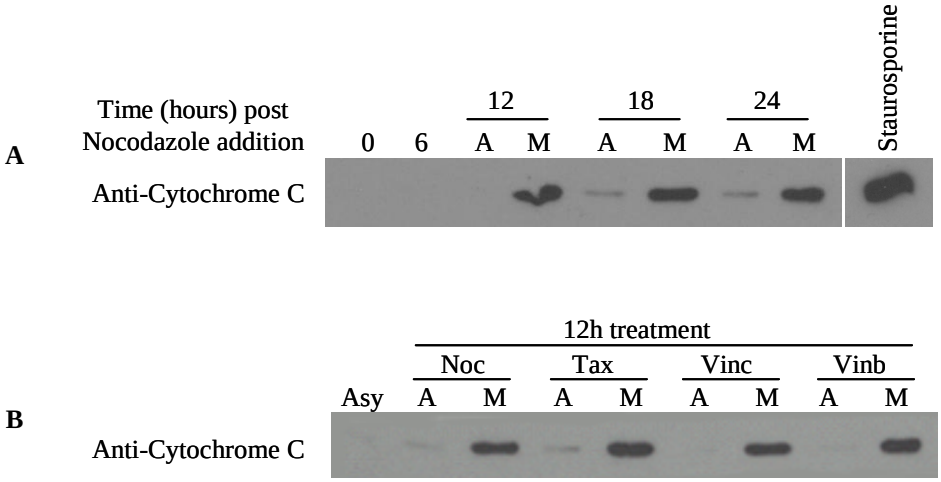


Figure 1.11: Cytochrome C is released in mitotic cells. A - A time course of Hela cells treated with Nocodazole for 12, 18 and 24 hours. Control cells were either untreated (0) or treated with Staurosporine (1mg/ml, 6 hours). Cytochrome C was detected using an anti-cytochrome C antibody on a western blot. **B –** Hela cells were treated with Nocodazole, Taxol, Vinblastine or Vincristine for 12 hours. Cytochrome C was detected on a western blot as above.

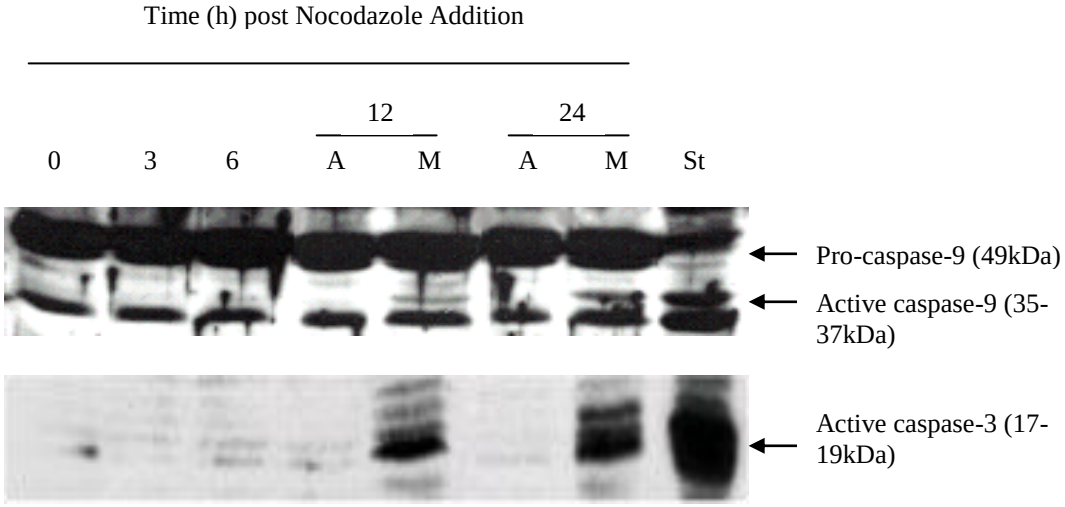


Figure 1.12: Caspase Activation in Response to Nocodazole. Treatment with 1µg/ml Nocodazole yielded mitotic (M) and attached (A) populations. Control cells were either untreated (0) or treated with Staursporine (1mg/ml, 6 hours). Antibodies that detect active and inactive forms of caspases-9 and caspases-3 were used for immunoblotting.

In additional preliminary experiments, caspase-8 cleavage in response to Nocodazole treatment was also investigated (data not shown). Unlike caspase-3 and caspase-9, caspase-8 cleavage was not seen in mitotically arrested cells following 12 or 24 hour treatment with Nocodazole, suggesting that the extrinsic pathway of apoptosis is not involved in bringing about cell death. Although further work is required to accurately establish the mechanism of cell death, this data suggests that following mitotic cell cycle arrest, cells undergo apoptosis via activation of the mitochondrial pathway.

Published data from the laboratory (Deacon *et al.,* 2003) showed that ERK1/2 is activated primarily in non-mitotic populations of Hela cells after treatment with Nocodazole, Taxol, Vinblastine or Vincristine. Cells arrested in mitosis undergo apoptosis where ERK1/2 was not found to have reduced activity (Deacon *et al.,* 2003), leading to suggestions that active ERK1/2 may have a role in regulating cell survival.

Another observation in the laboratory (unpublished) indicated that suppressing ERK1/2 activation, using the MEK inhibitor U0126, during MIA-treatment increased MIA-induced apoptosis. This was consistent with published data where a MEK inhibitor was added with Nocodazole or Taxol to inhibit activation of ERK1/2. An increase in MIA-induced apoptosis was observed (McDaid and Horwitz., 2001; McDaid *et al.,* 2005; MacKeigan *et al.,* 2000), further supporting a role for ERK1/2 in cell survival. However, the mechanism by which ERK1/2 may regulate cell survival is yet unknown.

Possible targets of ERK1/2 include caspase-9 and the Bcl-2 protein Bim. Recently it has been suggested that caspase-9 and Bim can be directly phosphorylated by active ERK1/2 (Allan *et al.,* 2003; Luciano *et al.,* 2003).

ERK1/2 has been shown to phosphorylate caspase-9 on threonine residue 125 in HEK293, Hela and NIH3T3 cells (Allan *et al.,* 2003). This phosphorylation was proposed to block

caspase-9 processing and subsequent caspase-3 activation, thereby promoting cell survival (Allan *et al.*, 2003).

The phosphorylation of Bim on serine residue 69 by ERK1/2 (Luciano *et al.,* 2003) has been shown to inhibit Bim activity by targeting Bim for proteasomal-dependent degradation resulting in cell survival (Ley *et al.,* 2003). Luciano *et al* (2003) investigated this phosphorylation in various cell lines including the human B lymphoma cell line Ramos and chronic myelogenous leukaemia cells K562 and found similar results. ERK's effect on Bim has also been observed in human mammary epithelial cell line, MCF10A (Marani *et al.,* 2004), therefore the phosphorylation of Bim by ERK1/2 may also occur in Hela cells.

1.8 Aims and Objectives

Treatment of cancer cells with chemotherapeutic MIAs such as Taxol reduces tumour growth by causing cell death. However, the mechanism by which these drugs induce cell death is unclear. Our preliminary studies and work by other groups has shown that ERK1/2 is activated by MIAs such as Taxol (Boldt *et al.,* 2002, McDaid *et al.,* 2005, MacKeigan *et al.,* 2000). Furthermore, our initial studies (unpublished) and reports by other groups have found that inhibition of MEK1/2 increases Taxol-induced apoptosis (McDaid and Horwitz., 2001, McDaid *et al.,* 2005, MacKeigan *et al.,* 2000). However, the mechanism responsible for MIA-induced cell death remains unclear.

Therefore the aims of this project were:

- To investigating the time course of ERK1/2 activation by MIAs using an anti-active ERK1/2 antibody;
- To examine whether a MEK inhibitor enhances apoptosis induced by MIAs;
- To asses whether inhibition of ERK1/2 by the MEK1/2 inhibitor induces apoptosis via the intrinsic or extrinsic pathway;
- To examine whether ERK1/2 acts as a survival signal by phosphorylating and inactivating two known substrates of ERK1/2, caspases-9 and Bim.

Figure 1.13 summarises the pathways that will be investigated in this project. The results of this study will provide further insight into the mechanism by which ERK1/2 may inhibit MIA-induced apoptosis and provide a rational basis for future therapeutic strategies.

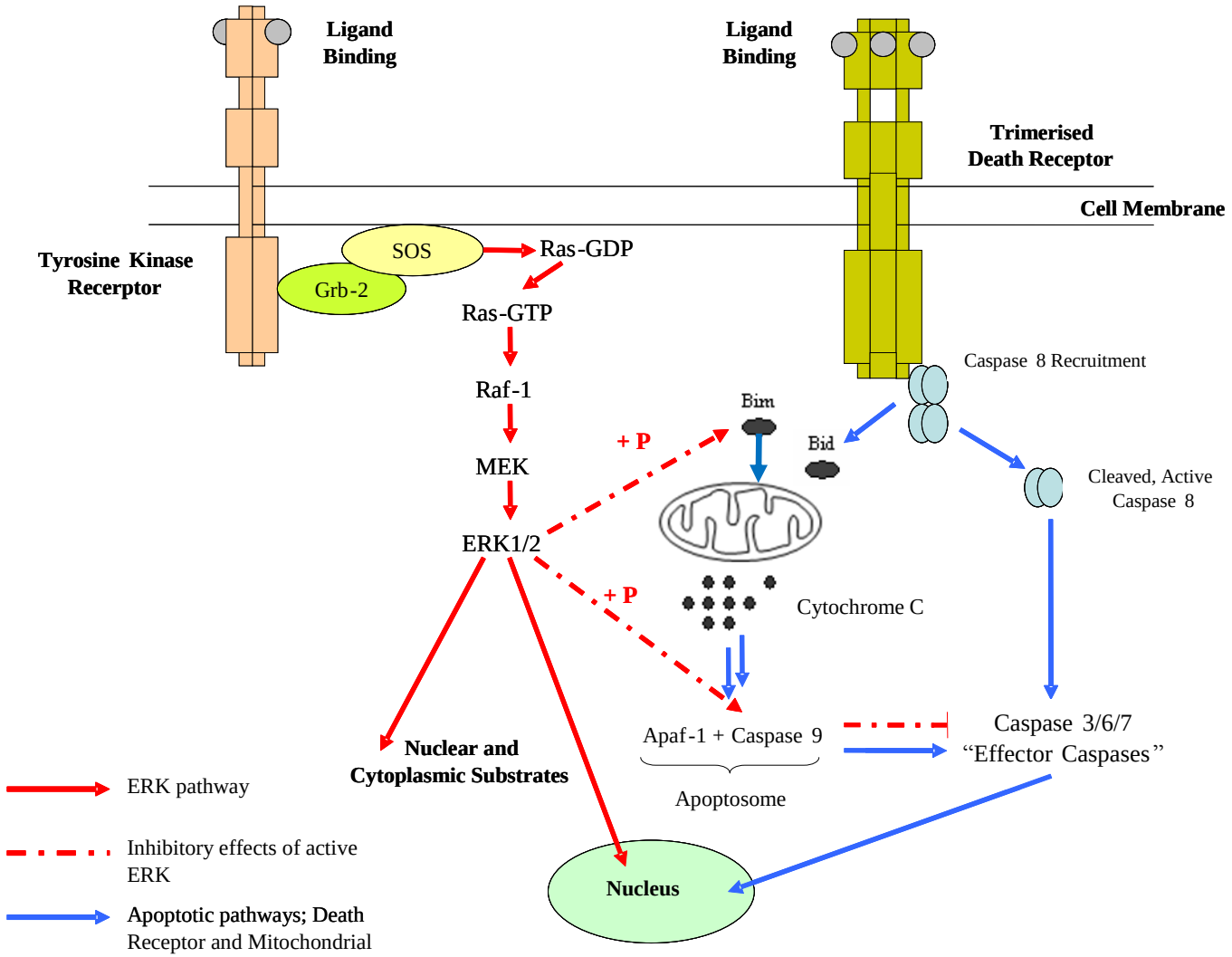


Figure 1.13: Summary of the pathways involved in drug-induced cell survival and death. As suggested by many studies, active ERK1/2 impinges on the apoptotic pathway to increase cell survival by targeting Bim and caspase-9. However, the apoptotic pathway involved in response to anti-cancer drugs is still unclear. This scheme illustrates some of the pathways that will be investigated in this project (highlighted in red).

CHAPTER 2
MATERIALS AND METHODS

2.1 Materials

2.1.1 Source of reagents

Reagent	Supplier
Protogel	Geneflow (Staffordshire, UK)
Foetal Bovine Serum, x100 Penicillin/Streptomycin, SeeBlue Plus 2^R prestained protein molecular weight markers	Invitrogen (Paisley, UK)
Ampicillin	Melford Laboratories (Suffolk, UK)
Prolong Gold Anti-Fade mounting medium	Molecular Probes (Invitrogen, Paisley, UK)
Agar, Yeast Extract, Tryptone	Oxoid (Basingstoke, UK)
Enhanced Chemiluminescence (ECL) reagent	Pharmacia Life Sciences (Kent, UK)
Coomassie Protein Assay Kit	Pierce (Rockford, USA)
High efficiency competent JM109 E-Coli cells	Promega (Southampton, UK)
Fugene 6 transfection reagent	Roche (Lewes, UK)
Dulbecco's Modified Eagles Medium (DMEM), Dulbecco's Phosphate Buffered Saline (PBS), without Ca^{2+} and Mg^{2+} ions,	Sigma (Poole, UK)

Cell Dissociation Medium, TEMED, Hoechst 33342 (1µM)) Poly-L-Lysine	

2.1.2 Drugs

All drugs used were purchased from Sigma apart from those indicated in the table below:

Drug	**Supplier**
U0126 MEK Inhibitor Staurosporine Okadaic Acid Etoposide Anisomysin	Calbiochem (Nottingham, UK)

2.1.3 Antibodies

Primary Antibodies	**Working Concentration**	**Application**	**Supplier**
Anti-Bim, polyclonal	1:2000	Western Blotting	Calbiochem (Nottingham, UK)
Anti-Phospho ERK1/2 Polyclonal Anti-Phospho Caspase-9	1:1000 1:1000	Western Blotting Immunofluorescence Western Blotting	Cell Signalling Technology (New England

Polyclonal Anti-Caspase-3 Monoclonal	 1:1000	 Western Blotting	Biolabs, UK)
Anti-Caspase-8 Polyclonal	1:1000	Western Blotting	Pharmingen
Anti-active MAPK (ERK1/2) Polyclonal	1:5000	Western Blotting	Promega (Southampton, UK)
Anti-Caspase-9 Polyclonal	0.2µg/ml	Western Blotting	Research Diagnostics Inc. (Massachusetts, USA)
Anti-α-PARP Polyclonal Anti-Cytokeratin-18 M30 Monoclonal	1:2000 1:1000	Western Blotting Immunofluorescence	Roche (Lewes, UK)
Anti-ERK1/2 Polyclonal Anti-HA Epitope, Polyclonal and Monoclonal	0.2µg/ml	Western Blotting Western Blotting	Santa Cruz Biotechnology (Insight Biotechnology, Middlesex, UK)
Anti-FLAG Monoclonal Anti-γ-tubulin Monoclonal	1:1000 1:1000	Western Blotting Western Blotting	Sigma (Poole, UK)
Anti-Bim Polyclonal	1µg/ml	Western Blotting	Stressgen (Cambridge BioScience,

			Cambridge, UK)
Anti-phospho BIM-EL Polyclonal	0.5µg/ml	Western Blotting	Upstate Cell Signalling Solutions
Secondary Antibodies			
Anti-rabbit Alexa Fluor 488nm Goat Anti-mouse Alexa Fluor 488nm Goat	2µg/ml 2µg/ml	Immunofluorescence Immunofluorescence	Molecular Probes (Invitrogen, Paisley, UK)
Anti-rabbit Horse-radish Peroxidase (HRP) Conjugate Anti-mouse Horse-radish Peroxidase (HRP) Conjugate	1µg/ml 1µg/ml	Western Blotting Western Blotting	Sigma (Poole, UK)

2.1.4 Buffers and Solutions

Buffer	Composition
RIPA cell lysis buffer	0.01M Tris-HCl pH 7.0, 0.15M NaCl, 2mM EDTA, 0.1% w/v SDS, 1% v/v NP-40, 0.5% w/v Na Deoxycholate, 50mM NaF, 30mM Na Pyrophosphate, H_2O up to 100ml, 100µM Na Orthovanadate and Protease Inhibitor cocktail
10x SDS PAGE Running Buffer	30.3g Tris, 144g Glycine, 10g SDS, H_2O to 1 litre
2x Sample Buffer	2ml Glycerol, 2ml of 10% SDS, 0.25mg Bromophenol blue,

	2.5ml Tris-SDS buffer (6.06g Tris, 4ml of 10% SDS, H_2O to 100ml, pH 8.8), 0.5ml β-mercaptoethanol, H_2O to 10ml
Transfer Buffer	3.6g Glycine, 0.75g Tris, 0.25 SDS, 50ml Methanol, H_2O to 250ml
10x Tris-Saline (pH 7.4)	0.1M Tris, 1.5M NaCl, H_2O
1x Tris-Saline-Tween (TST, pH 7.4)	100ml 10x Tris-Saline, 1ml 0.1% Tween 20, H_2O to 1 litre
Blocking Buffers	1 or 5% (w/v) BSA in TST 3 or 5% (w/v) Milk in TST 1% (w/v) BSA in PBS
Antibody Dilutants	1or 5% (w/v) BSA in TST 3 or 5% (w/v) Milk in TST
Sterile SOC medium (pH 7.0)	2g Tryptone, 0.5g Yeast Extract, 1ml of 1M NaCl, 0.25ml of 1M KCl, 1ml of 2M Mg^{2+}, 1ml 2M Glucose, H_20 to 100ml
Sterile LB Medium (pH 7.0)	10g Tryptone, 5g Yeast Extract, 10g NaCl, H_20 to 1 litre

2.1.5 Plasmids

Plasmid Vector	Application	Supplier
pcMV5-GFP-Erk2	Eukaryotic protein expression	Gift from Dr. M. Cobb, University of Texas
Flag-caspase-9	Eukaryotic protein expression	Gift from Dr. G. Nunez,

		University of Michigan
HA-Bim-EL	Eukaryotic protein expression	Gift from Dr. S. Cook, Babraham Institute, UK

2.1.6 Miscellaneous

Product	**Supplier**
Human Annexin V-FITC Kit	Bender MedSystems (UK)
SmartSpec 3000 spectrophotometer	Bio-Rad (Herts, UK)
Nikon 300 inverted microscope ORCA ER charge coupled device camera Openlab 5.09 software Adobe Photoshop for image editing	Nikon (Kingston upon Thames, UK) Hamamatsu (Shizuoka, Japan) Improvision (Coventry, UK) Adobe Systems (San Jose, CA)
Hoeffer Semi-Dry Blotter, Hybond-C Extra Nitrocellulose Membrane	Pharmacia Life Sciences
Qia Filter Plasmid Maxiprep Kit	Qiagen (Sussex, UK)

2.2 Methods

2.2.1 Cell Culture and Preparation of Cell Extracts

Human cervical carcinoma cells (HeLa) were cultured in DMEM (supplemented with 10% v/v FBS and 1% v/v Penicillin/Streptomycin) in a humidified incubator at 37 ^{o}C with 5% CO_2. To obtain mitotically arrested cells, an asynchronous population of Hela cells was treated with Nocodazole (3μM), Taxol (1μM), Vinblastine (1μM), or Vincristine (1μM) for various time points (0 to 24 hrs). Mitotic cells were collected by a mechanical shake-off, washed in cold PBS, and lysed in Radioimmunoprecipitation (RIPA) assay buffer as described previously (Patel *et al.*, 1998). Non-mitotic cells that remained attached to the plates were washed three times with cold PBS and lysed by scraping into RIPA buffer. Cell lysates were centrifuged at 14,000 x g for 10 minutes at 4^{o}C and the protein content was determined using the Coomassie protein assay reagent before normalising in RIPA buffer and solubilising in 2x SDS-PAGE sample buffer.

Alternatively, cell extracts were also made by lysing cells directly into 2x sample buffer after washing in cold PBS. The cell extracts were then sonicated for approximately 15 seconds prior to analysis.

2.2.2 Immunocytochemistry and Microscopy

HeLa cells were grown on coverslips in 6-well plates 24 hours before use and treated with Nocodazole (3μM), Taxol (1μM), Vinblastine (1μM), or Vincristine (1μM), with or without U0126 (10μM), for various times (0 to 24 hrs). The cells were washed three times in cold PBS and fixed in cold (-20˚C) methanol for 30 minutes. Mitotic cells were recovered by “shake-off”

and attached to poly-L-lysine (1mg/ml in H_2O)-coated coverslips. The mitotic cells were washed three times in cold PBS and fixed in cold (-20ºC) methanol for 30 minutes.

Fixed cells were then washed three times with PBS and blocked with 1% BSA in PBS for 45-60 minutes at room temperature. Cells were incubated with primary antibodies diluted in blocking buffer for one hour at room temperature, washed three times with PBS before incubation with 2˚ Alexa Fluor 488 goat anti-rabbit or anti-mouse IgG (diluted 1:1000 in blocking solution), for one hour at room temperature. The cells were washed three times with PBS and the nuclei labelled by staining with Hoechst 33342 (1μM in PBS) for 5 minutes at room temperature. Finally, the cells were washed three times with PBS before the coverslips were mounted onto slides using the mounting medium and sealed with clear nail varnish. Cells were observed and captured under a Nikon 300 inverted microscope using an ORCA ER camera. Images were sorted using Openlab 5.09 software and processed in Adobe Photoshop (see 2.1.6).

2.2.3 Transient Transfection

HeLa cells were grown in 6-well plates (either on coverslips for use in immunofluoresence or directly on the plate for lysing) and 24 hours later, cells were transfected with plasmid DNA using FuGENE 6 according to the manufacturer's protocol. 18 to 24 hours after transfection, the cells were treated with Nocodazole (3μM), Taxol (1μM), Vinblastine (1μM) or Vincristine (1μM) for various times (0 to 24 hours) and were either prepared for immunofluorescence (see 2.2.2) or lysed to produce cell extracts for Western blotting (see 2.2.1 and 2.2.4).

2.2.4 SDS PAGE and Western Blotting

Cell lysates solubilised in SDS-PAGE sample buffer were resolved by SDS-PAGE (using the appropriate percentage SDS gel) and electro-blotted onto nitrocellulose membrane using a Hoeffer semi-dry blotting apparatus. Membranes were blocked in the appropriate blocking buffer recommended by the manufacturer for one hour at room temperature. Incubation with 1˚ antibody was overnight at 4˚C. The membranes were washed three x 5 minutes in TST before incubation with 2˚ antibody for one hour at room temperature. Proteins were visualised using ECL reagent and developed on X-ray film.

2.2.5 Bacterial Transformation

To 1µl of plasmid, 50µl of JM109 E-coli cells were added and incubated on ice for 20 minutes. The cells were then heat-shocked for 45-50 seconds in a water bath set to 42°C, after which the cells were returned to ice for a further 2 minutes. 950µl of room temperature SOC medium was added to the bacterial cells and the suspension incubated for 1.5 hours at 37°C, with mixing every 15 minutes. The bacteria were pelleted by centrifugation at 14,000rpm for 15 seconds at room temperature. 900µl of the supernatant was discarded and the bacterial pellet resuspended in the remaining medium. This bacterial suspension was plated onto an agar plate containing Ampicillin (1µg/ml) and incubated overnight at 37°C. A single colony was picked and used to inoculate 500ml LB medium containing Ampicillin (1µg/ml). The plasmid was extracted and purified using the Qiagen maxi-prep kit and protocol. To measure the concentration of DNA, 5µl of purified plasmid was diluted in 495µl of water and its absorbance measured at 260nm, using a spectrophotometer (BioRad), where an absorbance reading of 1.0 corresponded to 50µg/ml of DNA.

2.2.6 Flow Cytometry

To analyse apoptosis using FITC-labelled annexin-V, flow cytometry was used. Hela cells were grown on 10cm tissue cultured plates and treated with Nocodazole (3μM), with and without a 15 minute pre-treatment with U0126, for various time points (0 to 24 hours). As a positive control, cells were treated with Etoposide for 24 hours. Two populations of cells were collected. First, the non-mitotic, attached population was lifted off by washing once with warm PBS and incubating with cell dissociation medium for 20 minutes. Once cells had detached fresh warm DMEM (with FBS and antibiotics) was added to incubated for 30 minutes to allow the cells to recover from detachment and spun down at 11,000 rpm for 5 minutes to pellet the cells. The medium was discarded and the cells were washed in 5ml PBS and pelleted by centrifugation (11,000rpm, 5min at room temperature). The cells were then resuspended in 500μl of Annexin-binding buffer and 5μl FITC-Annexin-V added.

Secondly, the mitotic population were collected by mechanical shake-off and spun at 11,000 rpm for 5 minutes. The medium was discarded and spun again in PBS. As above, the cells were resuspended in 500μl of Annexin-binding buffer and 5μl FITC-Annexin-V added. The cells were then prepared for FACS analysis using the reagents and protocol provided by the manufacturer. An additional 300μl of cold PBS was added to each sample to give a minimum of 500μl required for FACS analysis.

CHAPTER 3
RESULTS

3.1 ERK1/2 Activation and Intracellular Localisation in Response to MIAs

3.1.1 Activation State of ERK1/2

To investigate whether therapeutic concentrations of MIAs activate ERK1/2, an asynchronous population of Hela cells was treated with 0.1, 1, 10 or 100 µM of Taxol, Vinblastine or Vincristine or 0.3, 3, 33 and 330 µM of Nocodazole, for one hour and the cells lysed in RIPA buffer (section 2.2.1). Control cells were treated with an equivalent volume of DMSO alone as a negative control and positive control cells were treated with Phorbol Myristate Acetate (PMA, 1µg/ml) for one hour, a known activator of ERK1/2 (Anselmo *et al.,* 2001). The cell extracts were normalized using the Coomassie protein assay kit, resolved by SDS PAGE and Western blotted (section 2.2.4) with an anti-active ERK1/2 (or P-ERK1/2) antibody according to the manufacturers protocol. The P-ERK1/2 antibody detects ERK1/2 that is phosphorylated on Thr183 and Tyr185 (Widman *et al.,* 1999). Figure 3.1 shows the effect of varying concentrations of MIAs on ERK1/2 activation. All four drugs also activated ERK1/2 in a concentration-dependent manner (Figure 3.1), however, Nocodazole activated ERK1 and ERK2 equally whereas Taxol, Vinblastine and Vincristine appeared to preferentially activate ERK2 at low concentrations (0.1 and 1µM). Cells treated with PMA showed strong phosphorylation of ERK1/2 compared to DMSO-treated control cells. The same blots were washed and immunoblotted with a polyclonal antibody to detect total ERK1/2 protein. The data indicate that the level of total ERK1/2 protein was uniform in each sample which shows that the changes observed in the levels of phosphorylated ERK1/2 are not the result of differences in the amount of ERK1/2 in each lane. As 1µM concentration of Taxol, Vinblastine and Vincristine, and a 3µM concentration of Nocodazole were able to activate ERK1/2, these concentrations were used in subsequent experiments.

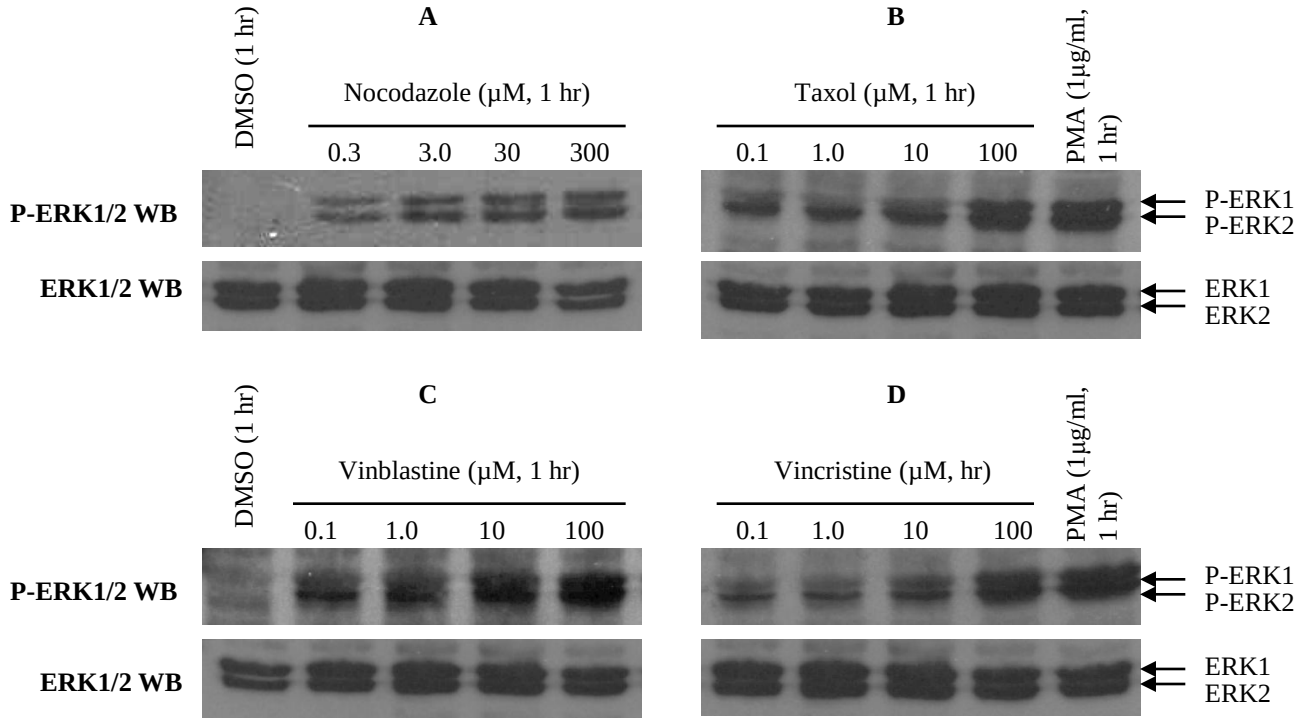


Figure 3.1: The effect of MIAs on ERK1/2 activity. An asynchronous population of HeLa cells was treated with varying concentrations of either (A) Nocodazole, (B) Taxol, (C) Vinblastine, (D) Vincristine, DMSO alone (0.01% v/v) or PMA alone (1μg/ml) for one hour. Cell extracts were prepared as described in Materials and Method (see section 2.2.1) and immunoblotted with an anti-P-ERK1/2 antibody (Upper panel, A - D) or with an anti-ERK1/2 antibody (Lower panel, A – D). WB = Western Blot

Next, a time course analysis of ERK1/2 activation by the MIAs was performed. An asynchronous population of Hela cells was treated with either Taxol, Vinblastine, Vincristine (all at 1μM) or Nocodazole (3μM) for varying times. Cells were first separated into non-mitotic and mitotic populations (see section 2.2.1), lysed in RIPA buffer and protein concentrations normalized. The cell lysates were resolved by SDS-PAGE and Western blotted using a P-ERK1/2 antibody (Figure 3.2). All four drugs caused rapid activation of ERK1/2, as ERK1/2 was phosphorylated within 30 minutes of drug addition. Phosphorylation of ERK1/2 was seen up to 16 hours following MIA treatment in the attached, non-mitotic populations, whereas ERK1/2 phosphorylation was reduced in the mitotic populations (12 and 16 hours after MIA addition). The reason for this is not understood but this outcome was consistently observed in the response to all four drugs.

3.1.2 Intracellular Localisation of ERK1/2 Following Activation by PMA

ERK1/2 translocation into the nucleus is an indicator of ERK1/2 activation (Lenormand *et al.*, 1998) therefore ERK1/2 localisation was determined following drug addition using the same P-ERK1/2 antibody that was used for Western blotting. Hela cells were grown on coverslips 24 hours before use and were treated with PMA (1μg/ml) for either 30 minutes or one hour. The coverslips were processed for immunofluorescence as described in section 2.2.2 and then incubated with varying concentrations of the P-ERK1/2 antibody to determine the optimum antibody concentration required to detect active ERK1/2 in cells. The results indicate that the P-ERK1/2 antibody was able to detect active ERK1/2 at all concentrations tested in cells that had been treated with PMA for either 30 minutes or one hour (Figure 3.3). However, clear translocation of P-ERK1/2 into the nucleus was only seen following treatment of Hela cells with PMA for 60 minutes when the P-ERK1/2 antibody was used at a dilution of 1:200.

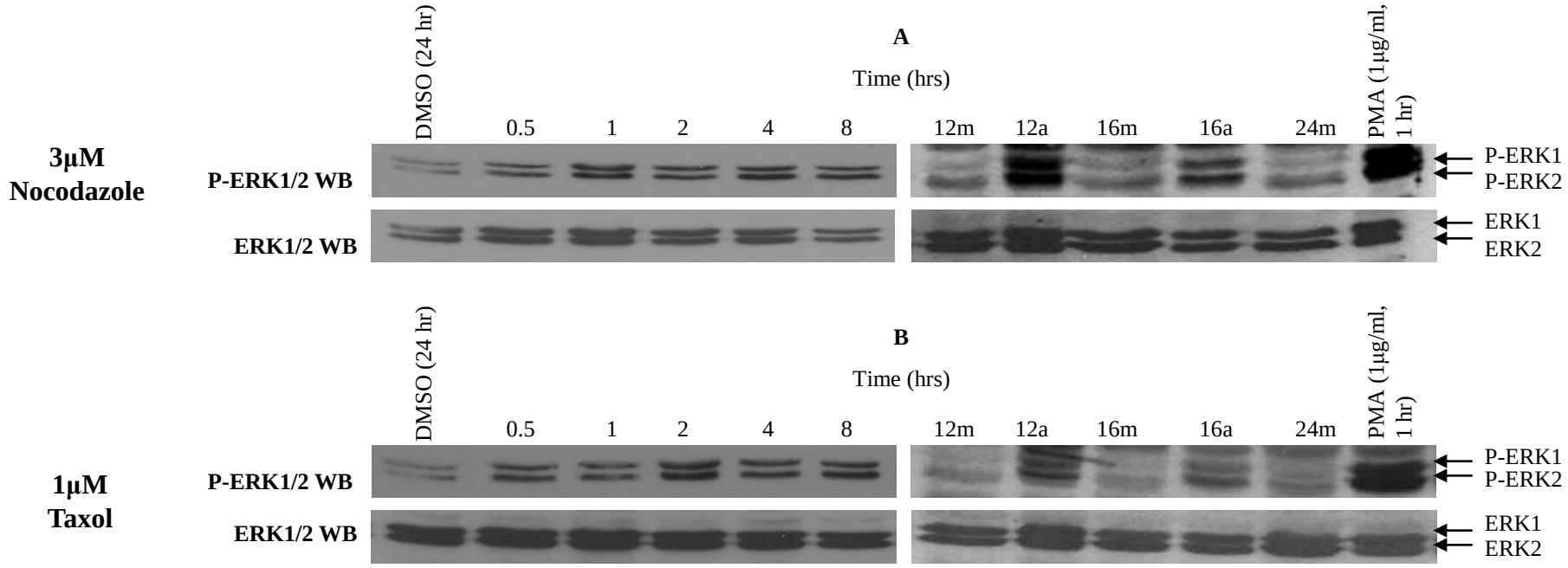
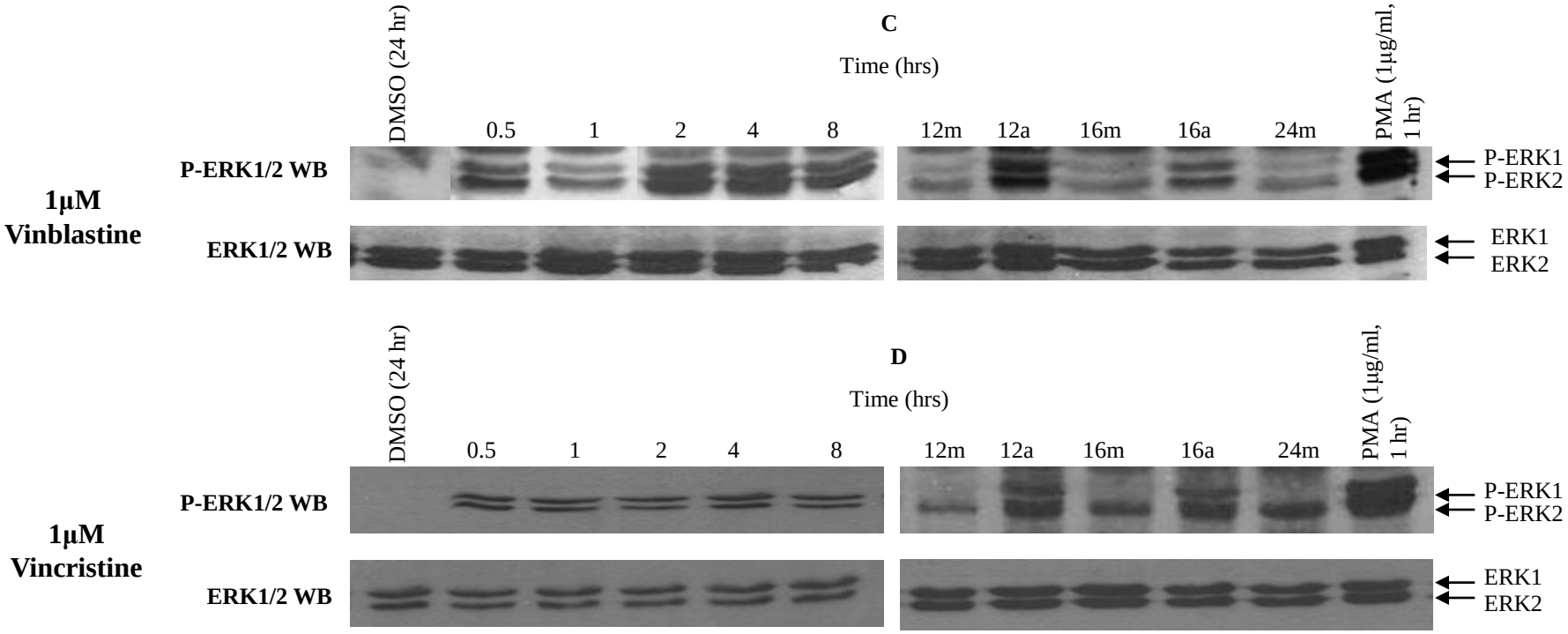


Figure 3.2: Time course of ERK1/2 activation by Nocodazole, Taxol, Vinblastine and Vincristine – An asynchronous population of HeLa cells was treated with either (A) Nocodazole, (B) Taxol, (C) Vinblastine, (D) Vincristine, DMSO alone (0.01% v/v) or PMA alone (1µg/ml) and cell extracts prepared at the indicated times post drug addition. After 8hr drug treatment the mitotic cells (m = mitotic population) were separated by shake-off from the non-mitotic, attached cells (a = attached population) prior to cell lysis. The cell lysates were immunoblotted with a P-ERK1/2 antibody (Upper panel, A – D) and an anti-ERK1/2 antibody (Lower panel, A – D). WB = Western Blot


C
Time (hrs)
DMSO (24 hr)
0.5
1
2
4
8
12m
12a
16m
16a
24m
PMA (1µg/ml, 1 hr)
1µM Vinblastine
P-ERK1/2 WB
ERK1/2 WB
P-ERK1
P-ERK2
ERK1
ERK2
D
Time (hrs)
DMSO (24 hr)
0.5
1
2
4
8
12m
12a
16m
16a
24m
PMA (1µg/ml, 1 hr)
1µM Vincristine
P-ERK1/2 WB
ERK1/2 WB
P-ERK1
P-ERK2
ERK1
ERK2

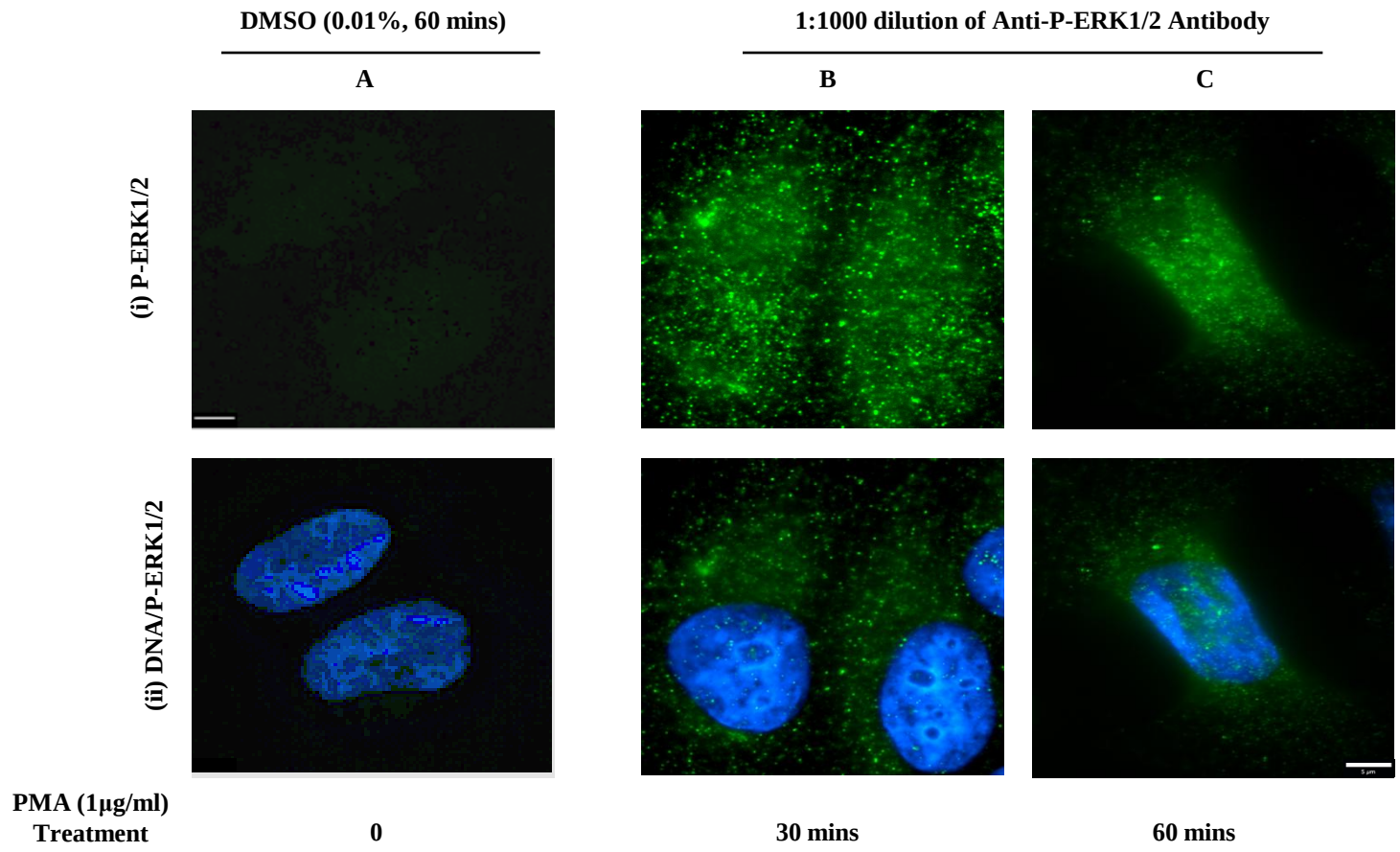
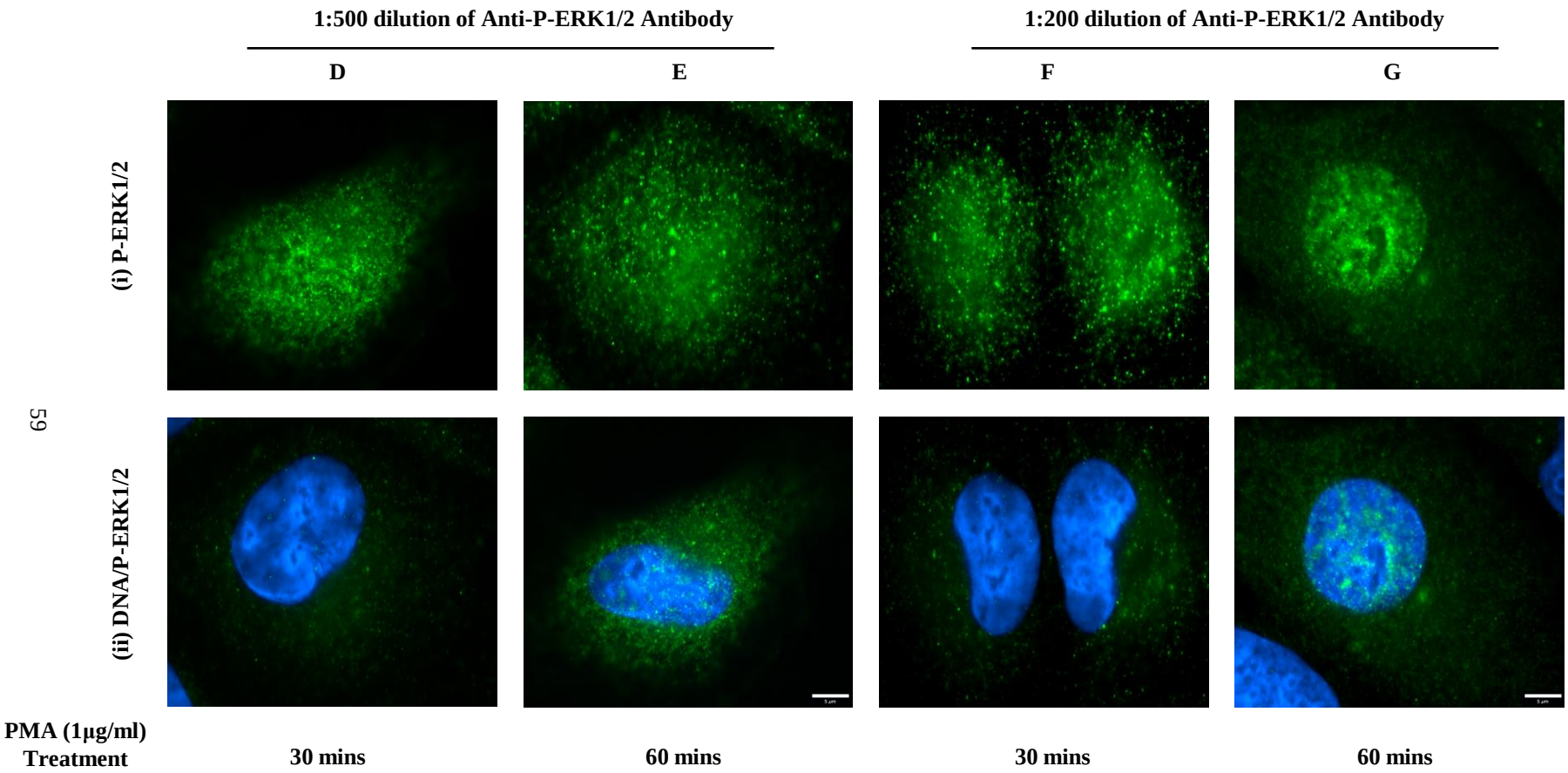


Figure 3.3: Optimisation of the anti-P-ERK1/2 antibody for immunocytochemistry – Hela cells were grown on coverslips and treated with either (A) DMSO alone (0.01% v/v) or 1µg/ml PMA for either 30 minutes (B, D and F) or one hour (C, E and G). The cells were then fixed and incubated with anti-P-ERK1/2 antibody at various dilutions (1:1000, 1:500 and 1:200). The images in the upper panel (i) show P-ERK1/2 staining (green) and the images in the lower panel (ii) show the superimposed image of Hoechst 33342-stained DNA (blue) and P-ERK1/2. Scale bar = 5µm.


1:500 dilution of Anti-P-ERK1/2 Antibody
1:200 dilution of Anti-P-ERK1/2 Antibody
D
E
F
G
(i) P-ERK1/2
(ii) DNA/P-ERK1/2
PMA (1µg/ml) Treatment
30 mins
60 mins
30 mins
60 mins

To ensure that the staining observed with the P-ERK1/2 antibody was specific, Hela cells were treated with PMA (1µg/ml) for 30-60 minutes or with an equivalent volume of DMSO alone. The results (Figure 3.4) indicated that no staining was observed in the absence of ERK1/2 activation (Figure 3.4A) whereas in the PMA-treated cells there was a time-dependent translocation of P-ERK1/2 from the cytoplasm to the nucleus (Figure 3.4B, C).

The results of this experiment were quantitated by counting 100 cells in random fields to determine the number of cells that displayed a nuclear localisation of P-ERK1/2 at various intervals following PMA addition. The results (Figure 3.5) showed that within 30 minutes of PMA addition, approximately 38% of the cells displayed P-ERK1/2 in the nucleus. Translocation of P-ERK1/2 into the nucleus was maximum at 60 minutes post PMA addition (60% of cells displaying nuclear P-ERK1/2 after staining) after which there was a gradual decrease in the number of cells showing nuclear P-ERK1/2. Therefore the results of this experiment provided evidence of P-ERK1/2 translocation into the nucleus following activation with PMA.

3.13 Intracellular Localisation of ERK1/2 Following Activation by MIAs

Two methods were used to investigate the intracellular distribution of ERK1/2 in response to MIA treatment. First, Hela cells were treated with Taxol, Vinblastine, Vincristine (all at 1µM) or Nocodazole (3µM) for various times. P-ERK1/2 was detected by immunofluorescence microscopy using the anti-P-ERK antibody. In the second method, Hela cells were transfected with GFP-ERK1 as described in Materials and Methods (section 2.2.3). The transfected cells were then treated with Taxol, Vinblastine, Vincristine (all at 1µM) or Nocodazole (3µM) for varying times.

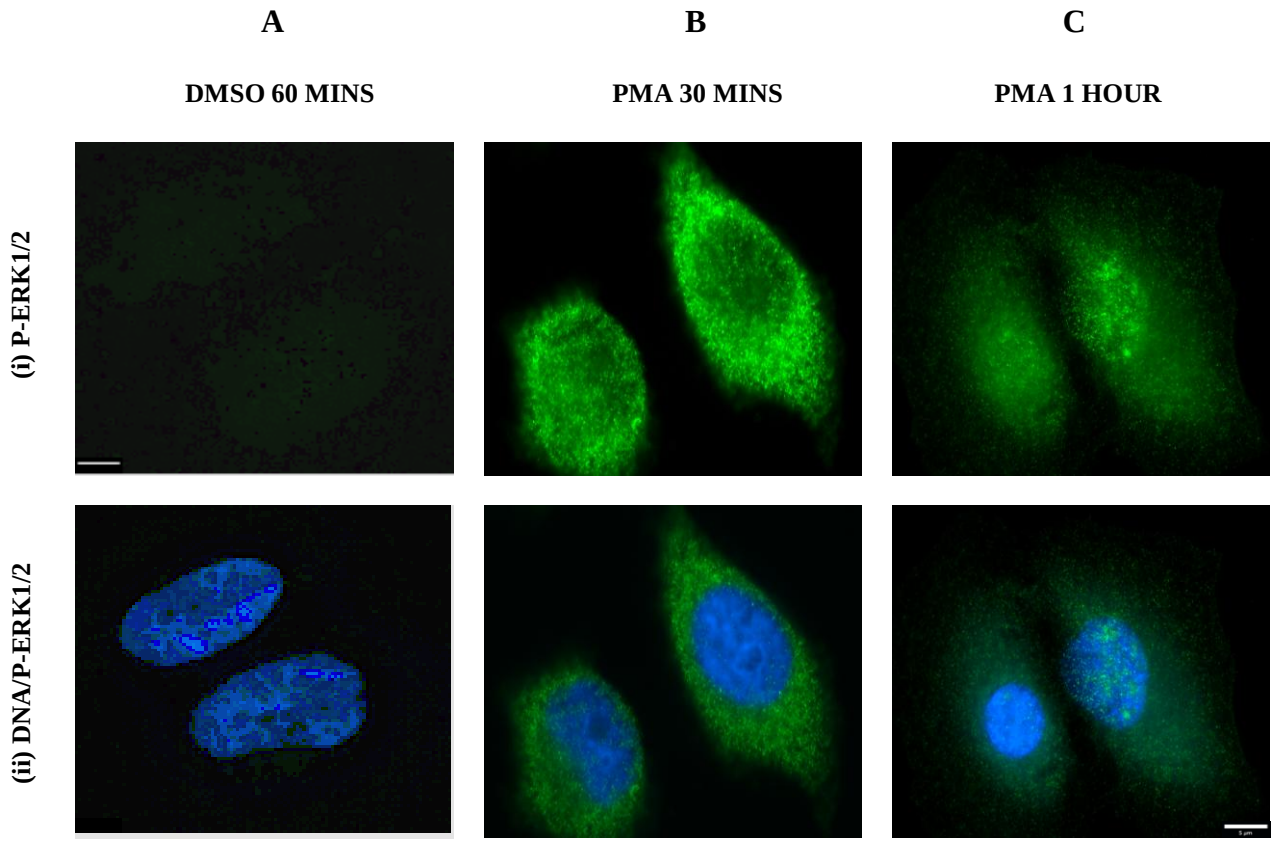


Figure 3.4: PMA induces translocation of P-ERK into the nucleus – An asynchronous population of Hela cells was treated with either PMA (1μg/ml) or DMSO (0.01% v/v) alone for 30-60 minutes. The cells were fixed and stained with an anti-P-ERK1/2 antibody as described in Materials and Methods (see section 2.2.2). (A) DMSO-treated cells, (B) PMA treatment for 30 minutes, (C) PMA treatment for 60 minutes. Images in the upper panel (i) show P-ERK1/2 staining (green) and images in the lower panel (ii) show a merge of P-ERK1/2 and DNA-stained with Hoechst 33342 (blue). Scale bar = 5μm.

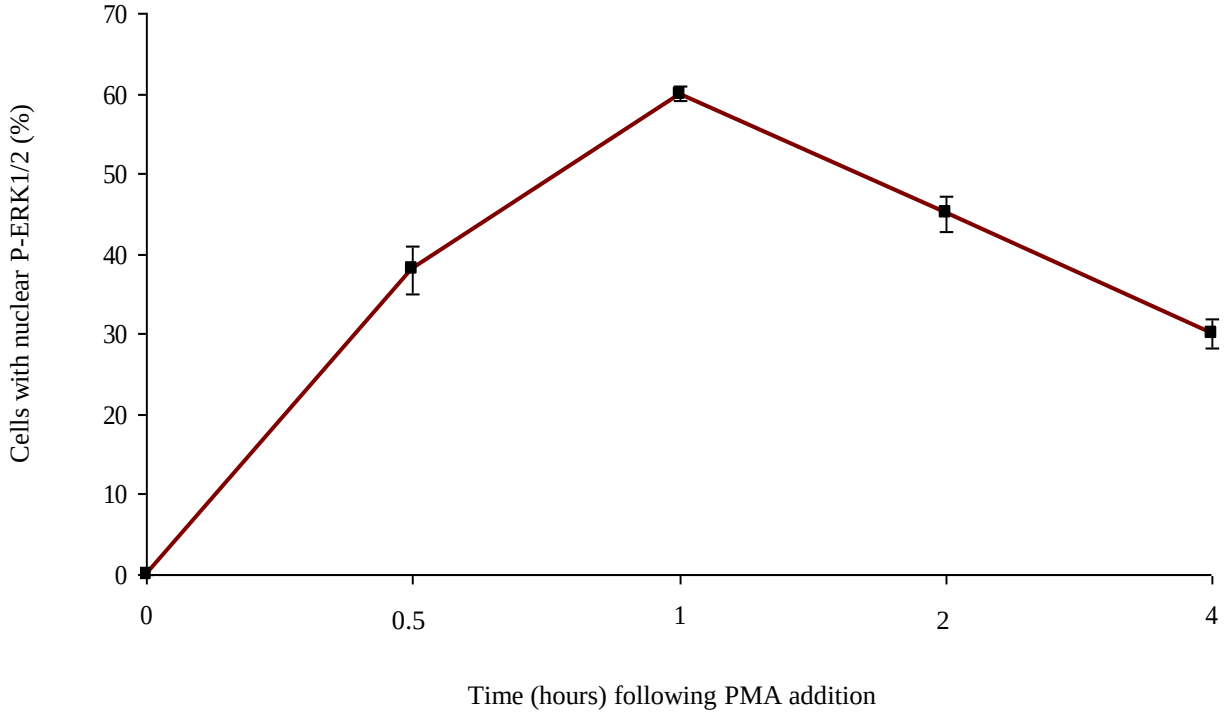


Figure 3.5: Time course of PMA-induced ERK1/2 translocation into the nucleus – An asynchronous population of Hela cells were treated with PMA (1μg/nl) for the indicated times. The cells were then fixed and stained with the anti-P-ERK1/2 antibody as described in Materials and Methods (see section 2.2.2). 100 cells were counted in random field at each time point and the percentage of cells displaying P-ERK predominantly in the nucleus was determined. The plot shows the mean (SD) of 3 separate experiments.

The results (Figure 3.6 - Figure 3.9) showed that all four drugs caused rapid ERK1/2 phosphorylation (within 30 minutes to one hour) and its translocation from the cytoplasm to the nucleus. However, P-ERK1/2 translocation was more clearly observed when assessed using GFP-ERK1. Treatment with Nocodazole induced high levels of ERK1/2 phosphorylation that was detected by both methods (Figure 3.6). At 30 minutes post Nocodazole addition, P-ERK1/2 was present throughout the cell whereas at one hour P-ERK1/2 appeared to be localised in the nucleus. After four hours of Nocodazole treatment, antibody detection indicated that P-ERK1/2 remained inside the nucleus (Figure 3.6 A, B) whereas GFP-ERK1 detection showed P-ERK1/2 located throughout the cell (Figure 3.6 C, D). Results for Taxol treatment (Figure 3.7) also indicated that ERK1/2 is rapidly activated and located throughout the cell after 30 minutes. At one hour post Taxol addition, P-ERK1/2 had translocated to the nucleus and at four hours, P-ERK1/2 reappeared throughout the cell as seen by both methods of detection. Treatment with Vinblastine (Figure 3.8) and Vincristine (Figure 3.9) indicated that both drugs affected the distribution of P-ERK1/2 in a manner similar to that observed with Taxol treatment. In summary, all four drugs induced phosphorylation and translocation of ERK1/2 within one hour of treatment.

The results of this experiment were quantitated by counting 100 cells in random fields to determine the percentage of cells with P-ERK1/2 in the nucleus at intervals after each drug addition. The results (Figure 3.10) indicate that all four drugs induced rapid translocation of P-ERK1/2 within 30 minutes to one hour following drug treatment. After one hour, the percentage of cells with nuclear P-ERK1/2 decreases indicating P-ERK1/2 translocated back to the cytoplasm.

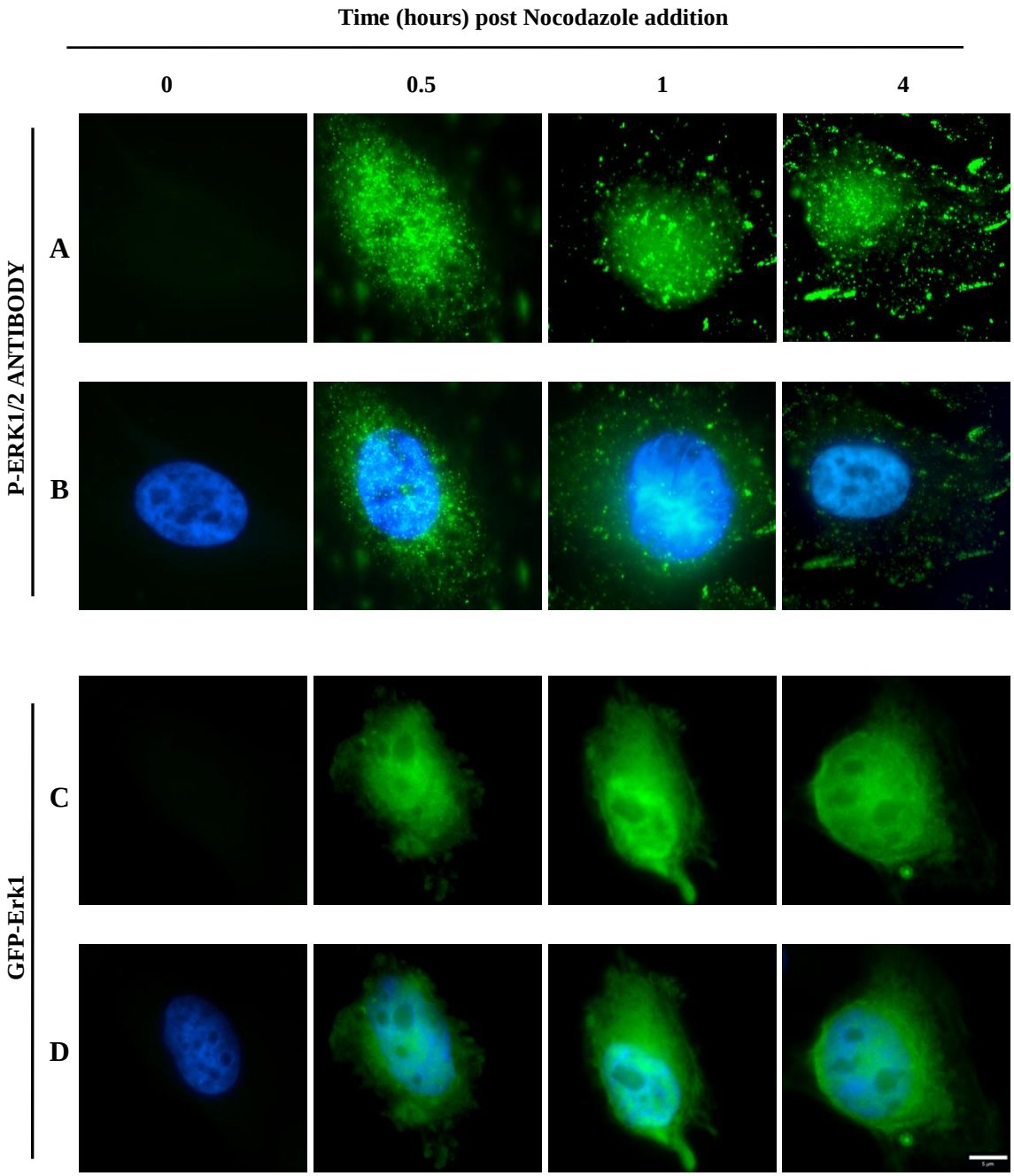


Figure 3.6: Time course of ERK1/2 translocation into the nucleus after treatment of Hela cells with Nocodazole. Hela cells were treated with Nocodazole (3μM) for the indicated times. The intracellular localisation of active ERK1/2 was determined using either the anti-P-ERK1/2 antibody (A, B) or GFP-Erk1 (C, D). (B) Merged images of DNA (blue) and anti-P-ERK1/2 (green), (D) merged images of GFP-Erk (green) and DNA (blue). Scale bar = 5μm.

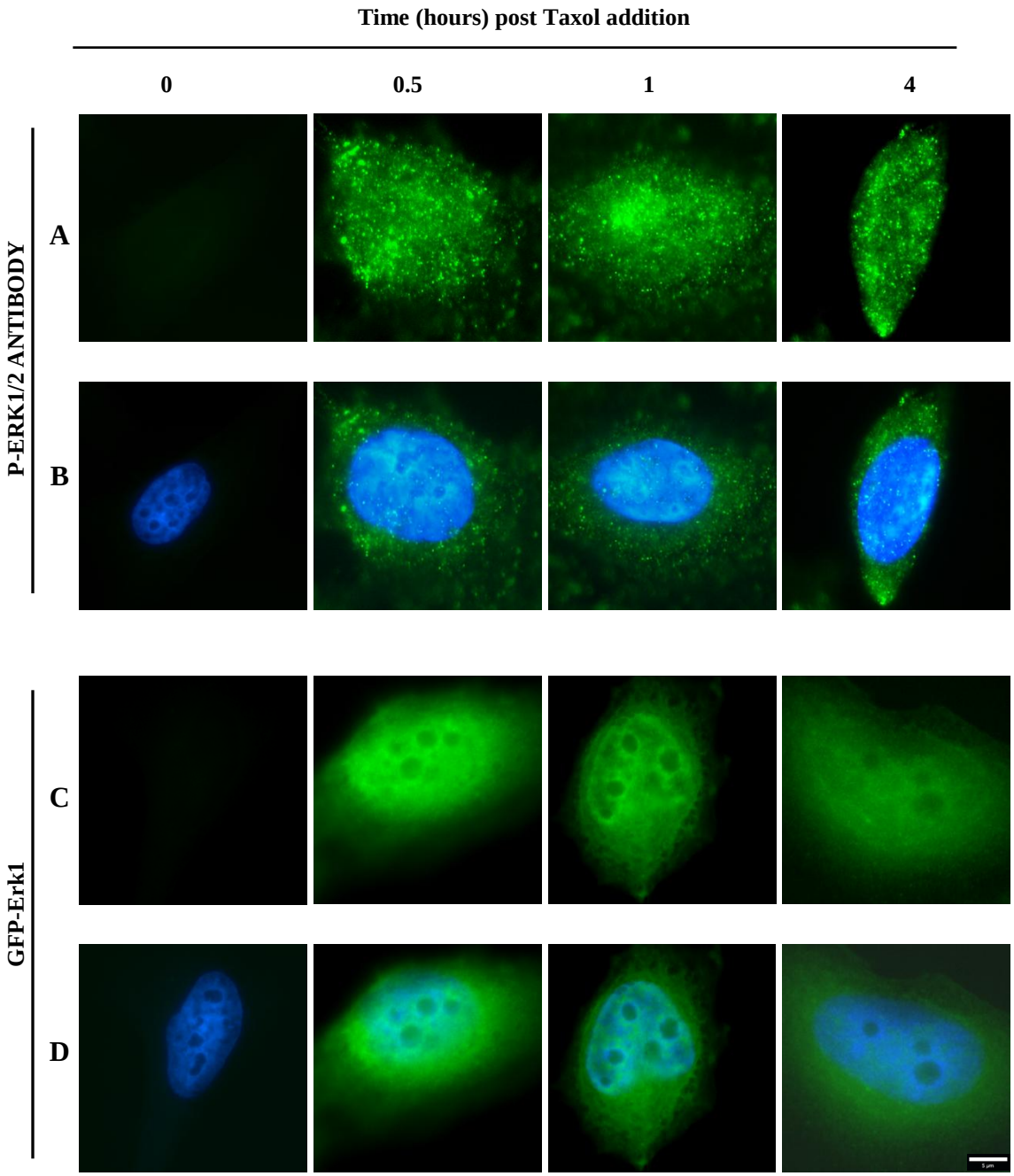


Figure 3.7: Time course of ERK1/2 translocation into the nucleus after treatment of Hela cells with Taxol. Hela cells were treated with Taxol (1μM) for the indicated times. The intracellular localisation of active ERK1/2 was determined using either the anti-P-ERK1/2 antibody (A, B) or GFP-Erk1 (C, D). (B) Merged images of DNA (blue) and anti-P-ERK1/2 (green), (D) merged images of GFP-Erk (green) and DNA (blue). Scale bar = 5μm.

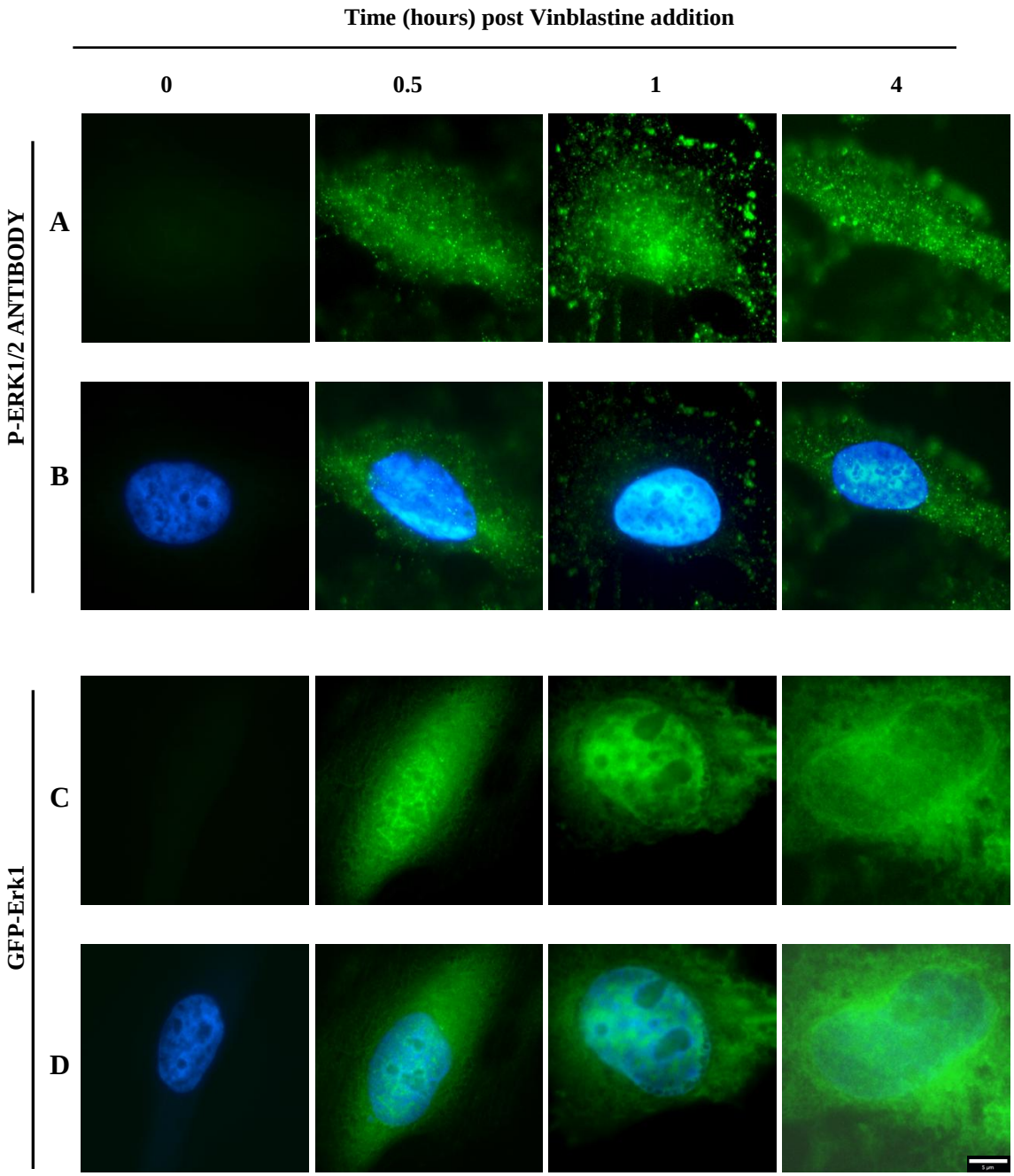


Figure 3.8: Time course of ERK1/2 translocation into the nucleus after treatment of Hela cells with Vinblastine. Hela cells were treated with Vinblastine (1μM) for the indicated times. The intracellular localisation of active ERK1/2 was determined using either the anti-P-ERK1/2 antibody (A, B) or GFP-Erk1 (C, D). (B) Merged images of DNA (blue) and anti-P-ERK1/2 (green), (D) merged images of GFP-Erk (green) and DNA (blue). Scale bar = 5μm.

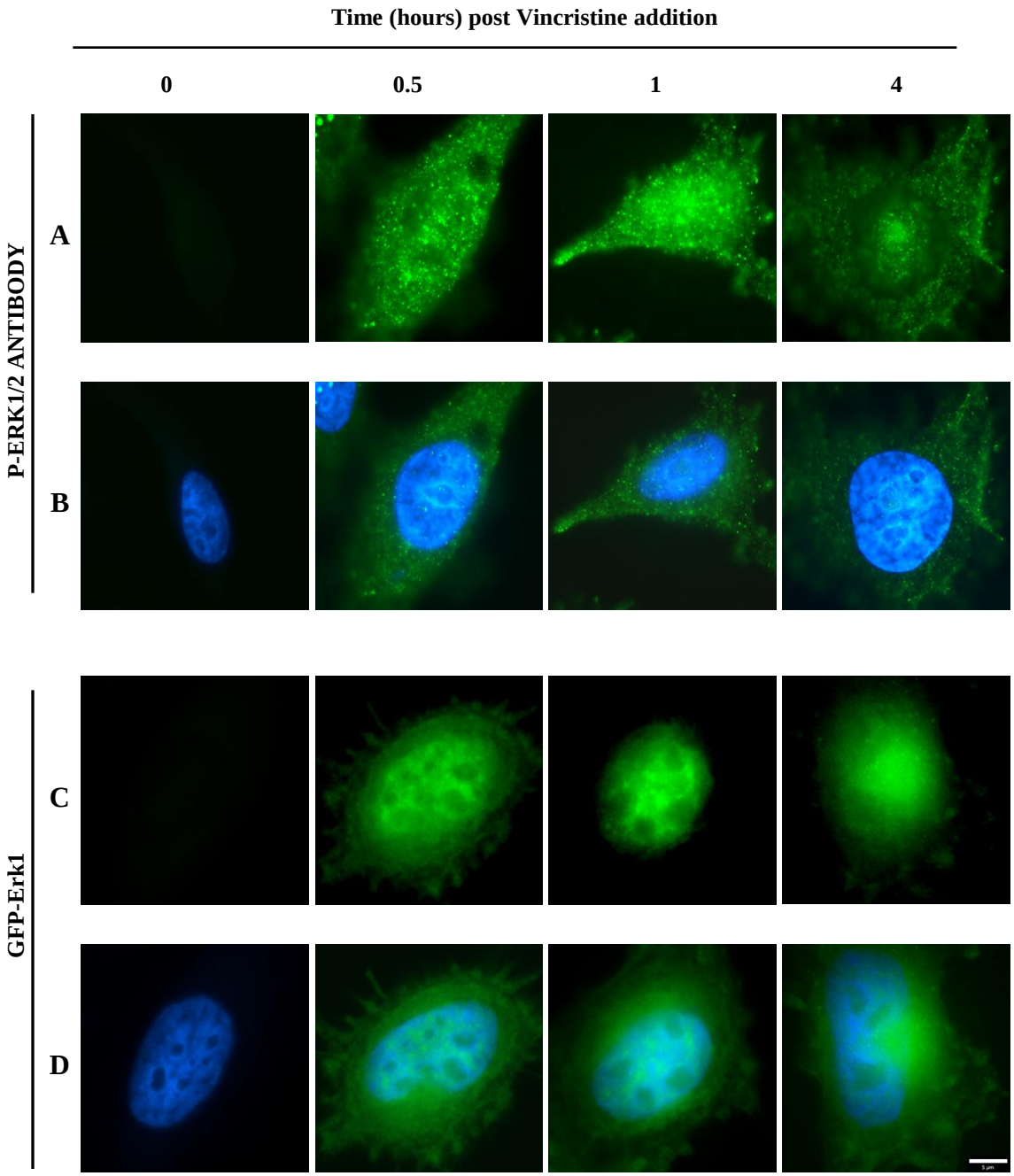


Figure 3.9: Time course of ERK1/2 translocation into the nucleus after treatment of Hela cells with Vincristine. Hela cells were treated with Vinbcristine (1μM) for the indicated times. The intracellular localisation of active ERK1/2 was determined using either the anti-P-ERK1/2 antibody (A, B) or GFP-Erk1 (C, D). (B) Merged images of DNA (blue) and anti-P-ERK1/2 (green), (D) merged images of GFP-Erk (green) and DNA (blue). Scale bar = 5μm.

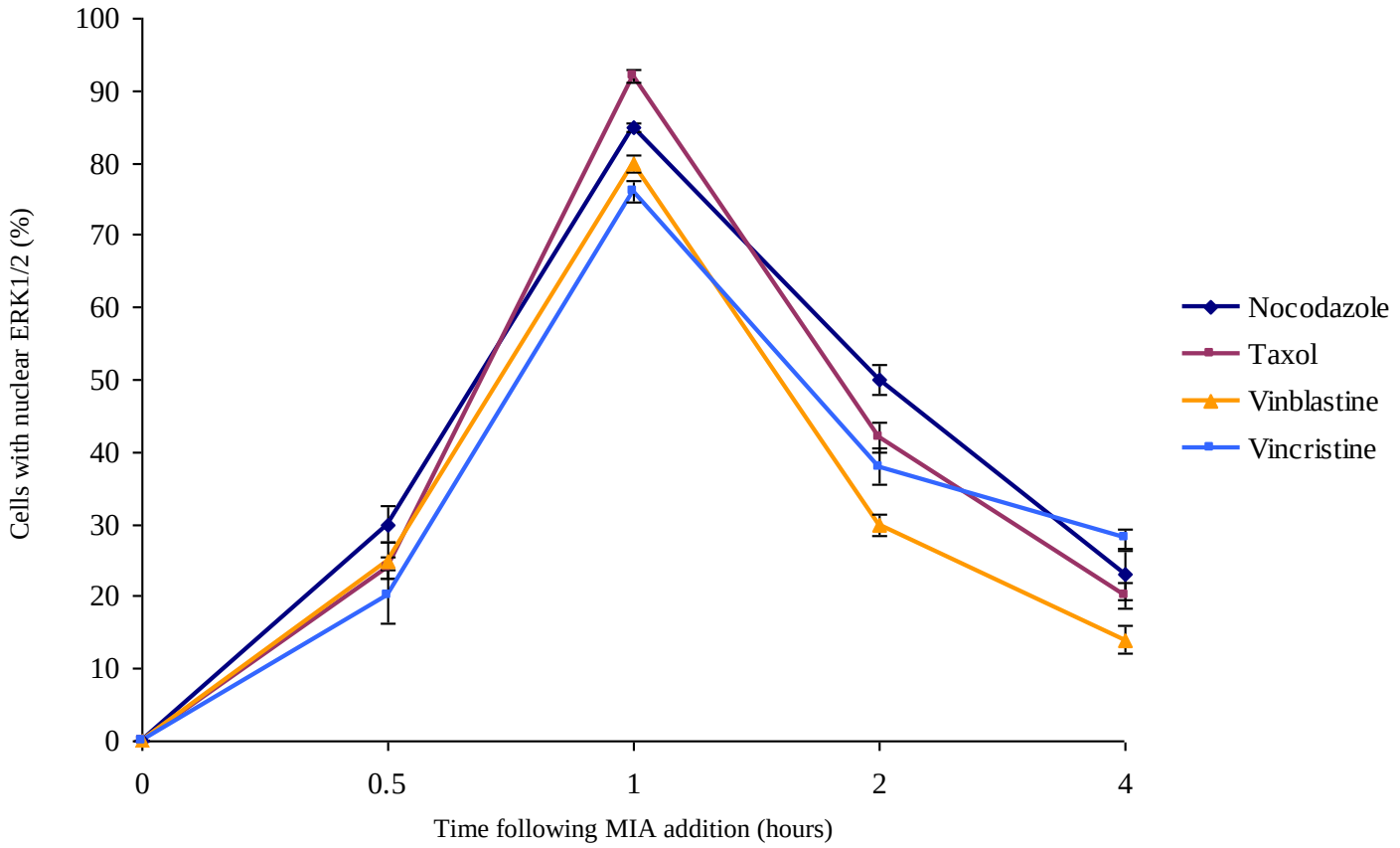


Figure 3.10: MIAs induce translocation of ERK1/2 into the nucleus. Hela cells were treated with either Nocodazole (3μM), Taxol (1μM), Vinblastine (1μM) or Vincristine (1μM) for the indicated times. Cells were then stained with anti-P-ERK1/2 antibody. 100 cells were counted in random fields at each time point and the percentage of cells displaying P-ERK predominantly in the nucleus was determined. The plot shows the mean (SD) of 3 separate experiments.

3.2 The Effect of Inhibiting ERK1/2 Activation on Cell Death

3.2.1 Inhibiting ERK1/2 Using U0126

After determining that ERK1/2 is rapidly phosphorylated and translocated to the nucleus in response to MIAs, I investigated the consequence of inhibiting MIA-induced ERK1/2 activity. First, the effect of a range of concentrations of U0126 (0 to 20μM) on PMA-induced ERK1/2 activation was examined. Cells were pre-incubated with U0126 (0 to 20μM) or DMSO alone for 15 minutes before the addition of PMA (1μg/ml final concentration). After further 60 minutes incubation, the cells were lysed and the activation state of ERK1/2 was examined by immunoblotting with the P-ERK1/2 antibody. The results (Figure 3.11A, top panel) indicate that U0126 caused a dose-dependent inhibition of PMA-induced ERK1/2 activation. However, U0126 inhibited ERK1 more potently than ERK2. An immunoblot with an ERK1/2 antibody confirmed that the changes in ERK1/2 phosphorylation were not as a result of variations in the levels of ERK1/2 protein (Figure 3.11A, bottom panel).

Next, the effect of U0126 (0 to 20μM) on MIA-induced ERK1/2 activation was examined. Cells were pre-treated with U0126 (0 to 20μM) for 15 minutes before the addition of either Nocodazole (3μM), Taxol (1μM), Vinblastine (1μM) or Vincristine (1μM). After further 60 minutes incubation, the cells were lysed and the activation state of ERK1/2 was examined with the P-ERK1/2 antibody. As with PMA treatment, U0126 caused a dose-dependent inhibition of MIA-induced ERK1/2 activation (Figure 3.11 B, C, D and E, top panels). ERK1 was inhibited more potently than ERK2 in Nocodazole, Taxol, Vinblastine and Vincristine-treated cells. The levels of ERK1/2 protein remained constant throughout drug-treatment, as seen with the anti-ERK1/2 antibody (Figure 3.11 B, C, D and E, bottom panels), confirming that the activation state of ERK1/2 varied in response to the MIAs and U0126.

The effect of U0126 on ERK1 inhibition was observed at concentrations of U0126 between 10 and 20μM. Therefore, a time course experiment was performed to determine whether 10μM of U0126 could sustain ERK1/2 inhibition following the addition of Nocodazole or Taxol. Hela cells were pre-treated with U0126 (10μM) for 15 minutes prior to the addition of either Nocodazole (3μM) or Taxol (1μM). Cells were incubated with the MIAs for 2 to 8 hours before examining ERK1/2 activity by immunoblotting with the P-ERK1/2 antibody. The results (Figure 3.12, top panel) shows that U0126 (10μM) gradually inhibited Nocodazole-induced activation of ERK1 and ERK2 over the time points examined, whereas U0126 (10μM) inhibited Taxol-induced activation of ERK1 and ERK2 at 2 and 4 hours with some ERK1/2 activation visible after 8 hours of treatment. Although ERK1/2 activation can be seen after 8 hours of treatment with Taxol plus U0126, this ERK1/2 activation seems significantly reduced in comparison to cells treated with Taxol in the absence of U0126, suggesting that U0126 is effective in inhibiting ERK1/2 activation.

The reduction in ERK1/2 activation in response to U0126 again was not the result of variations in the levels of ERK1/2 protein, as shown by the immunoblot with the anti-ERK1/2 antibody (Figure 3.12, bottom panel).

The results described above confirm that U0126 can inhibit ERK1/2 activation by MIAs; since U0126 inhibits MEK1/2, this suggests that MIA-induced activation of ERK1/2 is MEK1/2 dependent.

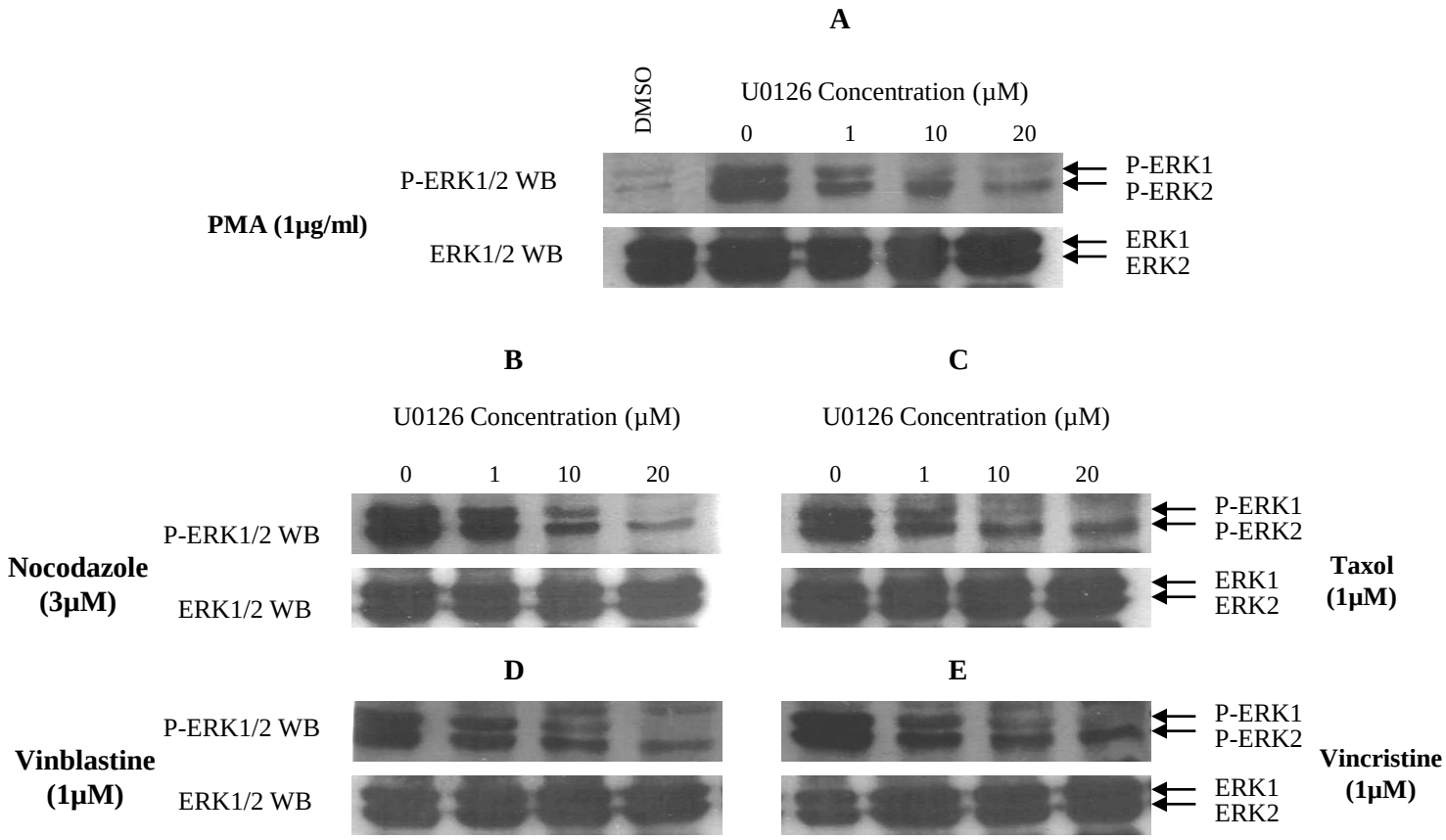


Figure 3.11: U0126 inhibits ERK activation – An asynchronous population of HeLa cells were treated with the indicated concentrations of U0126 for 15 minutes prior to the addition of either (A) PMA, (B) Nocodazole, (C) Taxol, (D) Vinblastine or (E) Vincristine. Cells were also treated with DMSO (0.1% v/v) alone. The cells were incubated for a further hour before preparation of cell extracts as described in Materials and Methods (see section 2.2.1) and immunoblotted with either anti_-ERK1/2 antibody (Upper panel, A-E) and an anti-ERK1/2 antibody (Lower panel, A-E). WB = Western Blot

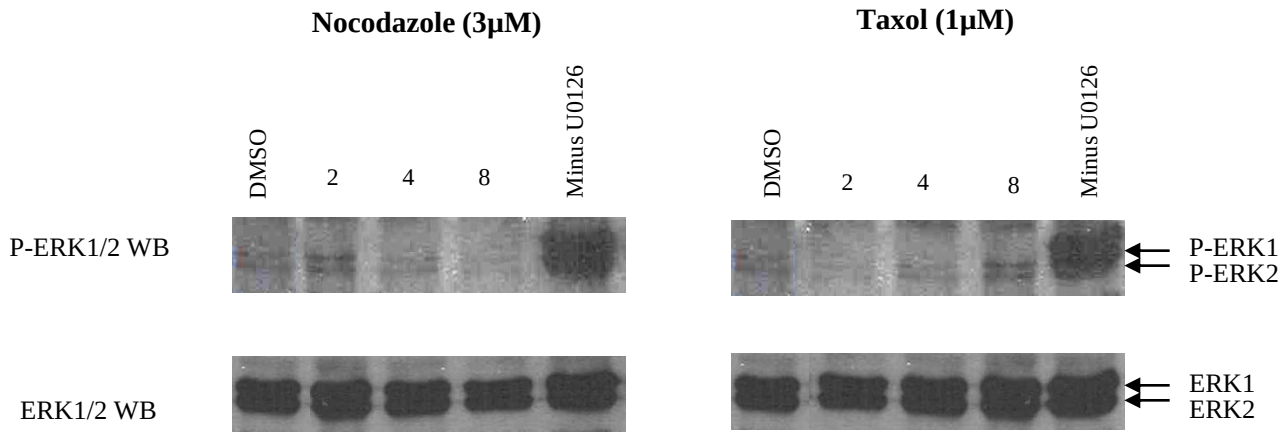


Figure 3.12: Time course of Nocodazole and Taxol-induced ERK1/2 activation by U0126. An asynchronous population HeLa cells were treated with U0126 (10µM) for 15 minutes prior to the addition of either Nocodazole (3µM) or Taxol (1µM). Cells were also treated with DMSO (0..05% v/v) alone for 8 hours and Nocodazole or Taxol (minus U0126) for 60 minutes. At the indicated time points (hours) cells extracts were prepared (section 2.2.1) and immunoblotted using anti-P-ERK1/2 antibody (Upper panel) and an anti-ERK1/2 antibody (Lower panel). WB = Western Blot

3.2.2 ERK1/2 Inhibition and Cell Death

As explained in section 1.3 (page 20), ERK1/2 has been implicated in regulating cell survival because its inhibition promotes apoptosis in response to growth factor deprivation (Erhardt *et al.,* 1999). ERK1/2 has also been implicated as a survival signal because its inhibition enhances Taxol-induced cell death (MacKeigan *et al.,* 2000; McDaid and Horwitz, 2001; McDaid *et al.*, 2005). To examine whether the inhibition of MIA-induced ERK1/2 activation by U0126 affects cell survival, various methods were applied: (i) examination of cytokeratin-18 cleavage using the M30 antibody; (ii) determination of PARP cleavage, (iii) Annexin-V binding, (iv) measuring apoptosis is mitotic cells using the M30 antibody and (v) Western blotting to determine the activation state of caspases involved in apoptosis.

3.2.2i M30 Staining

First, the level of cell death was measured using the M30 antibody (Deacon *et al.,* 1993). The M30 antibody only detects caspase-3 cleaved cytokeratin-18 (Kohler *et al.,* 2002). In a preliminary experiment, Hela cells were grown on coverslips and treated with either DMSO or Staurosporine ((1μM), a broad specificity protein kinase inhibitor, known to induce apoptosis (Weil *et al.,* 1996)) for 6 hours. The cells were then fixed and stained with the M30 antibody. Examination of the cells by fluorescent microscopy indicated that DMSO-treated cells showed no cytokeratin-18 cleavage (Figure 3.13Ai), the DNA intact and the chromatin present in a decondensed state (Figure 3.13Aii), whereas cells treated with Staurosporine showed cytokeratin-18 cleavage (Figure 3.13Bi) and chromatin condensation (Figure 3.13Bii).

Next, Hela cells were pre-treated with 10μM U0126 for 15 minutes, followed by Nocodazole (3μM) or Taxol (1μM) for 2 to 8 hours. The cells were then fixed and the levels of apoptosis assessed by staining with the M30 antibody. Nocodazole and Taxol induced apoptosis,

assessed by cytokeratin-18 cleavage, both in the presence and absence of U0126 (Figure 3.14). Quantifying the levels of apoptosis in this experiment (Figure 3.15) indicated that both Nocodazole and Taxol treatment alone caused a time-dependent increase in the number of apoptotic cells. However, in the presence of U0126 (10μM) there was an approximate 2-fold increase in the number of apoptotic cells at each time point examined, suggesting that U0126 may potentiate Nocodazole or Taxol-mediated apoptosis by blocking MEK-dependent ERK1/2 activation. Furthermore, statistical analysis (based on a T-test) indicates a p value below 0.05 for the values obtained, confirming that the increase in cell death upon the addition of U0126 was statistically significant.

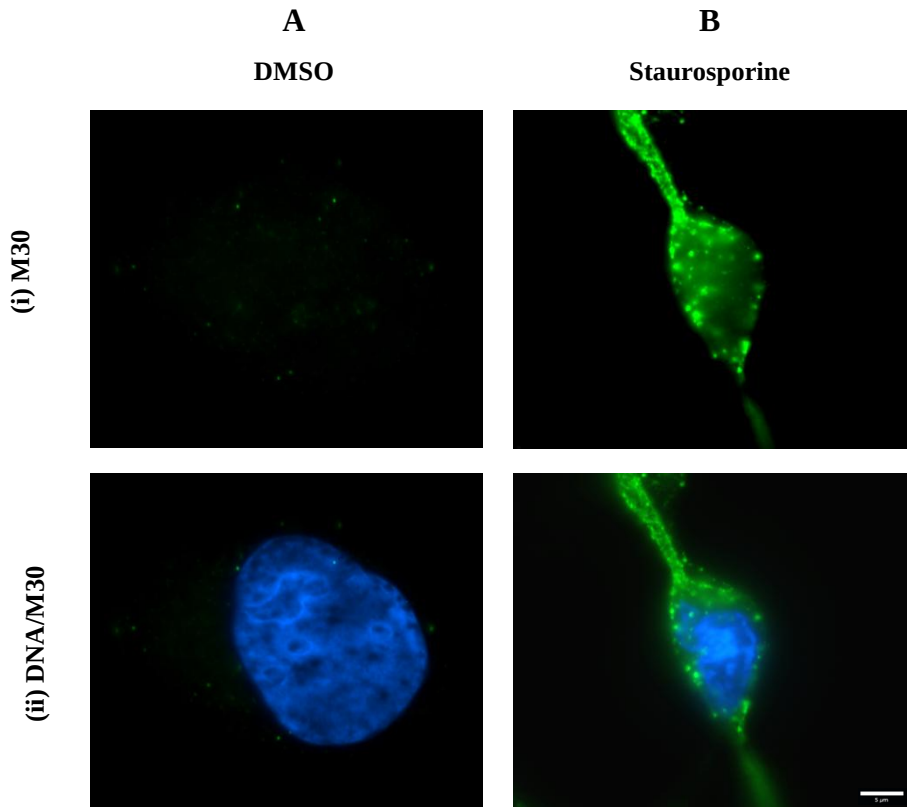


Figure 3.13: Staurosporine-induced apoptosis in Hela cells. A – DMSO-treated cell (6 hrs), **B** – Hela cells treated with Staurosporine (1µM) for 6 hours. The cells were then fixed and stained with the M30 antibody. (i) Cytokeratin-18 cleavage (green), (ii) DNA (blue) merged with cytokeratin-18 cleavage. Scale bar = 5µm.

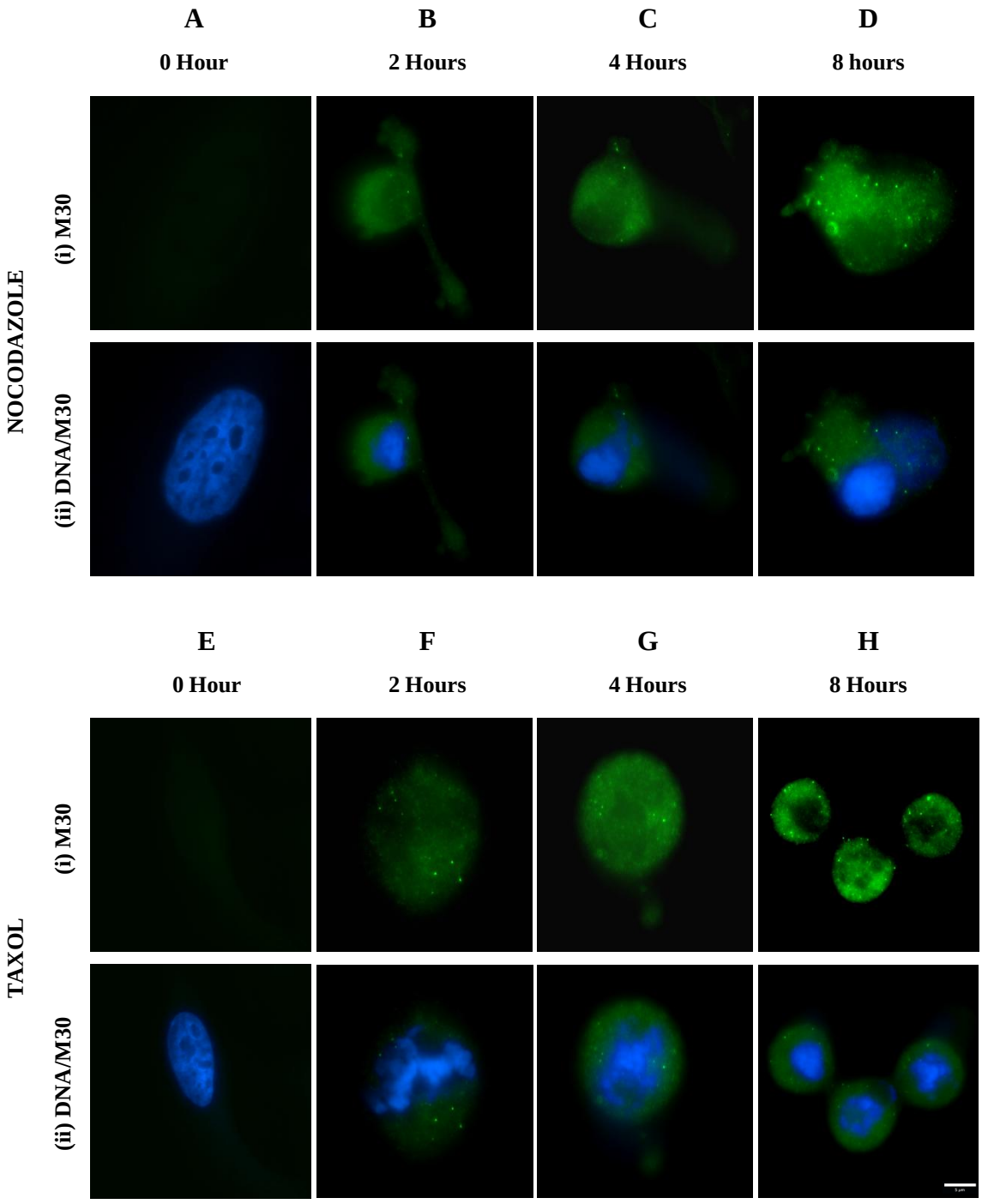


Figure 3.14: Apoptosis in response to U0126. HeLa cells were pre-treated with U0126 (10µM) for 15mins prior to the addition of Nocodazole (3µM) or Taxol (1µM) for the indicated times. The cells were then fixed and stained with the M30 antibody to detect cleaved cytokeratin-18. (i) Cleaved cytokeratin-18 (green), (ii) DNA (blue) + cleaved cytokeratin-18. Scale bar = 5µm.

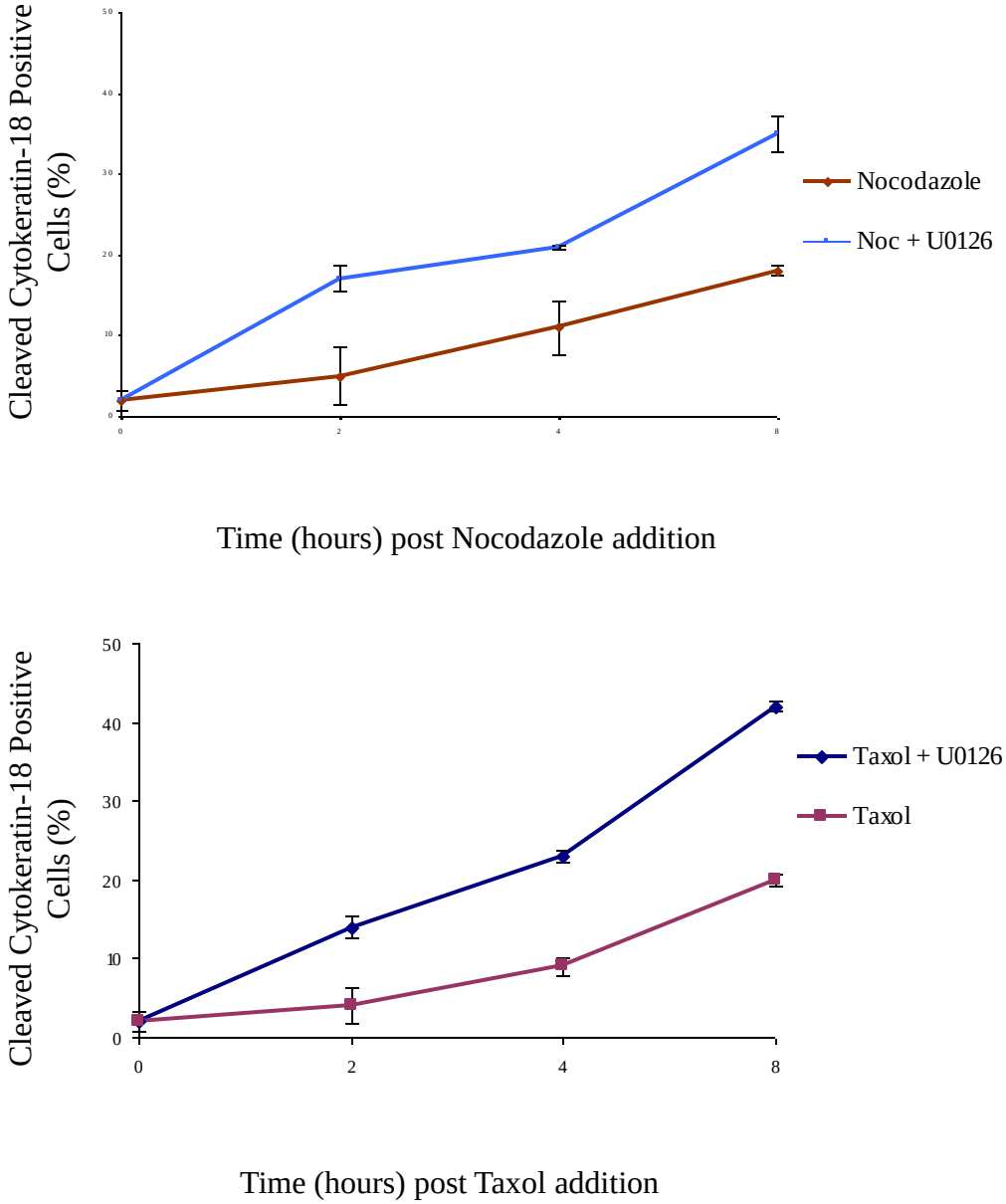


Figure 3.15: The effect of U0126 on Nocodazole and Taxol-induced apoptosis. HeLa cells were pre-treated with U0126 for 15mins prior to the addition of Nocodazole (3µM) or Taxol (1µM) for the indicated times. Cells were fixed and stained with the M30 antibody. 100 cells were counted in random fields at each time point and the percentage of cleaved cytokertain-18 positive cells were determined. The plot shows the mean (SD) of 3 separate experiments. Based on a two-sample T-test, $p<0.05$ for each time-point of cells treated with MIA + U0126 compared to MIA alone.

3.2.2ii PARP Cleavage

The second method investigated the effects of U0126 on Nocodazole and Taxol-induced apoptosis using PARP cleavage as an indicator of apoptosis (Boulares *et al.,* 1999). Hela cells were treated with either Nocodazole (3μM) or Taxol (1μM), with or without a 15 minute pre-treatment with U0126, for 2 to 24 hours. The results (Figure 3.16) indicated that PARP cleavage was not observed in cells treated with either Nocodazole or Taxol alone for 2 to 8 hours. The addition of U0126 (10μM) to either Nocodazole or Taxol had no effect on PARP cleavage at 2 to 8 hours after treatment. PARP cleavage was only observed in the mitotic populations of both Nocodazole and Taxol-treated cells (12 and 24 hours). These results suggest that U0126-mediated ERK1/2 inhibition does not induce apoptosis in the attached population of cells at 2 to 8 hours following MIA addition. Apoptosis is only induced in mitotic cells after 12 and 24 hours of treatment with Nocodazole or Taxol, either with and without U0126. No significant difference in PARP cleavage was observed in the mitotic cell populations that were treated with or without U0126, suggesting that U0126 had no effect on the levels of apoptosis in mitotic cells as assessed by PARP cleavage.

3.22iii Annexin-V binding

The third method used to analyse the effect of U0126 on Taxol-induced apoptosis was Annexin-V binding. Hela cells were treated with either Taxol (1μM) alone or pre-treated for 15 minutes with U0126 (10μM) before addition of Taxol (1μM, final concentration). Control cells were treated with either DMSO alone (0.05% v/v) or etoposide (100μg/ml - a topoisomerase inhibitor, known to induce apoptosis in Hela cells (Torriglia *et al.,* 1999). The cells were labelled with annexin-V and propidium iodide (PI) as described in Materials and Methods (see 2.2.6) and analysed by flow cytometry (as described in Figure 3.17).

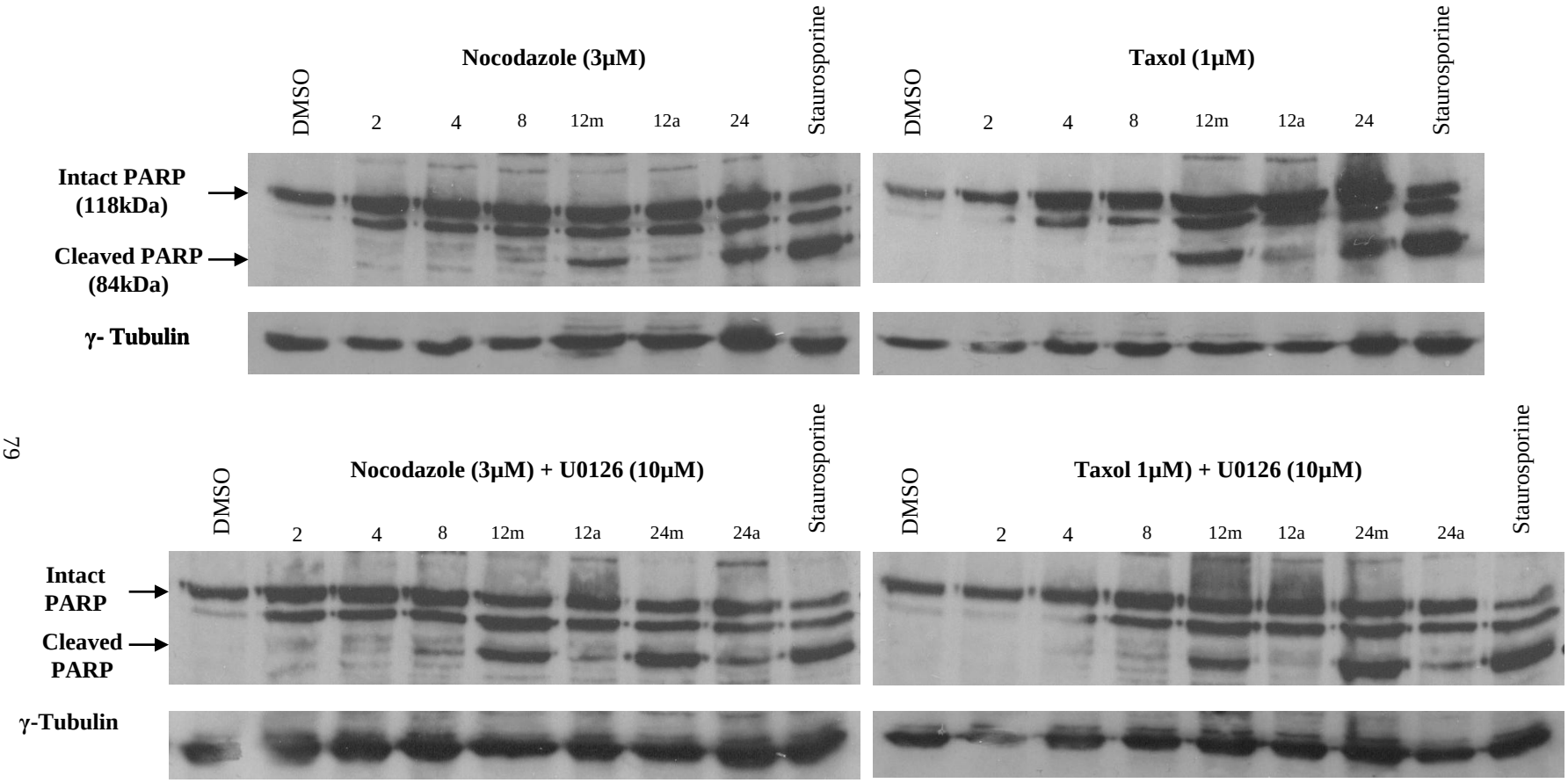


Figure 3.16: The effect of U0126 on Nocodazole and Taxol-induced PARP cleavage. Hela cells were treated with Nocodazole (3μM) or Taxol (1μM), with and without a 15 minute pre-treatment with U0126 (10μM). After 12 and 24 hours, the mitotic cells (m) were separated from the non-mitotic, attached cells (a) by "shake-off". Control cells were treated with DMSO (0.05% v/v) alone for 24 hours or with Staurosporine (1μM) for 6 hours. The level of PARP cleavage was assessed by immunoblotted with an anti-α-PARP antibody.

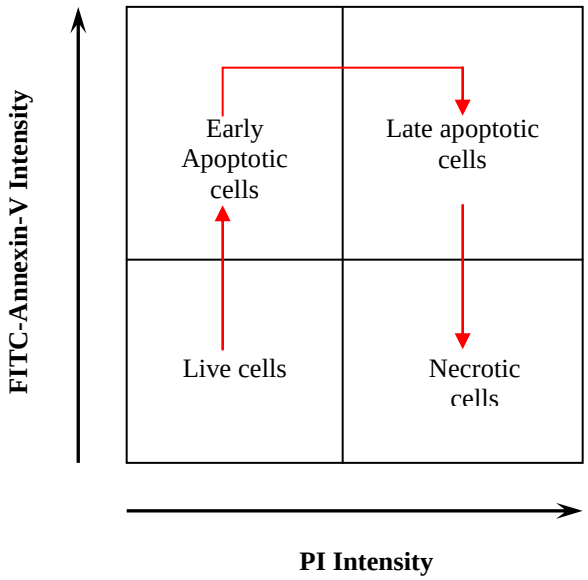


Figure 3.17: Analysis of cell death using Annexin-V and flow cytometry. Data created by the FACS machine are arranged by the ability of cells to bind annexin-V and incorporate PI. The apoptotic nature of the cells is determined by which quadrant they appear in and follows the path indicated by the red arrows.

As shown in Figure 3.18, cells treated with DMSO alone (0.05% v/v) and U0126 alone (10µM) for 24 hours did not affect cell survival and only 9.1% and 11.1% of the cells, respectively, were found in the upper left quadrant indicating apoptotic cells. For cells treated with etoposide (100µg/ml for 24 hours), 26.7% of cells were found in the upper left quadrant, with 16.1% in the upper right quadrant indicating early and late apoptotic cells respectively.

When Hela cells were treated with Taxol (1µM), either with or without U0126 (10µM) and assayed for apoptosis, it was found that U0126 did not significantly affect the level of apoptosis at 2, 6 and 24 hours after Taxol treatment (Figures 3.18 & 3.19). However, at 16 hours of Taxol treatment, U0126 increased the level of Taxol-induced apoptosis. At 16 hours 41.1% of Taxol-treated cells were apoptotic whereas in the presence of U0126 and Taxol, 61.4% of the cells were apoptotic. The results of this experiment indicate that apoptosis occurs

significantly in mitotic cells rather than attached cells, as treatment with Taxol for more than 12 hours causes the majority of cells to arrest in mitosis. Quantitation of the results of two independent experiments (shown in figure 3.19) suggest that the effect of U0126 on Taxol-induced apoptosis were variable, with only one experiment showing that U0126 increased Taxol-induced apoptosis in the mitotic population (16 hours post drug treatment).

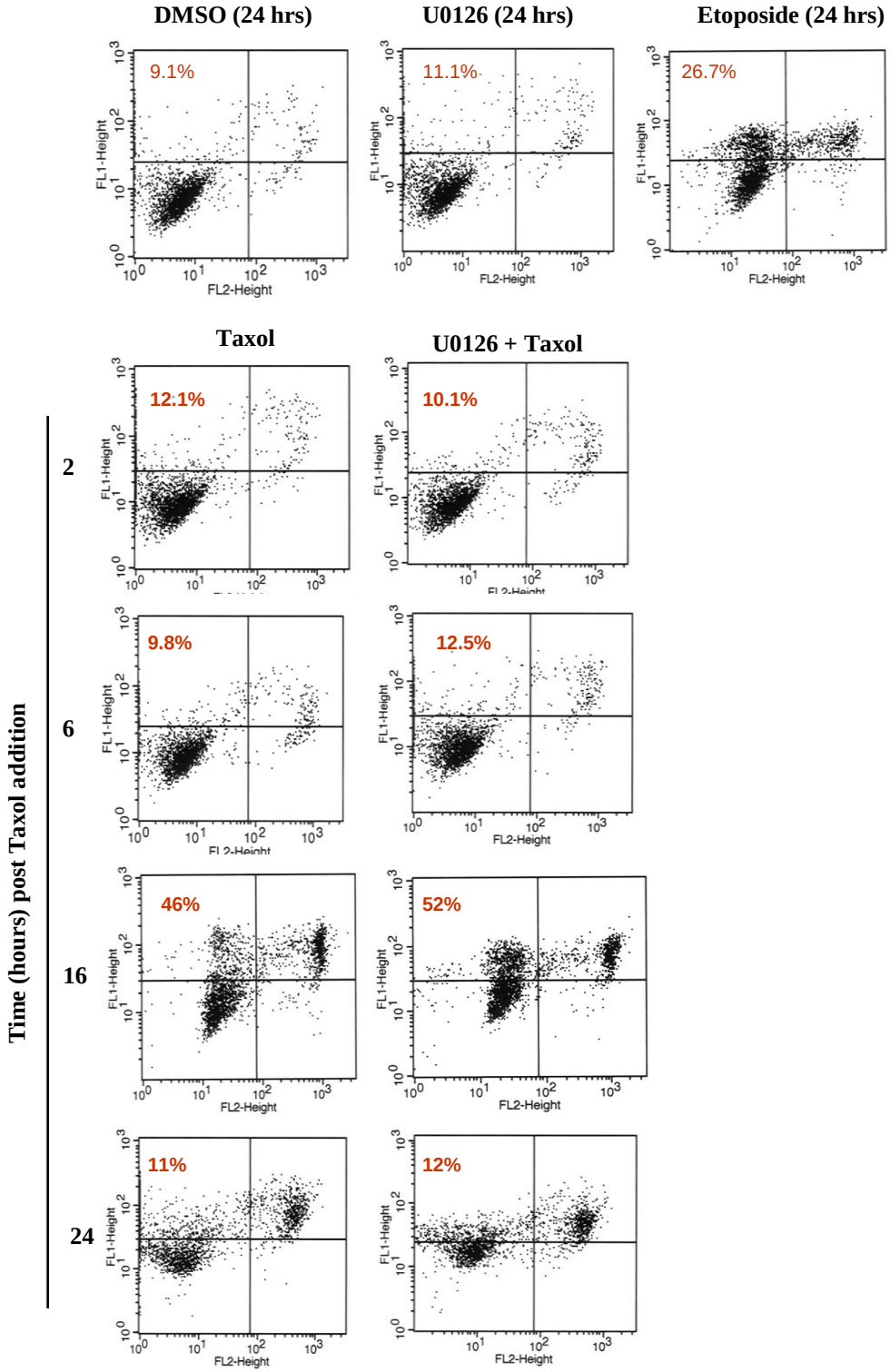


Figure 3.18: The effect of U0126 on Nocodazole and Taxol-induced apoptosis by flow cytometry. Hela cells were treated with Taxol (1μM) alone or with a 15 minute pre-treatment with U0126 (10μM). Control cells were treated with DMSO (0.05% v/v), U0126 (10μM) or Etoposide (100μg/ml) for 24 hours. The cells were prepared for analysis as described in Materials and Methods (see section 2.2.6). This data represents one set of FACS analysis carried out with the percentage apoptosis shown in red.

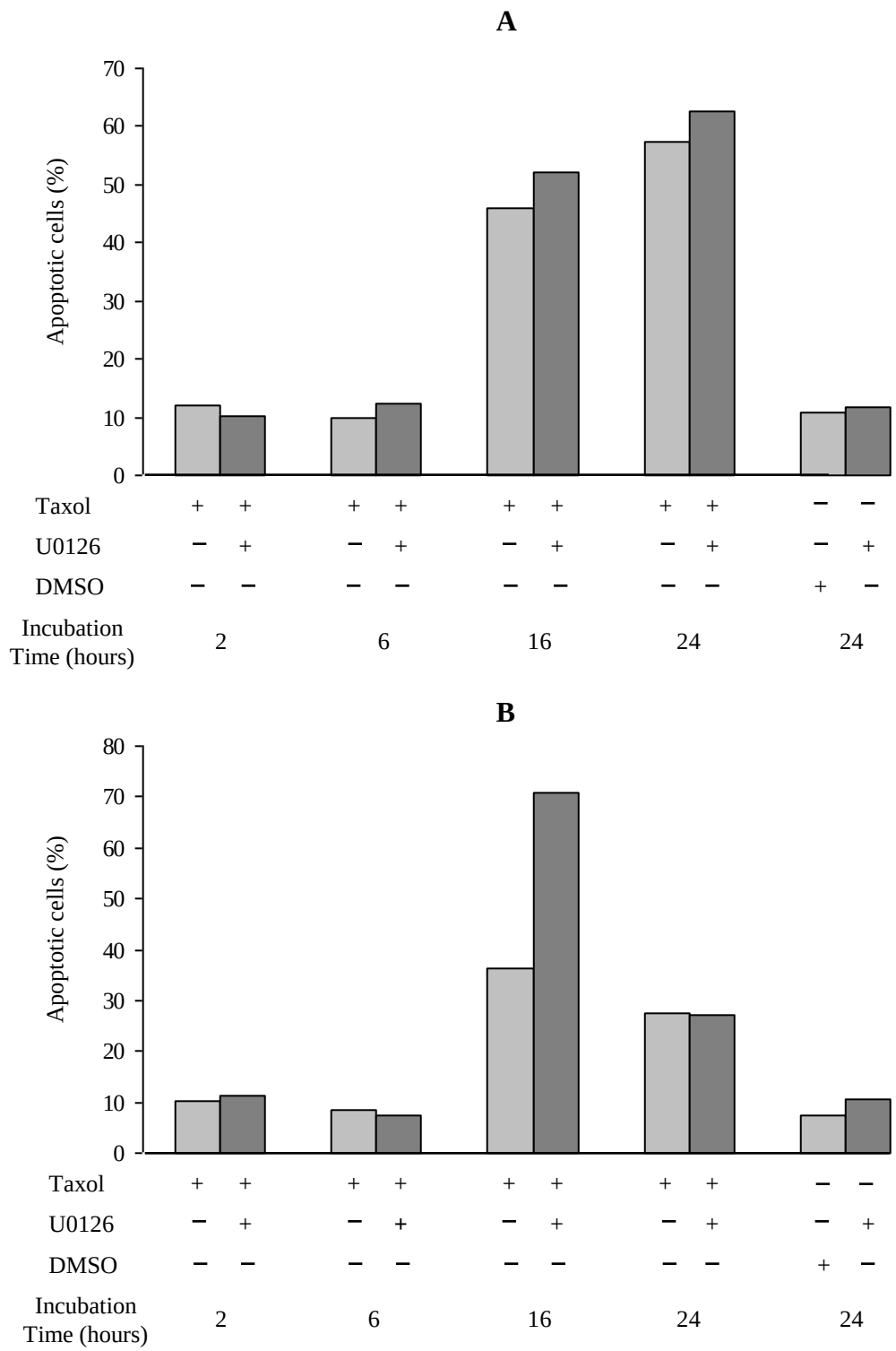


Figure 3.19: Quantifying the effect of U0126 on Nocodazole and Taxol-induced apoptosis by flow cytometry . Hela cells were treated with 1 μM Taxol with and without a 15 minute pre-treatment with 10μM U0126 for the indicated time points. As controls, Hela cells were treated with DMSO or U0126 alone for the maximum time point. Cells were then prepared for FACS analysis as described in chapter 2.2.6. A and B represent results of two independent experiments.

3.2.2iv Apoptosis in Mitotic Cells

As the addition of U0126 did not greatly affect MIA-induced apoptosis at the early time points following addition of the MIAs, I decided to examine more closely the effect of U0126 on apoptosis in just the MIA-arrested mitotic population of cells. First I examined the state of ERK1/2 phosphorylation in Taxol-arrested mitotic cells.

An asynchronous population of Hela cells was treated with Taxol (1μM) for 12 and 24 hours and the cells arrested in mitosis were collected by "shake-off" and attached to poly-L-lysine-coated coverslips. The cells were fixed and stained with an anti-γ-tubulin antibody to stain the centrosomes (green), the anti-P-ERK1/2 antibody (red) and the DNA-binding dye Hoechst 33342 (blue). The results show that P-ERK1/2 localises to a specific subcellular location of the cell that is co-stained by γ-tubulin (Figure 3.20) and represent the centrosomes. Unfortunately, there appears to be one centrosome in one of the images (Figure 3.20, 12 hour, γ-tubulin image). This was because the second centrosome was out of the plane of focus. Despite this, P-ERK1/2 appears co-localised with the centrosomes in mitotic cells, suggesting that a small fraction of P-ERK1/2 must be active in the mitotically-arrested cells.

Since ERK1/2 was found to be activated in the mitotic cells, I next examined the effect of inhibiting ERK1/2 on the survival of the mitotic cells by treating the cells with various concentrations of U0126. An asynchronous population of Hela cells was pre-treated with U0126 (0, 5, 10 or 20μM) for 15 minutes prior to the addition of Taxol (1μM) and incubated for a further 24 hours. Mitotic cells were collected by "shake-off" and attached to poly-L-lysine-coated coverslips. The cells were fixed and stained with the M30 antibody and the DNA dye Hoechst. The results indicate that there is a concentration-dependent increase of apoptosis in response to U0126 (plus Taxol) in comparison to treatment with Taxol alone (Figure 3.21). To support this further, statistical analysis (as measured by a T-test) shows that the values

obtained for cell death are overall statistically significant compared to the control and also compared to Taxol alone (Figure 3.21). This not only suggests that U0126-mediated ERK1/2 inhibition plays an important role in enhancing apoptosis in Hela cells but also suggests that ERK1/2 activation itself has a role in regulating cell survival in response to Taxol in mitotic cells.

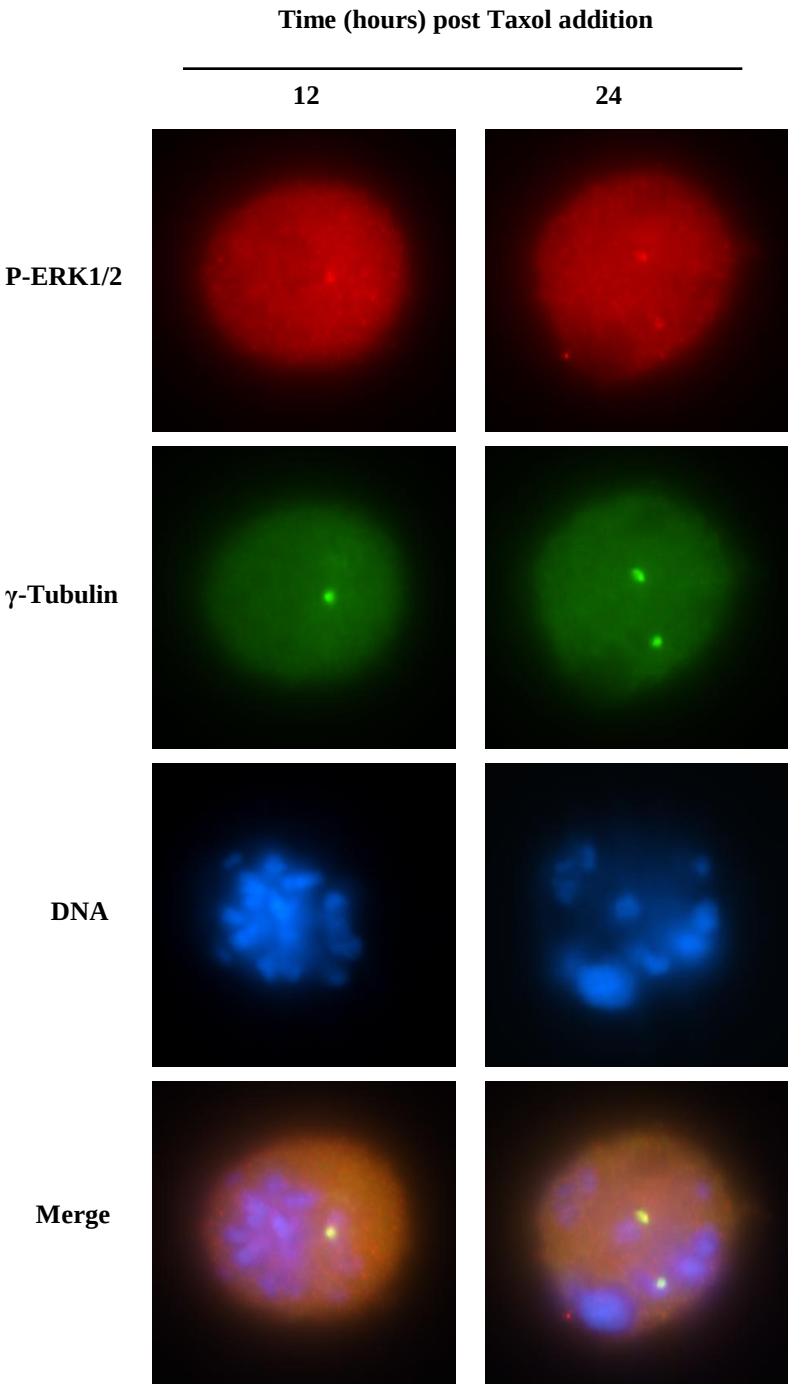


Figure 3.20: Determining P-ERK1/2 localisation in Taxol-arrested mitotic cells. Hela cells were treated with 1 μM Taxol for 12 and 24 hours. Cells arrested in mitosis were collected by "shake-off" (see section 2.2.1, page 47) and attached to poly-L-lysine-coated coverslips. The localisation of ERK1/2 was determined using P-ERK1/2 (red) and γ-tubulin (green) antibodies and Hoechst-dyed DNA. Scale bar = 5μm.

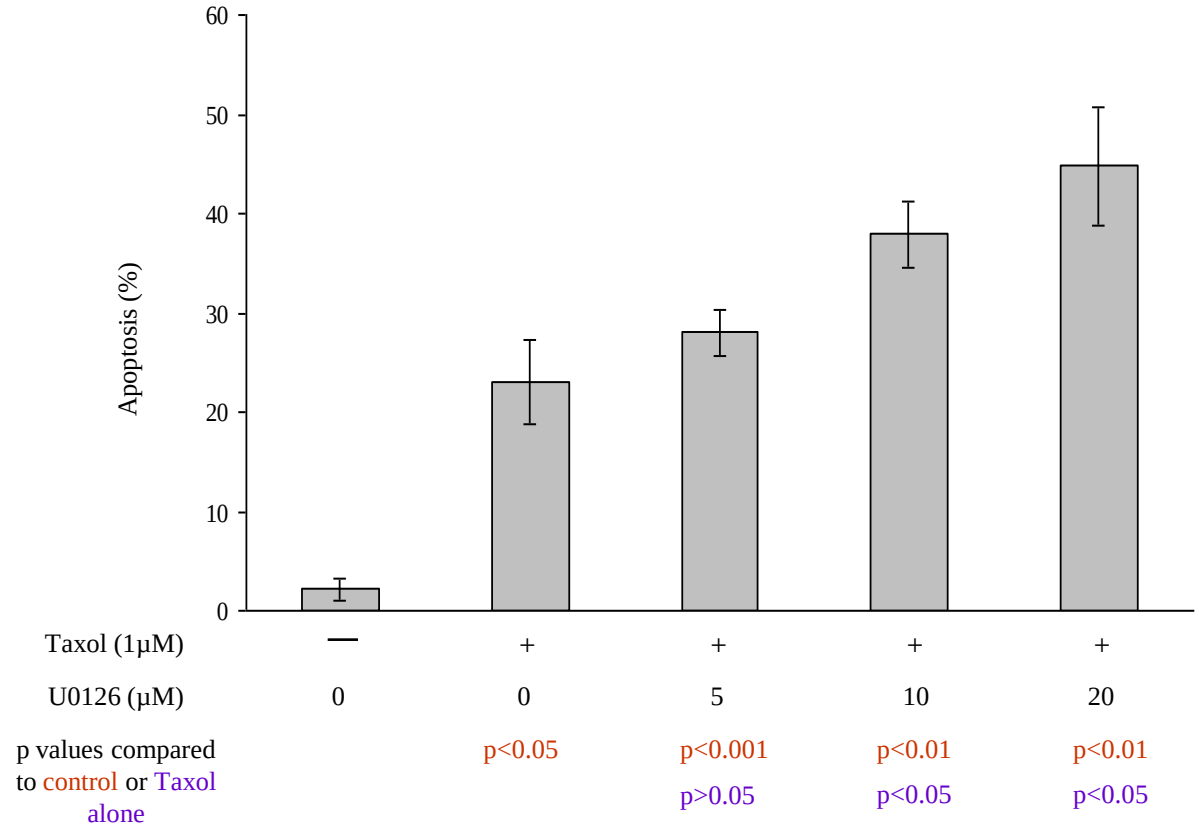


Figure 3.21: Levels of U0126-mediated apoptosis in Taxol-induced mitotic cells. Hela cells were pre-treated with varying concentrations of U0126 (as indicated) for 15 minutes prior to the addition of Taxol (1µM) and incubated for a further 24 hours. Mitotic cells were collected by "shake-off", fixed onto poly-L-lysine-coated coverslips and stained with the M30 antibody. Cells were counted in random fields for each experiment and the percentage of apoptotic cells was determined. The plot shows the mean (SD) for 3 separate experiments. p values are based on a two-sample T-test assuming unequal variances.

3.2.2v Activation state of apoptotic proteins

To understand how ERK1/2 may be involved in MIA-induced cell death, I investigated the extrinsic and the intrinsic pathways (section 1.6) of apoptosis. To determine whether the extrinsic pathway is involved in MIA-induced cell death, the activation state of two mediators of the extrinsic pathway, caspase-8 and Bid, was investigated.

Hela cells were treated with Nocodazole (3μM) or Taxol (1μM), either with or without a 15 minute pre-treatment with U0126 (10μM) for 2 to 8 hours. Cells were also treated with DMSO (0.25%, v/v) for 8 hours or Staurosporine (1μM) for 6 hours. The cell extracts were immunoblotted with an anti-caspase-8 antibody that detects both full-length (55-53kDa) and cleaved (41-43kDa) caspase-8. The results (Figure 3.22) indicated that treatment with Staurosporine resulted in caspases-8 cleavage. However, treatment with Nocodazole or Taxol did not result in caspases-8 cleavage, either in the presence or absence of U0126, suggesting that the extrinsic pathway may not be involved in MIA-induced cell death.

The cell extracts used in the preceding experiment were then immunoblotted with an anti-Bid antibody that detects both the intact (25kDa) and truncated (15kDa, tBid) forms of Bid. Treatment with Staurosporine resulted in Bid cleavage whereas treatment with Nocodazole indicated that Nocodazole did activate Bid (compared to DMSO-treated sample with sample treated with Nocodazole for 2 hours). Treatment with Taxol also resulted in Bid cleavage at 4 and 8 hours post Taxol addition (Figure 3.23A). The addition of U0126 did not greatly alter the levels of tBid when compared to Nocodazole or Taxol treatment alone (Figure 3.23B), suggesting that Bid cleavage and therefore the extrinsic pathway is not required to mediate MIA-induced ERK1/2-mediated cell death.

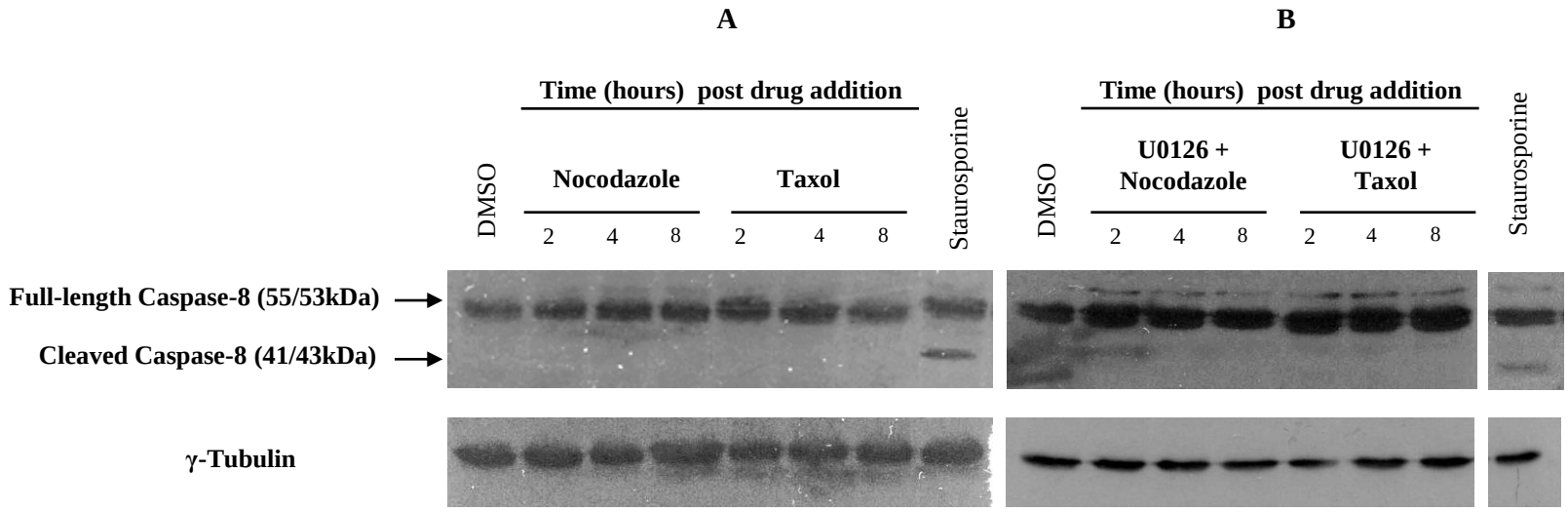


Figure 3.22: The effect of U0126 on Caspase-8 cleavage. (A) Hela cells were treated with Nocodazole (3μM) or Taxol (1μM) for the indicated times (hours) or with Staurosporine (1μM) for 6 hours or DMSO (0.05% v/v) for 8 hours. Cells were lysed and immunoblotted with an anti-caspase-8 antibody (upper panel) and a γ-tubulin antibody (lower panel). (B) Hela cells were pre-treated with U0126 (10μM) for 15mins prior to the addition of Nocodazole (3μM) or Taxol (1μM) for the indicated times. Cells were lysed and immunoblotted with an anti-caspase-8 antibody (upper panel) and a γ-tubulin antibody (lower panel).

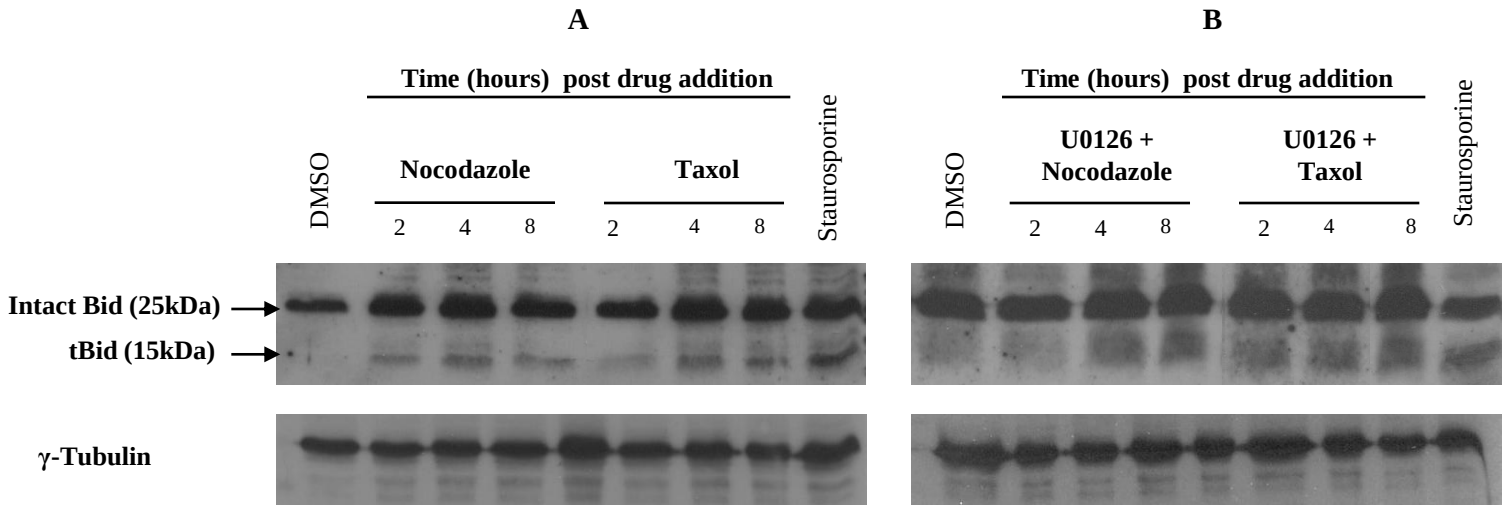


Figure 3.23: The effect of U0126 on Bid cleavage. (A) Hela cells were treated with Nocodazole (3μM) or Taxol (1μM) for the indicated times (hours) or with Staurosporine (1μM) for 6 hours or DMSO (0.05% v/v) for 8 hours. Cells were lysed and immunoblotted with an anti-Bid antibody (upper panel) and a γ-tubulin antibody (lower panel). (B) Hela cells were pre-treated with U0126 (10μM) for 15mins prior to the addition of Nocodazole (3μM) or Taxol (1μM) for the indicated times. Cells were lysed and immunoblotted with an anti-Bid antibody (upper panel) and a γ-tubulin antibody (lower panel).

To investigate whether U0126 enhances Nocodazole/Taxol-induced apoptosis through the activation of the intrinsic pathway, the activation state of caspase-9 and caspase-3 was examined.

Hela cells were treated with Nocodazole (3μM) or Taxol (1μM), either with or without a 15 minute pre-treatment with U0126 (10μM) for various times. Control cells were treated with DMSO (0.25%, v/v) for 24 hours or Staurosporine (1μM) for 6 hours. Caspase-9 cleavage was detected using an antibody that recognised the full-length inactive form (47kDa) and the cleaved active form (35-37kDa) of caspases-9. In the presence of Nocodazole or Taxol alone, only the full-length caspase-9 was observed at 2 to 8 hours after drug treatment. Prolonged treatment (24 hours) resulted in the cleavage of caspase-9 to the active form (Figure 3.24). The same cell extracts were immunobloted with an anti-caspase-3 antibody. The results (Figure 3.25) showed that caspase-3 was not activated in response to treatment with Nocodazole alone at 2 to 8 hours. Caspase-3 was also inactive after 2 to 4 hours treatment with Taxol alone. This was consistent with the data obtained for caspase-9 cleavage. However, Nocodazole treatment in the presence of U0126 indicated that caspase-3 was cleaved at 4 to 8 hours after drug treatment. In addition, Taxol plus U0126 treatment also resulted in caspases-3 cleavage between 2 to 8 hours after drug treatment. This is a clear indication that U0126 sensitises both Nocodazole and Taxol-induced cell death by activation of the intrinsic apoptotic pathway.

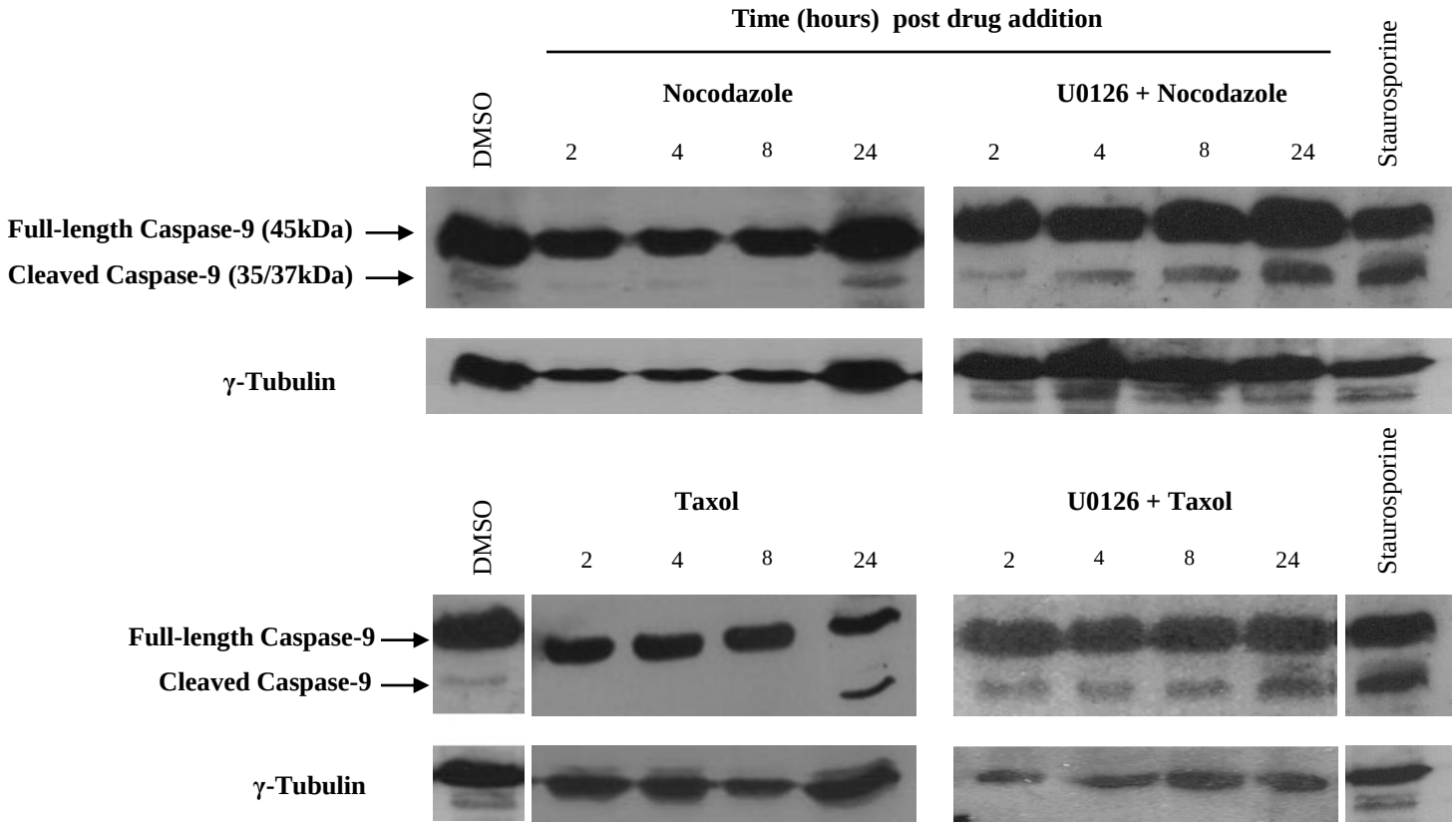


Figure 3.24: The effect of U0126 on caspase-9 cleavage. Hela cells were treated with Nocodazole (3μM) or Taxol (1μM) (left panel) and with a 15 minute pre-treatment with U0126 (10μM, right panel) for the indicated times. Control cells were treated with either DMSO (0.05% v/v) for 24 hours or Staurosporine (1μM) for 6 hours. The cell extracts were immunoblotted with anti-caspase-9 antibody (upper panels) and γ-tubulin antibody (lower panels).

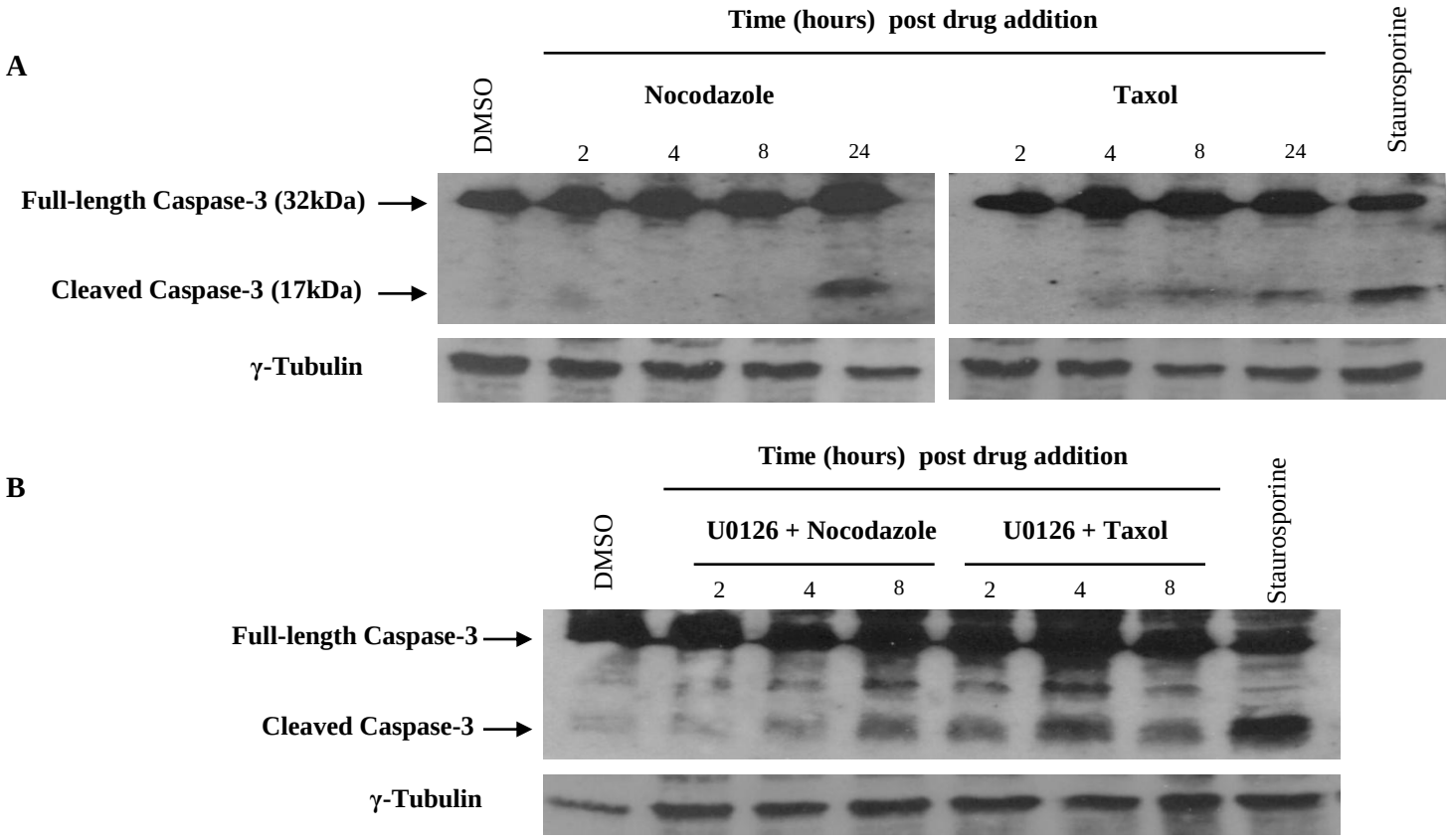


Figure 3.25: The effect of U0126 on caspase-3 cleavage. Hela cells were treated with Nocodazole (3μM) or Taxol (1μM) alone (A) or with a 15 minute pre-treatment with U0126 (10μM, B) for the indicated times (hours). Control cells were treated with either DMSO (0.05% v/v) for 24 hours or with Staurosporine (1μM) for 6 hours. The cells extracts were immunoblotted with an anti-caspase-3 antibody (upper panel) and a γ-tubulin antibody (lower panel).

3.3 How ERK1/2 May Regulate Cell Survival

To understand how ERK1/2 may be involved in the regulation of cell survival, I investigated the phosphorylation of two possible ERK1/2 substrates; caspase-9 and Bim (see Figure 1.13, page 39).

3.3.1 Regulation by Caspase-9 Phosphorylation

A study by Allan *et al* (2003) demonstrated that ERK1/2 phosphorylates caspase-9 on its threonine residue 125 in both mouse NIH3T3 and human HEK293 cells. To investigate this, Hela cells were treated with Taxol (1μM), with and without U0126 (10μM). Control cells were treated with either DMSO (0.25%, v/v) for 24 hours or Staurosporine (1μM) for 6 hours. The cells were lysed in SDS sample buffer as described in Materials and Methods (see section 2.2.1) and immunoblotted with an anti-phospho caspase-9 (P-caspase-9) antibody and an anti-caspase-9 antibody. The results (Figure 3.26, left panel) indicate that the P-caspase-9 antibody did not detect a band at the expected molecular weight of 49kDa in response to Taxol, either with or without U0126. A band at approximately 44kDa was detected by the antibody but it did not correspond to the size of any form of caspase-9.

An alternative method to increase the likelihood of detecting caspase-9 phosphorylation was also used. Hela cells were transfected with Flag epitope-tagged-caspase-9 as described in Materials and Methods (see section 2.2.3), in order to overexpress caspase-9. The transfected cells were treated with either DMSO (0.01%, v/v) or PMA (1μg/ml) for one hour. The results (Figure 3.26, right panel) indicated that the P-caspase-9 antibody did not detect the expected band at 49kDa in response to PMA.

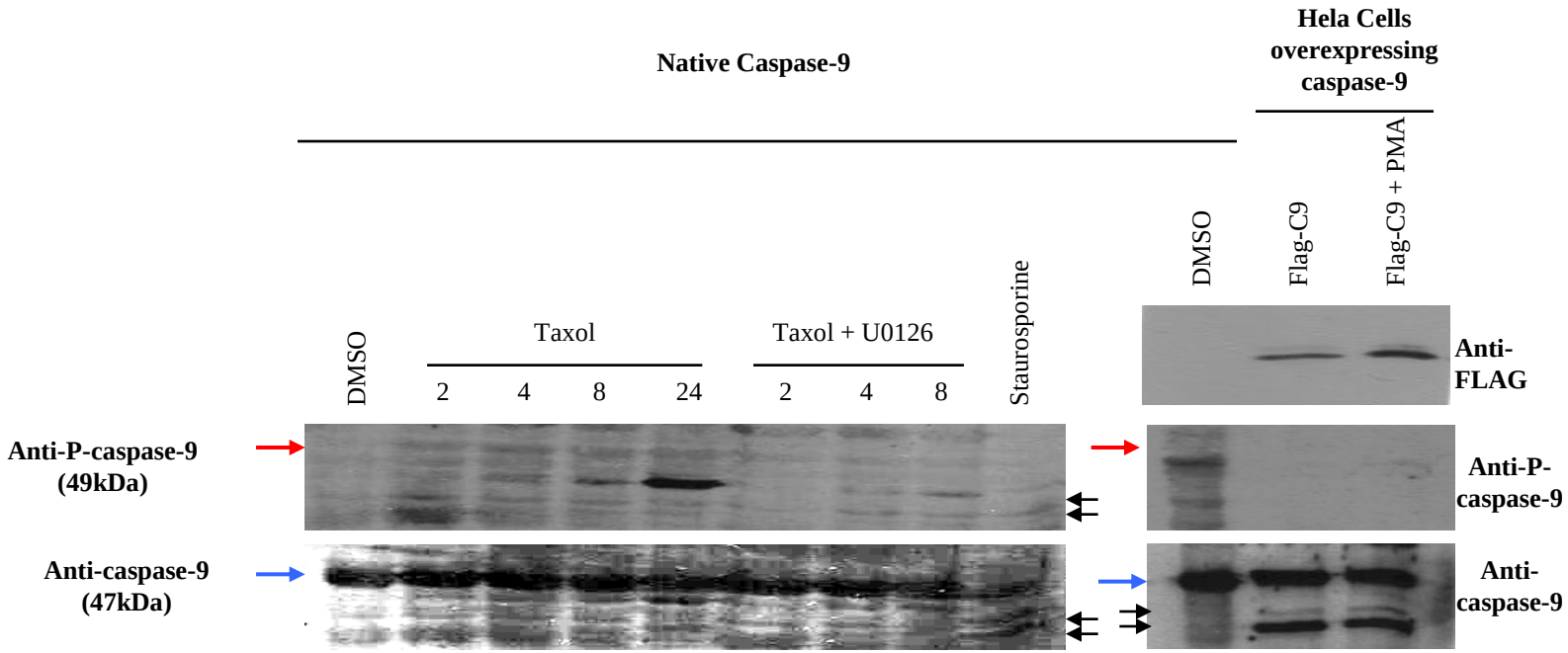


Figure 3.26: ERK1/2-dependent phosphorylation of caspase-9: Hela cells were initially treated with Taxol (1μM), either with and without a 15 minute pre-treatment with U0126 (10μM) for the indicated times (hours) or with DMSO (0.25% v/v) for 24 hours or with Staurosporine (1μM) for 6 hours. The cells were lysed directly into 2x SDS sample buffer as described in Materials and Methods (see section 2.2.1). Cell extracts were immunoblotted with an anti-P-caspase-9 antibody and also an anti-caspase-9 antibody (left panel). Hela cells were also transfected (see section 2.2.3) with Flag--caspase-9 to overexpress caspase-9. Transfected Hela cells were treated with either DMSO (0.01% v/v) or PMA (1μg/ml) for one hour and lysed in 2x sample buffer. Cell extracts were first immunoblotted with anti-Flag antibody to determine successful transfection, then with an anti-P-caspase-9 antibody and an anti-caspase-9 antibody (right panel). *Red arrows* indicate the expected position of P-caspase-9 (~49kDa), the *blue arrows* show native caspase-9 (47kDa) and the *black arrows* indicate the position of the large fragment of cleaved caspase-9 (35-37kDa).

I requested the manufacturers' cell extract to use as a positive control. The positive control was a cell lysate made from MCF-7 cells treated with epidermal growth factor (EGF) and PMA. As advised by the manufacturer, the P-caspase-9 antibody detects phosphorylated caspase-9 at approximately 60kDa in this positive control. Surprisingly, the P-caspase-9 antibody did not consistently detect a positive 60kDa product in the positive control lysate (data not shown) therefore I decided against further use of this antibody. Further transfections of Hela cells, followed by treatment with Nocodazole (3μM) or Taxol (1μM) were performed and the cell extracts were immunoblotted with the anti-caspase-9 antibody in the hope of observing a mobility shift indicating the phosphorylation of caspase-9 but unfortunately, I encountered problems with the transfections of caspase-9 and so I was unable to complete the examination of the phosphorylation state of caspase-9 in MIA-treated cells.

3.3.2 Regulation by Bim Phosphorylation

It has also been reported that ERK1/2 is capable of phosphorylating the Bcl-2 protein Bim on serine residue 69 (Luciano *et al.,* 2003) which then targets it for degradation by the proteasome in most cell types, including CCl39, CC139 clonal derivatives CR1–11 and CM3 cells and CHO cells (Ley *et al.,* 2003).

Initially, Hela cells were treated with Nocodazole (3μM) or Taxol (1μM), either with and without a 15 minute pre-treatment with U0126 (10μM) for one hour. The cell extracts were resolved by SDS PAGE and immunoblotted with an anti-Bim antibody, which detects native Bim-EL protein at 25kDa, and an anti-P-Bim antibody which recognises Bim-EL when it is phosphorylated on serine residue 69. Unfortunately, the anti-Bim antibody failed to detect native levels of Bim-EL in these extracts and the anti-P-Bim antibody gave high levels of non-specific binding (data not shown). As with caspase-9, we decided to transfect Hela cells with

an HA epitope-tagged Bim-EL (HA-Bim) plasmid, in order to overexpress the protein and increase the probability of detecting phosphorylated Bim-EL. However, overexpressed Bim caused rapid cell death before treatment with MIAs could even begin. I decided to optimise the procedure and found that reducing the quantity of the HA-Bim plasmid and also reducing the plasmid incubation time minimised cell death. The transfected cells were then treated with Nocodazole (3μM) or Taxol (1μM), with and without pre-treatment with U0126 (10μM) for one hour. Unfortunately, due to the sensitivity of the anti-P-Bim antibody, high levels of non-specific binding made it difficult to detect P-Bim-EL (data not shown). Eventually, we used an alternative anti-Bim antibody (Stressgen) shown to be very effective in detecting even low levels of Bim to identify Bim-EL phosphorylation by a mobility shift. To test this antibody, Hela cells were treated with PMA (1μg/ml) or Anisomycin (0.1μg/ml and 1μg/ml); a compound that activates JNK (Curtin & Cotter, 2002), which in turn phosphorylates Bim-EL on Threonine reside 56 (Lei & Davis, 2002). MCF-7 breast cancer cells were also used because they express high basal levels of Bim (O'Reilly *et al.,* 2000). MCF-7 cells were treated with either DMSO (0.01%, v/v) or PMA (1μg/ml). The results (Figure 3.27 upper panel) suggest that MCF-7 cells express higher levels of Bim than Hela cells. Treatment with PMA in both Hela cells and MCF-7 cells caused a mobility shift of Bim-EL from 23kDa to approximately 25kDa. Treatment with anisomysin in Hela cells also caused a similar mobility shift of Bim-EL, suggesting that Bim-EL is phosphorylated in response to PMA and anisomycin.

The γ-tubulin blot (Figure 3.27, lower panel) shows that protein levels are equal in Hela cells. In MCF-7 cells, the protein content is lower than in Hela cells which suggest that Bim-EL must be expressed at considerably higher levels in MCF-7 cells.

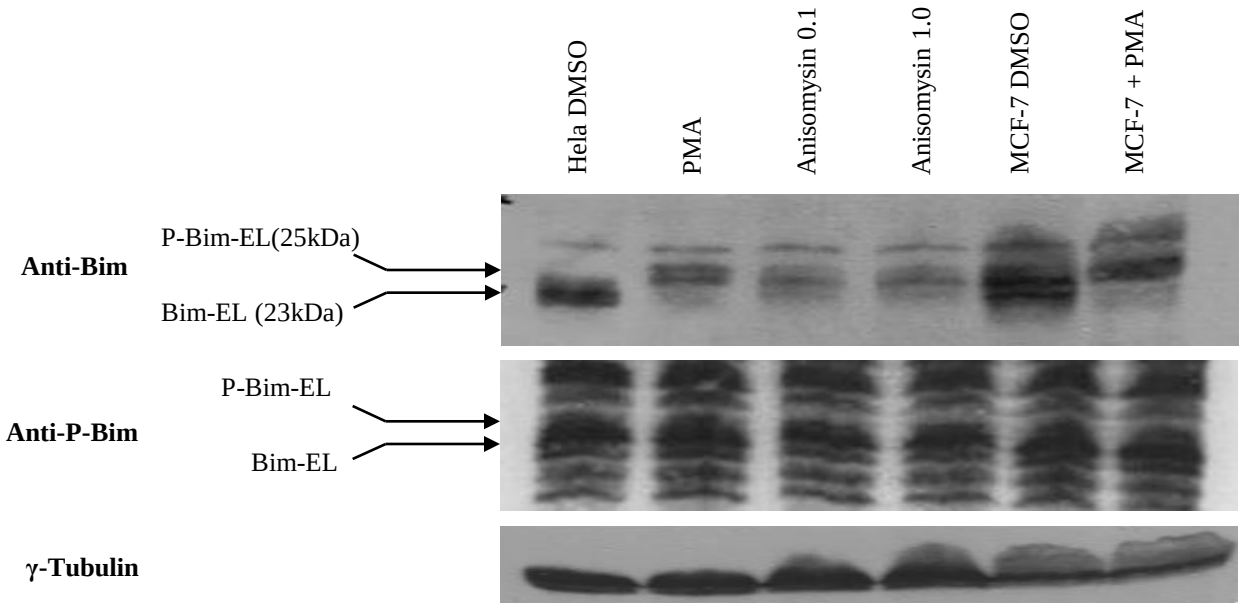


Figure 3.27: Bim undergoes a shift in mobility in response to either PMA or Anisomysin treatment. Hela cells were treated with PMA (1μg/ml), Anisomysin (0.1 μg/ml and 1.0 μg/ml) for 30 minutes, or DMSO (0.01% v/v). MCF-7 cells were also treated with PMA (1μg/ml) for 30 minutes or DMSO (0.01% v/v). The cells were lysed into 2x SDS sample buffer as described in Materials and Methods (see section 2.2.1). Cell extracts were immunoblotted with an anti-Bim antibody (upper panel), anti-P-Bim antibody (middle panel) and anti-γ-tubulin antibody (lower panel).

The next step was to detect phosphorylation of Bim-EL in response to Nocodazole and Taxol using this highly specific antibody. Hela cells were treated with Taxol (1μM), with or without a 15 minute pre-treatment with U0126 (10μM) for various times (Patel, R & Patel, V). Control cells were either untreated or treated with PMA (1mg/ml) for an hour. The cell extracts were immunoblotted with anti-Bim (Stressgen), anti P-ERK1/2 and anti-ERK1/2 antibodies. The anti-Bim antibody (Stressgen) effectively demonstrated the effects of Taxol and U0126 on Bim-EL phosphorylation via mobility shift (Figure 3.28). The results show Bim-EL phosphorylation at 6 and 9 hours post Taxol treatment, whereas after 18 and 24 hour treatment with Taxol (mitotic population of cells), very small fragments of Bim-EL can be seen, indicating possible Bim-EL degradation (Figure 3.28A, upper panel). The corresponding P-ERK1/2 blot shows ERK1/2 phosphorylation at 6 and 9 hours post Taxol treatment, whereas at 18 or 24 hours, ERK1/2 phosphorylation cannot be seen (Figure 3.28A, middle panel). The phosphorylation of ERK1/2 and Bim-EL seen at 6 and 9 hours post Taxol treatment suggests that ERK1/2 may be involved in mediating Bim phosphorylation. However, although ERK1/2 phosphorylation cannot be seen at 18 and 24 hours after Taxol treatment, Bim fragments can be seen. This may suggest that Bim-EL was degraded and therefore contributing to the survival of mitotic cells.

Treatment with Taxol plus U0126 successfully inhibited ERK1/2 phosphorylation (Figure 3.28B, middle panel) and also Bim-EL degradation, as fragmentation of Bim-EL cannot be seen (Figure 3.28B, upper panel). However, a slight shift in mobility of Bim-EL can still be seen although this shift does not reach the level seen after PMA treatment (Figure 3.28B, upper panel). Taking into account these results, it seems that ERK1/2 may have a role in regulating Bim-EL phosphorylation and possibly Bim-EL degradation.

In addition, equal protein loading confirmed by anti-ERK1/2 (Figure 3.28, lower panels) confirms that the changes observed in Bim-EL and ERK1/2 phosphorylation is not due to varying levels of ERK1/2 protein.

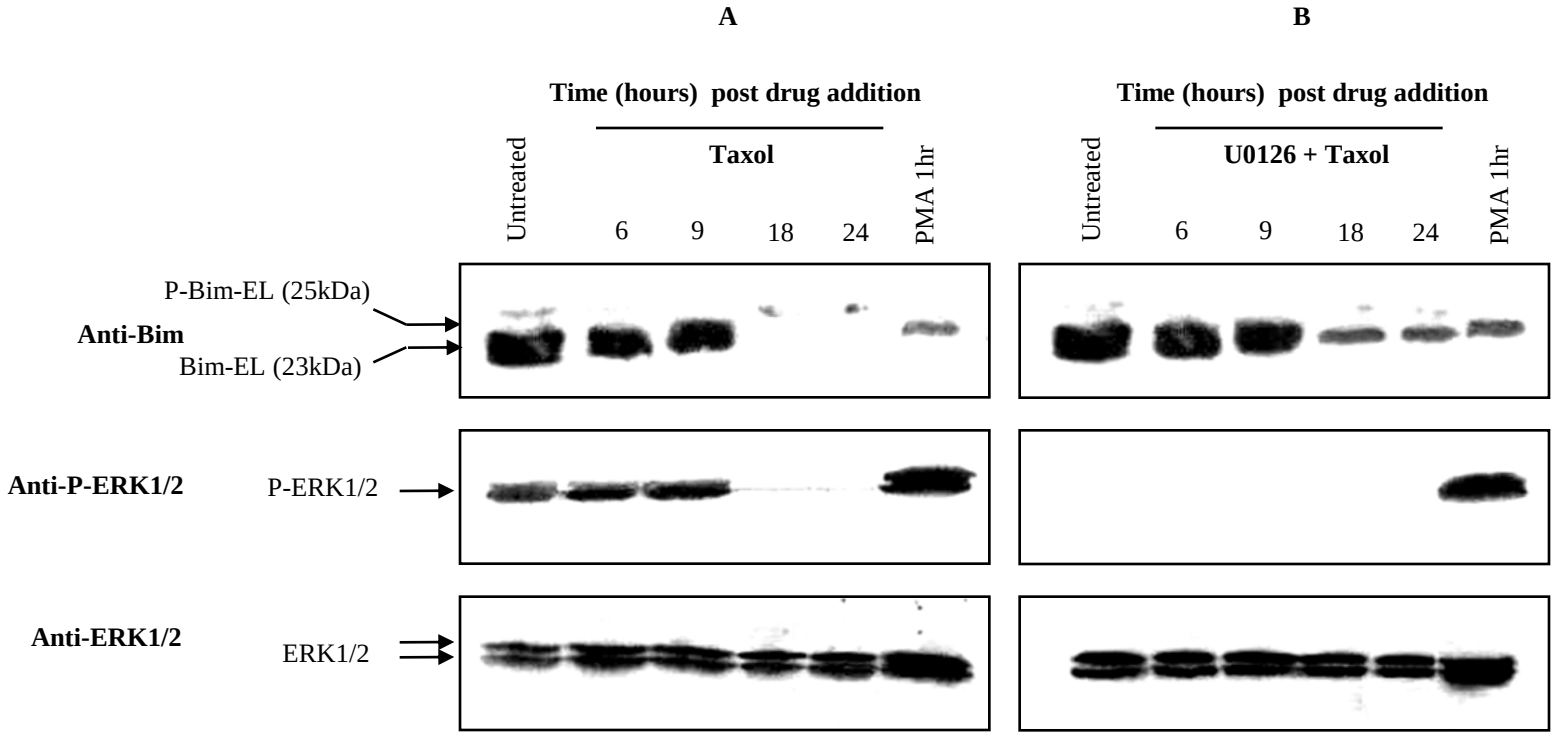


Figure 3.28: The effect of Taxol (U0126) on the phosphorylation state of Bim-EL. Hela cells were treated with Taxol (1µM), with and without a 15 minute pre-treatment with U0126 (10µM) for the indicated times (hours). Control cells were either untreated or treated with PMA (1mg/ml) for one hour. The cells were lysed into 2x SDS sample buffer as described in Materials and Methods (see section 2.2.1). The cell extracts were immunoblotted with anti-Bim (Stressgen, upper panel), anti-P-ERK1/2 (middle panel) or anti-ERK1/2 antibodies (lower panel). Bim-EL phosphorylation was measured via a mobility shift.

CHAPTER 4
DISCUSSION

The focus of this project has been to determine whether ERK1/2 activation by MIAs such as Taxol acts as a survival signal and if so, to determine the mechanism by which it may prevent or delay cell death. Therapeutic agents such as Taxol have been used to treat cancers such as ovarian, breast and non-small cell lung cancers. Taxol and other MIAs have been reported to activate the ERK1/2 pathway (see section 1.5) which belongs to the mitogen-activated protein kinase (MAPK) family of signalling pathways (see section 1.2). There have been reports that activating the ERK1/2 pathway contributes to cell survival. However, the mechanism by which ERK1/2 mediates cell survival remains unknown. Therefore the aims of this project were: firstly, to investigate ERK1/2 activation in response to MIAs; secondly, to examine whether inhibiting ERK1/2 activation induces cell death and finally to investigate how ERK1/2 may regulate cell survival by examining the phosphorylation states of the possible ERK1/2 substrates; caspases-9 and Bim. It is hoped that understanding the role of ERK1/2 in cell survival may lead to the development of more effective anti-cancer therapies.

4.1 ERK1/2 Activation and Intracellular Localisation in Response to MIAs

I investigated ERK1/2 activation in response to MIAs in two ways; the first was by Western blotting with an anti-P-ERK1/2 antibody and secondly by examining ERK1/2 translocation into the nucleus.

Initially I determined both the optimum concentrations and incubation times required for MIAs to activate ERK1/2 in Hela cells. The MIAs used in this project were Nocodazole, Taxol, Vinblastine and Vincristine. Nocodazole is not used therapeutically but has been used extensively in the laboratory as a microtubule-depolymerising agent (Hayne *et al.,* 2000, Deacon *et al.,* 2003). ERK1/2 activation was observed at low concentrations of each drug

(1μM or 3μM) and this level of ERK1/2 activation increased with the concentration of MIAs used (see Figure 3.1). In this study, it was ensured that the concentrations of Taxol, Vinblastine and Vincristine used were within the therapeutic range found in the plasma following drug administration (see section 1.5, pages 26 to 28), as reported in other studies (Boldt *et al.,* 2002, McDaid *et al.,* 2001, MacKeigan *et al.,* 2000 & Seidman *et al.,* 2001). Therefore the majority of experiments in this study were carried out using 3μM of Nocodazole and 1μM of Taxol, Vinblastine and Vincristine.

To determine their effect on ERK1/2 activation, Hela cells were incubated with either Nocodazole (3μM), Taxol (1μM), Vinblastine (1μM) or Vincristine (1μM) for a varying times. Although ERK1/2 was activated from 1 hour to 8 hours after drug treatment, prolonged treatment (12 and 24 hours) caused the majority of cells to arrest in mitosis where ERK1/2 activation was reduced as assessed by using a phospho-specific ERK1/2 antibody. This result is consistent with previous studies (Shapiro *et al.,* 1998, Harding *et al.,* 2003, Bogre *et al.,* 1999) that have investigated ERK1/2 activation and found that ERK1/2 activation is reduced in mitotic cells. However, the reason why ERK1/2 activation is reduced in the mitotic cell population remains unclear. There has been no direct evidence that suggests ERK1/2 is required in mitosis but there have been studies attempting to link activated ERK1/2 to the events of mitosis (Shapiro *et al.,* 1998, Zecevic *et al.,* 1998). Although immunoblotting using phospho-specific ERK1/2 antibodies shows no apparent P-ERK1/2, immunocytochemical staining has the advantage of detecting levels of P-ERK1/2 that may be below the threshold of detection by immunoblotting. Using an anti-active ERK1/2 antibody, Shapiro *et al* (1998) demonstrated that during the normal cell cycle, ERK1/2 is active throughout mitosis but at each phase, its location changes. Active ERK1/2 localised to the spindle poles between prophase and anaphase, and during metaphase, active ERK1/2 is localised in the chromosome

periphery. During cytokinesis, active ERK1/2 localises to the midbody (Shapiro *et al.,* 1998). Zecevic *et al* (1998) reported very similar data, again using phospho-specific ERK1/2 antibodies. However, they also identified a possible substrate for ERK1/2, the kinetochore motor protein CENP-E. Zecevic *et al* (1998) proposed that ERK1/2 alters the ability of CENP-E to mediate interactions between chromosomes and microtubules, an important event in mitosis.

The second method that I used to assay ERK1/2 activation by MIAs involved determination of its intracellular localisation. This was done using both an anti-P-ERK1/2 antibody and Green Fluorescent Protein (GFP)-tagged Erk1 (GFP-Erk1). Nuclear translocation of P-ERK1/2 has been reported by many groups (Khokhlatchev *et al*, 1998, Brunet *et al.*, 1999, Horgan and Stork, 2002) in cell lines such as REF 25 (rat embryo fibroblast) cells, Swiss 3T3, Chinese hamster lung fibroblasts, Neuronal PC12 cells and COS-7 cells.

GFP-Erk2 transfection into PC12 neuronal cells allowed detection of rapid GFP-ERK2 translocation to the nucleus upon cell stimulation by NGF and EGF (Horgan and Stork, 2002). Microinjection of phosphorylated ERK2 (P-ERK2) protein into the cytoplasm of REF 25 cells resulted in rapid (within one minute after microinjection) P-ERK2 translocation to the nucleus, as detected using anti-P-ERK antibodies (Khokhlatchev *et al.*, 1998). Further work by Khokhlatchev *et al* (1998) involved using kinase-deficient K52R Myc-epitope tagged ERK2 to demonstrate that ERK2 was phosphorylated and translocated to the nucleus upon cell stimulation with platelet-derived growth factor (PDGF). By introducing a mutant ERK2 protein, where the activating phosphorylation sites, threonine 183 and tyrosine 185, were mutated into alanine (T182A) and phenylalanine (Y185F), respectively, Khokhlatchev *et al* (1998) also

found that phosphorylation at these two sites is the important factor that determines nuclear translocation of ERK2 (Khokhlatchev *et a.,* 1998).

One aim of the present study was to confirm that endogenous ERK1/2 was phosphorylated when cells were stimulated with MIAs and that the P-ERK1/2 translocates to the nucleus in Hela cells. The results of studies using both immunocytochemistry, using antibodies against P-ERK1/2, and GFP-ERK1 indicated that P-ERK1/2 translocated from the cytoplasm to the nucleus within 60 minutes of treatment with the MIAs. Although immunostaining shows moderate levels of P-ERK within the nucleus, the data obtained by GFP-ERK1 allowed clear observation of P-ERK1/2 translocation into the nucleus upon activation by the MIAs. P-ERK1/2 translocation into the nucleus occurred predominantly at one hour following the addition of MIAs. This suggests that P-ERK1/2 is rapidly translocated to the nucleus upon activation, presumably to initiate the expression of proteins required by the cell (such as cyclin D), and then exported back to the cytoplasm between 2 to 4 hours after treatment.

The data showing ERK1/2 phosphorylation and translocation in response to MIAs supports previous studies that ERK1/2 can be phosphorylated and rapidly translocated to the nucleus (Khokhlatchev *et a.,* 1998; Brunet *et al.,* 1999; Horgan & Stork, 2003). The mechanisms involved in ERK1/2 translocation are much less understood. Khokhlatchev *et al* (1998) reported that microinjected mutants (T183A and Y185F)) of ERK2 can dimerise with phosphorylated wild-type ERK2 to enter the nucleus but further studies are required to clarify dimerisation as part of a mechanism for ERK2 nuclear translocation. Another study suggested the possibility that ERK1/2 translocation requires ERK1/2 activation and the synthesis of short-lived proteins, possibly nuclear anchors (Lenormand *et al.,* 1998). Lenormand *et al* (1998) inhibited protein synthesis with cycloheximide or inhibited the proteasome-dependent protein degradation pathway with LLnL (a membrane-permeable inhibitor of cysteine proteases that

acts upon proteasomes), which then lead to the inhibition of ERK1/2 nuclear translocation. Although Lenormand *et al* (1998) did not investigate the mechanisms involved in nuclear anchor-mediated ERK1/2 nuclear translocation, their study suggested that ERK1/2 activation leads to the synthesis of MAPK nuclear anchor proteins which somehow allows ERK1/2 to localise inside the nucleus.

Further work is essential to understand how ERK1/2 is transported to the nucleus and exported out but taking into account the results obtained in this project and previous published reports, the phosphorylation and activation of ERK1/2 is essential for the relocation of a proportion of ERK1/2 from the cytoplasm to the nucleus.

4.2 Inhibition of ERK1/2 and Cell Death

The idea that ERK1/2 may be involved in cell survival in response to MIAs originated from the observation that inhibiting ERK1/2's activity using a MEK inhibitor such as U0126 or CI-1040, in combination with MIAs such as Taxol, resulted in greater cell death than that caused by MIAs alone (McDaid and Horwitz., 2001, McDaid *et al.,* 2005, MacKeigan *et al.,* 2000). The mechanism by which ERK1/2 may regulate cell survival is currently unclear. Therefore, in the present study U0126, a specific inhibitor of MEK (Favata *et al.,* 1998), was used to inhibit ERK1/2 activity.

Pre-incubation of cells with U0126 at a range of concentrations before the addition of Nocodazole, Taxol, Vinblastine or Vincristine indicated that 10μM U0126 suppressed MIA-induced ERK1/2 activation. This result was consistent with the data published by Favata *et al*, 1998 and MacKeigan *et al,* 2000. The inhibition of ERK1/2 was dose-dependent and sustained

as long as the cells were incubated with the drug (up to 8 hours). This property may be useful when considering the role of a MEK inhibitor, such as U0126, in cancer chemotherapy.

Before investigating the mechanism by which ERK1/2-inhibition may regulate cell death, I first aimed to reproduce what had been shown by previous studies (MacKeigan *et al.*, 2000; McDaid and Horwitz, 2001; McDaid *et al.*, 2005), mainly that inhibiting ERK1/2 increase cell death in response to MIAs in Hela cells. Previous studies have reported an increase in cell death after treatment with an MIA in combination with a MEK inhibitor for 16 hours or more in cultured cell lines (MacKeigan *et al*, 2000; Stadheim *et al*, 2001; McDaid and Horwitz, 2001) and also *in vivo* using human heterotransplanted non-small cell lung cancer (NSCLC) tumours in nude mice (McDaid *et al*, 2005). In comparison to their treatment with Taxol alone, the introduction of a MEK inhibitor more than doubled the number of cells undergoing apoptosis. However, in this project, I wanted to determine the kinetics of MIA-induced cell death in the presence of U0126 at earlier time points to see if ERK1/2 inhibition had similar effects.

The effect of ERK1/2 inhibition on the survival of Hela cells treated with MIAs was assessed by a number of assays: (i) Cleavage of cytokeratin-18 using an M30 antibody; (ii) PARP cleavage; (iii) Annexin binding assay; (iv) measuring apoptosis specifically in mitotic cells using the M30 antibody; (v) cleavage of caspase-8 and Bid; (vi) cleavage of caspase-9 and caspase-3. Due to time constraints, for this part of the study, it was decided to examine the effects of MEK inhibition using just Nocodazole (a microtubule depolymerising agent) and Taxol (a microtubule-stabilising agent).

Immunostaining with the M30 antibody to detect cytokeratin-18 indicated an approximate 2-fold increase in cell death at each time point in response to U0126 compared to treatment with

Nocodazole or Taxol alone. Cell death in response to treatment with MIAs alone was seen after 12 hours, but in this study, the presence of U0126 increased the number of cells undergoing apoptosis between 2 to 8 hours, as measured by cytokeratin-18 cleavage. Unfortunately, the controls required for results shown in figures 3.14 and 3.15 did not include treatment with U0126 alone for the time points investigated. However, the results obtained using Annexin-binding (see figure 3.18, page 81) shows that treatment with U0126 alone for 24 hours did not affect the number of cells undergoing apoptosis in comparison to treatment with DMSO alone. Therefore, I would presume that treatment with U0126 alone would not affect cytokeratin-18 cleavage. This suggests that ERK1/2 may represent a survival signal in Hela cells as inhibiting ERK1/2 activity increases apoptosis.

To confirm the results obtained with the M30 antibody I also examined PARP cleavage following Nocodazole and Taxol treatment (with and without U0126) over the same time course. PARP cleavage was observed after 12-hour treatment with Nocodazole or Taxol alone where the majority of cells had arrested in mitosis, showing consistency with the M30 antibody above. However, in contrast to the M30 data, the presence of U0126 did not affect the levels of PARP cleavage confirming that in response to MIAs, cells arrest in mitosis before undergoing apoptosis.

Annexin-V-binding assay was employed to measure the number of cells undergoing apoptosis at each time point in response to Taxol alone or in the presence of Taxol and U0126. Annexin-V is a calcium-dependent phospholipid binding protein which binds phoshatidylserine that is exposed to the surface of the cell during apoptosis. Exposure of phoshatidylserine is an early marker for apoptosis, therefore a useful tool for this assay. The data obtained were variable but indicated that cell death does not increase between 2 to 8 hours of treatment with Taxol plus U0126, whereas longer periods of treatment (16 hours) resulted in increased cell death (up to

20%) of the mitotic population in response to ERK1/2 inhibition. 24 hour treatment with Taxol plus U0126 however, gave an unusual result which may have been due to errors in preparing the cells for flow cytometry. Unfortunately, due to limited time in the laboratory, I was unable to isolate and rectify the error.

The results obtained so far indicates that even in the presence of a MEK inhibitor, cell death only increases significantly once cells have entered mitosis. There is the possibility that the activation state of ERK1/2, which differs in the different phases of the cell cycle, could be the key. Immunocytochemical studies using P-ERK1/2 antibodies have shown that ERK1/2 is found active in the nucleus during pro-metaphase of the cell cycle (Shapiro *et al.*, 1998; Zecevic *et al.*, 1998). In response to MIAs such as Taxol, active ERK1/2 in cells that are in pro-metaphase could possibly bring about survival of these cells, therefore, the inhibition of ERK1/2 in mitotic populations of cells by U0126 may increase the number of cells undergoing apoptosis. These results contradict the data obtained with cytokeratin-18 staining as a marker for apoptosis. The increase in the number of apoptotic cells observed at early time points following the addition of U0126 suggests that the M30 antibody is a highly sensitive assay in comparison to PARP cleavage assay or the Annexin-V assay. However, there is scope for further improving and duplicating the Annexin-V assay to obtaining reliable, accurate and informative results.

I next decided to assess the effects of U0126 on apoptosis in Hela cells arrested in mitosis by Taxol. The reason for this was because P-ERK1/2 was found to co-localise with the centrosomes, suggesting an important function for ERK1/2 during mitotic cell cycle arrest. It has been reported that ERK1/2 co-localised with γ-tubulin at the centrosomes in Swiss 3T3 cells (Willard & Crouch, 2001), which then lead to the conclusion that ERK1/2 may act to coordinate passage through mitosis in Swiss 3T3 cells. To investigate whether P-ERK1/2 that

is co-localised with the centrosomes is involved in cell survival, mitotic Hela cells were treated with Taxol, either with or without U0126 for 24 hours (Patel, V & Patel, R). My results indicated that there was a dose-dependent increase in apoptosis in response to increasing concentrations of U0126, suggesting that inhibiting ERK1/2 that is located at the centrosomes is one of the key steps to bringing about cell death in Taxol-treated cells. Previous studies of ERK1/2 inhibition were based on prolonged (12 to 16 hours) treatments with Taxol and a MEK inhibitor (MacKeigan *et al,* 2000; Stadheim *et al,* 2001; McDaid and Horwitz, 2001) where the majority of cells would have arrested in mitosis and the results of this experiment are consistent with this by showing that the effects of a MEK inhibitor can be seen mainly after prolonged treatment.

Following this, the mechanism responsible for bringing about cell death in response to Taxol and MEK/ERK1/2 inhibition was investigated. Previous unpublished work in our laboratory (Mistry, P & Patel, R) investigated the cleavage and activation of caspase-3 and caspase-9 (see section 1.7, figures 1.13 & 1.15). Cleavage of both caspase-3 and caspase-9 was observed only in the mitotic cells after treatment with Nocodazole for 12 and 24 hours. This suggested that the intrinsic pathway of apoptosis may be involved in MIA-induced apoptosis. To extend these observations I investigated the activation states of caspase-8, Bid, caspase-9 and caspase-3.

In response to Taxol or Nocodazole alone or in the presence of U0126, there was no cleavage of caspase-8. There were however, basal levels of cleaved Bid in cells treated with Nocodazole and Taxol alone which then increased slightly once U0126 was introduced. Bid is a direct substrate of caspase-8 (Li *et al.,* 1998) but in recent years there has been speculation regarding Bid cleavage by caspase-3 (Esposti *et al.,* 2003) and also caspase-2 (Guo *et al.,* 2002). Caspase-3 and caspase-2 are proteins of the intrinsic pathway (Budihardjo *et al.,* 1999; Guo *et*

al., 2002) therefore it is unlikely that cell death occurs via the extrinsic pathway. Unfortunately, the anti-caspase-2 antibody available to me failed to detect caspase-2 in control cells and Nocodazole or Taxol-treated cells (data not shown). Due to limited time I was unable to examine the activation state of caspase-2 which may have given further insight into the reason why cleaved Bid was detected in my samples.

Caspase-9 and caspase-3 cleavage were only observed after treatment with Nocodazole or Taxol alone for 24 hours which supports our previous unpublished data which showed caspase-9 and caspase-3 cleavage in the mitotic cells after 12 and 24 hour treatment with Nocodazole (Figure 1.16). In response to U0126 these levels increase slightly, suggesting that both caspase-9 and caspase-3 are involved in apoptosis. The caspase-9 and caspase-3 processing seen in response to U0126 does not cause increased apoptosis at earlier time points, as assessed by PARP cleavage and the Annexin-V-binding data, but it may play a role after treatment with MIAs and U0126 for more than 12 hours (i.e. when cells have arrested in mitosis).

These results show that mitotic cell cycle arrest is followed by apoptosis and that centrosomal ERK1/2 may act as a survival signal in mitotically-arrested Hela cells. This is further supported by U0126-mediated increase in apoptosis of mitotic cells which is mediated by the intrinsic pathway of apoptosis.

4.3 Mechanism of ERK1/2 Mediated Cell Survival

4.3.1 Caspase-9

It has been reported that caspase-9 is phosphorylated by activated ERK1/2 on its threonine 125 residue thereby rendering it inactive by inhibiting the processing of caspase-9 associated with

its enzymatic activation (Allan *et al.,* 2003). Although Allan *et al* (2003) used mouse NIH3T3 and human HEK293 cells, it is possible that activation of ERK1/2 by MIAs may also lead to the phosphorylation of caspase-9 in Hela cells.

To detect caspase-9 phosphorylation, I used a phospho-specific antibody against phospho-caspase-9 (P-caspase-9 on thr125). Initially, Hela cells were treated with PMA (1μg/ml) for 60 minutes and the cell extracts were immunoblotted with the anti-P-caspase-9 antibody. Although ERK1/2 was successfully activated in response to PMA, phosphorylation of caspases-9 was not observed with the antibody (data not shown). To improve our chances of detecting P-caspase-9, Hela cells were then treated with PMA (1μg/ml) and Okadaic acid (1μM), a specific inhibitor of protein phosphatases 1 and 2A (Haystead *et al.,* 1989). These cell extracts were also immunoblotted with the anti-P-caspase-9 antibody and again ERK1/2 was activated but P-caspase-9 was not detected (data not shown).

It has since been reported that caspase-9 is also phosphorylated in cells arrested in mitosis, in response to Nocodazole or Taxol, on threonine 125 by CDK1/cyclin B as a way of regulating the onset of apoptosis (Allan & Clarke, 2007). It was proposed that cells arrested in mitosis eventually bypass the spindle assembly checkpoint (called "mitotic checkpoint slippage") caused by gradual inactivation of cyclin B, thereby causing a slow net dephosphorylation of caspase-9 and initiating apoptosis (Allan and Clarke, 2007). In a final attempt to detect caspase-9 phosphorylation, cell extracts made from Taxol-treated cells (with or without pre-treatment with U0126), including mitotic cells after 24 hour treatment with Taxol, were immunoblotted with the anti-P-caspase-9 antibody but again the antibody failed to detect caspase-9 phosphorylation (Figure 3.24, left panel).

From all of the experiments that were attempted, it was concluded that the anti-P-caspase-9 antibody was not working. After contacting the manufacturers of this antibody, they sent an

aliquot of their positive control; MCF-7 cells treated with PMA and epidermal growth factor (EGF). The manufacturers advised that in this cell extract, the P-caspase-9 antibody detected phosphorylated caspase-9 at 60kDa. Surprisingly, the anti-P-caspase-9 antibody did not consistently detect P-caspase-9 so we decided against further use of this antibody.

I then decided to overexpress caspases-9 by transfecting Hela cells with Flag-tagged caspase-9 and treating them with PMA (1μg/ml). Although the transfections were successful, as shown using an anti-Flag antibody, the anti-P-caspase-9 antibody did not detect P-caspase-9 in the transfected control cells or in the transfected cells following PMA treatment. Immunoblotting with the anti-caspase-9 antibody showed that caspase-9 was expressed in the transfected cells but no mobility shift was observed to indicate that caspase-9 had been phosphorylated (Figure 3.24, right panel). In these experiments, we were unable to obtain a reliable positive control showing caspase-9 phosphorylation and due to the time taken to perform initial tests, I was unable to continue investigating the role of ERK1/2 in regulating caspase-9 phosphorylation.

4.3.2 Bim

The pro-apoptotic Bcl-2 protein Bim has also been suggested to be a target for regulation by ERK1/2. Three independent studies have shown that Bim is phosphorylated by ERK1/2, thereby targeting it for proteasomal degradation (Ley *et al.,* 2003, Luciano *et al.,* 2003 and Marani *et al.,* 2004).

Initially, Hela cells were treated with PMA (1μg/ml) and the cell extracts were immunoblotted with a phospho-specific antibody against phospho-Bim (P-Bim) and also an anti-Bim antibody (Calbiochem). The anti-P-Bim antibody was very sensitive so a lot of background was observed (data not shown). The antibody was diluted further but the result was the same. The commercial anti-Bim antibody did not detect any protein at the various concentrations tested

(data not shown). Instead it was decided to overexpress Bim by transfecting HA-epitope-tagged Bim (HA-Bim) into Hela cells using Fugene 6 transfection reagent. After transfection, the cells were treated with PMA (1μg/ml), and the cell extracts were immunoblotted with both anti-P-Bim and anti-Bim antibodies. Transfection was confirmed using an anti-HA antibody but again the anti-P-Bim antibody produced a lot of non-specific binding whereas the anti-Bim antibody failed to detect any protein (data not shown). I varied the incubation times of the HA-Bim plasmid plus transfection reagent mix with the cells but it seemed that overexpressing Bim caused cell death quite rapidly, leaving very few live cells to treat with the drugs.

A second anti-Bim antibody (Stressgen) was kindly provided (by Roger Snowden, MRC Toxicology Unit, University of Leicester) as an alternative and with this antibody, we hoped to detect native Bim and also observe P-Bim by a mobility shift as a result of phosphorylation as has been reported previously (Marani *et al*., 2004). Hela cells were treated with DMSO, PMA and anisomycin and MCF-7 cells were treated with DMSO and PMA. It has been established that Bim expression levels vary between cell types; with Hela cells expressing very low levels of Bim protein (O'Reilly *et al.,* 2000). MCF-7 cells were found to express high levels of Bim protein and therefore it was used as a positive control in this experiment. The anti-Bim antibody (Stressgen) was used to immunoblot cell extracts prepared from Hela cells and MCF-7 cells. The results showed that upon either PMA treatment or anisomycin treatment, the mobility of Bim increased from 23kDa to 25kDa (Figure 3.21). This mobility shift of Bim has been shown to be as a result of phosphorylation (Ley *et al.,* 2003). Therefore, in Hela cells Bim is phosphorylated in response to PMA and anisomycin. In MCF-7 cells, a mobility shift of the Bim protein was also seen in response to PMA.

Recent publications (Ley *et al.,* 2003, Luciano *et al.,* 2003 and Marani *et al.,* 2004) have reported Bim phosphorylation mediated by ERK1/2 in a variety of cell lines, suggesting the possibility that Bim phosphorylation by ERK1/2 may be universal to all cell types. To investigate the possibility that Bim is phosphorylated by ERK1/2 in response to MIAs in Hela cells, Hela cells were treated with Taxol (either with or without U0126) and immunoblotted with the anti-Bim (Stressgen) and anti-P-ERK1/2 antibodies in the hope to observe a mobility shift to indicate Bim phosphorylation in response to ERK1/2 phosphorylation (Patel, V & Patel, R). The phosphorylation of Bim seen in response to Taxol (6 and 9 hours post treatment) directly correlated with the phosphorylation of ERK1/2. In addition, inhibiting ERK1/2 with U0126 prevented significant Bim phosphorylation, thereby supporting previous publications (Ley *et al.,* 2003; Luciano *et al.,* 2003; Marani *et al.,* 2004) that have suggested a role for ERK1/2 in mediating Bim phosphorylation. Furthermore, fragments of Bim were seen after 18 and 24 hours post Taxol treatment whereas upon ERK1/2 inhibition with U0126, these fragments of Bim did not appear. This result corroborates the notion that ERK1/2-mediated phosphorylation of Bim targets Bim for degradation (Ley *et al.,* 2003).

The main uncertainty is whether these effects on Bim are mediated by ERK1/2 or rather ERK5. Similarities between ERK5 and ERK1/2 have been reported and reviewed by Nishimoto & Nishida (2006). ERK5 has been shown to to be activated in response to serum, one of the well-known activators of ERK1/2 (Kato *et al*., 1997). ERK5 (as well as ERK1/2) can also induce immediate early genes such as c-Fos and c-Jun (Kato *et al*., 1997; Kamakura *et al*., 1999) and the most important similarity with ERK1/2 is that ERK5 activity can be inhibited by U0126, which was identified as MEK1/2-specific inhibitor (Kamakura *et al*., 1999). Taking these factors into account, it seems very likely that ERK5 has the potential to mediate Bim phosphorylation. Further work needs to be carried out in order to confirm this but the results

that have been presented in this project cannot be overlooked. It could be a possibility that both ERK1/2 and ERK5 are required to work together in order to regulate cell survival in response to MIAs but again, some verification is required.

4.4 Conclusion and Future Work

This project has examined the activation state of ERK1/2 in response to MIAs and has shown that ERK1/2 translocates to the nucleus in response to MIAs. Its inactivation has proved to be a very useful strategy in cancer therapy so far by increasing the number of cancer cells undergoing apoptosis. We have found that in Hela cells cell death does not occur between 2 and 8 hours of incubation with U0126. The data obtained in this project has however, given an indication that U0126 increases cell death after extended treatment (16 hours), when cells have arrested in mitosis, as assessed by Annexin-V-binding, which so far, supports the results of previous studies. An interesting prospect arose from the possibility that a small amount of ERK1/2 is active in early mitosis, suggesting that it may be this population of ERK1/2 proteins that are responsible for cell survival. The inhibition of this population of ERK1/2 proteins may increase cell death. Subsequent analysis of apoptosis in mitotic Hela cells treated with Taxol and U0126 showed that U0126-mediated increase in apoptosis is highly dependent on cells being held in mitosis, where the inhibition of ERK1/2 (co-localised with the centrosomes) plays a crucial role. So far, no previous reports have demonstrated the importance of inhibiting centrosomal ERK1/2 specifically in order to increase cell death of MIA-treated cancer cells. This is a vital piece of evidence that may be useful for future therapeutic strategies.

Proteins of the apoptotic pathways were then investigated and from the data presented, I found that the intrinsic pathway is most likely to be involved in bringing about apoptosis (see figure

4.1 for summary). This is the first report showing that the intrinsic pathway may mediate cell death in response to inhibiting ERK1/2 activation using U0126 in Hela cells. Further work here is required to confirm this hypothesis; for example by knocking out caspase-9 and caspase-3 and observing the effects on cell death in the presence of MIAs plus U0126. If the intrinsic pathway is involved, the expected result, having knocked out both caspases, would be an overall decrease in the number of cells undergoing apoptosis in the presence of U0126.

These result may be used for further studies in other cell lines and eventually be a target for improving existing cancer therapies.

This project also investigated the mechanisms of regulation by which ERK1/2 may regulate cell survival in mitotically-arrested cells. Based on published data, we aimed at finding whether caspase-9 and Bim are phosphorylated by active ERK1/2 in Hela cells, making them incapable of initiating apoptosis. Unfortunately this was not resolved in this study and this work needs to continue in order to clarify this potential mechanism. As previous publications have found caspase-9 and Bim to be phosphorylated by active ERK1/2, there is some confidence that similar results may be obtained from further experiments in Hela cells.

So far, alternative anti-P-caspase-9 antibodies to detect phosphorylation on thr125 have not been found from other commercial sources therefore a different approach to detect caspases-9 phosphorylation on thr125 will be required. There is the possibility of phosphorylating recombinant caspases-9 protein with recombinant, active ERK1/2 *in vitro* which then could be used as a positive control for the anti-P-caspase-9 antibody.

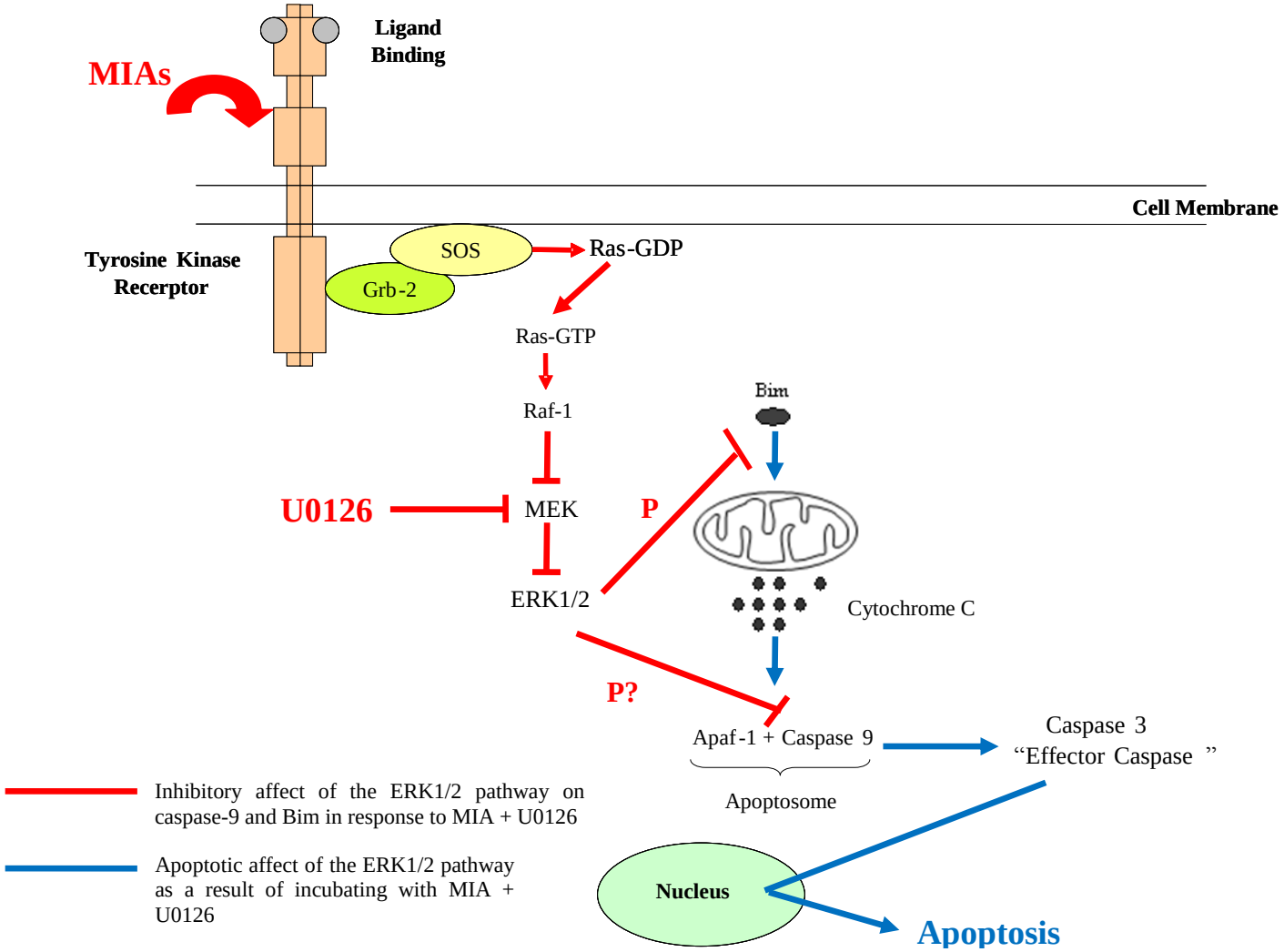


Figure 4.1: Summary of the pathways involved in ERK-mediated cell death in response to MIAs + U0126s. Based on the results of this project, it seems that the mechanism involved in MIA-induced, ERK1/2-mediated cell death requires the components of the intrinsic apoptotic pathway and not of the extrinsic apoptotic pathway. The effect of MIAs with U0126 on the phosphorylation state of caspase-9 was not concluded in this study but the cleavage of caspase-9 observed in response to ERK1/2 activation suggests the involvement of the intrinsic apoptotic pathway.

Alternatively, to increase the probablity of detecting P-caspase-9, caspase-9 could be immunoprecipitated post treatement with MIAs and then western blotted using the anti-P-caspase-9 antibody. If caspase-9 is found to be phosphorylated by ERK1/2, it will be a further indication that the intrinsic pathway is involved in mediating cell death.

The anti-Bim antibody (Stressgen) showed Bim-EL phosphorylation by a mobility shift in response to Taxol and also suggested that this phosphorylation causes degradation of Bim-EL. In addition, further work needs to continue to determine whether Bim-EL phosphorylation is regulated by ERK1/2 or ERK5. One possibility would be to knock out ERK1/2 and observe the effects of MIAs and U0126 on ERK5 using a specific anti-ERK5 antibody and also observe Bim-EL phosphorylation using the anti-Bim antibody (Stressgen). There is a possibility that ERK5 may be the MAPK involved in regulating cell survival as well as ERK1/2 but this cannot be assumed as yet.

Finding how ERK1/2 regulates cell survival will be highly beneficial in designing new drugs to target cancer cells. Likewise, establishing the mechanism by which cell death occurs upon ERK1/2 inhibition will also provide new insights into developing highly specific drugs to target cancer cells.

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