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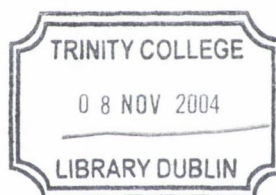
**Investigation into the mechanism underlying the
antiproliferative and apoptotic effects of pyrrolo-1,5-
benzoxazepine compounds**

**Thesis submitted to the
University of Dublin
For the Degree of
Doctor of Philosophy**

By

**Jude Mulligan
Department of Biochemistry
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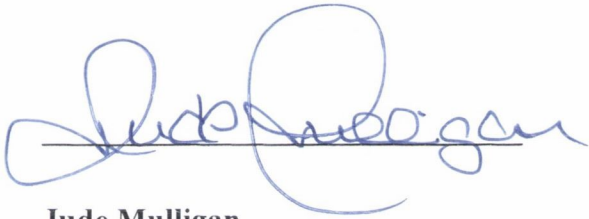
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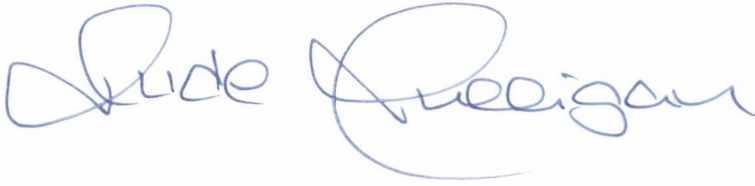
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Abbreviations

AIF	Apoptosis Inducing Factor
APC	Anaphase-Promoting Complex
APS	Ammonium Persulphate
ATP	Adenine Triphosphate
BSA	Bovine Serum Albumin
CAK	CDK-Activating Kinase
CBR	Central-type Benzodiazepine Receptor
CDK	Cyclin Dependent Kinase
CDKI	CDK Inhibitor
CIP/KIP	CDK Interacting Protein/Kinase Inhibitory Protein
CML	Chronic Myeloid Leukaemia
DMEM	Dulbeco's Modified Eagle's Medium
DMSO	Dimethyl Sulphoxide
DPM	Disintegrations Per Minute
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethylene Glycol-bis(β -aminoethyl Ether) N,N, N',N'-Tetraacetic Acid
ERK	Extracellular Signal-regulated Kinase
FACS	Fluorescence Activated Cell Sorter
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
GDP	Guanine Diphosphate
GFP	Green Fluorescent Protein
GTP	Guanine Triphosphate
HCL	Hydrochloric Acid
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HPLC	High Performance Liquid Chromatography
HRP	Horse Radish Peroxidase
HTLV-1	Human T-cell Leukaemia Virus 1

ICAD	Inhibitor of Caspase-Activated Deoxyribonuclease
IP	Immunoprecipitation
JIP	JNK Interacting Protein
JNK	c-Jun N-terminal Kinase
KDa	Kilodalton
LDH	Lactate Dehydrogenase
MAP	Microtubule-Associated Protein
MAP	Mitogen-Activated Protein
MAPK	Mitogen-Activated Protein Kinase
MAPKK	Mitogen-Activated Protein Kinase Kinase
MAPKKK	Mitogen-Activated Protein Kinase Kinase Kinase
MDR	Multidrug Resistance
MEK	MAP kinase/ERK kinase
MEM	Minimum Essential Medium
MKK	Mitogen Kinase Kinase
MLK	Mixed Lineage Kinase
MPF	Maturation-Promoting Factor
MTT	4,5-dimethylthiazol-2, 5-diphenyl tetrazolium bromide
NuMA	Nuclear Mitotic Apparatus Protein
PAK	p21-Activated Kinase
PARP	Poly (ADP-ribose) Polymerase
PBOX	Pyrrolobenzoxazepine
PBR	Peripheral-type Benzodiazepine Receptor
PBS	Phosphate Buffered Salin
PI	Propidium Iodide
PIPES	Piperazine-N,N'-bis[2-ethanesulfonic acid] sequisodium salt
PKC	Protein Kinase C
PMSF	Phenylmethylsulfonyl Fluoride
PVDF	Polyvinylidene Difluoride
Rb	Retinoblastoma Protein
ROI	Reactive Oxygen Intermediate
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean

TBS	Tris Buffered Saline
TEMED	<i>N, N, N', N'</i> ,-Tetramethylethylenediamine
TNF	Tumour Necrosis Factor
TUNEL	Terminal deoxynucleotidyl Transferase mediated bio-dUTP Nick Labelling
UV	Ultraviolet

Abstract

A novel series of pyrrolo-1,5-benzoxazepine (PBOX) compounds have potential as anti-cancer agents. The PBOX compounds appear to be subdivided into two groups, those that exert antiproliferative effects (e.g. PBOX-21) by arresting cancerous cells in the G1 phase of the cell cycle without eliciting cytotoxic effects, and those that induce apoptotic cell death (e.g. PBOX-6). A fuller understanding of the molecular mechanisms underlying the oncostatic and apoptotic effects of these PBOX compounds is essential to their development as therapeutic agents in the treatment of cancer.

The first part of the study concerned the elucidation of the molecular mechanism underlying the oncostatic effect of PBOX-21, a representative member of the antiproliferative subset of PBOX compounds. PBOX-21 induces G1 arrest in a dose- and time-dependent manner in both the human 1321N1 astrocytoma and MCF-7 breast carcinoma cell lines. The PBOX compounds are high affinity ligands of the peripheral-type benzodiazepine receptor (PBR). However, the PBOX-21 induced G1 arrest does not appear to be mediated through binding to the PBR, as saturation of the PBR binding sites with the specific PBR ligand, PK 11195, prior to treatment of the cells with PBOX-21 does not prevent the arrest. The G1 arrest is preceded by a rapid decrease in CDK2 kinase activity and cyclin D₃ expression and is concomitant with a decrease in CDK4 kinase activity. The timing of these events would suggest they are likely to be part of the mechanism by which PBOX-21 initiates the G1 arrest. The inhibition of both CDK2 and CDK4 kinase activities was found to be at least in part due to their early downregulation in protein expression following PBOX-21 treatment. The decrease in CDK4 activity may also be due to a decreased complex association of CDK4 with cyclin D₃. Molecular events found to be elicited subsequent to the onset of arrest were a decrease in CDK6 kinase activity, an upregulation in the CDK inhibitor, p21, and a downregulation of cyclin D₂. Association of CDK6 with cyclin D₃ was also found to be abrogated following treatment with PBOX-21. These events may be involved in maintaining the G1 arrest. Furthermore, Rb becomes hypophosphorylated in response to PBOX-21 treatment. These results indicate that PBOX-21 exerts its growth inhibitory effects through the

modulation of the expression and/or activity of several key G1 regulatory proteins.

The second part of this study concentrated on elucidating the molecular mechanisms underlying the apoptotic effects of a representative pro-apoptotic PBOX compound, PBOX-6. It was demonstrated that PBOX-6 induces apoptotic cell death in the MCF-7 human breast carcinoma cell line. Apoptosis in response to PBOX-6 is preceded by an accumulation of cells in the G2/M phase of the cell cycle. PBOX-6 induces a sequential upregulation in JNK activity and cyclin B₁ associated CDK1 kinase activity. The rapid activation, within 5 minutes, of the JNK kinase signalling pathway would appear to be important in the mechanism of PBOX-6 induced arrest and apoptosis as its inhibition protected against these effects. PBOX-6 also elicits similar effects upon MCF-7 cell cycle kinetics, CDK1 kinase activity and G2/M regulatory proteins as two representative antimicrotubule agents, nocodazole and paclitaxel, suggesting that PBOX-6 may target the microtubule network.

Indirect immunofluorescence demonstrated that PBOX-6 and another pro-apoptotic PBOX compound, PBOX-15, cause a depolymerisation of the microtubule network of MCF-7 cells. They also cause a disassembly of purified tubulin *in vitro*, whereas the non-apoptotic PBOX-21 has no effect on either the cellular microtubule network or purified tubulin. This suggests that the molecular target of the pro-apoptotic PBOX compounds is tubulin. PBOX-6 does not bind to either the vinblastine or the colchicine binding site on tubulin, suggesting that it binds to, an as yet, uncharacterised novel site on tubulin.

The ability of PBOX-6 to bind tubulin and cause microtubule depolymerisation, suggests it as a novel candidate for antineoplastic therapy. This is all the more significant, as both the clinical success of, and increasing resistance to, antimicrotubule agents in the treatment of a variety of cancers has prompted the search for new agents with a similar mechanism.

1 GENERAL INTRODUCTION

1.1 Overview of the eukaryotic cell cycle

The cell cycle can be considered as a collection of highly ordered processes that results in the duplication of the cell, thus underlying the growth and development of all living organisms. It is a universal process whose function is to ensure that individual cells respond properly to environmental cues in order to maintain tissue homeostasis. This requires a precise spatial and temporal control of cell proliferation. The molecular basis of this control is highly conserved from simple unicellular eukaryotes such as yeast, to complex metazoans such as humans. The precision with which cell cycle events are executed ensures the survival of living organisms, while loss of this precision increases genomic instability, an important factor in the development of cancer.

The eukaryotic cell cycle consists of four distinct phases (Fig 1.1). Early light microscopic studies recognised that cell division was preceded by mitosis. Little could be seen during the interval between succeeding mitoses (known as interphase) except that cells grew in volume. Interphase remained a black box until chromosome duplication was detected and shown to occur during a narrow window of time during interphase (Howard and Pelc, 1951). This split interphase into three intervals: G1, the gap between the M phase (mitosis) and the onset of DNA synthesis and replication; S phase, the period of DNA synthesis and replication; and G2, the gap between S and M phases.

In the G1 phase, cells monitor both their internal and external environments to determine whether to divide or to remain in a state of quiescence (G0). Once a threshold size has been reached and specific proteins have been activated, the cell progresses from the G1 to the S phase. Here cells replicate their genome in a restricted time. After this point mitogenic signals are no longer required for cells to progress through the cell cycle. After the DNA has been replicated, cells progress through the second much shorter growth phase, G2. Finally, the cell is ready to divide into two daughter cells, which will start a new cycle.

Precise coordination of the progression through cell cycle phases is critical not only for normal cell division but also for effective growth arrest under

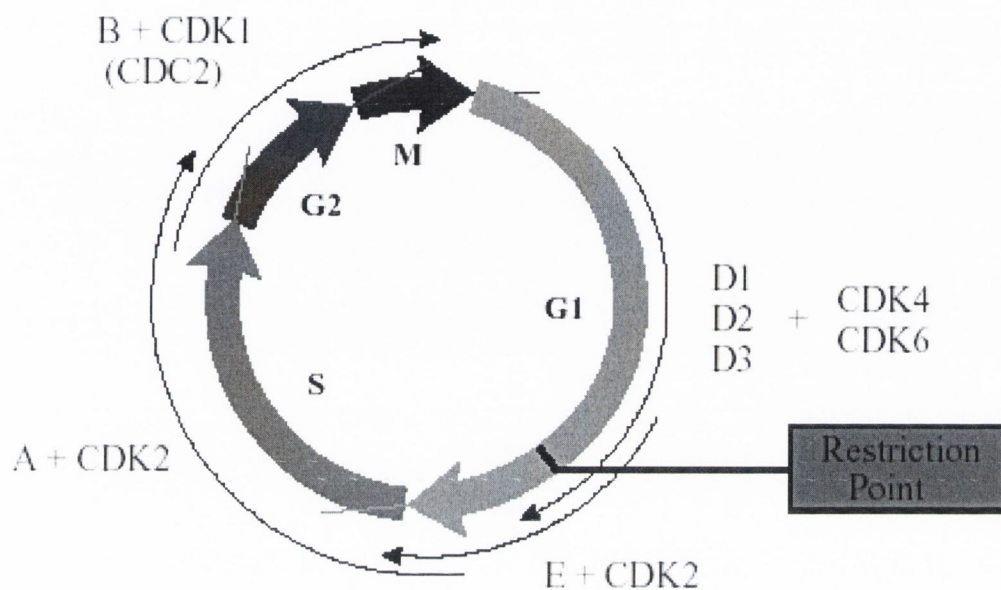


Figure 1.1 The Phases of the Cell Cycle

The cell cycle consists of 4 distinct phases: (1) G1 phase, the first gap or growth phase and the phase in which the cell spends the majority of its time; (2) S phase, the period during which the cell duplicates its DNA content; (3) G2 phase, the second gap or growth phase; (4) M phase, during which time the cell undergoes mitosis/meiosis and divides into two daughter cells. The Restriction Point is a point in late G1 after which the cell is irreversibly committed to completion of the cell cycle. This figure also illustrates the combinatorial interactions of cyclins and CDKs during the cell cycle.

conditions of stress or after DNA damage. As a consequence, a derangement in the cell cycle machinery may contribute to uncontrolled cell growth, which is the principal feature of cancer. An important conceptual advance in understanding the ordering and control of the cell cycle came with the idea that the cell cycle should be considered as a temporally organised sequence (Hartwell *et al.*, 1974). This thinking focused attention on the way in which different cell cycle events were linked together in an orderly sequence. Later events were often found to be dependent upon the successful completion of earlier events, and it was reasoned by Hartwell and Weinert (1989) that these dependencies could be of two types: (1) directly coupled, i.e. the product of an early event is the substrate for a later event (the so-called substrate-product relationship) and (2) regulatory mechanisms, termed ‘checkpoints’. Checkpoints are biochemical pathways that ensure dependence of one process on another process that is otherwise biochemically unrelated (Hartwell *et al.*, 1994). These checkpoints monitor DNA status and integrity and prevent inappropriate transitions into S phase and mitosis when problems are detected.

1.2 Cell Cycle Machinery

The identification of the four distinct cell phases set the stage for the discovery of the core machinery that controls the order and timing of the events. Early cell cycle research revealed the factor that stimulated progression through the meiotic cell cycle in *Xenopus* oocytes. This protein complex was termed maturation-promoting factor (MPF) (Masui *et al.*, 1971). The purification of MPF revealed two components required for activity: a protein called a cyclin and a protein serine/threonine kinase known as a cyclin-dependent kinase (CDK).

1.2.1 Cyclin Dependent Kinases (CDKs)

Cyclin Dependent Kinase 1 (CDK1, also known as cdc2), in combination with cell cycle specific cyclins, is able to single-handedly drive the yeast cell cycle; however, the high level of complexity of mammalian systems dictates that multiple CDKs control various phases of the cell cycle. CDKs are characterised by a number of features including the necessity of cyclin binding and specific activation phosphorylations and dephosphorylations to attain full kinase activity (these are discussed in length below).

CDKs are typically 300 amino acids in length containing certain recognisable motifs that help classify the kinase as a CDK. The T-loop is an 18 amino acid motif containing the conserved activating threonine phosphorylation site. The T-loop acts to block the catalytic cleft of the kinase in the absence of cyclin binding thereby making the cleft inaccessible to potential substrates. The PSTAIRE region is a 16 amino acid stretch representing another highly conserved CDK motif. Cyclin specificity is dictated by the specific PSTAIRE motif of each CDK molecule (Morgan, 1995). In humans to date, nine CDKs (CDK1-9) (Table 1.1) have been identified. Not all of these CDKs participate in cell cycle control and instead participate in basic cellular functions such as transcription.

In mammals, four separate CDKs (CDK1, CDK2, CDK4 and CDK6) are responsible for controlling the various stages of the cell cycle (Elledge, 1996) (Table 1.1). At the G1/S transition, CDK2 and CDK4/6 govern the entry into S-phase. CDK2 continues to be active through S-phase. Lastly, CDK1 becomes active in G2 and its activity persists through mitosis (Sherr, 1996). Another CDK, CDK7 also has ties to the cell cycle in that it is responsible, in combination with cyclin H, to act as the CDK-activating kinase (CAK) (see later). The remaining mammalian CDKs have yet to be shown to play a crucial role in normal cycle progression. Cyclin dependent kinases, as their name implies, require the binding of a cyclin subunit during these phases to begin to achieve kinase activity.

1.2.2 Cyclins

The cyclins earned their names due to their periodic expression in the cell cycle (Evans *et al.*, 1983). Complexes of cyclins with CDKs play a central role in the control of the cell cycle by phosphorylating specific substrates, and this is thought to be critical in the regulation of an ordered sequence of events leading to DNA replication and chromosomal segregation. Therefore, these cyclin/CDK complexes have been deemed the 'cell cycle engines.' To confer specificity to this mechanism, during evolution, eukaryotic cells have developed

Table 1.1 Description of known mammalian cyclins and CDKs

Cell Cycle Protein	Involvement in the Cell Cycle	Function of protein	Regulation of activity
Cyclin A	Through S progression into M	Acitvates CDK2 (G1) and CDK1 (G2)	Transcriptional and ubiquitin degradation
Cyclin B ₁	Progression into M	Activates CDK1 (G2)	Transcriptional and ubiquitin degradation
Cyclin B ₂	Progression into M (G2)	?	Ubiquitin degradation
Cyclin C	Throughout cell cycle	Binds to CDK8	?
Cyclin D ₁ , D ₂ , D ₃	Through G1	Acitvates CDK4 and CDK6	Transcriptional and ubiquitin degradation
Cyclin E	Progression into S	Activates CDK2	Transcriptional and ubiquitin degradation
Cyclin G	Progression into G2/M	?	?
Cyclin H	Part of CAK complex	Activates CDK7 in CAK complex	?
Cyclin I	Throughout cell cycle	Homolog of Cyclin G	?
Cyclin T ₁ , T _{2a} , T _{2b}	Throughout cell cycle	Activates CDK9	?
CDK1	Progression into M	Phosphorylates Histone H1, lamins etc.	Activated by cyclins A and B
CDK2	Progression past G1 restriction point and through S	Phosphorylates pRb	Activated by cyclins A and E
CDK3	Through G1	?	?
CDK4	Through G1	Phosphorylates pRb	Activated by cyclins D1, D2 and D3
CDK5	Post-mitotic neurons	Binds p35	?
CDK6	Through G1	Phosphorylates pRb	Activated by cyclins D1, D2, and D3
CDK7	Part of CAK complex	Activate CDKs and phosphorylate RNA pol II	Activity dependent on presence of substrate?
CDK8	Throughout cell cycle	Binds cyclin C	?
CDK9	Throughout cell cycle	Phosphorylates largest subunit of RNA polymerase II	Activated by Cyclins T1, T2a and T2b

Table from ‘The Molecular Basis of Cell Cycle and Growth Control’ 1999

a large number of functionally different cyclins which control various parts of the cell cycle when complexed with a CDK partner. Their expression patterns dictate at which point in the cell cycle they play a role. Similar to prokaryotes, and also in higher organisms, the new synthesis and the accumulation of a specific cyclin dictates the formation of a cyclin/CDK(s) complex at a distinct point of the cell cycle. The rapid destruction of this cyclin and the synthesis of a new one will ensure the progression into the subsequent phase. Thus, the transient appearance of different cyclin/CDK complexes drives critical cell cycle events such as cell growth (G1), DNA replication (S), and cell division (G2/M) (Figure 1.1). Many different cyclins with their partner CDKs have been cloned and characterised (Table 1.1).

Other ‘cyclins’ exist that do not show cell cycle fluctuations. These proteins are involved in other cellular processes but do not appear to play a role in cell cycle control. They are only deemed cyclins due to their overall structural homology and conserved cyclin domains. These two types of cyclins, cell cycle related (A, B, D and E) and non-cell cycle related (H and C) make up the two cyclin subgroups which are evolutionarily divergent. The recently discovered cyclin T (Peng *et al.*, 1998), cyclin G (Okamoto *et al.*, 1994) and Cyclin I (Nakamura *et al.*, 1995) have not yet been classified into either of these two groups as of yet.

1.2.2.1 Domain Structure

Within the cyclin structure are domains which are required for cyclin activity and degradation. The ‘cyclin box’ is the only criterion by which proteins are judged in order to deem them cyclins. This conserved domain spans approximately 100 amino acids located in the middle of the protein and appears to play a role in CDK binding. Cyclins also possess degradation sequences. The ‘destruction box’ is a protein sequence at the amino-terminus which is required for efficient cyclin proteolysis at the end of its designated cycle. This proteolysis is ubiquitin-mediated. The destruction box is only characteristic of the mitotic cyclins, cyclin B₁, cyclin B₂ and cyclin A (Harper *et al.*, 2002). The D-type and E-type cyclins contain PEST sequences rich in proline, glutamic acid, serine and threonine at their carboxy-terminus (Rechsteiner *et al.*, 1996). These sequences make the proteins inherently unstable. Even though these proteins do not contain

destruction boxes, their degradation is also mediated by ubiquitin-mediated proteolysis.

During ubiquitin degradation proteins are targeted for degradation by the ATP-dependent 26S proteasome complex by ligation to ubiquitin, a 76 amino acid polypeptide. This degradation is initiated by the phosphorylation of threonine 286, which signals the binding of ubiquitin to a lysine residue of the target cyclin. A polyubiquitin chain is formed, and proteins ligated to multiple ubiquitins are degraded to small peptides by the proteasome complex. The Anaphase-Promoting Complex (APC) functions as a cell cycle-regulated ubiquitin ligase that mediates the destruction of the mitotic cyclins, triggering entry into anaphase and ultimately exit from mitosis, and thus, entry into a new cell cycle (Harper *et al.*, 2002) (Figure 1.2).

1.2.3 Cyclin/CDK subcellular targets

One of the subcellular targets of the active cyclin/CDK complexes is believed to be the retinoblastoma susceptibility gene product Rb (Ewen *et al.*, 1993; Kato *et al.*, 1993). Rb becomes phosphorylated at a time close to and perhaps concomitant with the restriction checkpoint. Rb binds to (and negatively regulates the activities of) transcription factors such as E2F-DP1 heterodimers, whose functions are important for the G1/S transition. Phosphorylation of Rb reverses the growth suppressive effects of Rb by releasing E2F from its inhibitory constraint, thereby enabling the untethered factor to activate genes required for DNA replication and thus progression through the cell cycle (reviewed by Nevins *et al.*, 1997; Weinberg, 1995). For more detail see Section 1.3.

1.2.4 Cyclin/CDK structure, function and regulation

1.2.4.1 Complex assembly and associated structural changes

Cyclins bind to the amino terminal portion of the CDK by contacting the middle of the PSTAIRE motif through hydrophobic interactions and by hydrogen bonding to the remainder of the PSTAIRE motif. All protein kinases possess a tertiary structure comprising of a small N-terminal lobe and a larger C-terminal lobe. ATP fits snugly in the cleft between the lobes, in such a way that the

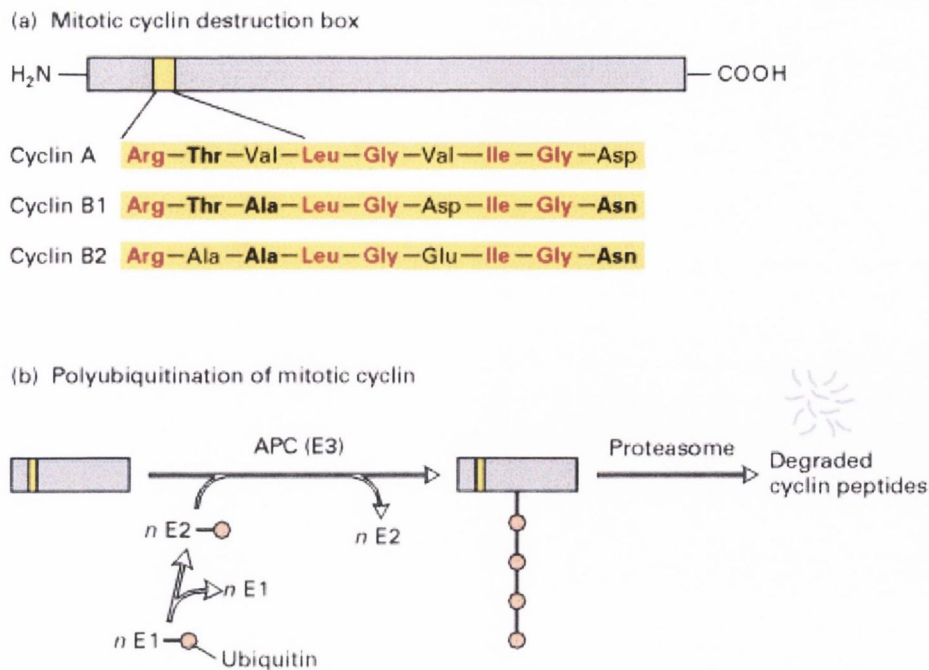


Diagram taken from Molecular Cell Biology, 4th Edition (Lodish, 1999)

Figure 1.2 Polyubiquitination of mitotic cyclins

(a) All mitotic cyclins have a homologous destruction box (yellow) near the N terminus. The destruction box sequences of *Xenopus* mitotic cyclins, cyclin B₁, cyclin B₂ and cyclin A, are shown with amino acid residues conserved in all three proteins in red; residues conserved in two of the three proteins are in black boldface.

(b) Late in anaphase, multiple ubiquitin molecules are added one at a time to lysine residues C-terminal to the destruction box in mitotic cyclins. A ubiquitin first is linked to the activating enzyme E1, which then passes the activated ubiquitin to one of several ubiquitin-conjugating enzymes (E2). After E2, together with a ubiquitin ligase (E3), called the anaphase-promoting complex (APC), recognises a destruction-box sequence, the attached ubiquitin is transferred to the substrate protein. Repetition of this process polyubiquitinates mitotic cyclins, which then are rapidly degraded by a multiprotein proteasome complex.

phosphates are oriented outward, toward the mouth of the cleft. The protein substrate binds at the entrance of the cleft, interacting mainly with the surface of the C-terminal lobe. Nearby residues catalyse the transfer of the γ -phosphate of ATP to a hydroxyl oxygen in the protein substrate.

Monomeric CDKs are completely inactive in the absence of binding with cyclins. This is due to at least two reasons:

(1) Key residues in the catalytic cleft are incorrectly positioned, so that when ATP binds to the monomeric CDK, it does so in a conformation that prevents nucleophilic attack by ATP on the substrate hydroxy group because the phosphates of the ATP are not ideally oriented for the kinase reaction. This prevents the breaking of the phosphate bond and phosphorylation of the substrate. Thus CDK activation requires extensive structural changes in the CDK catalytic cleft.

(2) The T-loop domain of the C-terminus blocks the catalytic cleft. Following the interaction of the PSTAIRE region of the CDK with the 'cyclin box' of the cyclin, the PSTAIRE helix is reoriented, thus allowing the ATP phosphate bond to become susceptible to the attack from a bound substrate. This is also facilitated by the binding of the partner cyclin to the N-terminal portion of the T loop. This interaction displaces the T loop that partially obscures the substrate-binding site. Finally, the interaction between the cyclin and the T loop exposes the threonine residues (e.g. Thr-160 in CDK2 and Thr 161 in CDK1, Thr-172 in CDK4, and Thr-170 in Cdk7 itself) in the T loop, which becomes accessible for phosphorylation by another cyclin/CDK complex known as CDK-activating kinase (CAK). Phosphorylation by CAK completes the full activation of the cyclin/CDK complex. The Thr-phosphorylation by CAK promotes further structural changes in the CDK-subunit leading to stabilisation of the complex with cyclin and affecting the putative substrate-binding site (Russo *et al.*, 1996).

CAK is composed of cyclin H, CDK7 and a third protein of 36kD that is referred to as MAT1 (Adamczewski *et al.*, 1996). Most cyclin/CDK complexes are probable substrates for CAK. However, the observations that cyclin H/CDK7 is a component of TFIIH (a protein belonging to the basal transcriptional machinery) and that it can phosphorylate the C-terminal domain of the RNA polymerase II (Feaver *et al.*, 1994) (thereby participating in the induction of RNA

transcription more than in the activation of cyclin/CDK complexes) raise the question of whether another CDK-specific activating protein exists. Nevertheless, given its role in both transcription and CDK activation, CAK could provide a link between transcription and cell cycle control.

1.2.4.2 Inhibitory phosphorylation

After exerting its activity, the cyclin/CDK complexes need to be inactivated and replaced by other complexes in the next phase of the cell cycle. Tyrosine and/or threonine phosphorylation is suggested to prevent premature kinase activation and to maintain it within tightly regulated limits. The importance of CDK phosphorylation, in terms of regulation of CDK activity was worked out mainly in yeast on CDK1.

During G2, mammalian cyclin B/CDK1 complexes are held in an inactive state by phosphorylation of CDK1 at two negative regulatory sites, threonine 14 (Thr14) and tyrosine 15 (Tyr15), located in the ATP-binding pocket of the kinase. Phosphorylation of Tyr15 by Wee1 and Myt1 protein kinases, interferes with phosphate transfer to a bound substrate (Atherton-Fessler *et al.*, 1993). In contrast, Thr14 phosphorylation inhibits CDK1 by interfering with ATP binding (Endicott *et al.*, 1994).

Dephosphorylation of Thr-14 and/or Tyr-15 is necessary to achieve maximal CDK1 and CDK2 activity. The molecules responsible for these dephosphorylations belong to the CDC25 family of dual specificity phosphatases. In humans, three CDC25 genes (A, B and C) serve the same function in the more complex regulation of mammalian cell in which multiple CDKs exist (Bartek *et al.*, 2001). CDC25a acts on the G1 cyclin/CDK complexes, while CDC25c acts on the cyclin B/CDK1 complex. Even though CDK4 and CDK6 have no Thr14-like residue, both of these molecules undergo similar regulation via CDC25a. CDK4 and CDK6, instead, have only the tyrosine residue in this region (Matsushime *et al.*, 1992). Figure 1.3 represents a generalised model of CDK activation.

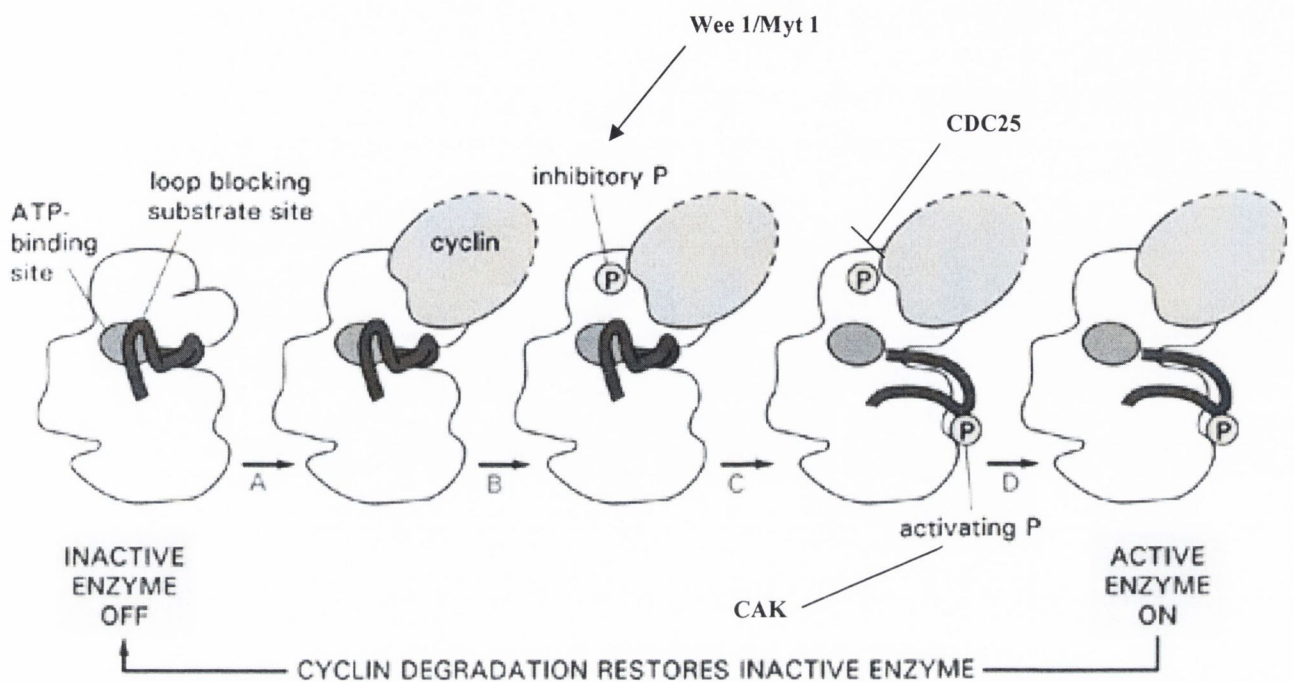


Diagram taken from 'Molecular Biology of the Cell', 3rd Edition, (Alberts, 1994).

Figure 1.3 Model of CDK activation

In step A, the cyclin subunit binds, leading to the addition of the inhibitory phosphate by Wee1/Myt 1 kinase in step B. The activating phosphorylation by CAK occurs in step C, but the enzyme only becomes active after the inhibitory phosphate is removed in step D by a member of the family of CDC25 phosphatases. The ubiquitin-mediated degradation of cyclin after step D causes enzyme inactivation, including the loss of the activating phosphate, which returns the CDK to its initial inactive state.

1.3 Regulation of the G1/S Transition

The G1 phase is the interval in which cells respond to extracellular cues that ultimately determine whether cells will make the decision to replicate DNA and divide or, alternatively, to exit the cell cycle into a quiescent state (G0).

1.3.1 D-type cyclins

The D-type cyclins consist of three family members, cyclins D₁, D₂ and D₃. They are highly conserved between species (Inaba *et al.*, 1992; Sherr *et al.*, 1992; Xiong *et al.*, 1991), suggesting that each member of the family has important roles in cell cycle regulation that constrain evolutionary divergence.

The expression of the D-type cyclins is induced in G1 by mitogenic growth factors, and they are synthesised as long as the stimulation persists. Their levels oscillate moderately during the cell cycle with peak levels achieved near the G1/S transition (Ajchenbaum *et al.*, 1993). However, they are rapidly degraded via the ubiquitin-dependent proteasome pathway (Pagano, 1997) when mitogens are withdrawn regardless of the position of the cell in the cycle.

As the D-type cyclins behave as cell cycle regulatory molecules, the D-type cyclins are prime targets for genetic aberrations leading to carcinogenesis. Cyclin D₁ has been identified as the PRAD-1/bcl-1 proto-oncogene that undergoes gene rearrangements, gene amplification and deregulated expression in a variety of tumour types, (reviewed in Motokura *et al.* (1993)). Abberant expression of cyclin D₂ has been linked to human male germ cell tumourigenesis (Houldsworth *et al.*, 1997) and Cyclin D₃ over-expression has been associated with retinoblastomas (Squire *et al.*, 1984), and malignant lymphomas (Schouten *et al.*, 1990).

D-type cyclins mediate their regulatory function by assembling into holoenzymes with either CDK4 or CDK6 primarily (Figure 1.4), and to a lesser extent CDK2. On the basis of early studies a model of G1 cell cycle progression has emerged whereby cyclins D and E were thought to mediate functionally equivalent sequential inactivating phosphorylation events on pRb (Harbour *et al.*, 1999). However, Lundberg and Weinberg (1998) show that endogenous cyclin D-CDK4/6 complexes are able to only partially phosphorylate pRb, and complete

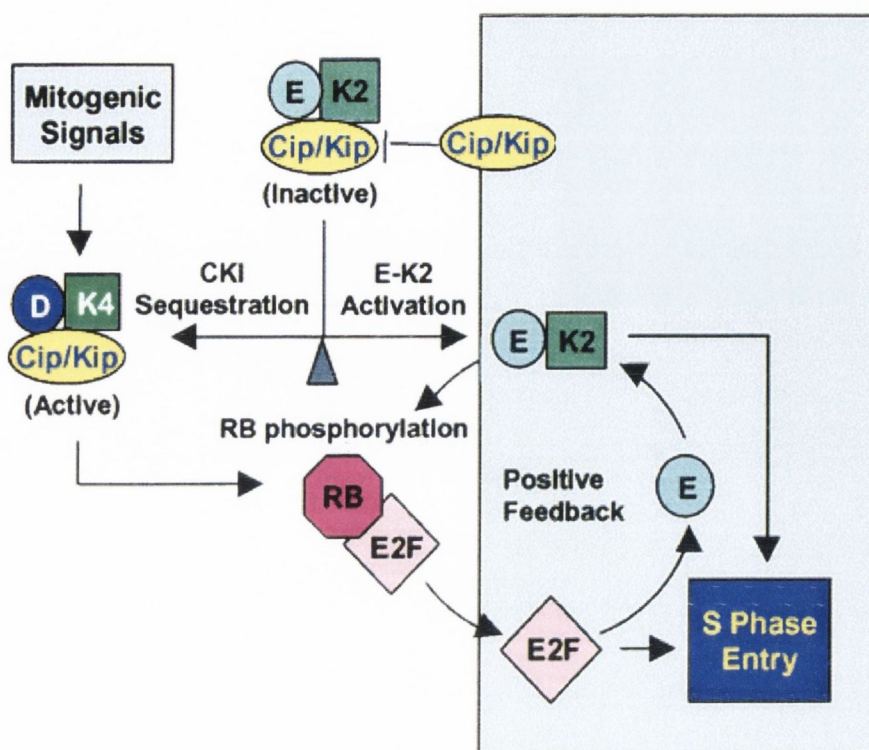


Diagram taken from (Sherr *et al.*, 1999)

Figure 1.4 Regulation of the G1/S transition

Mitogenic signals promote the assembly of active cyclin D-dependent kinases containing either CDK4 (or CDK6; not shown) and a Cip/Kip protein. Sequestration of Cip/Kip proteins lowers the inhibitory threshold and facilitates activation of the cyclin E/CDK2 complex. The cyclin D- and E-dependent kinases contribute sequentially to Rb phosphorylation, canceling its ability to repress E2F family members and activating genes required for entry into S phase. The latter include cyclins E and A, as well as a bank of gene products that regulate nucleotide metabolism and DNA synthesis (for review, see Nevins 1998). The induction of cyclins by E2F (highlighted by background shading) contribute to mitogen independence and to the irreversibility of the transition.

phosphorylation can only be completed by cyclin E-CDK2 complexes.

1.3.2 Cyclin E

The expression of cyclin E in mammalian cells occurs later than the D-type cyclins. Cyclin E expression is periodic with accumulation in late G1 and downregulation in S (Koff *et al.*, 1992b; Lew *et al.*, 1991). Free cyclin E is highly unstable and is subject to ubiquitin-mediated proteolysis, whilst active cyclin E/CDK2 complexes eventually lead to their own destruction by phosphorylating threonine 380 on cyclin E, resulting in dissociation of cyclin E/CDK2 and degradation of cyclin E (Won *et al.*, 1996).

Cyclin E expression is also regulated by pRb via E2F transcription factors (Geng *et al.*, 1996). Cyclin E transcription is activated when pRb is hyperphosphorylated and no longer exerts repression of the cyclin E promoter via E2F transcription factor complexes. Because the cyclin E gene is itself E2F-responsive, cyclin E-CDK2 acts through positive feedback to facilitate progressive rounds of Rb phosphorylation and E2F release (Figure 1.4).

In mammalian cells, cyclin E preferentially forms complexes with CDK2. Maximal levels of cyclin E-dependent kinase activity are found at the G1/S transition. Lundberg and Weinberg (1998) suspect that there is a particular subset of phosphorylation sites upon Rb that are targeted specifically by cyclin E-CDK2.

1.3.3 Cyclin-CDK inhibitors during G1

Whereas cyclin-CDK complexes are key regulators of G1 cell cycle progression, evolution has added yet another layer of G1 cell cycle regulation in the form of cyclin-CDK inhibitory proteins. Two classes of CDKI have been defined, based on their sequence similarity and specificity of action. One class is the CIP/KIP family (for CDK interacting protein/Kinase inhibitory protein), also known as the 'universal CDK inhibitors'. They indifferently inhibit all of the CDKs, but preferentially inhibit CDKs of the G1/S phase. The second class of CDKI is the INK4 family. Members of this family of CDKIs are specific inhibitors as their inhibitory activity is restricted to CDK4 and CDK6.

1.3.3.1 CIP/KIP family

The CIP/KIP family consists of three members: p21^{CIP1,WAF1,Sdi1} (el-Deiry *et al.*, 1993); (Noda *et al.*, 1994), p27^{KIP1} (Toyoshima *et al.*, 1994) and p57^{KIP2} (Lee *et al.*, 1995). The CIP/KIP family members associate with and inhibit the activity of CDK-cyclin complexes through their N-terminal domains that are more conserved than their C-terminal domains (Fotadar *et al.*, 1996). Their inhibitory action is achieved by first anchoring to the cyclin, extending across the amino-lobe of the CDK, and finally invading the active site, culminating in displacement of critical β -strands and occupation of the adenine-binding pocket of the substrate-binding site. Although it was initially assumed that the CIP/KIP proteins would inhibit both CDK4/6 and CDK2, it is now recognised that the CIP/KIP bound cyclin D/CDK complexes remain catalytically active (Cheng *et al.*, 1999). Even more surprisingly, it turns out that CIP/KIP proteins are required for the assembly of the active cyclin D-dependent holoenzymes Cheng *et al.*, 1999; LaBaer *et al.*, 1997). It appears that Cip/Kip proteins act in a stoichiometric fashion. The conversion of active complexes to inactive complexes is achieved by changing the ratio of CIP/KIP protein to cyclin/CDK, such that active complexes contain a single CIP/KIP molecule (Figure 1.4), whereas inactive complexes include multiple CIP/KIP subunits.

1.3.3.2 p21

The first CDK inhibitor to be discovered was p21. It is the prototype of universal inhibitors of CDK enzyme activity. p21 expression has been shown to be regulated largely at the transcriptional level by both p53-dependent and independent mechanisms (Gartel *et al.*, 1999). p53 is a sequence specific DNA binding protein able to activate transcription leading to either cell cycle arrest or apoptosis. Hence p53 is referred to as a tumour suppressor protein. In response to radiation and chemotherapy, p53 protein is stabilised and mediates cell cycle arrest and apoptosis. The p21 promoter contains two conserved p53-binding sites, and at least one of these is required for p53 responsiveness after DNA damage (Macleod *et al.*, 1995). p21 is induced by p53 in response to genotoxic stressors and serves to buffer cyclin/CDK activity to block cell cycle progression and allow DNA repair.

1.3.3.3 INK4 family

The INK4 family contains four closely related members, which all specifically inhibit the activity of CDK4 and CDK6 (Figure 1.4). They do not inhibit any other kinase throughout the cell cycle. The members of the INK4 family are p16^{INK4A,MTS1} (Serrano *et al.*, 1993), p15^{INK4B,MTS2} (Hannon *et al.*, 1994), p18^{INK4C} and p19^{INK4D} (Guan *et al.*, 1994); (Hirai *et al.*, 1995). p15 and p16 contain four ankyrin repeats, whereas p18 and p19 have five (Serrano *et al.*, 1993); (Hannon *et al.*, 1994); (Guan *et al.*, 1994); (Hirai *et al.*, 1995); (Russo *et al.*, 1998).

Structural studies of the p16-CDK6 and p19-CDK6 complexes suggest that association with INK4 family members prevents ATP binding of CDK6, and that p16 indirectly prevents CDK6 from binding to cyclin D (Russo *et al.*, 1998). Thus INK4-bound CDKs cannot support catalysis and are isolated as CDK-INK4 heterodimers (Russo *et al.*, 1998).

1.3.4 The Rb pathway

Rb is the product of the retinoblastoma tumour suppressor gene, and operates in the midst of the cell cycle clock apparatus. Its main role is to act as a signal transducer connecting the cell cycle clock with the transcriptional machinery. In this role, Rb allows the clock to control the expression of banks of genes that mediate the advance of the cell through a critical phase of its growth cycle (Figure 1.4). Loss of Rb function deprives the clock and thus the cell of an important mechanism for braking cell proliferation through modulation of gene expression. As stated previously, Rb exerts its antiproliferative function by regulating a number of downstream effectors. The best-characterised Rb targets are members of the E2F/DP family of transcription factors, generically referred to as E2F.

Two other proteins, p130 and p107, are members of the Rb 'pocket protein' gene family. The pocket-protein family shares structural and biochemical properties and interacts with a number of common cellular proteins (Mulligan *et al.*, 1998). Like Rb, p107 and p130 bind and modulate the activity of E2F transcription factors, and they can interact with histone deacetylases (Ferreira *et al.*, 1998) suggesting that they can also regulate transcription by altering chromatin structure and availability of E2F.

1.4 Regulation of the G2/M Transition

The entry of a cell from the G2 phase into mitosis is regulated by an elaborate network of kinases and phosphatases that control both the timing of cell division and the complete reorganisation of the cellular architecture. The enzyme complexes responsible for driving the cell into mitosis are composed of the mitotic cyclins, cyclin A and cyclin B₁ and their association with CDK1.

1.4.1 Cyclin A

Cyclin A is unique among the cyclin family because it can activate two different CDKs, and functions in both S phase and mitosis. Cyclin A synthesis commences at the start of S phase and the protein is predominantly nuclear (Pines *et al.*, 1991). It associates with CDK2 during S phase and CDK1 throughout G2 and mitosis. In association with CDK1, cyclin A appears to actively prepare the cell for mitosis in the absence of significant cyclin B/CDK1 activity (Pines *et al.*, 2001; Strausfeld *et al.*, 1996). It may also contribute to the control of cyclin B stability (Yam *et al.*, 2002). Cyclin A starts to accumulate during S phase and is abruptly destroyed before metaphase. The synthesis of cyclin A is mainly controlled at the transcription level, involving E2F and other transcription factors. Removal of cyclin A is carried out by ubiquitin-mediated proteolysis, but whether the same anaphase-promoting complex/cyclosome targeting subunits are used as for cyclin B is unclear (Yam *et al.*, 2002). Consistent with its role as a key cell cycle regulator, expression of cyclin A is found to be elevated in a variety of tumours. For example, elevated cyclin A expression is associated with hepatocellular carcinoma (Zhou *et al.*, 2003), oral squamous cell carcinoma (Chen *et al.*, 2003) and human invasive breast carcinomas (Bukholm *et al.*, 2003).

1.4.2 B-Type Cyclins

There are two different cyclin B proteins in mammalian cells, cyclin B₁ and cyclin B₂. In human cells, the B-type cyclins start to be synthesised from the end of S phase, mainly due to cell cycle regulated transcription (Pines *et al.*, 1989). Both of these cyclins are located within the cytoplasm during interphase due to the presence of a cytoplasmic retention signal at the N-terminus (Pines *et al.*, 1994) of both cyclins. However, at the start of mitosis, cyclin B₁ is

dramatically relocated to the nucleus, while cyclin B₂ remains within the cytoplasm (Pines *et al.*, 1994). Cyclin B₂ is a non-essential protein that associates with the Golgi apparatus and probably plays a role in Golgi remodelling during mitosis (Brandeis *et al.*, 1998; Jackman *et al.*, 1995).

Mitosis is the most dramatic period of the cell cycle, involving a major reorganisation of virtually all cell components. During mitosis, the chromosomes condense, the nuclear envelope of most cells breaks down, the cytoskeleton reorganises to form the mitotic spindle, and the chromosomes move to opposite poles. The entry into and events of mitosis are triggered by the cyclin B/CDK1 complex. Regulation of cyclin B/CDK1 complexes at multiple levels ensures the tight regulation of the timing of mitotic entry. After association of cyclin B with CDK1, the complex is regulated by a number of phosphorylation events, resulting either in activation or inhibition of kinase activity. The events involved in regulation of cyclin/CDK complexes is discussed in Section 1.2.3. In particular the regulation of cyclin B₁/CDK1 kinase activity is summarised in Figure 1.5.

1.4.2.1 Cyclin B₁/CDK1 Targets: Its role in the progression to metaphase

As cells enter mitosis, phosphorylation of key components of the subcellular structures causes the complete reorganisation of the architecture of the cell. One of the most remarkable features of cell-cycle control is that a single protein kinase, cyclin B₁/CDK1, is capable of orchestrating these diverse and complex rearrangements that occur in the early stages of mitosis. Besides regulating the activity of itself by phosphorylating CDC25c and cyclin B₁ (see Figure 1.5), cyclin B₁/CDK1 also acts directly by phosphorylating some of the structural proteins involved in the mitotic cellular reorganisation.

Figure 1.5 Regulation of cyclin B/CDK1 kinase activity

CDK1 (Cdc2) complexes with cyclin B and then undergoes a positive phosphorylation by CAK on Thr161, and negative phosphorylations by the Wee1/Myt1 family of protein kinases on Tyr15 (and Thr14 in vertebrate cells) (Atherton-Fessler *et al.*, 1993; Endicott *et al.*, 1994). Dephosphorylation of Thr14 and Tyr15 by CDC25c activates cyclin B/CDK1 at the G₂/M transition (Millar *et al.*, 1992). Active cyclin B/CDK1 phosphorylates and further activates CDC25c (Hoffmann *et al.*, 1993). Cyclin B/CDK1 also phosphorylates cyclin B₁ itself, promoting cyclin B₁ nuclear translocation, and thus activation (Borgne *et al.*, 1999). These phosphorylations form a positive autocatalytic loop. Cyclin B/CDK1 activity is then terminated toward the end of mitosis by ubiquitination and subsequent proteolytic degradation of cyclin B by the Anaphase Promoting Complex (APC) (Harper *et al.*, 2002; Zachariae *et al.*, 1999), which is essential for progression into anaphase and subsequent mitotic exit. The activating phosphorylation on Thr 161 is removed, in part at least, by Protein Phosphatase 1 and/or 2 (Lorca *et al.*, 1992).

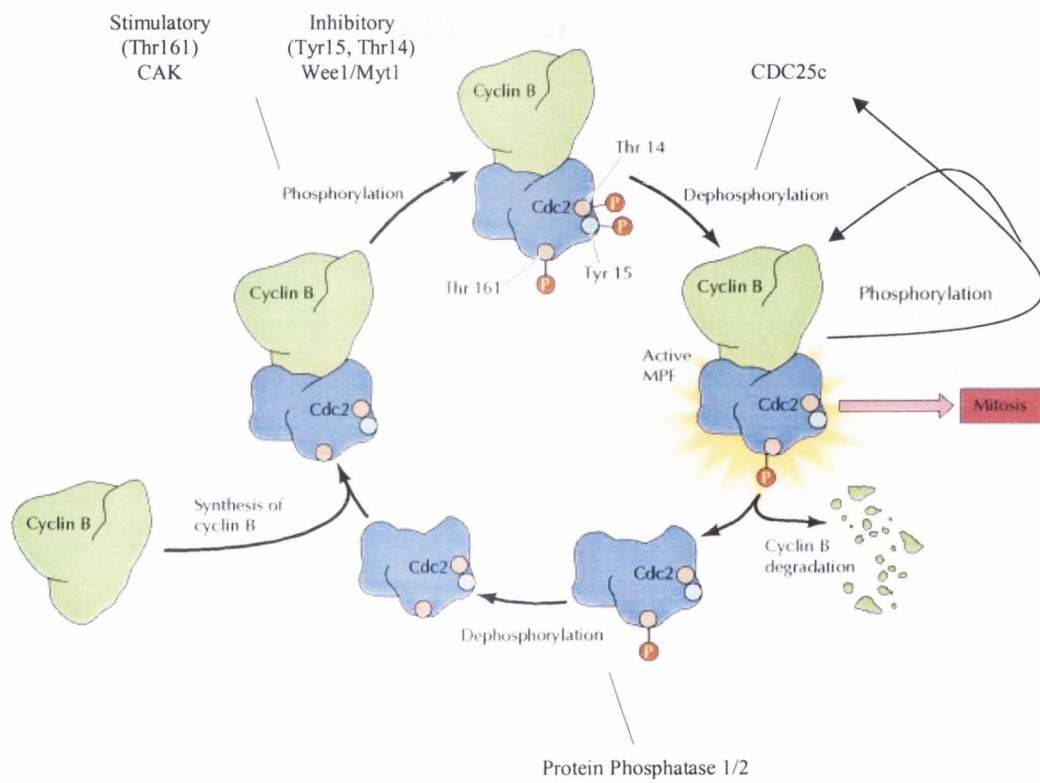


Diagram taken from 'The Cell' 2nd Edition, (Cooper, 2000)

Chromatin Condensation: The condensation of interphase chromatin to form the compact chromosomes of mitotic cells is a key event in mitosis, critical in enabling the chromosomes to move along the mitotic spindle without becoming broken or tangled with one another. A complex of five proteins, known as the condensins, are required for chromosome condensation in extracts of mitotic cells and appear to function by wrapping DNA around itself, thereby compacting chromosomes into the condensed mitotic structure. Condensins are phosphorylated and activated by cyclin B₁/CDK1 (Kimura *et al.*, 2001), providing a direct link between activation of cyclin B₁/CDK1 and mitotic chromosome condensation.

Nuclear Envelope Breakdown: Breakdown of the nuclear envelope, which is one of the most dramatic events of mitosis, represents the most clearly defined target of cyclin B₁/CDK1 action. Nuclear envelope disassembly involves changes in all three of its components: Disassembly of the nuclear lamina results from phosphorylation by cyclin B₁/CDK1 of the lamins (Zhai *et al.*, 1996), causing nuclear filaments to break down into their individual component lamin dimers.

Mitotic Spindle Formation: The reorganisation of the cytoskeleton that culminates in the formation of the mitotic spindle results from dynamic instability of cellular microtubules (See Section 1.5.1). Cyclin B₁/CDK1 initiates the formation of the mitotic spindle by phosphorylation of a class of proteins known as the microtubule-associated proteins (MAPs) that control microtubule dynamics. Cyclin B₁/CDK1 is capable of controlling the microtubule length of the mitotic spindle by phosphorylating a MAP protein, and reducing its ability to stabilise microtubules, increasing the dynamic instability of the microtubules required for the formation of the mitotic spindle (Chang *et al.*, 2001; Ookata *et al.*, 1995). Thus cyclin B₁/CDK1 is directly responsible for the regulation of mitotic spindle assembly and dynamics.

1.4.3 Mitotic Checkpoint Control

As cells undergo mitosis, their replicated genetic material must be distributed evenly between two daughter cells. In prophase, a bipolar spindle is formed between two microtubule organising centres. Condensed sister chromatids are attached to the spindle via their kinetochores in prometaphase and metaphase, and are pulled to opposite poles as they undergo anaphase. As the missegregation of sister chromatids leads to aneuploidy, this process must be tightly controlled by checkpoints that monitor the completion of critical steps in the pathway. In human cells, entry into mitosis is delayed by the Chfr checkpoint. This checkpoint delays the activation of CDC25c and thus the inactivation of Wee1/Myt1 kinases, leading to a delay in CDK1 activation (Scolnick *et al.*, 2000). The metaphase-anaphase transition is under the control of the spindle assembly checkpoint, which inhibits the separation of sister chromatids until all of the kinetochores are attached to microtubules (Amon, 1999; Burke, 2000; Wassmann *et al.*, 2001). Finally, exit from mitosis is controlled by a Bub2-dependent checkpoint pathway that inhibits the mitotic exit network (Burke, 2000; Hoyt, 2000).

1.4.3.1 The Spindle Assembly Checkpoint

The spindle assembly checkpoint prevents cells from entering anaphase until all chromosomes are properly aligned. Eukaryotic cells arrest if chromosomes fail to become bioriented on the spindle and have not congressed to the metaphase plate (Rieder *et al.*, 1994). Several studies have implicated unattached kinetochores as the source of the checkpoint signal and it is thought that mechanical tension at the kinetochore determines whether or not the checkpoint signal is initiated (Stern *et al.*, 2001; Zhou *et al.*, 2002c). In prophase and prometaphase, when kinetochores are not attached and under tension, the 3F3/2 antibody recognises a phosphoepitope at the kinetochore. This phosphorylation may be the trigger for activation of the spindle assembly checkpoint. It is thought that dephosphorylation occurs when the kinetochores attach to the spindle, leading to inactivation of the checkpoint (Nicklas, 1997; Stern *et al.*, 2001).

The transition into anaphase takes place after the degradation of an ‘anaphase inhibitor’ called Pds1 in *Saccharomyces cerevisiae* and Securin in mammalian cells (Clarke *et al.*, 2000). Loss of Pds1/Securin via ubiquitination

and degradation by the APC complex, in turn, leads to the liberation of the enzyme Separase/Esp1, which digests the protein complex Cohesion that binds the sister chromatids together. Thus Separase/Esp1 allows for sister chromatid separation (Yeong *et al.*, 2000). The APC must be in a complex with Cdc20 in order to ubiquitinate Pds1/Securin, as it acts as an adaptor molecule to bind substrates. The spindle assembly checkpoint causes inactivation of the APC by binding to Cdc20 preventing ubiquitination and degradation of Pds1/Securin and thus preventing progression into anaphase and mitotic arrest. Genetic studies have identified Mad1-3, Bub1-3 and Mps1 as the main molecular components induced after activation of the checkpoint. The human homologue of at least one of these proteins, hsBub1, has been shown to be mutated in two colorectal tumours (Cahill *et al.*, 1998). Furthermore, the human homologue for Mad1, HsMad1, may be a cellular target of the human T-cell leukaemia virus 1 (HTLV-1) oncoprotein, Tax (Jin *et al.*, 1998). Thus, perturbation or mutation of the checkpoint elements that negatively regulate the onset of anaphase might contribute to the development of cancer. Figure 1.6 summarises the molecular events forming the activated spindle assembly checkpoint complex.

1.5 Microtubules

Microtubules are dynamic cytoskeletal components that function in the development and maintenance of cell shape and polarity, in cell division and in cellular movement. Microtubules are formed from protein subunits of tubulin. The tubulin subunit is itself a heterodimer formed from two closely related globular proteins called α - and β -tubulin, tightly bound together by noncovalent bonds. A microtubule is a stiff, hollow cylindrical structure built from 13 parallel protofilaments, each composed from the polymerisation of alternating α - and β -tubulin monomers. Since the 13 protofilaments are aligned in parallel with the same polarity, the microtubule itself is a polar structure, and it is possible to distinguish a plus (fast-growing) and a minus (slow-growing) end.

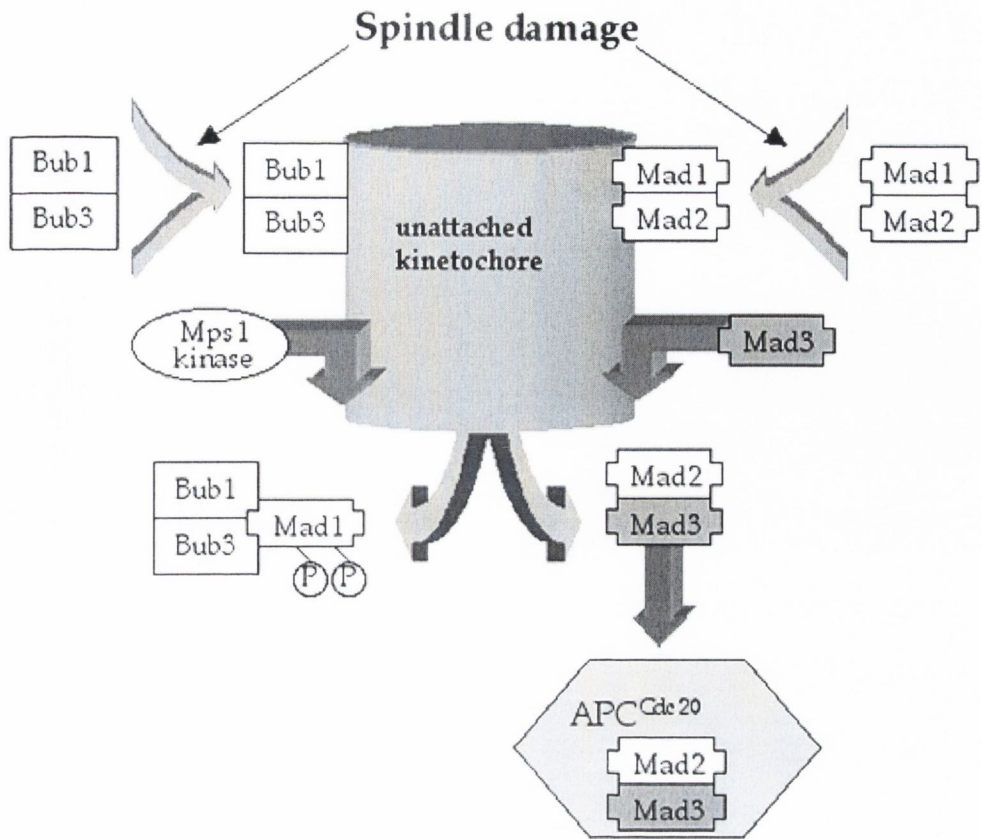


Diagram taken from 'Eurekah Biosciences Collection' (Duncan J. Clarke, 2000).

Figure 1.6 A model for the formation of the activated spindle assembly checkpoint complex.

Checkpoint activation is thought to rely on the recognition of unattached, phosphorylated kinetochores following spindle damage, by Mad2 and the formation of an activated Mad1/Mad2 complex. Association of Mad1/Mad2 and Bub1/Bub3 complexes with kinetochores is stimulated by the onset of spindle assembly and/or spindle aberrations. Mad2 is displaced from Mad1/Mad2 complexes, induced by Mps1 kinase, simultaneously stimulating the formation of the Bub1-Bub3-Mad1 complex on the one hand and an active Mad2 complex on the other (Brady *et al.*, 2000; Hardwick *et al.*, 2000). The activated Mad2 complex may include Mad3 (Hardwick *et al.*, 2000). The cell cycle arrest is the result of inhibition of APC activity by binding of the activated Mad2 complex to the Cdc20 component of the APC, preventing destruction of anaphase inhibitor Pds1/Securin (Yeong *et al.*, 2000).

1.5.1 Microtubule Assembly Dynamics

Tubulin dimers can depolymerise as well as polymerise, and microtubules can undergo rapid cycles of assembly and disassembly. Each α and β monomer has a binding site for one molecule of GTP. The α monomer bound GTP is physically trapped at the dimer interface and is not subject to hydrolysis or exchange. The nucleotide β monomer, in contrast, may be in either the GTP or the GDP form, and it is exchangeable. GTP hydrolysis to GDP weakens the binding affinity of tubulin for adjacent molecules, thereby favoring depolymerisation and resulting in the dynamic behavior of microtubules. One such dynamic behavior is 'treadmilling', in which tubulin molecules bound to GDP are continually lost from the minus end and replaced by the addition of tubulin molecules bound to GTP to the plus end of the same microtubule. In microtubules, GTP hydrolysis also results in a second type of dynamic behavior known as dynamic instability, in which individual microtubules alternate between cycles of growth and shrinkage (See Figure 1.7).

Because of their inherent dynamic instability, most microtubules are frequently disassembled within the cell. This dynamic behavior can, however, be modified by the interactions of microtubules with other proteins. Some cellular proteins act to disassemble microtubules, either by severing microtubules or by increasing the rate of tubulin depolymerisation from microtubule ends. Other proteins, called microtubule-associated proteins or MAPs, bind to microtubules and increase their stability. Such interactions allow the cell to stabilise microtubules in particular locations and provide an important mechanism for determining cell shape and polarity. A large number of MAPs have been identified, and they vary depending on the type of cell. The best-characterised are MAP-1, MAP-2, and tau, isolated from neuronal cells, and MAP-4, which is present in all non-neuronal vertebrate cell types. The tau protein has been extensively studied because it is the main component of the characteristic lesions found in the brains of Alzheimer patients. The activity of MAPs is regulated by phosphorylation, allowing the cell to control microtubule stability.

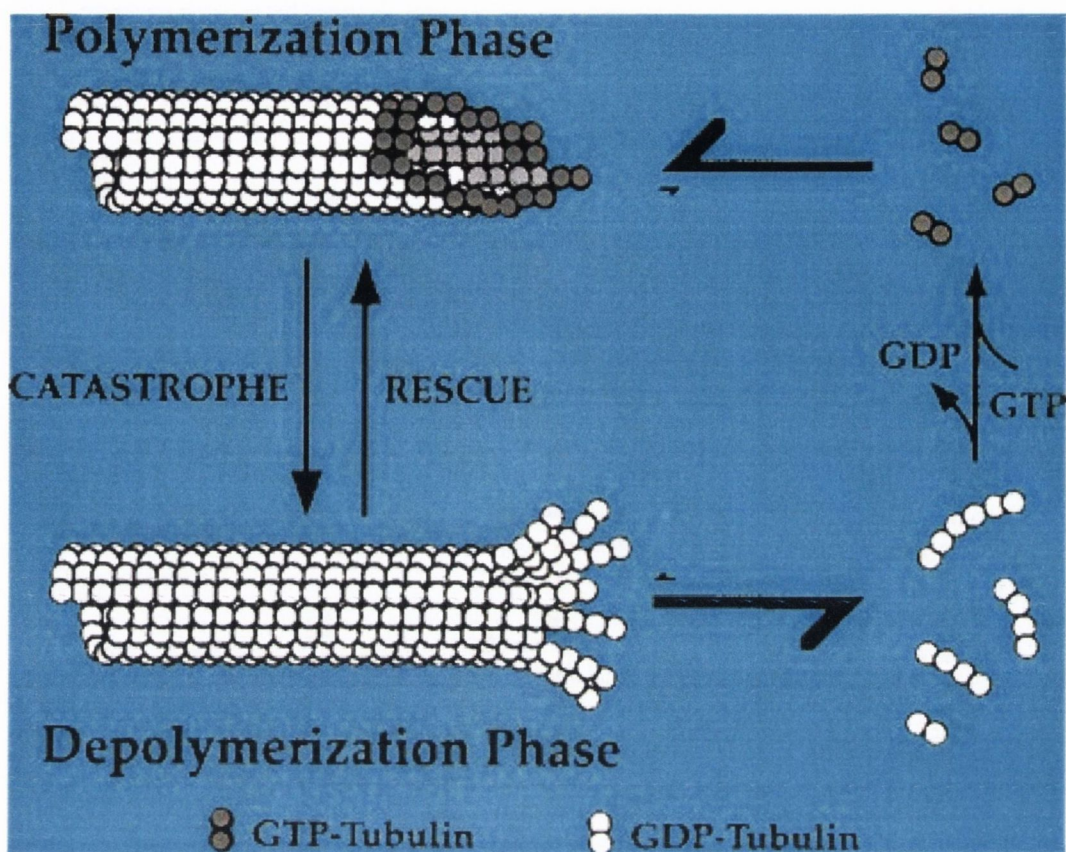


Diagram taken from (McEwen, 2002)

Figure 1.7 GTP-Cap Model for Microtubule Dynamic Instability

Whether a microtubule grows or shrinks is determined by the rate of tubulin addition relative to the rate of GTP hydrolysis. As long as new GTP-bound tubulin molecules are added more rapidly than GTP is hydrolysed, the microtubule retains a GTP cap at its plus end and microtubule growth continues. However, if the rate of polymerisation slows (catastrophe), the GTP bound to tubulin at the plus end of the microtubule will be hydrolysed to GDP. If this occurs, the GDP-bound tubulin will dissociate, resulting in rapid depolymerisation and shrinkage of the microtubule. The reverse of this process is known as rescue.

1.5.2 Reorganisation of Microtubules during Mitosis

Microtubules completely reorganise during mitosis, providing a dramatic example of the importance of their dynamic instability. The microtubule array present in interphase cells disassembles and the free tubulin subunits are reassembled to form the mitotic spindle. This restructuring of the microtubule cytoskeleton is directed by duplication of the centrosome. In animal cells, the major microtubule-organising center is the centrosome, to form two separate microtubule-organising centers at opposite poles of the mitotic spindle.

As the cell enters mitosis, the dynamics of microtubule assembly and disassembly also change dramatically. First, the rate of microtubule disassembly increases about tenfold, resulting in overall depolymerisation and shrinkage of microtubules. This is caused by phosphorylation of MAPs by cyclin B1/CDK1, which reduces its ability to stabilise microtubules, thus leading to increasing dynamic instability of the microtubules (See Section 1.4.2.2). At the same time, the number of microtubules emanating from the centrosome increases by between five and tenfold. In combination, these changes result in disassembly of the interphase microtubules and the outgrowth of large numbers of short microtubules from the centrosomes.

The increased dynamic instability of the mitotic spindle lends to it a labile nature. It is this lability which determines its function, and the mitotic spindle assembly checkpoint ensures that the mitotic spindle maintains its dynamic and labile nature. Because of the central role of microtubules in mitosis, drugs that affect microtubule assembly are useful not only as experimental tools in cell biology but also in the treatment of cancer.

1.6 The Cell Cycle as a Target for Cancer Therapy

Proliferative disorders such as cancer are recognised as diseases of the cell cycle. Invariably, components of the cell cycle are deregulated in cancer resulting in uncontrolled cellular proliferation. The selective growth advantage of cancer cells also stems from amplification of positive growth signals, mutation of checkpoint and surveillance genes as well as deregulation of programmed cell death or apoptosis. Deregulation of these components offers the cell a selective

growth advantage as compared to its nearest neighbours. These advantages allow the cell to escape the normal growth constraints such as contact inhibition, substrate adherence, growth factor requirements and quiescence in response to negative intracellular growth signal. Major research efforts are aimed at discovery of molecular targets that are specific for cancer cells. One tier of this anticancer research is the development of therapies that target checkpoint signalling pathways.

1.6.1 CDK Inhibitors

Timely and coordinated progression through the cell cycle is controlled by the sequential activation and deactivation of CDKs. Disrupted cell-cycle control due to aberrant CDK activity has been linked directly to the molecular pathology of cancer (Kamb, 1995; Malumbres *et al.*, 2001; Pavletich, 1999). For example, loss of function of endogenous CDK inhibitors such as p16, over-expression of cyclin D₁ and CDK4, and alterations to CDK substrates such as Rb are frequently observed in human tumours (Shapiro *et al.*, 1999). These deregulations have provided the impetus to the active search for pharmacological inhibitors of these kinases.

The first generation of CDK inhibitors includes several classes of drugs, all derived from microbial and plant sources (Meijer *et al.*, 1997). Of these agents, flavopiridol and UCN-01 (7-hydroxystaurosporine) are currently in clinical trials. Flavopiridol, a chemical CDK inhibitor, arrests cancer cells at the G1/S and G2/M transitions by inhibiting CDK2, CDK4 and CDK1 activity (Buolamwini, 2000). Flavopiridol produced favourable clinical responses in Phase 1 and Phase 2 studies of patients with renal, colorectal, gastric, lung and esophageal carcinomas, and current clinical trials are evaluating the drug in non-Hodgkin's lymphoma, breast and prostate cancers (Elsayed *et al.*, 2001; Senderowicz *et al.*, 1998).

UCN-01 is a derivative of staurosporine, a non-specific Protein Kinase C (PKC) inhibitor. Although, UCN-01 inhibits certain PKC isozymes, its strong anti-proliferative effects are thought to be unrelated to PKC inhibition. UCN-01 has been reported to induce G1 cell cycle arrest at low nanomolar concentrations, associated with an induction of endogenous p21 and p27 (Akiyama *et al.*, 1997). UCN-01 has also been shown to abrogate the G2 DNA damage checkpoint by

inhibiting phosphorylation of CDC25c by the kinase Chk1 and leading to CDK1 inhibition (Graves *et al.*, 2000).

1.6.2 Antimicrotubule Agents

Antimicrotubule agents, as the name suggests, target cellular microtubules, thereby disrupting the normal dynamic reorganisation of the microtubule network required for mitosis and cell proliferation. This disruption leads to the failure of mitotic spindle formation and thus failure of spindle attachment to the kinetochores of chromosomes, engaging the spindle assembly checkpoint (See Section 1.4.3.1) (Sorger *et al.*, 1997). A tremendous interest in small organic molecules that modulate the dynamics of microtubules has developed over the years since the discovery of the first tubulin binding agent, colchicine, in the 1960s (Borisy *et al.*, 1967), due to their potential use as chemotherapeutic agents.

There are two classes of antimicrotubule agents: those that prevent the assembly of / depolymerise tubulin, and those that promote the assembly / polymerise tubulin. Depolymerisers of tubulin include the *vinca* alkaloid family of compounds (vinblastine, vincristine, vinorelbine), nocodazole and colchicine. At high concentrations, these agents reduce or abolish the microtubule content of cells in culture and prevent polymerisation of purified tubulin *in vitro*. Polymerisers of tubulin include the taxanes (paclitaxel, docetaxel) and the epothilones. These agents increase the fraction of polymerised tubulin within the cell.

In the field of antineoplastic chemotherapy, antimicrotubule agents constitute an important class of compounds, with broad activity both in solid and in hematologic neoplasias. The taxanes are effective in the treatment of refractory ovarian cancer, metastatic breast cancer, non-small cell lung cancer and head and neck and bladder carcinomas (Crown *et al.*, 2000; Rowinsky, 1997). The *vinca* alkaloids have importance in the treatment of leukemia, Hodgkins's disease, non-Hodgkin's lymphomas, testicular cancer, Kaposi's sarcoma, breast cancer, small cell lung cancer, and other malignancies (Rowinsky, 1997; van Tellingen *et al.*, 1992).

This antineoplastic activity is rooted in these compounds' ability to induce mitotic arrest and subsequent apoptotic cell death via the spindle assembly checkpoint. The specific action of the antimicrotubule agents on the mitotic

spindle may be attributed to the difference in dynamicity of microtubules in interphase and mitosis. Mitotic spindle microtubules are 10-100 times more dynamic than microtubules in interphase cells. It is this dramatic increase in dynamicity that allows the mitotic spindle to function and also singles it out as a prime target for antimicrotubule agents. In addition, the absolute requirement of a functional spindle for the proper migration of chromosomes during mitosis may also explain why this stage of the cell cycle is particularly vulnerable to tubulin active agents, even though these compounds act on other cellular microtubules as well (Jordan *et al.*, 1998b).

Antimicrotubule induced mitotic arrest is associated with an upregulation and activation of cyclin B₁/CDK1 activity in a variety of cell lines (Donaldson *et al.*, 1994), as the spindle assembly checkpoint prevents ubiquitin degradation of cyclin B₁ via the APC and thus exit from mitosis (See Section 1.4.3.1). The majority of clinically effective anticancer drugs inhibit some aspect of the machinery responsible for DNA replication and chromosome segregation, giving rise to cell cycle arrest via induction of specific cell cycle checkpoints. These checkpoints ensure that subsequent cell cycle events are inhibited until the inflicted damage is repaired. The fidelity of checkpoint control and susceptibility of cells to cell death through initiation of programmed cell death pathways, known as apoptosis (Lockshin *et al.*, 2001) while repair is underway may be important factors in the success of chemotherapy (O'Connor *et al.*, 1992).

1.6.3 Apoptosis

It is now generally accepted that cell death in response to treatment with certain anticancer drugs, cytokines, and ionising radiation represents the culmination of a complex set of biochemical and molecular events that in many instances is due to apoptosis (Fisher, 1994). Apoptosis is an evolutionarily conserved and genetically regulated biological process that plays an important role in the development and homeostasis of multicellular organism (Lockshin *et al.*, 2001). Apoptotic death requires special machinery, which is always in place and only requires a trigger to activate it. The central component of this machinery is a proteolytic system involving a family of proteases called caspases (cysteine-dependent aspartate-directed proteases). Caspases have cysteine residues at their active sites and cleave after aspartic acid residues in their substrate proteins.

Activated caspase targets include cytoskeleton proteins (nuclear lamins, actin, gelsolin), regulators of DNA repair (poly ADP-ribose polymerase (PARP)), degradation of nuclear DNA by activation of DNA-dependent protein kinase, and deactivation of the inhibitor of caspase-activated deoxyribonuclease protein (ICAD), RNA splicing (U1 protein), nuclear mitotic apparatus protein (NuMA), and cell cycle proteins (including Rb and p21-activated kinase (PAK)) (Bratton *et al.*, 2000). Caspases are also involved in extracellular apoptotic events including fragmentation into apoptotic bodies and exposure of phosphatidylserine on the cell surface. However, the exclusive role of caspases in mediating the apoptotic response has been challenged recently with the discovery of caspase-independent cell death programmes (Lockshin *et al.*, 2002).

1.6.3.1 Bcl-2 Proteins and Apoptosis

The common mechanism of apoptosis is negatively regulated by several sets of genes, of which the best characterised is the still growing Bcl-2 family (Adams *et al.*, 1998). In recent years, it has been well established that Bcl-2 prevents most forms of apoptotic cell death as well as certain forms of necrotic cell death. A large number of Bcl-2-related proteins have been isolated (Adams *et al.*, 1998; Tsujimoto, 1998) and divided into three categories:

1. Anti-apoptotic members such as Bcl-2, Bcl-XL, Bcl-W, Mcl-1, A1 (Bfl-1) and Boo, all of which exert anti-cell death activity and share sequence homology, particularly within four regions, Bcl-2 homology (BH)1 through BH4, although some members lack an apparent BH4 domain.
2. Pro-apoptotic members such as Bax, Bak, Bad, Mtd (Bok), Diva, which share sequence homology in BH1, BH2 and BH3 but not in BH4, although significant homology at BH4 has been noticed in some members.
3. 'BH3-only proteins', the pro-apoptotic proteins which include Bik, Bid, Bim, Hrk (DP5), Blk and Bnip3, Bnip3L, and share sequence homology only in BH3.

The anti-apoptotic members of this family, such as Bcl-2 and Bcl-XL, prevent apoptosis either by sequestering proforms of caspases or by preventing the release of mitochondrial apoptogenic factors such as cytochrome c and apoptosis inducing factor (AIF) into the cytoplasm (Tsujimoto *et al.*, 2000). In contrast, pro-apoptotic members of this family, such as Bax and Bak, trigger the release of

caspases via heterodimerisation and also by inducing the release of mitochondrial apoptogenic factors into the cytoplasm by acting on the mitochondrial permeability transition pore, thereby leading to caspase activation (Tsujimoto *et al.*, 2000).

One of the unique features of Bcl-2 family proteins is heterodimerisation between anti-apoptotic and pro-apoptotic proteins, which is considered to inhibit the biological activity of their partners. However, reversible phosphorylation and dephosphorylation is another mechanism that directly regulates the functional activity of both antiapoptotic and proapoptotic Bcl-2 family members. The dynamic phosphorylation and dephosphorylation of Bcl-2 causes conformational change within the protein. Multi-site phosphorylation of Bcl-2 inhibits its anti-apoptotic function and is followed by cell death (Blagosklonny, 2001). Hyperphosphorylation of Bcl-2 by antimicrotubule agents has previously been shown (Fan *et al.*, 2000; Fan *et al.*, 2001; MacCarthy-Morrogh *et al.*, 2000; Park *et al.*, 2001; Wang *et al.*, 1999; Wu-Wong *et al.*, 2001; Yamamoto *et al.*, 1999). In addition, Bcl-2 has been shown to be phosphorylated by JNK (Fan *et al.*, 2000) (Yamamoto *et al.*, 1999). In addition to serine-70 (Ser70), antimicrotubule agents promote phosphorylation of serine-87 (Ser87) and threonine-69 (Thr69), inactivating Bcl-2. Among the kinases considered to mediate these inactivating kinases, is JNK kinase (Fan *et al.*, 2001) and cyclin B₁/CDK1 (Vantieghem *et al.*, 2002). A major obstacle for identification of the mitotic Bcl-2 kinase(s) is that inhibition of putative kinase(s) by any means (dominant-negative mutants, antisense oligonucleotides, pharmacological agents) may arrest the cell cycle, preventing mitosis and Bcl-2 phosphorylation.

1.6.3.2 Mitogen-Activated Protein Kinase Signalling

The mitogen-activated protein (MAP) kinases are a group of protein serine/threonine kinases that are activated in response to a variety of extracellular stimuli and mediate signal transduction from the cell surface to the nucleus. These signalling cascades have important roles in the regulation of cell growth, differentiation, cell survival, apoptosis, stress and inflammatory responses (Chan-Hui *et al.*, 1998). In mammalian systems, the biochemical properties of three MAP kinases have been characterised in detail, the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs) and the p38 MAP kinases

(Ichijo, 1999). Components and activators of the three MAP kinase cascades are summarised in Figure 1.8.

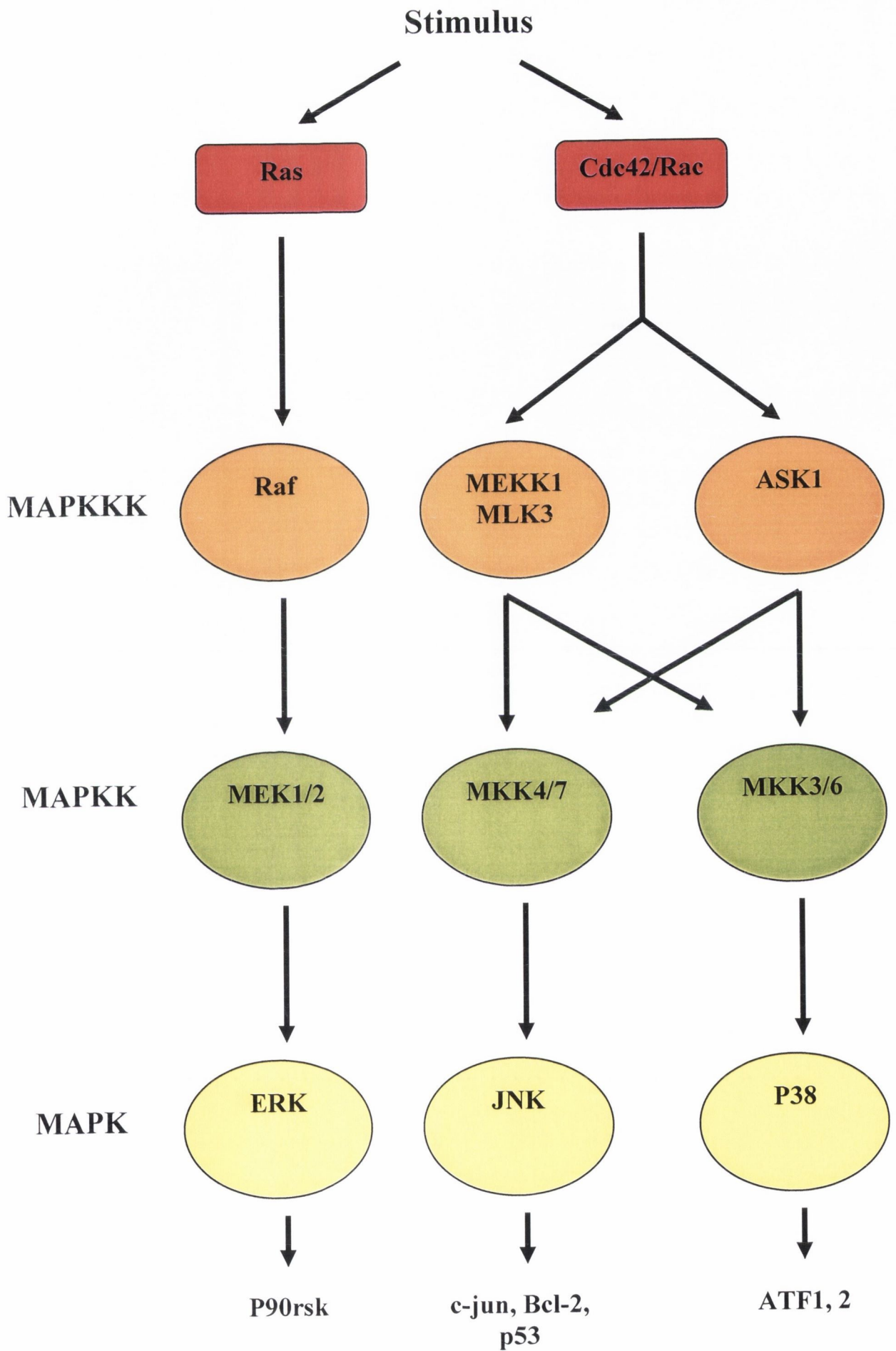
Activation of ERK is mediated by two upstream protein kinases, which are coupled to growth factor receptors by a GTP-binding protein called Ras. Activation of Ras leads to activation of the Raf protein-serine/threonine kinase, which phosphorylates and activates a second protein kinase called MAP kinase/ERK kinase (MEK). MEK1 or MEK2 is a dual-specificity protein kinase that activates members of the ERK family by phosphorylation of both threonine and tyrosine residues separated by one amino acid (e.g. threonine-183 and tyrosine-185 of ERK2). Once activated, ERK phosphorylates a variety of targets, including the serine/threonine kinase p90/rsk2, Bcl-2 and Bad (Bonni *et al.*, 1999; Klumpp *et al.*, 2002).

Unlike the ERK signalling pathway, the JNK and p38 pathways are preferentially activated by inflammatory cytokines such as TNF α and IL-1 β , and a diverse array of cellular stresses, including UV irradiation, heat, osmotic shock, DNA damaging and antimicrotubule agents. The JNK signalling pathway is involved in regulation of many cellular events, including growth control, transformation and apoptosis (Ip *et al.*, 1998).

Upstream regulation of JNK activation is poorly understood. The JNK and p38 pathways are activated upstream by two Ras related GTP-binding proteins of the Rho family, namely Rac and Cdc42 (Bagrodia *et al.*, 1995; Coso *et al.*, 1995). As in the ERK system, the immediate upstream activators of JNK are mitogen kinase kinases' (MKK's). While the ERK pathway is stimulated by MKKs 1/2, JNK appears to be activated by MKK4 (Yang *et al.*, 1997) or MKK7 (Holland *et al.*, 1997) depending upon the cell type, developmental stage and stress stimulus. Other kinases such as the Mixed Lineage Kinase 3 (MLK3) (Tibbles *et al.*, 1996), MEKK1 and ASK1 (Ichijo *et al.*, 1997) lie further upstream of the MKKs, and probably activate both MKK's and JNK in a similar cell type and stimulus dependent manner. ASK1 also activates p38 MAPK. JNK activity is further modified by JNK interacting proteins (JIP's), which are scaffolding proteins

Figure 1.8 Components and Activators of the MAP kinase signalling cascades

Three MAP kinase cascades have been characterised in mammalian cells. Activation of the signalling cascade involves phosphorylation and activation of MAP kinase kinase kinases (MAPKKKs), which in turn will phosphorylate MAPK kinases (MAPKKs). These in turn, phosphorylate and activate either the ERK, JNK or p38 MAP kinases (MAPKs) on specific tyrosine and threonine residues. These MAPKs activate transcription factors and increase their transcriptional activity, as well as phosphorylating other key cellular proteins. While MAPKs are specific for their substrates, a large extent of crosstalk takes place at the MAPKKK level. MAPK signalling cascades are regulated by upstream GTP-binding proteins from the Ras (primarily the ERK pathway) and Rho (Cdc42 and Rac, primarily regulators of the JNK and p38 pathways) subfamilies.



thought to sequester all of the necessary kinases for JNK activation (Whitmarsh *et al.*, 1998).

The downstream effectors of the JNK pathway are numerous and include both cytoplasmic and nuclear targets. In the nucleus, JNK's phosphorylate and activate the transcription factor c-jun (Derijard *et al.*, 1994), a component of the AP-1 transcription factor, which regulates genes involved in apoptosis. JNK has been shown to phosphorylate and inactivate the anti-apoptotic Bcl-2 protein (Yamamoto *et al.*, 1999), whilst phosphorylation of the pro-apoptotic protein p53 by JNK results in its activation (Hu *et al.*, 1997). However, the role of JNK activation in apoptosis is highly controversial, being suggested to have anti-apoptotic functions (Krause *et al.*, 2001; Liin, 2003) as well as pro-apoptotic functions. It appears that the JNK pathway functions in a cell-type and stimulus-dependent manner and its different components can sometimes play opposing roles in apoptosis. p38 MAPKs, like JNKs, are stress activated kinases that are highly conserved in evolution, exist in multiple isoforms, and are activated in response to numerous cellular stresses. Like other members of the MAP kinase family, upstream regulation involves phosphorylation by MKK's, specifically MKK3, MKK4, and MKK6 (Derijard *et al.*, 1995). The downstream targets of the p38 MAPK pathway are similar to the targets of JNKs, and involve transcription factors such as ATF1 and 2, kinases, and pathways influencing pro-inflammatory cytokines (Harper *et al.*, 2001).

1.6.3.3 Antimicrotubule Agent-Induced Apoptotic Cell Death

Studies in both normal and transformed human cells, treated with members of either the polymerising or depolymerising groups of antimicrotubule inhibitors, have shown that apoptosis can be initiated rapidly and directly from mitotic arrest (Fan *et al.*, 2001; Hayne *et al.*, 2000; Woods *et al.*, 1995; Zhou *et al.*, 2002a).

In many systems, JNK activation is a proapoptotic signal, a notion supported by the development of JNK1/JNK2-deficient mouse embryos (Tournier *et al.*, 2000). In particular, a role for JNK signalling in the apoptotic response of cells to antimicrotubule agents has been suggested. JNK activity is increased by antimicrotubule agents in a wide variety of cell types (Lee *et al.*, 1998; Shtil *et al.*, 1999; Wang *et al.*, 1999; Wang *et al.*, 1998), and inhibition of JNK inhibits

paclitaxel- (Lee *et al.*, 1998; Wang *et al.*, 1999), vinblastine- (Brantley-Finley *et al.*, 2003), nocodazole- and colchicine-induced apoptosis (Wang *et al.*, 1998). Because JNK has been implicated primarily in stress responses, JNK activation by antimicrotubule agents may represent an acute response to microtubule damage. The phosphorylation of Bcl-2 has also been described as an important step from microtubule damage to apoptosis (Blagosklonny *et al.*, 1997; Haldar *et al.*, 1995). Recently, it has been shown that Bcl-2 phosphorylation occurs normally at the G₂-M phase of the cell cycle, and that a pathway involving JNK may be involved (Yamamoto *et al.*, 1999). The possibility that JNK is responsible for Bcl-2 phosphorylation at G₂-M strengthens the connection between JNK signalling and apoptosis. The phosphorylation-induced mobility shift of Bcl-2 at the G₂/M transition involves only a proportion of the Bcl-2 molecules (Yamamoto *et al.*, 1999), whereas it has been shown that more highly phosphorylated Bcl-2 species are induced in response to vinblastine (Fan *et al.*, 2000). This difference in the extent and duration of substrate phosphorylation, leading to the accumulation of highly phosphorylated JNK substrates in antimicrotubule agent induced-mitotically arrested cells, may be a key factor in promoting apoptosis.

1.6.4 Pyrrolobenzoxazepine (PBOX) compounds

1.6.4.1 The Peripheral-Type Benzodiazepine Receptor

Benzodiazepines, such as valium, are among the most highly prescribed drugs due to their anxiolytic, relaxant and sedative effects. These pharmacological effects are mediated through their interaction with the neuronal GABA-gated chloride channel (Zisterer *et al.*, 1997). This is known as the central-type benzodiazepine receptor and is present exclusively in the central nervous system. This GABA/benzodiazepine receptor complex is a hetero-oligomer composed of five subunits, α -, β -, γ -, δ - and ρ -polypeptides. Benzodiazepines bind to the α -subunit and facilitate the inhibitory effect obtained by GABA. However, benzodiazepines also bind to another distinct recognition site known as the peripheral-type benzodiazepine receptor (PBR), so called because it was first discovered in peripheral tissues, but was subsequently found to be also present in the CNS (Braestrup *et al.*, 1977).

A number of compounds have been developed for studying the PBR. Among the best characterised of these compounds are PK 11195 and Ro5-4864

which bind to the PBR with high affinities. Although the 18 kDa protein responsible for binding to the isoquinoline component of these compounds has been cloned, little is known about the exact molecular structure and the true physiological role of the PBR. Benzodiazepine receptors have been found to be highly conserved throughout evolution suggesting some fundamental role within the cell. A wide variety of physiological functions have been attributed to the PBR over the years. These include involvement in immune response (Zavala *et al.*, 1991), behavioural conditions, cellular respiration (Gavish *et al.*, 1999), alteration in cardiac action potentials (Mestre *et al.*, 1984), and calcium channels (Mestre *et al.*, 1986), alterations of proto-oncogene expression (Curran *et al.*, 1985), stimulation of steroidogenesis and, more recently, an involvement in apoptosis (Hirsch *et al.*, 1998).

An involvement of the PBR in controlling cell growth has also been suggested. PBR ligands such as diazepam, PK 11195 and Ro5-4864, have been shown to inhibit cell growth in a variety of cell lines. Gorman *et al.* (1989) found that Ro5-4864, PK 11195, diazepam and clonazepam were capable of inhibiting the proliferation of both rat C6 glioma cells and mouse neuro-2A neuroblastoma cells in the micromolar range. However, the different potencies and specificities of these compounds for the anti-proliferative actions and binding affinities for the PBR suggested that these anti-proliferative actions are not mediated through the PBR itself. Micromolar concentrations are required to elicit these anti-proliferative effects, whilst the PBR is saturated by these ligands at nanomolar concentrations.

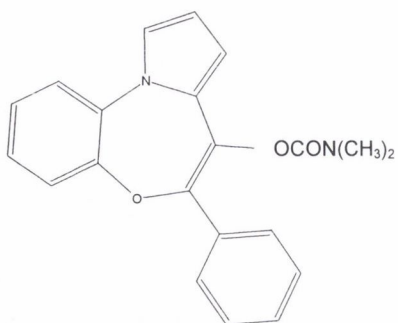
1.6.4.2 Pyrrolobenzoxazepine (PBOX) compounds as potential anticancer agents

Recently a series of novel compounds were synthesised as high affinity PBR ligands based upon the pyrrolobenzoxazepine skeleton (Campiani *et al.*, 1996) (Figure 1.9). These compounds, which are known as pyrrolobenzoxazepine (PBOX) compounds, are selective for the PBR and do not interact with the central-type benzodiazepine receptor (CBR) (Campiani *et al.*, 1996). In addition, these novel ligands have been shown to elicit similar biological effects, such as stimulation of steroidogenesis, to the classically used PBR ligands PK 11195 and Ro5-4864 (Campiani *et al.*, 2002). It was thought that these novel ligands would

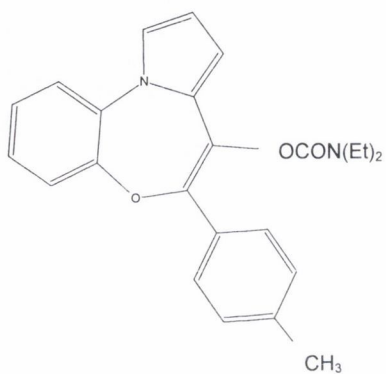
prove useful in the quest to further decipher the structure and function of the PBR.

Ligands to the PBR have previously been shown to elicit cytostatic effect on cancerous cell lines. Gorman *et al.*, (1989) found that Ro5-4864, diazepam, clonazepam and PK 11195, the classically used PBR ligands, inhibited the proliferation of rat C6 glioma and mouse neuro-2A neuroblastoma cells in the micromolar range, whilst Camins *et al.*, (1995) demonstrated that Ro5-4864, PK 11195 and diazepam inhibited in a concentration-dependent manner the proliferation of V79 Chinese hamster lung cells. Similar to the classical PBR ligands, a subset of the PBOX compounds have been shown to have anti-proliferative effects upon the C6 rat glioma and human 1321N1 astrocytoma cell lines at micromolar concentrations (Zisterer *et al.*, 1998). These PBOX compounds were found to induce a specific accumulation of C6 glioma and 1321N1 cells in the G1 phase of the cell cycle. In both cell types, there was a significant decrease in the proportion of cells in the S and G2/M phases of the cell cycle, with a concomitant increase in the proportion of cells in the G1 phase (G1 arrest). Significantly the anti-proliferative effects of these compounds upon both these cell lines was found to be entirely due to this G1 arrest as the PBOX compounds were not cytotoxic to the cells. Also, the anti-proliferative effects did not appear to be mediated through binding to the PBR, as 1000-fold higher concentrations of the drugs were required to produce the anti-proliferative effects than were required to saturate the PBR (Zisterer *et al.*, 1998).

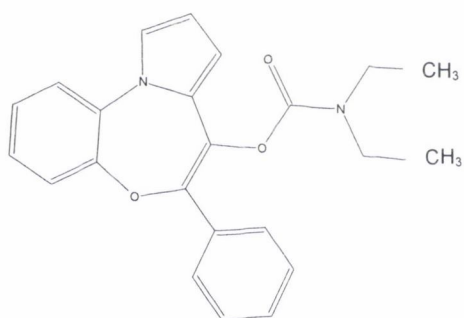
It has recently been found that a second subset of these PBOX compounds are capable of inducing apoptosis in a number of cancerous cell lines derived from the haematopoietic system such as HL-60 promyelocytic leukaemia, Jurkat acute lymphoblastic leukaemia and Hut-78 lymphoblastic leukaemia cells, and the normally resistant chronic myeloid leukaemia (CML), K562 cells. It has also been shown that PBOX-6, a representative member of these pro-apoptotic PBOX compounds, potently induces apoptosis in the human MCF-7 breast carcinoma, LNCaP prostate carcinoma and the Hela cervical carcinoma cells. This suggests the potential of these drugs as novel anticancer therapeutics in the treatment of



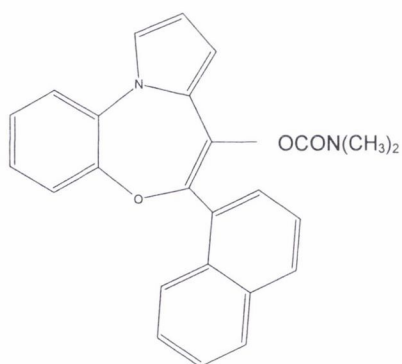
PBOX-1
(formerly NF-182)



PBOX-21
(formerly NF-262)



PBOX-60
(formerly NF-490)



PBOX-6
(formerly NF-225)

Figure 1.9 **Structure of pyrrolo-1,5-benzoxazepine compounds**

both solid tumours and haematologic neoplasias. Preliminary results suggest that the apoptotic response induced by the PBOX compounds is not associated with a general toxicity phenomenon both *in vitro* and *in vivo*. Apoptosis *in vitro* is not accompanied by signs of primary necrosis (Mc Gee, PhD thesis, 2001) and these compounds have no effect on normal haematopoietic cells as judged from *ex vivo* cultures of human CFU-GM cells, a human clonogenic progenitor of multiple lineages of mature haematopoietic cells (unpublished results). Furthermore, results from an *in vivo* toxicity study demonstrated that healthy Balb/c mice treated daily with PBOX-6 for 8 days exhibited no lethality, no difference in weight and no effect on haematology parameters at the end of the treatment regimen (unpublished results).

It has been demonstrated that the mechanism in which PBOX compounds induce apoptosis does not involve their specific interaction with the mitochondrial peripheral-type benzodiazepine receptor (PBR), which they bind to with high affinity, due to a lack of correlation between their affinities for the receptor and their apoptotic potencies, their high apoptotic activity in PBR deficient cells such as Jurkat acute lymphoblastic leukaemia cells and their lack of apoptotic induction in PBR-rich rat Leydig cells (Zisterer *et al.*, 2000). The involvement of reactive oxygen intermediates in PBOX-induced apoptosis (Mc Gee *et al.*, 2001; Zisterer *et al.*, 2000) has also been ruled out. It has been shown that PBOX compounds do not function as farnesyl protein transferase inhibitors or as topoisomerase inhibitors (Mc Gee, PhD thesis).

Part of the mechanism involved in the induction of apoptosis by PBOX-6 has, however, been elucidated. Activation of the JNK signaling pathway is essential during PBOX-6-induced apoptosis in CML cells. Treatment of K562 cells with PBOX-6 resulted in the early (within minutes) and transient activation of JNK. Furthermore, treatment of K562 cells with the JNK inhibitors, dicoumarol or JIP-1, inhibited PBOX-6-induced JNK activity and apoptosis, demonstrating that JNK activation is an essential component of the apoptotic pathway induced by PBOX-6 (Mc Gee *et al.*, 2002a). The importance of JNK in the apoptotic signaling pathway has also been confirmed in a number of other cell lines derived from both the haematopoietic system and from solid tumours, such as HL-60 and LNCaP cells respectively (unpublished results). However, the

signaling components lying upstream of JNK and activated by PBOX compounds remain to be elucidated.

Overexpression of the Bcl-2 proto-oncogene in tumour cells confers resistance against apoptosis induced by chemotherapeutic drugs. PBOX-6 overcomes Bcl-2 mediated resistance to apoptosis in K562 cells by the time and dose-dependent phosphorylation and inactivation of anti-apoptotic Bcl-2 family members, Bcl-2 and Bcl-XL. PBOX-6 also induces Bcl-2 phosphorylation and apoptosis in wild type leukaemia CEM cells and cells over expressing Bcl-2. This is in contrast to chemotherapeutic drugs such as etoposide, actinomycin D and ultraviolet irradiation, whereby overexpression of Bcl-2 confers resistance against apoptosis. In addition, PBOX-6 induces Bcl-2 phosphorylation and apoptosis in wild type Jurkat cells and cells overexpressing Bcl-2. However, Jurkat cells containing a Bcl-2 triple mutant, whereby the principal Bcl-2 phosphorylation sites are mutated to alanine, demonstrate resistance against Bcl-2 phosphorylation and apoptosis. Finally, inhibition of JNK activity prevents Bcl-2 phosphorylation and apoptosis, implicating JNK in the upstream signaling pathway leading to Bcl-2 phosphorylation (unpublished results). Collectively these findings identify Bcl-2 phosphorylation and inactivation as a critical step in the apoptotic pathway induced by PBOX-6.

Cancer has always been attributed to an abnormal proliferation of cells and agents that interfere with cell cycle progression may have potential as anticancer therapeutics. The cytostatic and cytotoxic effects of PBOX compounds in human tumour cell lines suggests their potential as novel anticancer agents. As the validity of any new therapy and/or therapeutic is more easily established if a sound mechanistic basis can be shown, an understanding of the molecular mechanisms underlying the action of these PBOX compounds would further their potential as novel anticancer agents.

1.7 Aims of this study

- (1) To assess the cytostatic effects of a range of antiproliferative PBOX compounds in two human carcinoma cell lines.
- (2) To systematically analyse the changes that occur in kinase activities, expression levels, interactions and phosphorylation status of the cell-

cycle regulatory molecules in response to PBOX compound treatment and to compare them to the timing of onset of the induced growth arrested and apoptotic states, so as to attempt to characterise the pathway underlying the mechanism of action of these compounds.

- (3) To investigate the role played by JNK in mediating the arrest and apoptosis induced by a representative apoptotic PBOX compound, PBOX-6
- (4) To examine the hypothesis that PBOX-6 acts at a molecular level in a manner similar to antimicrotubule agents.

2 MATERIALS AND METHODS

2.1 Materials

The full names and addresses of the sources listed below are given at the end of the list.

1321N1 cell line	E.C.A.C.C
Anti- α -Tubulin antibody	Sigma
Anti- β -Actin antibody	Sigma
Anti-CDC25A antibody	NeoMarkers
Anti-CDK1 antibody	BD-Pharmingen
Anti-CDK2 antibody	BD-Pharmingen
Anti-CDK4 antibody	BD-Pharmingen
Anti-CDK6 antibody	BD-Pharmingen
Anti-Cyclin A antibody	BD-Pharmingen
Anti-Cyclin B ₁ antibody	BD-Pharmingen
Anti-Cyclin D ₁ antibody	Merck Biosciences
Anti-Cyclin D ₂ antibody	Merck Biosciences
Anti-Cyclin D ₃ antibody	BD-Transduction
Anti-Cyclin E antibody	Merck Biosciences
Anti-JNK1 antibody	Santa Cruz
Anti-p16 antibody	BD-Pharmingen
Anti-p21 antibody	BD-Pharmingen
Anti-PARP antibody	Merck Biosciences
Anti-Rb phospho antibody	BD-Pharmingen
Anti-Rb-(ser780) phospho antibody	MBL
Aprotinin	Merck Biosciences
Ammonium persulphate	Sigma
ATP	Sigma
β -Glycerophosphate	Sigma
β -Mercaptoethanol	Sigma
Bromophenol Blue	Sigma

BSA	Sigma
Colchicine	Sigma
Colchicine Binding Site Competitive Assay Kit	Cytoskeleton
Coomassie Brilliant Blue G-250	Sigma
Cytocentrifuge Filters	Shandon
DMSO	Sigma
DTT	Sigma
DMEM	Sigma
ECL detection system	Amersham
Ecoscint	National Diagnostics
EDTA	Sigma
EGTA	Sigma
Ethanol	Chemical Store, TCD
FACS Tubes	Becton Dickonson
FCS	Sigma
Filter paper	Whatman
FITC conjugated goat anti-mouse IgG antibody	Sigma
Formavar coated copper grids	CMA
G50 Sephadex TM	Pharmacia
G-PEM Buffer	Cytoskeleton
Gentamicin	Sigma
Glutamine	Gibco
Glycerol	Sigma
60% Glycerol Buffer	Cytoskeleton
Glycine	Sigma
Goat anti-mouse IgG peroxidase conjugate	Sigma
Goat anti-rabbit IgG peroxidase conjugate	Sigma
Goat anti-rat IgG peroxidase conjugate	Sigma
GST-c-Jun	New England Biolabs
GST-Rb	Santa Cruz
GTP	Cytoskeleton
Half Area 96-well plates	Corning Costar Inc
HEPES	Sigma
Histone H1	Sigma

Kodak X-Omat™ LS film	Sigma
Leupeptin	Sigma
MCF-7 cell line	E.C.A.C.C
MEM	Sigma
Methanol	Chemical Store, TCD
Nocodazole	Sigma
Non-Essential Amino Acids	Sigma
Paclitaxel	Sigma
Pepstatin A	Sigma
Phenylethylenediamine	Sigma
Pipettes, sterile	Sterilin
PMSF	Sigma
Poly-L-Lysine coated slides	Sigma
Ponceau S	Sigma
[γ - ³² P] ATP	Amersham
Propidium Iodide	Sigma
Protein molecular weight standards	New England Biolabs
Protogel™	National Diagnostics
PVDF	Sigma
RapiDiff Kit	Sigma
SDS	BDH
Sodium flouride	Sigma
Sodium orthovanadate	Sigma
TCA	Sigma
TEMED	Sigma
Tissue culture flasks and plates	Greiner
Trizma base	Sigma
Triton X-100	Sigma
Trypsin	Sigma
Tubulin	Cytoskeleton
Tween 20	Sigma
Uranyl acetate	CMA
Urea	Sigma
[³ H] Vinblastine	Amersham

Vinblastine

Sigma

Pyrrolo-1,5-benzoxazepine compounds were gifts from Professor Giuseppe Campiani, Department of Pharmaceutical Chemistry, University of Siena, Italy.

Addresses of Suppliers:

Amersham Biosciences UK Ltd., Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, UK.

BDH, British Drugs House, Chemicals Ltd., c/o Lennox Chemicals, J.F. Kennedy Drive, Dublin 12, Ireland.

BD-Pharmingen, Life Science Research, Tullastrasse 8-12, 69126 Heidelberg, Germany.

Centre for Microscopy and Analysis (CMA), Basement, East End 4, Trinity College Dublin, Dublin 2, Ireland.

Chemicon International, Inc., 28820 Single Oak Drive, Temecula, CA 92590, USA.

Cytoskeleton Inc., 1830 S. Acoma St., Denver, CO 80223, USA.

European Collection of Animal Cell Cultures (E.C.A.C.C.), PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury SP4 0JG, UK.

Gibco, Life Technologies Ltd., 3 Fountain Drive, Inchinnan Business Park, Paisley PA4 9RF, UK.

Greiner, GmbH, Maybachstrasse 2, P.O. Box 1162, D-7443 Frickenhause, Germany.

MBL, Medical and Biological Laboratories Co., Ltd., 5-10 Marunouchi 3 chome, Naka-ku Nagoya, 460-0002, Japan.

Molecular Devices Corporation, 1311 Orleans Drive, Sunnyvale CA 94089-1136, USA.

National Diagnostics, 305 Patton Drive, Atlanta, Georgia 30336, USA.

NeoMarkers, 47790 Westinghouse Dr., Fremont, CA 94539, USA.

New England Biolabs, Knowl Piece, Wilbury Way, Hitchin, Hertfordshire SG4 0TY, UK.

Santa Cruz Biotechnology Inc., Bergheimer Str. 89-2, 69115 Heidelberg, Germany.

Shandon Inc., 171 Industry Drive, Pittsburg, PA 15275, USA.

Sigma, Chemical Co., Ltd., Fancy Road, Poole, Dorset, UK.

Sterlin, Bibby Sterlin Ltd., Stone, Staffordshire, UK.

Whatman Ltd., Maidstone, Kent, UK.

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 Growth and maintenance of the human astrocytoma 1321N1 cell line and the human breast carcinoma MCF-7 cell line

The human astrocytoma 1321N1 and the human breast carcinoma MCF-7 cell lines were obtained from European Collection of Cell Cultures (ECACC). They were both grown at 37°C under a humidified atmosphere of 95% O₂ and 5% CO₂. The 1321N1 cells were maintained in Dulbecco's Modified Medium (DMEM), whilst the MCF-7 cells were maintained in Minimum Essential Medium (MEM). Both media were supplemented with 10% (v/v) Foetal Calf Serum (FCS), 2mM L-glutamine and 100µg/ml gentamicin (complete medium). The MEM was also supplemented with 1% (v/v) non-essential amino acids.

Both of the cell lines were passaged twice weekly. The cells were removed from the surface of the flask by incubation with 1ml of trypsin-Ethylenediaminetetraacetic Acid (EDTA) (0.05mg/ml) for 1-5 minutes depending on the adherency of the cells. 10 ml of complete medium was then added to the cells and they were centrifuged at 500xg for 5 minutes. The medium was removed and the pellet was resuspended in 1ml of complete medium. Confluent cultures of 1321N1 cells were passaged 1:10 and MCF-7 confluent cultures were passaged 1:3. Cells were seeded in either 75cm² flasks in 20ml of medium or in 175cm² flasks in 30ml of medium. When seeding cells for experiments, they were counted using a haemocytometer. This is achieved by pipetting a 10-20µl volume under a glass coverslip placed over the middle section of the haemocytometer (described in Section 2.2.2). Following approximately 30 passages, cells were discarded and a new batch grown from liquid nitrogen stocks.

2.2.1.2 Use of a haemocytometer

Haemocytometer counting allows the counting of cells in suspension. The haemocytometer is a modified microscope slide comprising two polished surfaces, each of which displays a precisely ruled, sub-divided grid. The grid consists of nine primary squares, each measuring 1mm on a side (area 1mm²) and 25 medium

squares, each measuring 0.2 mm on a side (0.04mm^2). The squares are limited by three closely spaced lines and are used to determine if cells lie within or outside the grid. The plane of the grid rests 0.1mm below two ridges that support a sturdy coverslip. A clean coverslip was placed over the haemocytometer surface, and a 10-20 μl volume of cell suspension was pipetted under the cover slip making sure not to overfill the volume underneath the coverslip so as to prevent it floating. This was drawn across the grid by capillary action. The number of cells within the 4 outer squares and center square were counted – specifically the cells that are within, or that touch, the left or top boundary are counted, while those that touch or are outside the lower or right hand boundary are not counted. When the haemocytometer is loaded properly, the volume of cell suspension that will occupy one medium square is 0.04 mm^3 ($0.02\text{ mm}^2 \times 0.1\text{ mm}$) or $4.0 \times 10^{-6}\text{ ml}$. There are 25 of the medium squares, which totals a volume of $1 \times 10^{-4}\text{ ml}$. Total cell concentration in the original suspension (in cells/ml) is then: cells/ml = total count in the 25 medium squares $\times 1 \times 10^4$. Viable cell number can be determined by counting the number of cells that exclude a dye such as Trypan Blue. A viable cell possesses an intact cell membrane and therefore does not allow uptake of Trypan Blue. A suspension of cells is diluted 1:5 with Trypan blue and the number of cells excluding and including the dye is counted giving a number of viable and non-viable cells.

2.2.1.3 Cryo-preservation of cells

1321N1 and MCF-7 cells were grown to a state of sub-confluency and were harvested and counted as previously described (Sections 2.2.1 and 2.2.2 respectively). The cells were centrifuged and the pellet was resuspended in 1ml of a mixture of FCS and dimethyl sulphoxide (DMSO) (9:1). The cells were then transferred into 1.5ml cryotubes and placed at -20°C for 2 hours and then at -80°C for 3 hours before storing them in a liquid nitrogen vessel.

When required, an aliquot of cells was removed from the liquid nitrogen vessel, rapidly thawed and resuspended in 10ml of complete medium. This cell suspension was centrifuged at 500xg for 5 minutes, the medium containing DMSO was discarded and the pellet was resuspended in complete medium. Cells were then seeded in tissue culture flasks as previously described (Section 2.2.1.1).

2.2.1.4 Determination of 1321N1 cell growth curve

1321N1 cells (2.5×10^6) were seeded in 175cm² flasks and following 48 hours were treated with or without PBOX-21 (25µM). Viable cell number was determined at the indicated times by counting the trypan blue-excluding cells using a haemocytometer as described in Section 2.2.1.2.

2.2.2 3-(4,5-dimethylthiazol-2, 5-diphenyl tetrazolium bromide (MTT) assay for cell proliferation

The MTT cell proliferation assay is based upon the fact that viable cells are metabolically active. The water soluble yellow tetrazolium MTT is reduced by metabolically active cells, in part by mitochondrial dehydrogenases, generating NADH and NADPH (van de Loosdrecht *et al.*, 1994). The resulting intracellular purple formazan can be solubilised and quantified by spectroscopic methods.

MCF-7 cells were seeded at 1.5×10^4 cells per well of a 96-well plate containing 200µl of growth medium per well. 1321N1 cells were seeded at 0.1×10^4 per well containing 200µl of medium. The MCF-7 cells were incubated for 24 hours prior to treatment with varying concentrations of drug. The 1321N1 cells were incubated for 48 hours following seeding before treating with varying concentrations of drug, so as to ensure they were in the exponential phase of growth. Both cell lines were then incubated for the indicated times.

Following incubation, the medium within the wells was removed and 100µl of phosphate buffered saline (PBS) was then added to the wells to remove any remaining medium. This is so as to avoid precipitation of serum protein from the medium following the later addition of organic solvent (Denizot *et al.*, 1986). MTT (50µl of 1 mg/ml) was then added to each well. The cells were then incubated for 3 hours after which time the formed purple formazan crystals were completely solubilised by the addition of 200µl of DMSO. The plates were then wrapped in aluminium foil and left for 20 minutes to ensure solubilisation of the purple formazan crystals. The optical density of each well was measured using an automated microplate reader (Molecular Devices) at a wavelength of 595nm.

2.2.3 Lactate dehydrogenase (LDH) assay for cytotoxicity

LDH is a cytosolic enzyme present within all mammalian cells. The normal plasma membrane is impermeable to LDH, but damage to the cell membrane results in leakage of LDH into the extracellular fluid (Rae, 1975). The

release of the cytoplasmic enzyme lactate dehydrogenase (LDH), used as a measure of cell death, was performed following a modification of a method by Youkin *et al.* (1993).

Cells were seeded down and treated as previously described for the MTT assay (Section 2.2.2). Following the specified period of treatment, 50µl of medium was removed from each well and transferred to a fresh 96-well plate. To this an equal volume of substrate mix from the Promega cytotoxicity assay kit was added. The substrate mix contains a tetrazolium salt (INT) which is converted into a red formazan product via a coupled enzymatic reaction catalysed by LDH. The presence of LDH in the extracellular fluid directly correlates to the amount of formazan formed. The plate was left covered at room temperature for 20 minutes. After this time, 50µl of stop solution (0.1M NaOH) was added to all wells before reading the optical density using an automated microplate reader (Molecular Devices) at a wavelength of 490nm.

A 100% lysis control was a set of untreated cells, which were lysed by adding 20 µl of lysis solution (9% (v/v) Triton X-100 in water) provided in the Promega Cytotoxicity Assay kit. This was necessary each time an assay was undertaken in order to calculate the percentage cytotoxicity relative to this 100% control.

2.2.4 Flow cytometry

2.2.4.1 Fixing of cells

1321N1 cells were seeded at a density of 5×10^5 cells in 25 cm² flasks in 5ml of complete medium. MCF-7 cells were seeded at a density of 1×10^6 cells also in 25 cm² flasks and 5ml of complete medium. Once the cells were growing exponentially they were treated with varying concentrations of drug and incubated for up to 48 hours. Following this time, the cells were harvested by scraping with a cell scraper and centrifuged at 500xg for 5 minutes. The cell pellet was resuspended in ice-cold PBS and then centrifuged again at 500xg for 5 minutes. The cell pellet was resuspended in 200µl of ice-cold PBS and then made up to a 2ml volume with ice-cold 70% ethanol in PBS so as to fix the cells. The fixed samples could be stored at 4°C for up to 1 month.

2.2.4.2 Nuclear staining of cells with propidium iodide

Stored samples were centrifuged at 500xg for 5 min and the ethanol/PBS supernatant was removed gently so as to not disturb the cell pellet. The pellet was then resuspended in 800µl of PBS and transferred to a Fluorescence Activated Cell Sorting (FACS) tube (5ml polystyrene round-bottomed tube, 12 x 75 mm). RNase A in water (100µl of a 5mg/ml stock) was added to the samples followed by 100µl of a 1mg/ml stock of Propidium Iodide (PI) in water. The samples were quickly vortexed and incubated for 30 minutes at 37°C in the dark.

2.2.4.3 Flow cytometric analysis

Flow cytometry is a means of measuring certain physical and chemical characteristics of cells or particles as they travel in suspension one by one past a sensing point. The modern flow cytometer consists of a light source, collection optics, electronics and a computer to translate signals to data. In most modern cytometers the light source of choice is a laser which emits coherent light at a specified wavelength. Scattered and emitted fluorescent light is collected by two lenses (one set in front of the light source and one set at right angles) and by a series of optics, beam splitters and filters, specific bands of fluorescence can be measured. Physical characteristics such as cell size, shape and internal complexity as well as any cell component or function that can be detected by a fluorescent compound can be examined. The DNA content of a cell can be measured via staining with the fluorescent DNA binding dye, propidium iodide. The measurement of the DNA content of cells provides information about the effect on the cell cycle of added stimuli e.g. transfected genes or drug treatment. Cells in the various phases of the cell cycle can be differentiated between using this method as they will possess a different amount of DNA depending on what stage of the cell cycle they are in.

Using a Becton Dickinson FACSCalibur flow cytometer, the samples were illuminated with 200mW of light at 488nm and the fluorescence was read using a 630/22 nm band-pass filter (FL2). The cells were gated so as to exclude debris and doublets using a pulse width versus pulse area dot plot (FL2W on x-axis, FL2A on y-axis). A constant number of 10,000 viable cells was read for each sample. The percentage of cells in the G1, S and G2/M phases was

determined by subjectively applied markers on a histogram plot and data was analysed using the Macintosh-based application Cellquest.

2.2.5 Western Blot Analysis

2.2.5.1 Preparation of cell lysates

1321N1 cells were grown as described previously (Section 2.2.1) and seeded at 2.5×10^6 cells in 175 cm² flasks. MCF-7 cells were seeded at 3×10^6 in 75cm² flasks. Once the cells were growing exponentially they were treated with varying concentrations of drug and incubated for up to 48 hours. Following treatment, the cells were harvested via scraping and centrifuged at 500xg for 5 minutes followed by washing in ice-cold PBS. The cells pellets were resuspended in lysis buffer (50-250µl, depending on cell number so that all samples would have approximately the same protein concentration). The lysis buffer contained 50mM Tris-HCl, pH 7.5, 250mM NaCl, 5mM EDTA, 0.1% (v/v) Triton, 50mM sodium fluoride, protease inhibitors (1µg/ml aprotinin, 1µg/ml leupeptin, 1µg/ml pepstatin A, 1mM sodium orthovanadate, 100µg/ml phenylmethylsulfonyl fluoride (PMSF)) and 1mM dithiothreitol (DTT). Samples were vortexed for 10 seconds and left on ice for 30 minutes, vortexing every 10 minutes. The homogenates were then centrifuged at 20,000xg for 20 minutes at 4°C in an Eppendorf mini-centrifuge, model 5417R, and the supernatants were removed. The protein concentration of the resultant supernatants were determined by a Bradford assay.

2.2.5.2 Preparation of cell lysates for detection of poly (ADP-ribose) polymerase (PARP) cleavage

MCF-7 cells were grown, and treated as described in Section 2.2.6.1. Following treatment, the cells were harvested by scraping and centrifuged at 500xg for 5 minutes, the supernatant was removed and the pellets resuspended in PARP sample buffer (200µl) (62.5mM Tris/HCL pH 6.8, 6M urea, 10% (v/v) glycerol, 2% (w/v) SDS, 0.00125% bromophenol blue and 5% (v/v) β-mercaptoethanol added immediately before use). Samples were sonicated using a DAWE soniprobe sonicator for 15 seconds and heated at 65°C for 15 minutes.

2.2.5.3 Protein determination – Bradford assay

Protein concentration of cell lysates was determined using a modification of the method of Bradford (Bradford, 1976).

200µl of Bradford solution (0.01% Coomassie Brilliant Blue G-250, 4.7% (v/v) ethanol and 8.5% (w/v) orthophosphoric acid) was added to 18µl of H₂O and 2µl of the protein sample in a 96-well microtitre plate and left at room temperature for 5 minutes. The absorbance at a wavelength of 595 nm was measured using an automated plate reader (Molecular Devices) and the results were compared to the standard curve prepared using known concentrations of BSA. All protein determinations were performed in triplicate.

2.2.5.4 Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out as described by Laemmli (Laemmli, 1970), using an ATTO electrophoresis unit (model AE 6450) with 8% resolving gels for detection of Ser780-phosphorylated pRb, 10% resolving gels for Cyclins A, B₁, D₁, D₂, D₃, E, CDK1, CDK2, CDK4, CDK6 and PARP, and 12% resolving gels for detection of p21, p16. All these gels used a 5% stacking gel. Table 2.1 details the contents of the various gel solutions. The volumes quoted are those required for the pouring of one gel.

For detection of pRb phosphorylated status, a 5-18% gradient gel was employed. This involved making up 5% and 18% acrylamide resolving gel solutions as detailed in Table 2.2. The volumes quoted are those required for one gel. These two resolving gel solutions were then mixed linearly by a LKB Bromma 2232 microperpex peristaltic pump system. The gel was run in a larger ATTO electrophoresis unit (model AE-6220).

Table 2.1 Contents of stacking and resolving gels

	5% stacking gel (volume, ml)	8% resolving gel (volume, ml)	10% resolving gel (volume, ml)	12% resolving gel (volume, ml)
Protogel™ (Ultra-pure 30% (w/v) acrylamide and 0.8% (w/v) bisacrylamide)	0.83	2.7	4	3.19
ddH₂O	3.19	4.6	3.19	4
1.5M Tris-HCL (pH 8.8)	-----	3.19	2.5	2.5
1M Tris-HCL (pH 6.8)	0.63	-----	-----	-----
10% SDS (w/v)	0.05	0.15	0.1	0.1
10% APS (w/v)	0.05	0.15	0.1	0.1
TEMED	0.005	0.006	0.004	0.004

Table 2.2 Contents of 5-18% gradient gel

	5% resolving gel (volume, ml)	18% resolving gel (volume, ml)
Protogel™ (Ultra-pure 30% (w/v) acrylamide and 0.8% (w/v) bisacrylamide)	1.7	6
DdH₂O	6.84	1.3
1.5M Tris-HCL (pH 8.8)	1.25	2.5
1M Tris-HCL (pH 6.8)	-----	-----
10% (w/v) SDS	0.1	0.1
10% (w/v) APS	0.1	0.1
TEMED	0.008	0.004

Equal amounts of protein (30-100µg), prepared as described previously (Section 2.2.6.1) were added to 5X SDS Sample Buffer (containing 125mM DTT, 10µl). The samples were then boiled for 3 minutes and then loaded onto the SDS-PAGE gel along with broad range molecular weight markers (6-175kDa) (NEB). The gel was run in buffer (0.025M Tris, 0.192M glycine and 0.1% (w/v) SDS) and the samples were electrophoresed at 80V until the tracker dye reached the resolving gel. The voltage was then increased so as to maintain a constant charge of 20-25 mAmps. The gel was electrophoresed until the tracker dye reached the bottom of the resolving gel. Proteins were then transferred/immunoblotted onto polyvinylidene difluoride (PVDF) membrane.

2.2.5.5 Western blotting

Proteins resolved by SDS-PAGE were transferred onto 0.2µm PVDF membrane using a wet transfer apparatus (BioRad mini trans blot). Prior to transfer the PVDF membrane was immersed in methanol for 60 seconds, washed with double distilled (dd) H₂O and was then equilibrated along with the gel in transfer buffer (0.025M Tris, 0.192M glycine and 10% (v/v) methanol) for 15 minutes. The gel was then laid on a sponge and 4 layers of filter paper and overlaid with the PVDF membrane. Another 4 layers of filter paper were laid on top of the membrane followed by a second sponge. The entire assembly was carefully placed in a cassette and an electric current applied. An ice block was used to transfer apparatus cool. The transfer apparatus was placed on a stirring box and the transfer was carried out at a constant current of 160 mAmps for between 40 – 90 minutes, depending on the size of the protein.

A semi-dry transfer apparatus (Hoefer Scientific Instruments, Semi-phor) was employed for the transfer of resolved proteins on the 5-20% gradient gels. Again, the PVDF membrane and gel were equilibrated in transfer buffer for 15 minutes. The PVDF membrane was laid on top of 2 layers of filter paper and overlaid by the gel. Another 2 layers of filter paper then covered the gel. The transfer was carried out at a constant current of 160 mAmps.

Following the transfer of proteins, the PVDF membrane was blocked on a rocking platform in blocking buffer containing 5% (w/v) dry milk in TBST (0.1% (v/v) Tween 20 in Tris Buffered Saline (TBS) at room temperature for 1-3 hours or overnight at 4°C. Once blocked, the membrane was then washed briefly in

TBST (2 x 2 min) followed by incubation in primary antibody. The antibody was made up in 2-4 mls blocking buffer within a sterilin and rotated on a roller (Denley Spiramax 5) for 1-2 hours at room temperature or overnight at 4°C. The membrane was then washed again in TBST (2 x 5min, 1 x 10min) followed by incubation in Horse Radish Peroxidase (HRP)-conjugated secondary antibody made up in blocking buffer for 1 hour at room temperature. The membrane was then washed in TBST (2 x 5min, 1 x 10min, 2 x 5min) before a final wash in TBS. In order to assess if transfer of proteins was achieved the PVDF membrane was stained using Ponceau S stain (0.3% (w/v) Ponceau S, 3% (v/v) TCA).

The membrane was developed using the Enhanced Chemiluminescence (ECL) detection system, which detected the secondary antibody by means of an HRP-catalysed oxidation of luminol under alkaline conditions resulting in emission of light. The light emitted is detected by exposing the membrane to Kodak X-Omat LS film and developed using an automated developer (Fuji x-ray film processor).

2.2.5.6 Stripping and re-probing of blots

Membranes were incubated with stripping buffer (62.5mM Tris-HCL, pH 7.8, 100mM β -mercaptoethanol, 2% (w/v) SDS) for 15-20 minutes in a 50ml sterilin and placed in a water bath set to 50°C followed by several washes in TBST at room temperature. The membrane was blocked on a rocking platform, washed and incubated with the primary and secondary antibodies as previously described (Section 2.2.6.5).

2.2.6 Immunoprecipitation (IP)

2.2.6.1 Preparation of cell lysates

1321N1 and MCF-7 cells were seeded as described in Section 2.2.1 and treated with drug once the cells were in the exponential phase of growth. Following treatment for the indicated times, the cells were harvested via scraping and centrifuged at 500xg for 5 minutes and washed in ice-cold PBS. The cell pellets were resuspended in 500 μ l of IP lysis buffer which contained the following: 50mM N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), pH 7.5, 150mM NaCl, 1mM EDTA, 2.5mM Ethylene Glycol-bis(β -aminoethyl Ether) N,N, N',N'-Tetraacetic Acid (EGTA), 10% (v/v) glycerol,

0.1% (v/v) Tween 20, 1mM DTT, 1mM NaF, 10mM β -glycerophosphate, and protease inhibitors (10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 0.1mM PMSF and 0.1mM sodium orthovanadate). The DTT, NaF, β -glycerophosphate and protease inhibitors were added fresh to the IP buffer. The cell suspension was vortexed vigorously for 10 seconds and kept on ice for 30 minutes, vortexing every 5-10 minutes. Following this time the samples were sonicated on ice using a Branson sonicator 250, output level at position 6 for 10 seconds and were then centrifuged at 20,000xg for 20 minutes and the supernatant was removed. The protein content was determined by a Bradford assay.

2.2.6.2 Immunoprecipitation

The appropriate antibody was added to equal amounts of protein (100-500 μ g) in each cell lysate sample. An aliquot (1 μ l/1 x 10⁶ cells) of CDK2, CDK4 and CDK6 and 1 μ g of CDK1 and JNK1 antibodies was used per sample. The volume of the incubation mixture was made up to 500 μ l with IP lysis buffer. The samples were then incubated on a rotating platform for 3 hours at 4°C. A 50% slurry of protein A beads in PBS (50 μ l) was then added to the samples and incubated overnight at 4°C on the rotating platform.

The samples were then centrifuged for 2 minutes at 500xg to pellet the beads. The supernatant was removed gently using a gel-loading tip making sure not to disturb the beads. The pellet beads were then washed 3 times in 1ml of ice-cold IP lysis buffer.

After the final wash, the pelleted beads were resuspended in 20 μ l of 3X SDS sample buffer and then boiled for 3 minutes. The protein samples were resolved by SDS-PAGE as described previously (Section 2.2.6.4) and were either immunoblotted with the pull-down antibody to assess immunoprecipitation efficiency, or immunoblotted with another primary antibody to assess complexation status.

2.2.6.3 *In vitro* cyclin-dependent and JNK kinase assay

Following the washing of the immune-complex beads with ice-cold IP lysis buffer, the beads were then washed 3 times with ice-cold kinase buffer which contained the following: 50mM HEPES, pH 7.5, 2.5mM EGTA, 10mM MgCl₂, 1mM DTT, 10mM β -glycerophosphate, 1mM NaF and 0.1mM sodium orthovanadate. The lysates were then resuspended in 30 μ l of kinase buffer

supplemented with 20 μ M unlabelled Adenosine Triphosphate (ATP), 2 μ Ci [γ^{32} P] ATP and either 1 μ g GST-Rb substrate for CDK4 and CDK6, 10 μ g Histone H1 substrate for CDK1 and CDK2, or 1 μ g of GST-c-Jun substrate for JNK1. The suspensions were incubated in a prewarmed Perspex box at 30°C for 30 minutes, gently agitating the tubes every 5 minutes. An aliquot of 3X SDS sample buffer (20 μ l) was then added to each tube and the samples were boiled for 3 minutes behind a perspex screen and then loaded and resolved with molecular weight standards onto a 12% SDS-PAGE gel for analysis of GST-Rb and Histone H1 and a 10% SDS-PAGE gel for analysis of c-jun. The gel was carefully removed from the gel-box and gel-plates and dried under a vacuum using a gel drier (Biorad, Model 583) for 2 hours at 55°C behind a Perspex screen. The dried gel was then exposed to Kodak X-Omat film at -70°C. Phosphorylation was quantitated using a densitometric scan using the Scion Image software package of the phosphorylated bands in the autoradiogram (© 2000 Scion Corporation, based on NIH Image for Macintosh).

2.2.7 Terminal deoxynucleotidyl Transferase mediated bio-dUTP Nick Labelling (TUNEL) staining of cells

TUNEL identifies apoptotic cells in situ by using terminal deoxynucleotidyl transferase to the free 3'-OH of cleaved DNA. The ApopTag Plus Kit (Chemicon) was used for detection of DNA fragmentation in apoptotic cells by direct end-labeling of cellular genomic DNA with a digoxigenin-nucleotide using terminal deoxynucleotidyl-transferase which then binds a peroxidase bound digoxigenin antibody. The bound peroxidase enzymatically generates an intense localized stain from an applied chromogenic peroxidase substrate. MCF-7 cells were seeded at a density of 1×10^5 in a volume of 1ml per well of a 24-well plate. Following treatment with either vehicle (0.5% (v/v) ethanol) or 10 μ M PBOX-6 the cells were washed in PBS, and fixed in 5% formaldehyde in PBS for 15 min at room temperature. Fixed cells were stored dry on slides (Sigma) at room temperature before processing according to the manufacturer's specifications. After mounting, slides were analysed by light microscopy.

2.2.8 Cytospin preparation and RapiDiff staining of cells

MCF-7 cells were seeded at a density of 1×10^5 in a volume of 1ml per well of a 24-well plate. Following treatment with the indicated compounds, the cells were harvested by scraping. A 500 μ l aliquot of the cells was treated with 1mM EDTA so as to prevent clumping of the cells. The cells were then resuspended thoroughly and cytocentrifuged on to poly-L-lysine coated slides at 700xg for 2 minutes using a Cytospin 3 (Shandon). The slides were removed and left to air-dry at room temperature for approximately 2 minutes. Staining was carried out using the RapiDiff kit, containing solution A, 100% methanol, solution B, eosin-Y and solution C, methylene blue. The cells were fixed by dipping them 10 times in solution A. The cytoplasm of the cells was stained pink by dipping the slide 10 times in solution B and the nucleus was stained blue by dipping the slide 8 times in solution C. Excess dye was washed off with H₂O, and the slides were observed under a light microscope (Nikon) using 40X magnification, and photographs subsequently taken.

2.2.9 Indirect Immunofluorescence

MCF-7 cells were seeded at a density of 1.5×10^5 cells upon sterile 22mm x 22mm coverslips in a 2ml volume per well of a 6-well plate. Following treatment with the indicated compounds, the medium was removed and the cells washed gently with 1ml of PBS. The cells were then fixed in 100% methanol at -20°C for 10 minutes, after which time they were washed three times with PBS. The cells were then blocked at room temperature for 30 minutes in 5% BSA made up in PBST (0.1% (v/v) Triton in PBS). The blocking buffer was removed and the cells were incubated for 1 hour at room temperature in primary antibody to α -tubulin (500 μ l/well, 1:1000 dilution in blocking buffer). The cells were washed 3 times with PBS and then incubated for a further hour at room temperature in Fluorescein Isothiocyanate (FITC)-conjugated goat anti-mouse IgG (500 μ l/well, 1:1000 dilution in blocking buffer) after which time they were washed a further 3 times with PBS. The cells were then incubated with 0.2 μ g/ml PI made up in blocking buffer for 2 minutes. The coverslips were then removed from the wells, drained and 5 μ l of a 2 μ g/ml phenylethylenediamine in a 50:50 solution of PBS and glycerol was applied to the surface of each slide. The slides were then immediately mounted onto glass slides and sealed with a thick coating

of clear nail varnish. The slides were viewed at a magnification of 1000x with an Nikon microscope and images captured using ImageCapture software.

2.2.10 Assay of Tubulin Assembly

Tubulin is composed of a heterodimer of two related polypeptides, α and β tubulin. Tubulin polymerises to form microtubules. Microtubules are intrinsically dynamic polymers, but do not have a simple equilibrium. They polymerise and depolymerise by the reversible addition and loss of tubulin dimers at the ends of the microtubules. The assembly of purified bovine tubulin was monitored using the CytoDYNAMIX ScreenTM3. This assay uses a 96-well plate format with 300 μ g of >99% purified bovine brain tubulin in each well.

The tubulin (1mg, in lyophilised format) was resuspended on ice in 330 μ l of ice-cold G-PEM buffer (80mM Piperazine-N,N'-bis[2-ethanesulfonic acid] sequisodium salt (PIPES) pH 6.9, 0.5mM MgCl₂, 1mM EGTA, 1mM Guanidine Triphosphate (GTP), 5% (v/v) glycerol) and left on ice for 1 minute to allow for complete resuspension. 10 μ l of 10X strength of each compound tested made up in G-PEM buffer was pipetted into a half area 96-well plate prewarmed to 37°C. A 100 μ l volume of tubulin was pipetted into the designated wells of the prewarmed plate. It is important for the total pipetting to be less than 30 seconds from start to finish. The assay was conducted at 37°C and tubulin polymerisation was followed turbidimetrically at 340nm in a Spectramax 340PC spectrophotometer (Molecular Devices). The absorbance was measured at 30 second intervals for 1 hour.

2.2.11 Colchicine Binding Site Competitive Assay

Tubulin is the target of a variety of therapeutic agents such as vinblastine, paclitaxel and colchicine. These three ligands form the pharmacological map of tubulin's surface, as they bind at separate sites and will not compete with each other's binding site. This assay determines the ability of a ligand to bind to the colchicine binding site. The assay utilises size exclusion chromatography to separate bound and unbound fluorescein-labelled colchicine. The tubulin, polymerised into microtubules, has a molecular weight of approximately 100,000 whilst colchicine has a molecular weight of 399, therefore, tubulin will elute from the size exclusion columns first. If the colchicine is bound to tubulin, the

fluorescent peak will occur in these fractions. If the ligand competes with colchicine for binding to the colchicine binding site, this fluorescent signal will be reduced relative to the vehicle control and the ligand is confirmed as a colchicine site binding moiety.

Each reaction was performed in a 160 μ l volume containing 1mg/ml >99% purified bovine brain tubulin in 0.25mM PIPES buffer at pH 6.9, containing 0.05mM GTP, and 0.25mM MgCl₂. The tubulin stock was prepared by resuspending tubulin in lyophilised format to a 1mg/ml concentration in the buffer described above on ice and then centrifuged for 10 minutes at 14,000xg to remove denatured tubulin. The supernatant was carefully removed into a clean eppendorf tube on ice, leaving approximately 10 μ l in order not to disturb the pellet. Each reaction contained 8 μ l of test compound at the appropriate concentration and 150 μ l of tubulin stock. This reaction mixture was mixed gently by pipetting up and down three times and then incubated at 37°C for 30 minutes. A 2 μ l volume of the fluorescent colchicine stock (2mM) was diluted to 200 μ l in buffer, and following the 30 minute incubation, 4 μ l of this was added to each reaction giving a final concentration of 0.5 μ M fluorescent colchicine. This was then incubated for a further 30 minutes at 37°C. Each reaction was then applied to a G25 sephadexTM chromatography column. The columns were prepared by allowing a 3ml volume of the G25 slurry (slurry composed of an excess volume of reaction buffer as described above) to settle and then washing the columns twice with 5mls of buffer. The eluent from the columns was collected in designated wells of a 96-well plate. When the eluent from the application of the reaction mixture had been collected, 160 μ l of buffer was applied to the top of the column and the eluent collected in a fresh well. This was continued until 12 wells were filled per reaction. The plate was then read on a Spectramax Gemini XS fluorescence 96-well spectrophotometer (Molecular Devices), at excitation and emission wavelengths of 485nm and 535nm respectively.

2.2.12 Vinblastine Binding Site Competitive Assay

The ability of a ligand to bind to the vinblastine binding site on tubulin was next examined. Each reaction was performed as described for determination of binding to the colchicine binding site, however, instead of a fluorescent

vinblastine analogue, the binding of radiolabelled [^3H]-vinblastine was used and its presence within the collected fractions measured by liquid scintillation counting as described in Section 2.2.14.1. The tubulin, polymerised into microtubules, has a molecular weight of approximately 100,000 whilst vinblastine has a molecular weight of 811, therefore, tubulin will elute from the size exclusion columns first. If the vinblastine is bound to tubulin, the peak of radioactivity will occur in these fractions. If the ligand competes with vinblastine for binding to the vinblastine binding site, this radioactivity signal will be reduced relative to the vehicle control and the ligand is confirmed as a vinblastine site binding moiety. Each reaction contained a final concentration of $0.5\mu\text{M}$ [^3H]-vinblastine, and purified ($>99\%$) bovine brain tubulin was used. Finally, vinblastine has a larger molecular weight than colchicine, so as to improve the separation of the bound [^3H]-vinblastine from the unbound [^3H]-vinblastine, G50 sephadexTM columns were used instead of the G25 sephadexTM columns used in the colchicine binding site competitive site assay.

2.2.13 Liquid scintillation counting

The [^3H]-radioactivity was measured using a Packard 1500 scintillation counter. The scintillant cocktail used was a commercial scintillant Ecoscint. The average counting efficiency for [^3H] counting in the Packard 1500 was calculated to be 45%, based on a quench-correction curve relating counting efficiency to channels ratio. This was calculated from a quench curve relating counting efficiency to the spectral index of the external standard.

3 INVESTIGATION INTO MECHANISM UNDERLYING THE ONCOSTATIC EFFECTS OF PBOX-21

3.1 Introduction

Cancer has always been attributed to an abnormal proliferation of cells and agents that interfere with cell cycle progression may have potential as anti-cancer therapeutics. The discovery of inhibitors of the cell cycle such as paclitaxel (Schrijvers *et al.*, 1998) and UCN-01 (Akiyama *et al.*, 1997) has led to rapid advances in the investigation and design of a variety of antiproliferative compounds that are used clinically or have the potential for development as antiproliferative agents. It has previously been shown that some members of a series of novel pyrrolo-1,5-benzoxazepines (PBOXs) elicit antiproliferative effects upon tumour cells including human 1321N1 astrocytoma cells (Zisterer *et al.*, 1998). The PBOX compounds were originally synthesized as high affinity ligands to the peripheral-type benzodiazepine receptor (PBR) (Campiani *et al.*, 1996). Benzodiazepines such as Valium, are among the most highly prescribed drugs due to their anxiolytic, relaxant and sedative effects. They mediate their pharmacological effects through their interaction with the neuronal GABA-gated chloride channel (Zisterer *et al.*, 1997). This is known as the central-type benzodiazepine receptor and is present exclusively in the central nervous system. However, benzodiazepines also bind to another distinct recognition site, now known as the PBR. A wide variety of functions have been attributed to the PBR over the years. These include involvement in immune response (Zavala *et al.*, 1991), behavioural conditions, effects on mitochondrial respiration, steroidogenesis and also on cell growth (Gorman *et al.*, 1989). However, the true physiological function of the receptor remains to be discovered (Gavish *et al.*, 1999). It was thought that this group of novel PBOX compounds would help in probing the true physiological function of the receptor.

Ligands to the PBR have previously been shown to elicit cytostatic effect on cancerous cell lines. Gorman *et al.*, (1989) found that Ro5-4864, diazepam, clonazepam and PK 11195, the classically used PBR ligands, inhibited the proliferation of rat C6 glioma and mouse neuro-2A neuroblastoma cells in the micromolar range, whilst Camins *et al.*, (1995) demonstrated that Ro5-4864, PK 11195 and diazepam inhibited in a concentration-dependent manner the proliferation of V79 Chinese hamster lung cells. However, both of these studies

concluded that it was unlikely that these antiproliferative effects were mediated through an interaction with the PBR, as far higher concentrations were required to elicit such effects (approximately 1000-fold) than are necessary to saturate the PBR. More recently, Zisterer *et al.*, (1998) found that some members of the PBOX compounds also induced antiproliferative effects within the human astrocytoma cell line, 1321N1. This inhibition of proliferation was found to be specifically through an accumulation of cells in the G1 phase of the cell cycle, although the time of onset of the G1 arrest and the molecular mechanism underlying the arrest remained unclear.

These cytostatic effects of PBOX compounds in a human tumour cell line indicated the potential of these compounds for use as novel anticancer drugs and indicated the need to examine the direct mechanisms responsible for the initiation and/or maintenance of the growth-arrested state. Because cell growth and proliferation are ultimately regulated by a highly conserved set of cell cycle regulatory proteins, a productive approach to the study of the antiproliferative mode of action of the PBOX compounds is to examine their influence on the regulation of the cell cycle-regulating machinery.

The G1 phase is the interval in which cells respond to extracellular cues that ultimately determine whether cells will make the decision to replicate DNA and divide or, alternatively, to exit the cell cycle into a quiescent state (G0). Once cells make the decision to begin DNA replication, they are irreversibly committed to complete the cycle, and the time late in the G1 phase at which this decision is made is designated the restriction point checkpoint (Figure 1.1). The eukaryotic cell cycle is regulated by the action of the cyclin-dependent kinases (CDKs) and their activating subunits, the cyclins (McDonald *et al.*, 2001). The expression of a subset of CDKs and cyclins occurs during the G1 phase of the cell cycle, placing them in a temporal position to influence the restriction point checkpoint and cell cycle progression. Among such candidate G1 control molecules are the D-type cyclins, cyclin E and the CDK inhibitors belonging to the CIP and INK4 families. Genetic lesions that disable key regulators of G1 phase progression in mammalian cells are present in most human cancers.

D-type cyclins consist of three family members, D₁, D₂ and D₃. They are the major downstream targets of extracellular signalling pathways. Their expression is induced by mitogenic growth factors, whilst conversely mitogen

withdrawal in G1 leads to rapid degradation of the D-type cyclins and failure of the cells to enter S-phase. D-type cyclins mediate their regulatory function by assembling combinatorily into holoenzymes with either CDK4 or CDK6 primarily, and to a lesser extent CDK2. Whereas CDK4 and CDK6 are relatively long-lived proteins, the D-type cyclins are unstable, and their induction, synthesis, and assembly with their catalytic partners all depend upon persistent mitogenic signalling. It is in this sense that the D-type cyclins act as growth factor sensors, forming active kinases in response to extracellular cues (Sherr, 1993). The mitogen-dependent accumulation of the cyclin D-dependent kinases triggers the phosphorylation of the retinoblastoma protein (Rb), thereby helping to cancel its growth-repressive functions (Ewen *et al.*, 1993; Kato *et al.*, 1993). Rb represses the transcription of genes whose products are required for DNA synthesis. Prior to the restriction point of G1, Rb is found in an underphosphorylated form. In this state, Rb binds to and negatively regulates the activities of transcription factors such as the E2Fs (Nevins, 1998). Rb also mediates its growth inhibitory role by recruiting repressors such as histone deacetylases (Brehm *et al.*, 1998; Luo *et al.*, 1998) and chromosomal remodeling SWI/SNF complexes (Zhang *et al.*, 2000) to E2F-responsive promoters on DNA. However, Rb phosphorylation by the G1 CDKs disrupts these interactions, releasing E2F from its inhibitory constraint, thereby enabling the untethered transcription factor to activate genes required for DNA replication. Thus the phosphorylation status of Rb is a critical determinant in the execution of the progression of the cell cycle from G1 to the S phase.

Apart from a battery of genes that regulate DNA metabolism, E2Fs induce the cyclin E and A genes. In mammalian cells, cyclin E preferentially forms a complex with its catalytic partner CDK2 (Dulic *et al.*, 1992; Koff *et al.*, 1992a). The activity of the cyclin E-CDK2 complex peaks at the G1-S transition, after which cyclin E is degraded and replaced by cyclin A during S phase. The cyclin E-CDK2 complexes can phosphorylate histone H1, Rb and the Rb family members p107 and p130 (Castano *et al.*, 1998; Woo *et al.*, 1997).

Whereas cyclin-CDK complexes are key regulators of G1 cell cycle progression, evolution has added yet another layer of G1 cell cycle regulation in the form of cyclin-CDK inhibitory proteins. Two classes of G1 cyclin/CDK inhibitors have been defined based on this sequence similarity and specificity of action. One class is the CIP/KIP (CDK interacting protein/Kinase inhibitory

protein). These proteins are also known as the ‘universal CDK inhibitors’. They indifferently inhibit all of the CDKs, but preferentially inhibit CDKs of G1/S phase. This family consists of three members: p21^{CIP1,WAF1,Sdi1} (el-Deiry *et al.*, 1993); (Noda *et al.*, 1994), p27^{KIP1} (Toyoshima *et al.*, 1994) and p57^{KIP2} (Lee *et al.*, 1995).

Another class of CDK inhibitors, the so-called INK4 proteins (named for their ability to inhibit CDK4), specifically target the cyclin D-dependent kinases (Ruas *et al.*, 1998). To date, four INK4 proteins have been identified. These include the founding member p16^{INK4a} (Serrano *et al.*, 1993) and three other closely related genes designated p15^{INK4b} (Hannon *et al.*, 1994), p18^{INK4c} (Guan *et al.*, 1994) , and p19^{INK4d} (Hirai *et al.*, 1995). Structural studies of the p16-CDK6 and p19-CDK6 complexes suggest that association with INK4 family members prevents ATP binding of CDK6, and that p16 indirectly prevents CDK6 from binding to cyclin D (Russo *et al.*, 1998). Other proteins have CDK inhibitory properties, but *bona fide in vivo* roles as CDK inhibitors remain to be established (Coats *et al.*, 1999). The classification of CDK inhibitors is based upon their structure as well as their CDK-inhibitory affinity.

Aims of this chapter

In the present study the antiproliferative action of a number of PBOX compounds in two human tumour cell lines was characterised. The changes that occurred in kinase activities, expression levels, interactions and phosphorylation status of the cell cycle regulatory molecules in human 1321N1 cells, in response to treatment with a potent antiproliferative PBOX compound, PBOX-21 was then systematically analysed and compared to the timing of onset of the growth arrest process. Thus, it was possible to distinguish between induced changes in cell cycle regulating molecules, which are possibly responsible for the growth arrest, from those that are as a result of the cell cycle arrest itself.

3.2 Results

3.2.1 Characterisation of the antiproliferative action of PBOX compounds in 1321N1 and MCF-7 cells

3.2.1.1 A range of PBOX compounds induce antiproliferative effects upon 1321N1 cells in a dose-dependent manner

In order to confirm the antiproliferative effects of PBOX-21 (previously NF 262) and PBOX-1 (previously NF 182) on 1321N1 astrocytoma cells as reported by Zisterer *et al.*, (1998), an MTT assay was performed. A third PBOX compound used, PBOX-60, was also tested because based upon molecular modelling studies, it was considered to be potentially as potent as either PBOX-21 or PBOX-1 (Campiani, G., personal communication). 1321N1 cells were seeded in each well of a 96-well plate, left for 48 hours, until they were in the exponential phase of growth and then treated with the indicated concentrations of PBOX compounds for a further 48 hours. The percentage cell proliferation was determined by performing an MTT assay as described previously (Section 3.2.3).

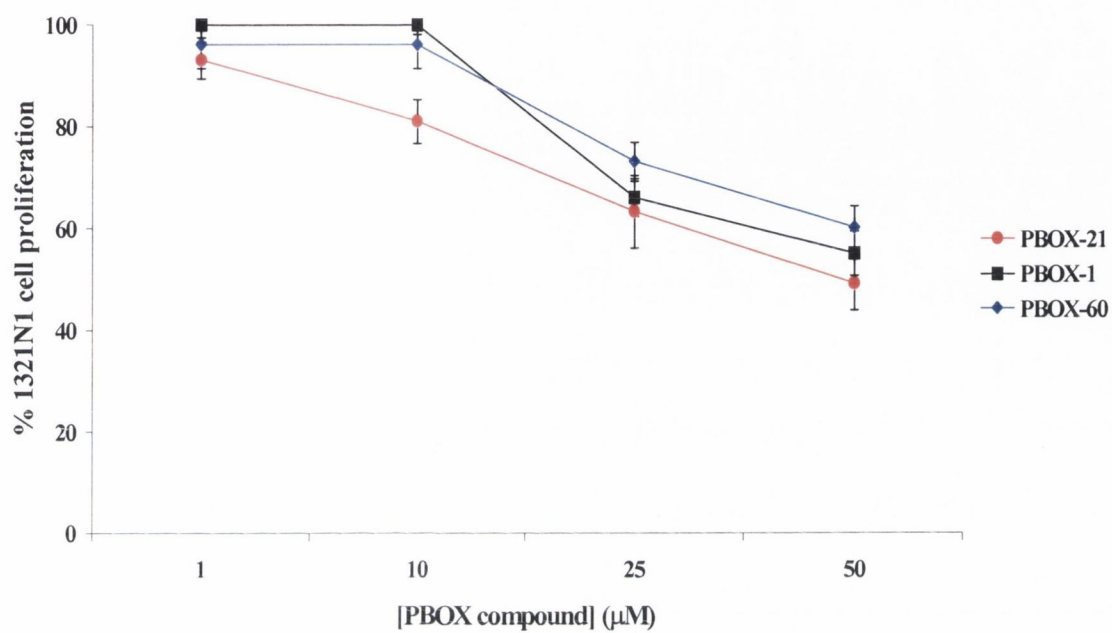
All three PBOX compounds screened inhibited cell proliferation in a dose-dependent manner (Figure 3.1). Anti-proliferative effects were observed with concentrations of less than 10 μ M for PBOX-21, whilst higher concentrations of PBOX-1 and PBOX-60 were required before they elicited an effect upon proliferation. The anti-proliferative effects were not associated with cytotoxicity (as measured by LDH release) until the highest concentration of drug (50 μ M) was used. The percentage of cytotoxicity associated with this dose was 9.3%, 10.2% and 8.7% for PBOX-21, PBOX-1 and PBOX-60 respectively.

3.2.1.2 A range of PBOX compounds induce antiproliferative effects upon MCF-7 cells in a dose-dependent manner

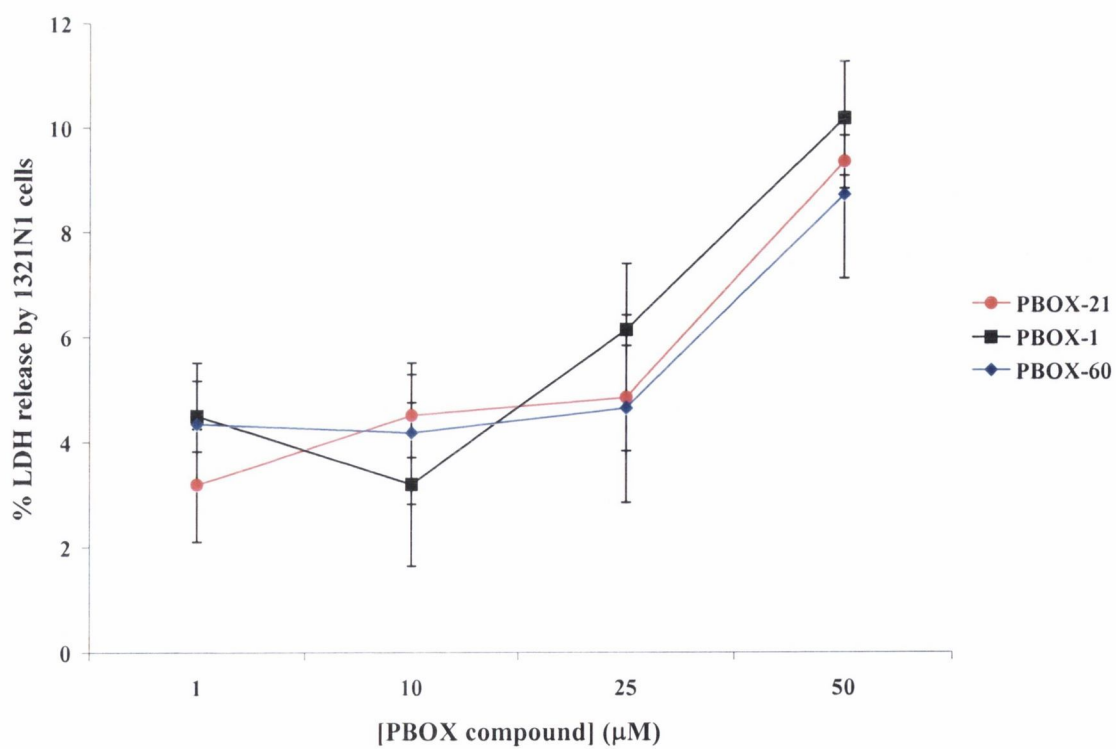
To determine if these antiproliferative effects exhibited by these PBOX compounds were cell-type specific, PBOX-21, PBOX-1 and PBOX-60 were also assessed in the MCF-7 human breast carcinoma cell line. MCF-7 cells were

Figure 3.1 PBOX compounds inhibit cell proliferation of 1321N1 cells in a dose-dependent manner with negligible cytotoxicity

1321N1 cells were seeded at a density of 0.1×10^4 in each well of a 96-well plate in 200 μ l of complete medium. The 1321N1 cells were left for 48 hours, until they were in the exponential phase of growth. After this time, the 1321N1 cells were treated for 48 hours with the indicated PBOX compounds giving a final concentration of 1, 10, 25 and 50 μ M. The percentage cell proliferation was determined by performing an MTT assay (a) as described previously (Section 2.2.2). The cytotoxicity of the compounds was determined by performing an LDH assay (b) as described previously (Section 2.2.3). The results shown represent the mean $\pm$ S.E.M. of three separate experiments.



(a)



(b)

seeded in each well of a 96-well plate, left for 24 hours, until they were in the exponential phase of growth and were then treated with the indicated concentrations of PBOX compounds. The MCF-7 cells were incubated for a further 48 hours and the percentage cell proliferation was determined by performing an MTT assay as described previously (Section 3.2.3).

As was found with the 1321N1 cells, all three PBOX compounds screened inhibited cell proliferation (Figure 3.2). Using an LDH assay as described previously (Section 2.2.4) the percentage of cytotoxicity associated with each dose of the three compounds was found to be negligible (less than 5%). It appears that both PBOX-1 and PBOX-21 elicited comparable antiproliferative effects in the MCF-7 cell line, with negligible cytotoxic effects. However, as PBOX-21 elicited the most potent antiproliferative effect within the 1321N1 cell line, it was chosen as the representative PBOX compound with which to carry out further mechanistic studies. It was decided that 25 μ M was the optimal concentration with which to treat the cells, because it was capable of effective inhibition of cell proliferation without a significant cytotoxic effect.

3.2.1.3 PBOX-21-treated 1321N1 cells display decreased proliferation

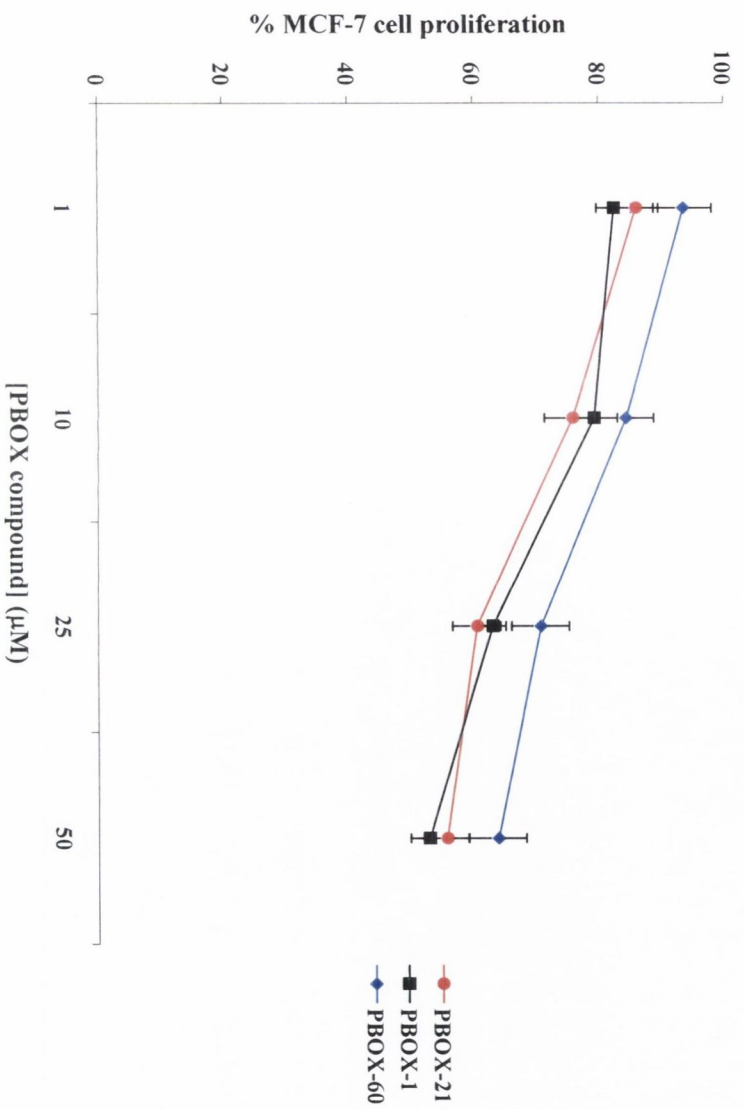
To assess the inhibitory effect of PBOX-21 on the growth of 1321N1 cells, their growth rate in the absence and presence of both vehicle (0.5% ethanol (v/v)) and 25 μ M PBOX-21 was determined (Figure 3.3). Proliferation of exponentially growing 1321N1 cells was inhibited following treatment with PBOX-21 with an inhibitory effect seen after 24 hours, and the cell number did not increase significantly after 48 hours. This indicates that the action of PBOX-21 is exerted rapidly. The vehicle itself inhibited cell proliferation slightly over time. A lower concentration of ethanol could not be used due to solubility problems with the PBOX compounds.

3.2.1.4 Analysis of cell cycle effects of PBOX-21 upon 1321N1 cell line

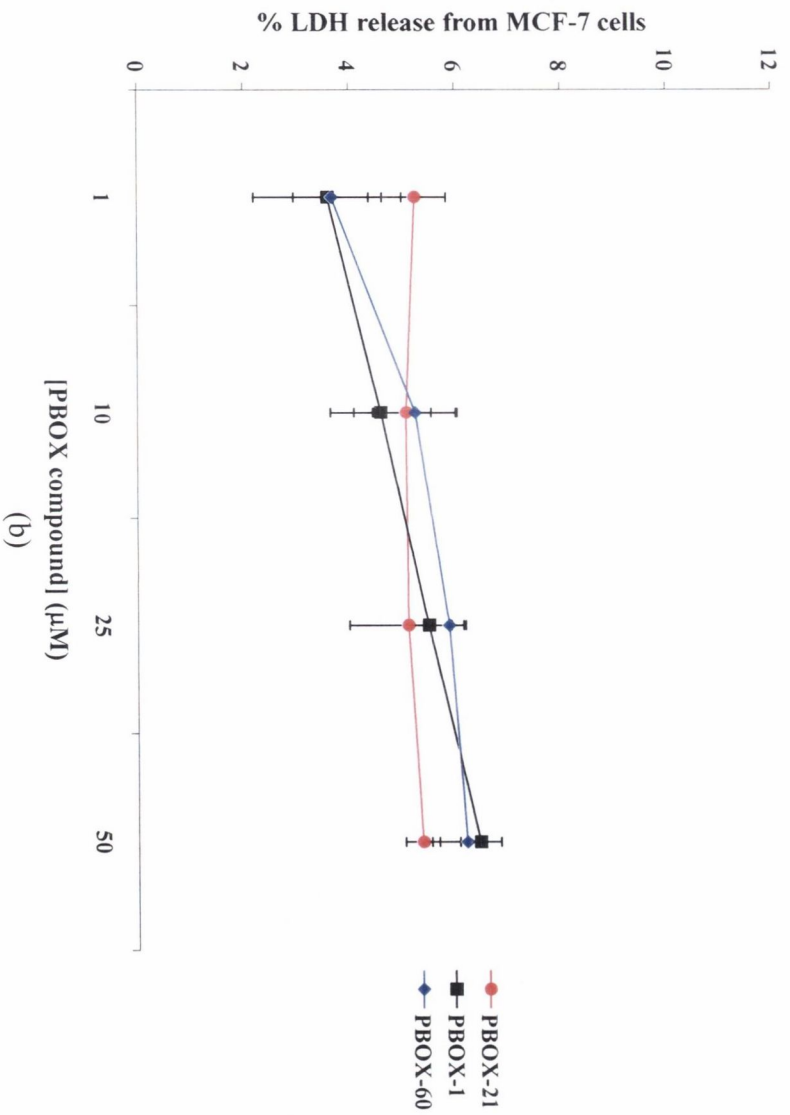
In order to characterise the antiproliferative action of PBOX-21, the distribution of the 1321N1 cells within the various phases of the cell cycle was analysed using flow cytometry. 1321N1 cells were seeded in 175cm² flasks, incubated for 48 hours until the cells were in the exponential phase of growth and then treated with either vehicle (0.5% ethanol (v/v)) or 25 μ M PBOX-21 for the

Figure 3.2 PBOX compounds inhibit cell proliferation of MCF-7 cells in a dose-dependent manner with negligible cytotoxicity

MCF-7 cells were seeded at a density of 1.5×10^4 in each well of a 96-well plate in 200 μ l of complete medium. The MCF-7 cells were left for 24 hours, until they were in the exponential phase of growth. After this time, the cells were treated for 48 hours with the indicated PBOX compounds giving a final concentration of 1, 10, 25 and 50 μ M. The percentage cell proliferation was determined by performing an MTT assay (a) as described previously (Section 2.2.2). The cytotoxicity of the compounds was determined using an LDH assay (b) as described previously (Section 2.2.3). The results shown represent the mean $\pm$ S.E.M. of three separate experiments.



(a)



(b)

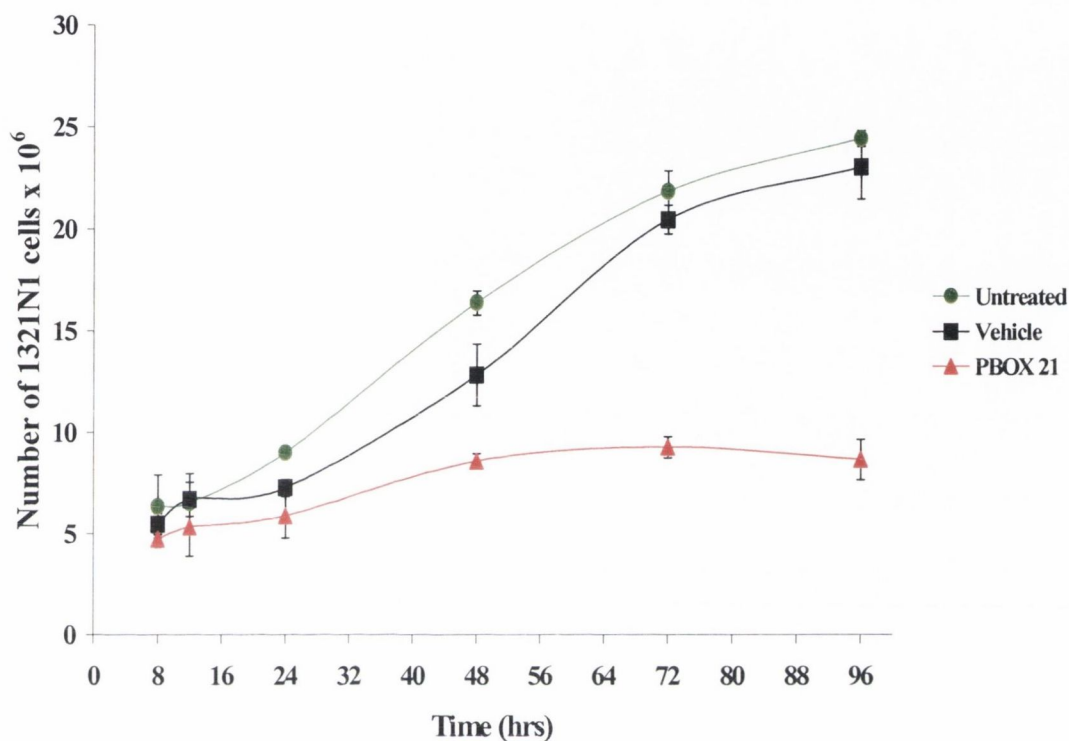


Figure 3.3 PBOX-21 causes a time-dependent decrease in 1321N1 cell proliferation

1321N1 cells were seeded at a density of 2.5×10^6 in 175cm² flasks in 30mls of complete medium and were incubated at 37°C under a humidified atmosphere of 95% O₂ and 5% CO₂ for 48 hours until the cells were in the exponential phase of growth. The cells were then either untreated (green) or treated with vehicle (0.5% ethanol (v/v), black) or PBOX-21 (25μM, red) and incubated for the indicated times. Cell number was counted at the indicated times using a hemocytometer. Results represent the mean $\pm$ S.E.M. of three separate experiments.

indicated times. The cells were harvested as described previously (Section 2.2.1) and an aliquot of the cells was prepared and analysed using flow cytometry as described previously (Section 2.2.4).

Treatment with PBOX-21 caused a time-dependent increase in the number of 1321N1 cells in the G1 phase of the cell cycle (Figure 3.4 (b)) and this first became significant at 12 hours. The percentage increase in the number of PBOX-21 treated 1321N1 cells in the G1 phase relative to the vehicle treated cells was maintained up to 24 hours of treatment. A maximum induction of G1 arrest was seen at 20 hours when there was 16% more PBOX-21 treated 1321N1 cells in G1 than vehicle treated cells. After this time, although a significant G1 arrest was still noticeable in response to PBOX-21 treatment, the vehicle itself (0.5% ethanol (v/v)) also appeared to elicit a small effect, relative to untreated cells, indicating that this arrest was not due to a cell density effect. This is in agreement with the effect elicited by the vehicle control upon 1321N1 cell proliferation (Figure 3.3).

This increase in the number of cells in the G1 phase following PBOX-21 treatment was associated with a concomitant reduction in the number of cells in both the S and G2/M phases (Figure 3.4 (c) and (d)). PBOX-21 did not cause any change in the number of cells in the pre-G1 phase of the cell cycle over the time course examined (Figure 3.4 (a)). As any necrotic and apoptotic cells are seen in the pre-G1 phase, this confirms the results of the LDH assay which indicated that PBOX-21 does not have a cytotoxic effect upon the 1321N1 cells at this concentration.

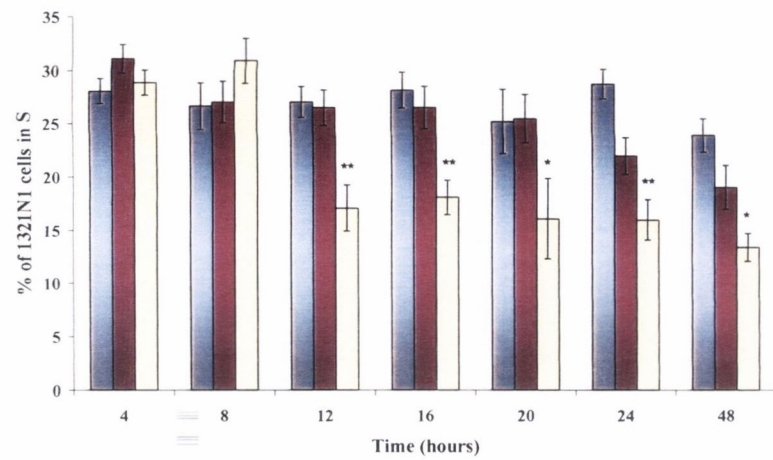
3.2.1.5 Analysis of cell cycle effects of PBOX-21 upon the MCF-7 cell line

MCF-7 cells were seeded in 25 cm² flasks and incubated for 24 hours until the cells were in the exponential phase of growth. The cells were then treated with either vehicle (0.5% ethanol (v/v)) or 25µM PBOX-21 for the indicated times. The cells were harvested as described previously (Section 2.1.1) and prepared and analysed using flow cytometry as described in Section 2.5.

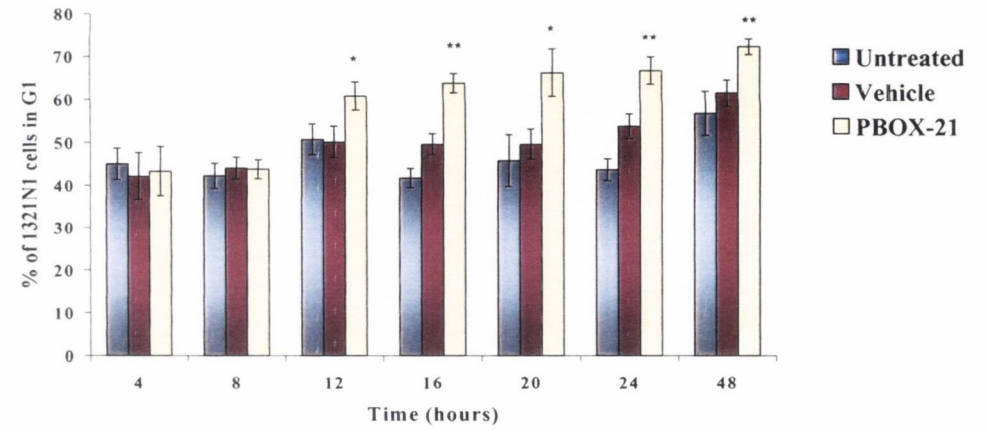
PBOX-21 (25 µM) caused a time-dependent increase in the percentage of MCF-7 cells within the G1 phase of the cell cycle (Figure 3.5 (b)). A significant

Figure 3.4 PBOX-21 causes a time-dependent increase in the number of 1321N1 cells within the G1 phase.

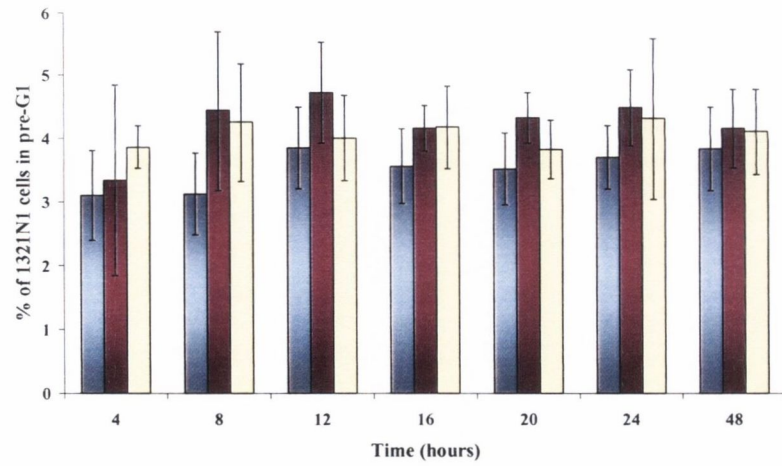
1321N1 (2.5×10^6) cells were seeded down in 175cm² flasks in 30mls of complete medium and were incubated at 37°C under a humidified atmosphere of 95% O₂ and 5% CO₂ for 48 hours until the cells were in the exponential phase of growth. The cells were then either untreated (blue) or treated with vehicle (0.5% ethanol (v/v), red) or PBOX-21 (25µM, white) and incubated for the indicated times. The cells were then harvested as described previously (Section 2.2.1) and an aliquot of the cells was prepared and analysed using flow cytometry as described previously (Section 2.2.4). The results represent the mean $\pm$ S.E.M of four separate experiments. Statistical analysis was carried out using the Instat computer program. * $p < 0.05$, ** < 0.01 with respect to vehicle treatment; Students t-test.



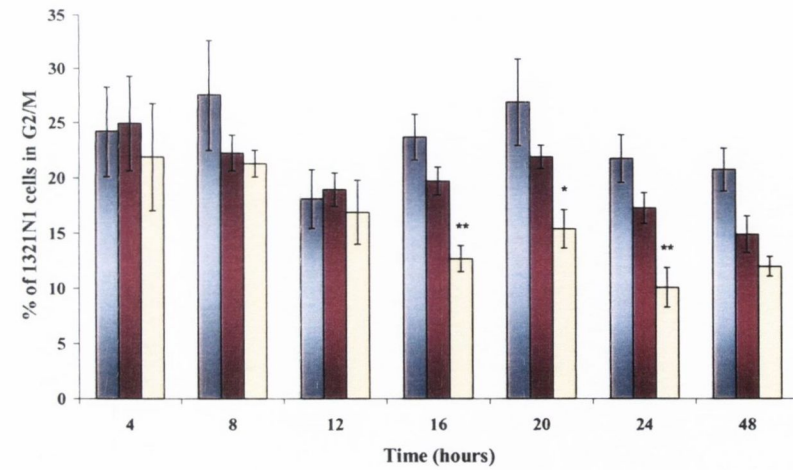
(a)



(b)



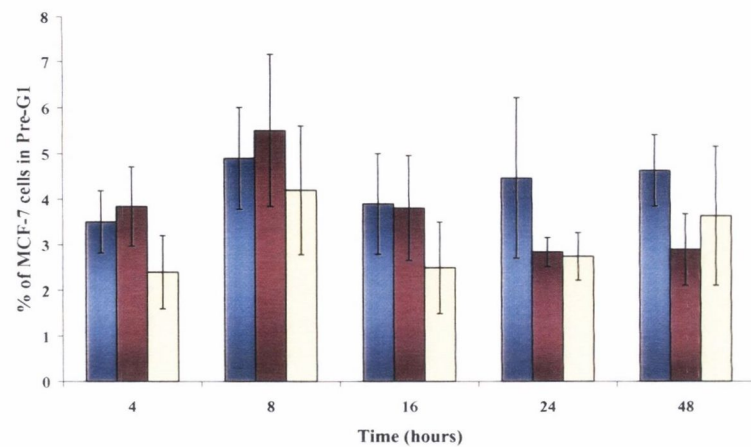
(c)



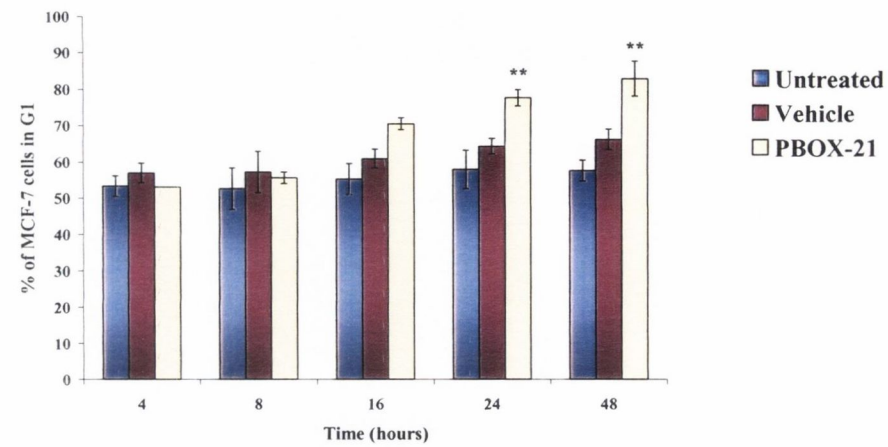
(d)

Figure 3.5 PBOX-21 causes a time-dependent increase in the number of MCF-7 cells within the G1 phase

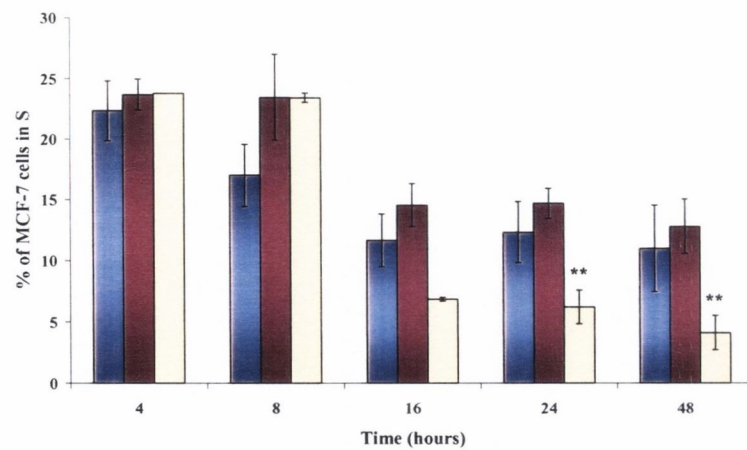
MCF-7 (1×10^6) cells were seeded down in 25cm^2 flasks in 5mls of complete medium and were incubated at 37°C under a humidified atmosphere of 95% O_2 and 5% CO_2 for 24 hours until the cells were in the exponential phase of growth. The cells were then either untreated (blue) or treated with vehicle (0.5% (v/v) ethanol, red) or PBOX-21 ($25\mu\text{M}$, white) and left for the indicated times. The cells were harvested as described previously (Section 2.2.1) and prepared and analysed using flow cytometry as described in Section 2.2.4. The results represent the mean $\pm$ S.E.M of four separate experiments. Statistical analysis was carried out using the Instat computer program. * $p < 0.05$, ** < 0.01 with respect to vehicle treatment; Students t-test.



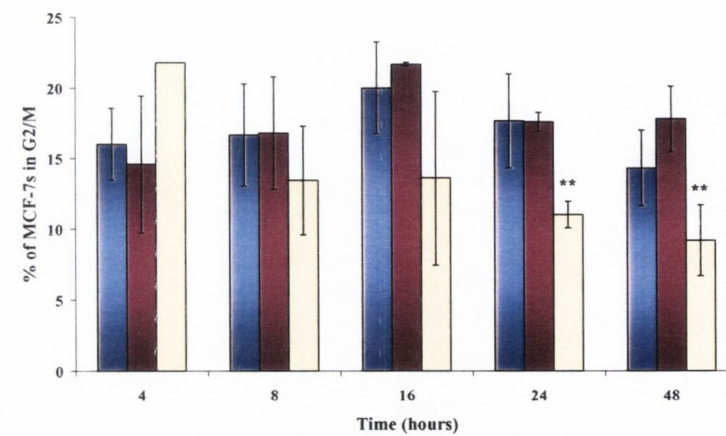
(a)



(b)



(c)



(d)

maintained up to 48 hours. Again, the vehicle itself (0.5% ethanol (v/v)) appeared to elicit a small G1 arrest relative to untreated cells. This induction of G1 arrest by 25 μ M PBOX-21 resulted in a time-dependent decrease in the percentage of cells within both the S and G2/M phases (Figure 3.5 (c) and (d)). PBOX-21 did not result in an increase in the percentage of cells within the pre-G1 phase of the cell cycle up to 48 hours of treatment (Figure 3.5 (a)). This indicates that 25 μ M PBOX-21 did not result in apoptotic or necrotic cell death, and supports the result of the LDH assays. Finally, it can be concluded that PBOX-21 elicits similar antiproliferative effects in both the 1321N1 and MCF-7 cell lines.

3.2.1.6 Dose-dependent PBOX-21 inhibition of 1321N1 and MCF-7 cell proliferation parallels the cell cycle inhibition

The dose-dependent effect of PBOX-21 upon the cell viability was assessed by MTT assay, so as to determine if the effect of PBOX-21 upon 1321N1 and MCF-7 cell cycle progression correlated with cell growth. Both 1321N1 and MCF-7 cells were seeded as described previously (Section 3.2.1.1) and then treated with the indicated concentrations of PBOX-21 for a further 48 hours. The percentage cell proliferation was determined by MTT assay as described previously (Section 3.2.3).

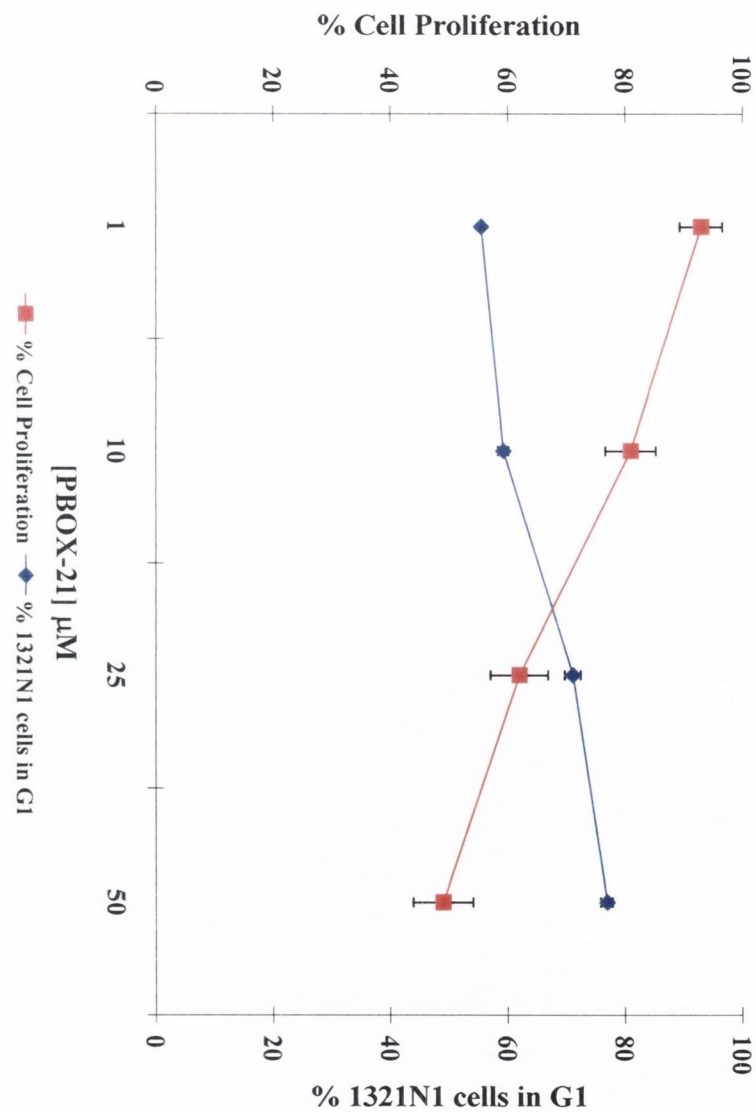
PBOX-21 was found to dose-dependently inhibit both 1321N1 and MCF-7 cell proliferation which paralleled the accumulation of these cells in the G1 phase of the cell cycle (Figure 3.6). Taken together, these results indicate that PBOX-21 inhibits cell growth by causing the G1 arrest of the cell cycle.

3.2.1.7 Reversibility of PBOX-21 induced G1 arrest in both MCF-7 and 1321N1 cell lines

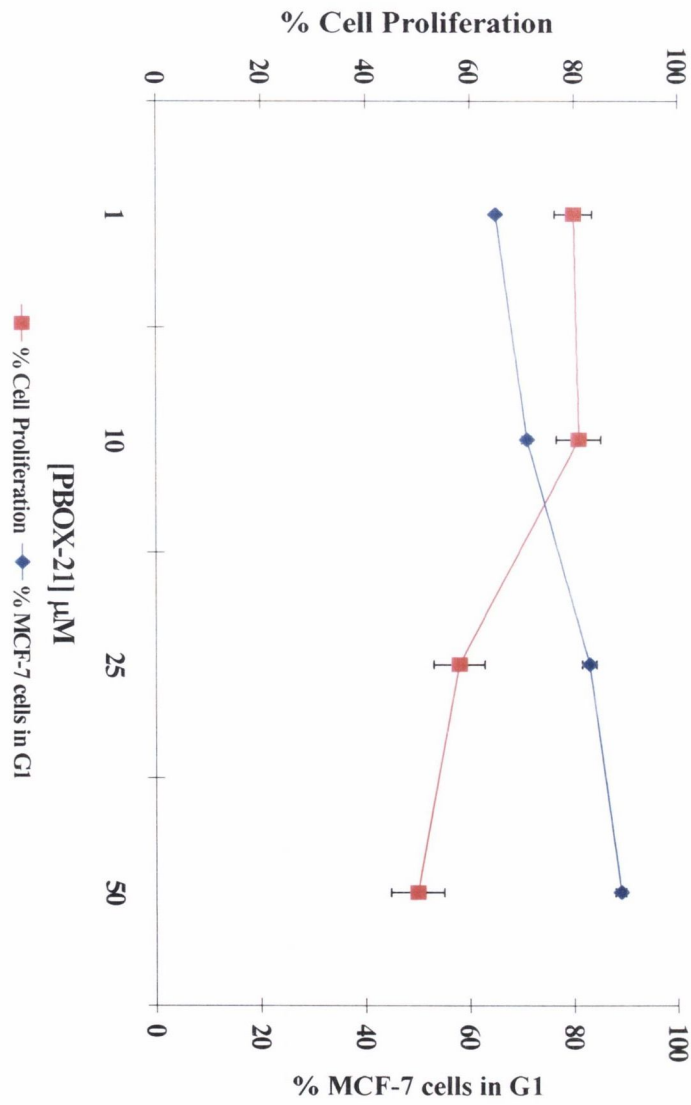
In order to confirm the lack of cytotoxicity of PBOX-21 to cells, the reversibility of PBOX-21 induced G1 arrest was investigated. Both 1321N1 and MCF-7 cells in the exponential phase of growth were treated with 25 μ M PBOX-21 for 24 hours. The cells from duplicate flasks were harvested as described previously (Section 2.1.1) and prepared and analysed using flow cytometry as described in Section 2.2.4. The medium in the remaining flasks was carefully removed and the cells washed in fresh medium. Another 5 mls of fresh complete medium in the absence of PBOX-21 was added to the flasks and the cells were incubated for a further 24 hours. The cells were harvested and prepared and

Figure 3.6 Dose-dependent PBOX-21 inhibition of 1321N1 and MCF-7 cell proliferation parallels the cell cycle inhibition

For the MTT assay, 1321N1 and MCF-7 cells were seeded as described in Section 3.2.1.1. For Flow cytometric analysis, 1321N1 and MCF-7 cells were seeded as described in Section 3.2.1.3. The cells were incubated until they were in the exponential phase of growth and then treated with the indicated concentrations of PBOX-21 for a further 48 hours. The cell proliferation and cell cycle arrest is expressed as a percentage of the vehicle (0.5% (v/v) ethanol) and was determined by performing an MTT assay (as described in Section 2.2.2) and flow cytometric analysis (as described in Section 2.2.5) respectively. The results represent the mean $\pm$ S. E. M. of three separate experiments.



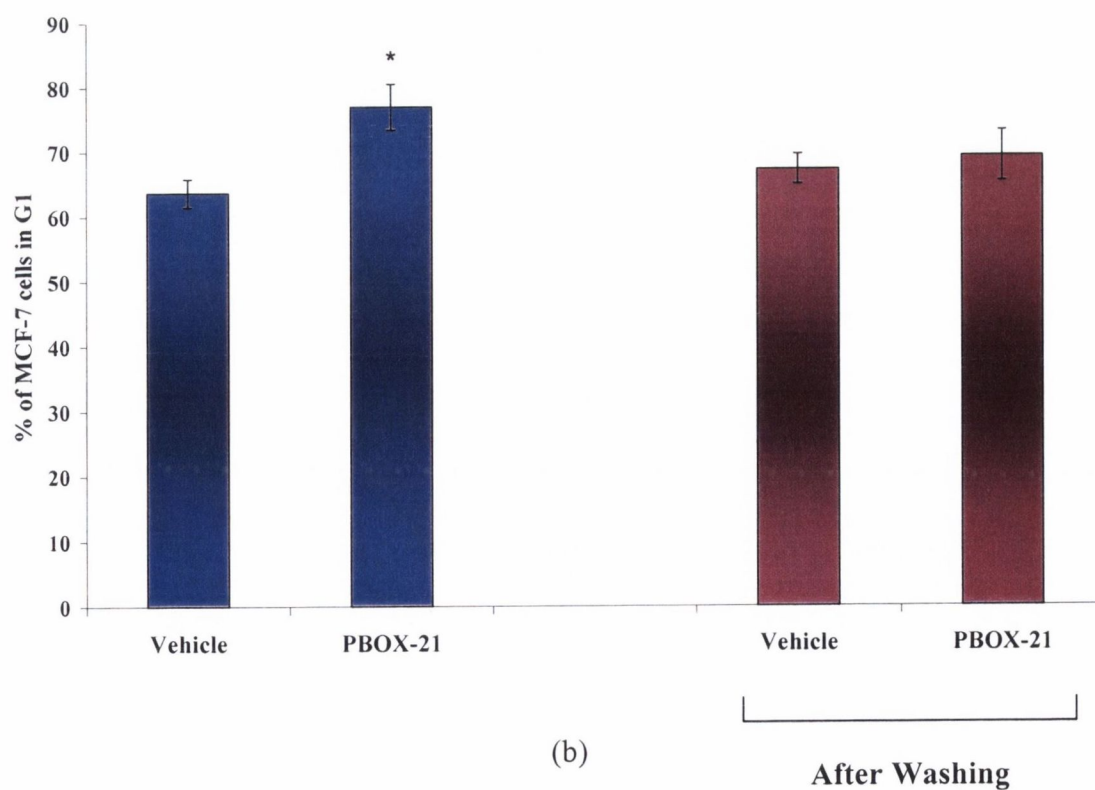
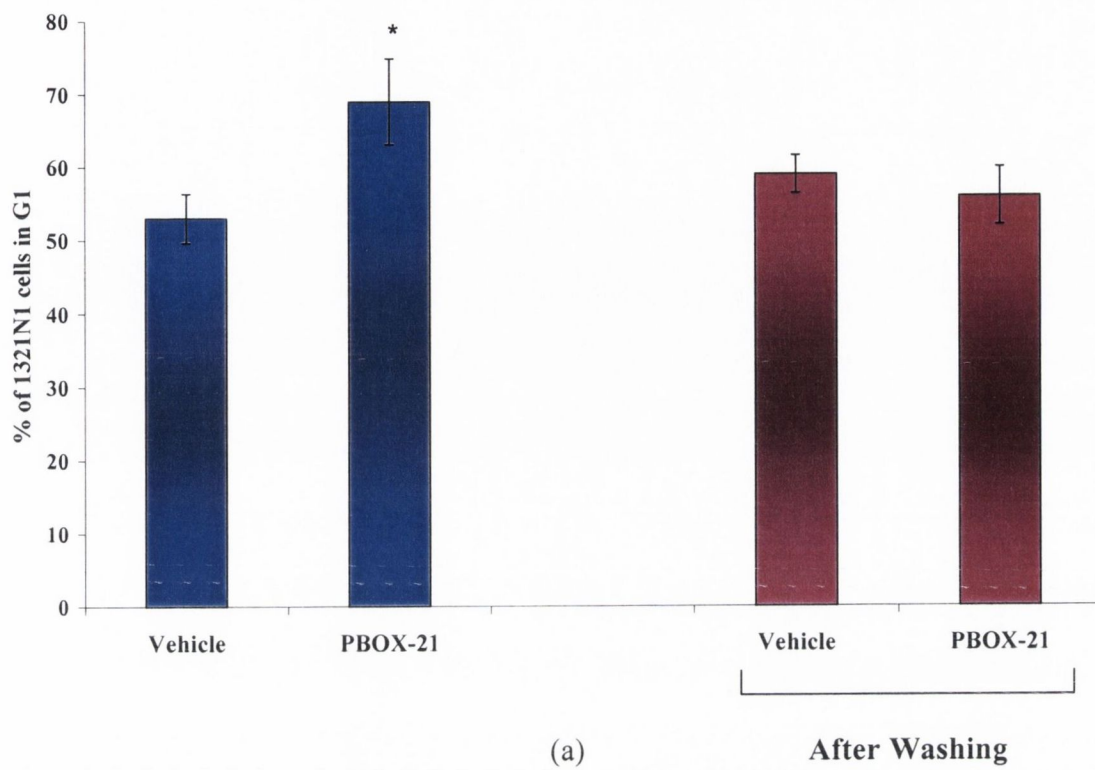
(a)



(b)

Figure 3.7 PBOX-21 induced G1 arrest in 1321N1 and MCF-7 cells is reversible

1321N1 cells (5×10^5) and MCF-7 cells (1×10^6) were seeded down in 25cm² flasks in 5mls of complete medium and were incubated at 37°C under a humidified atmosphere of 95% O₂ and 5% CO₂ until the cells were in the exponential phase of growth. The cells were then treated with either vehicle (0.5% (v/v) ethanol) or PBOX-21 (25µM) and left for 24 hours. The cells from one set of vehicle and PBOX-21 treated flasks were harvested as described previously (Section 2.1.1) and prepared and analysed using flow cytometry as described in Section 2.5. The medium in the remaining set of flasks was carefully removed and the cells washed in fresh medium. Another 5mls of fresh complete medium in the absence of PBOX-21 was added to the flasks and the cells were incubated for a further 24 hours. The cells were harvested, prepared and analysed using flow cytometry. The results represent the mean $\pm$ S.E.M of three separate experiments. Statistical analysis was carried out using the Instat computer program. * $p < 0.05$, ** < 0.01 with respect to vehicle treatment; Students t-test.



analysed using flow cytometry. The 24 hour treatment with PBOX-21 resulted in an increase of 16% of the 1321N1 cells (Figure 3.7 (a)) and 13% of MCF-7 cells in G1 relative to the vehicle control (Figure 3.7 (b)). Following removal of PBOX-21 and incubation in the drug-free medium for a further 24 hours, this G1 arrest was seen to have been reversed as the percentage of both cell lines within the G1 phase was comparable between the vehicle and treated samples. It would appear that incubation of the cells in drug-free medium for 24 h was sufficient for transition from the G1 block and allowed a return to a normal distribution of cells within the various phases of the cell cycle. This reversal of the G1 arrest indicates a specificity of action of PBOX-21 and confirms its lack of toxicity to the cells.

3.2.1.8 The mechanism of PBOX-21 induced cell cycle arrest does not appear to be mediated through the PBR

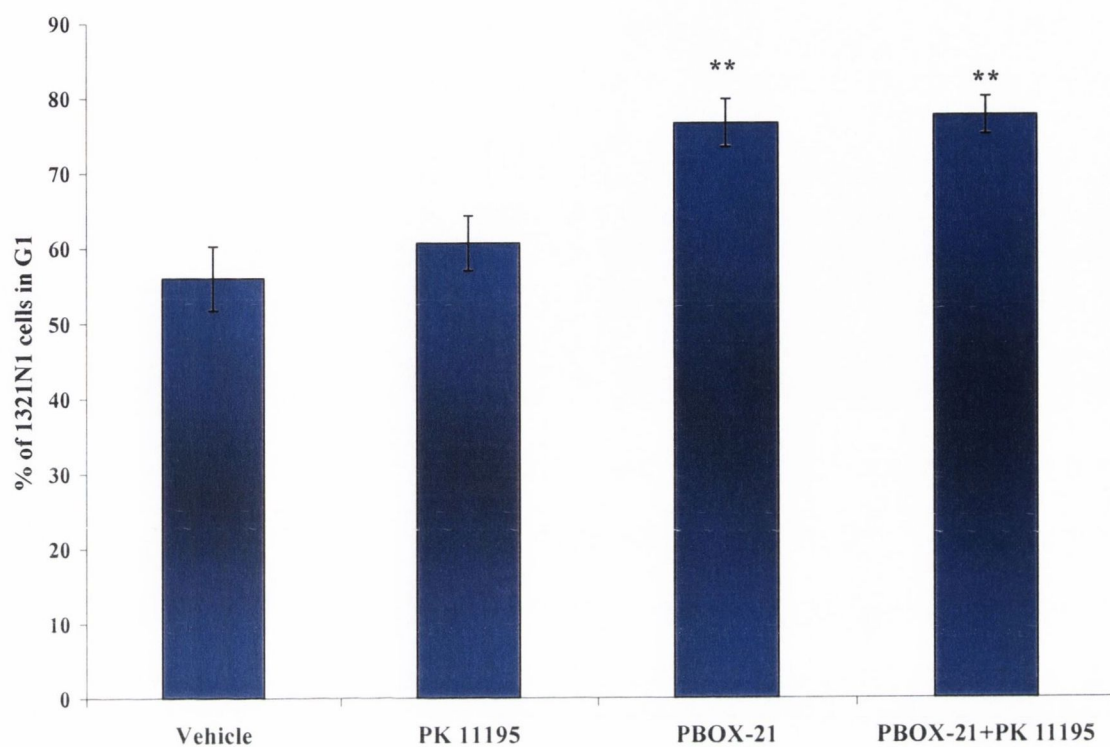
Zisterer *et al.*, (1998) suggested that the antiproliferative effects of PBOX-21 were not mediated by a specific interaction with the PBR. In order to confirm this, cells were pretreated for 1 hour with 10 μ M PK 11195, a well characterised ligand of the PBR. This concentration of PK 11195 would saturate the PBR as its K_d value in these cells is in the nanomolar range (Zisterer *et al.*, 1997), yet did not elicit a significant G1 arrest. After this time, the cells were then treated with 25 μ M PBOX-21 and incubated for a further 16 hours. The cells were harvested as described previously (Section 2.2.1) and prepared and analysed using flow cytometry as described in Section 2.2.4. It was found that saturating the PBR with PK 11195 did not prevent PBOX-21 induced G1 arrest in either the 1321N1 or MCF-7 cell lines (Figure 3.8). Therefore, it would appear that PBOX-21 does not mediate its antiproliferative effects through binding to the PBR.

3.2.2 The effect of PBOX-21 upon the CDK kinase activities

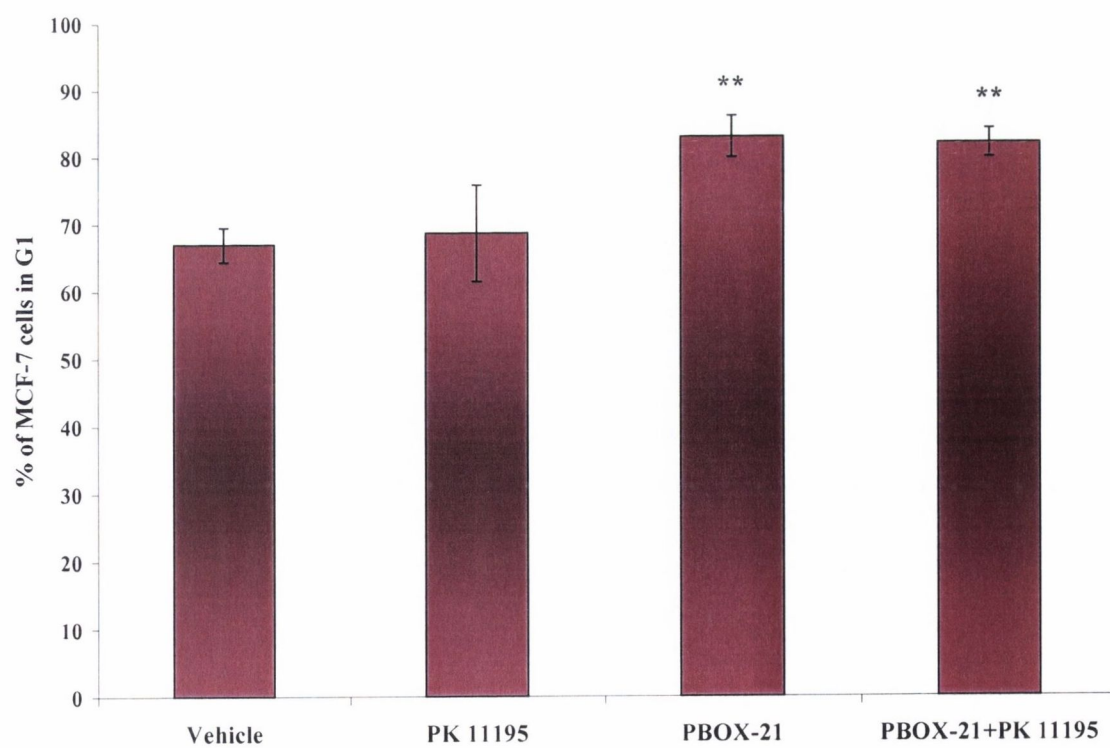
It was found that PBOX-21 mediates an antiproliferative effect via the induction of an arrest in the G1 phase of the cell cycle. This antiproliferative effect was not seen to be cell specific as PBOX-21 was capable of inducing a significant accumulation of cells in the G1 phase both in the human 1321N1 astrocytoma and MCF-7 breast carcinoma cell lines. However, this arrest was manifested much more rapidly within the 1321N1 cells compared to that within the MCF-7 cells, and as such the 1321N1 cells were chosen as the cell line with which to further characterise the mechanism underlying the PBOX-21 induced

Figure 3.8 PBOX-21 induced G1 arrest in the 1321N1 cells does not appear to be mediated through binding to the PBR

1321N1 cells (5×10^5) and MCF-7 cells (1×10^6) were seeded down in 25cm² flasks in 5mls of complete medium and were incubated at 37°C under a humidified atmosphere of 95% O₂ and 5% CO₂ until the cells were in the exponential phase of growth. The cells were then treated for 24 hours with either vehicle (0.5% (v/v) ethanol), PK 11195 (10µM), PBOX-21 (25µM) or PBOX-21 following one hour pretreatment with 10µM PK 11195. The cells were harvested, prepared and analysed using flow cytometry. The results represent the mean $\pm$ S.E.M of three separate experiments. Statistical analysis was carried out using the Instat computer program. * $p < 0.05$, ** < 0.01 with respect to vehicle treatment; Students t-test.



(a)



(b)

using an *in vitro* kinase assay to determine the activities of CDK2, CDK4 and CDK6.

3.2.2.1 PBOX-21 causes a reduction in CDK2, CDK4 and CDK6 kinase activity.

1321N1 cells were treated for the indicated times with PBOX-21 and total cell lysate was prepared as described (Section 2.2.6.1). The kinase of choice, was immunoprecipitated from the lysate as described (Section 2.2.6.2) and an *in vitro* kinase assay performed on the immunoprecipitate as described (Section 2.2.7).

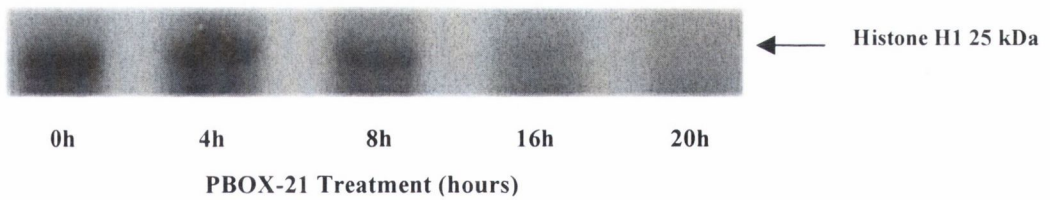
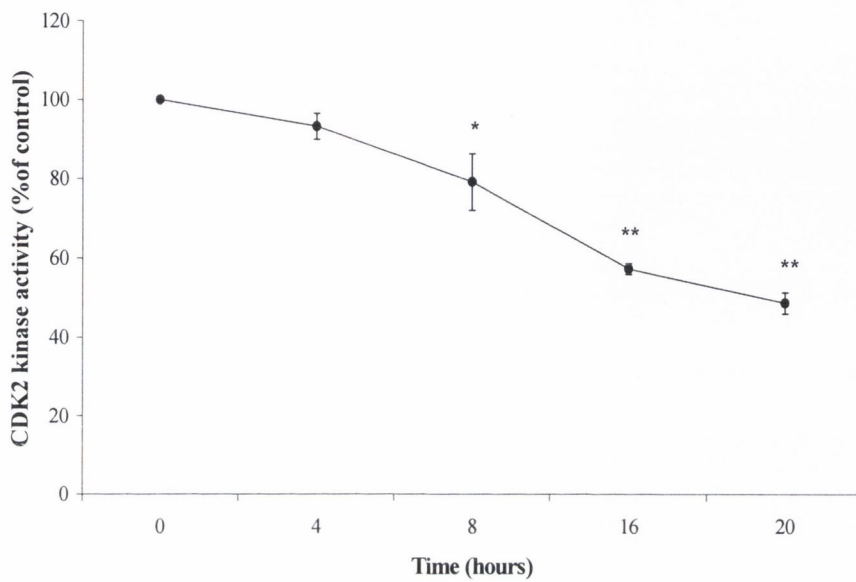
PBOX-21 treatment was found to cause a time-dependent reduction in the kinase activity of CDK2, CDK4 and CDK6 (Figure 3.9 (a), Figure 3.10 (a) and Figure 3.11 (a) respectively). CDK2 kinase activity was initially down regulated by approximately 20% after 8 hours of treatment, with the reduction of activity increasing up to 20 hours of treatment at which time the activity of CDK2 had been reduced by approximately 50%. As CDK2 kinase activity is reduced before any changes in cell cycle distribution could be detected, this suggests that this downregulation may be a cause of the G1 arrest. The level of CDK2 in the immunoprecipitates (Figure 3.9 (b)) was also seen to decline over time.

CDK4 activity declined later than that of CDK2, with a reduction in kinase activity of approximately 20% seen after 12 hours of treatment (Figure 3.10 (a)), which coincides with the onset of arrest. The reduction in the activity of CDK4, as with CDK2, increased over time, up to 48 hours at which time it had been reduced by approximately 60%. Again, as with CDK2, the level of CDK4 within the immunoprecipitates declined over time (Figure 3.10 (b)). As the reduction in CDK4 kinase activity was concomitant with the onset of arrest it was unlikely to be instrumental in its initiation but likely to be important to its maintenance.

A reduction in CDK6 activity did not become apparent until 20 hours with a reduction in activity of approximately 50% (Figure 3.11 (a)). The level of CDK6 within the immunoprecipitates was unchanged over time (Figure 3.11 (b)). As this decrease in CDK6 kinase activity is after the initial observation of G1

**Figure 3.9 PBOX-21 causes a time-dependent downregulation in CDK2
kinase activity**

Whole cell extracts were prepared from 1321N1 cells as described previously (Section 2.2.6.1) following treatment with PBOX-21 (25 μ M) for the indicated times. The protein (200-500 μ g) was immunoprecipitated with anti-CDK2 antibody and Protein A beads and then incubated with Histone H1 substrate and γ^{32} P-ATP and resolved by SDS-PAGE. Densitometry was performed on the resultant bands using Scion Image software with 0 hours set to 100%. The results shown are the mean $\pm$ S.E.M of three separate experiments. The result of a representative kinase assay has been included (a). A sample of the immunoprecipitated protein was also resolved using SDS-PAGE, transferred to a PVDF membrane and probed with anti-CDK2 antibody (b). Statistical analysis was carried out using the Instat computer program. * $p < 0.05$, ** < 0.01 with respect to 0 hours treatment; Students t-test.



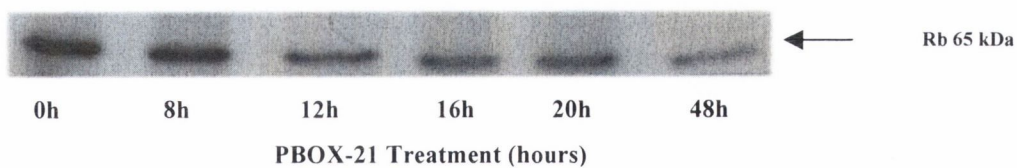
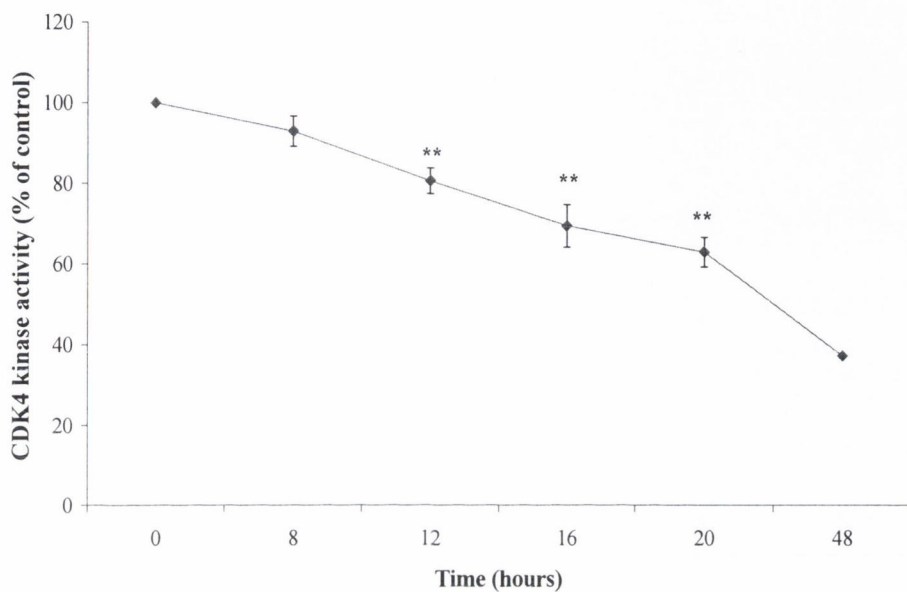
(a) CDK2 kinase activity



(b) Immunoprecipitated CDK2

Figure 3.10 PBOX-21 causes a time-dependent downregulation in CDK4 kinase activity

Whole cell extracts were prepared from 1321N1 cells as described previously (Section 2.2.6.1) following treatment with PBOX-21 (25 μ M) for the indicated times. The protein (200-500 μ g) was immunoprecipitated with anti-CDK4 antibody and Protein A beads, then incubated with Rb substrate and γ^{32} P-ATP and resolved by SDS-PAGE. Densitometry was performed on the resultant bands using Scion Image software with 0 hours set to 100%. The results shown are the mean $\pm$ S.E.M of three separate experiments. The result of a representative kinase assay has been included (a). A sample of the immunoprecipitated protein was also resolved using SDS-PAGE, transferred to a PVDF membrane and probed with anti-CDK4 antibody (b). Statistical analysis was carried out using the Instat computer program. * $p < 0.05$, ** < 0.01 with respect to 0 hours treatment; Students t-test.



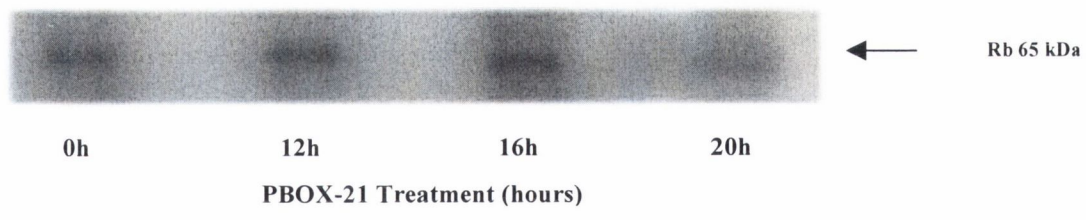
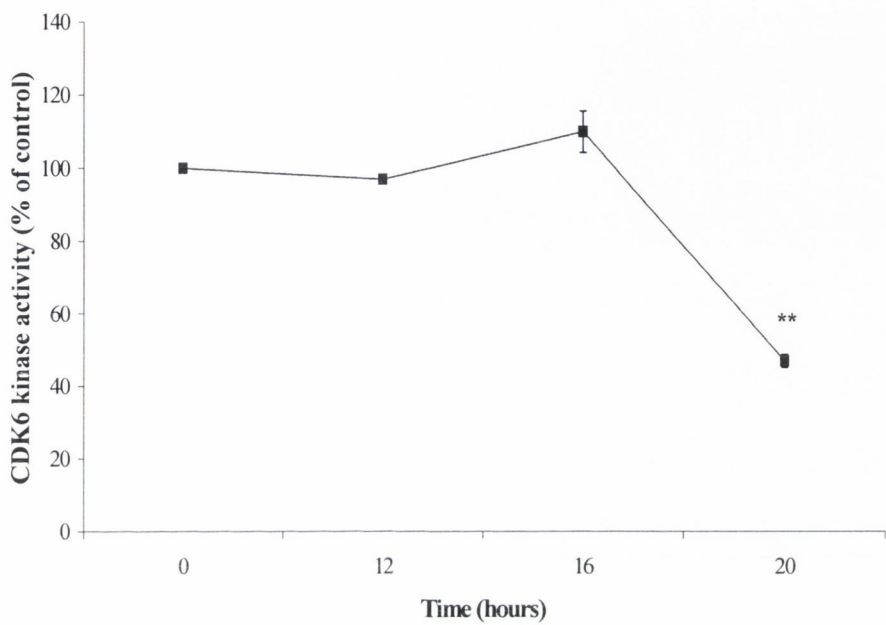
(a) CDK4 kinase activity



(b) Immunoprecipitated CDK4

Figure 3.11 PBOX-21 causes a time-dependent downregulation in CDK6 kinase activity

Whole cell extracts were prepared from 1321N1 cells as described previously (Section 2.2.6.1) following treatment with PBOX-21 (25 μ M) for the indicated times. The protein (200-500 μ g) was immunoprecipitated with anti-CDK6 antibody and Protein A beads, then incubated with Rb substrate and γ^{32} P-ATP and resolved by SDS-PAGE. Densitometry was performed on the resultant bands using Scion Image software with 0 hours set to 100%. The results shown are the mean $\pm$ S.E.M of three separate experiments. The result of a representative kinase assay has been included (a). A sample of the immunoprecipitated protein was also resolved using SDS-PAGE, transferred to a PVDF membrane and probed with anti-CDK6 antibody (b). Statistical analysis was carried out using the Instat computer program. * $p < 0.05$, ** < 0.01 with respect to 0 hours treatment; Students t-test.



(a) CDK6 kinase Activity



(b) Immunoprecipitated CDK6

arrest, it was possibly a consequence of the arrest and a mechanism of maintaining it.

3.2.3 The effect of PBOX-21 upon the level of expression of key cell cycle regulatory proteins

Cyclins assemble with CDKs to form holoenzymes that drive the cell through the cell cycle. The CDK does not become catalytically active until it binds its cyclin partner. Whilst the expression level of CDKs themselves is relatively stable throughout the cell cycle, cyclins have a periodic pattern of expression, thus earning themselves their name. Thus the both the expression of the CDK itself and the expression of its cyclin partner serves to regulate the activity of the cyclin/CDK complexes. Therefore, the expression levels of the G1 CDKs (CDK2, CDK4 and CDK6) as well as the G1 cyclins, i.e. the D-type cyclins (cyclin D₁, D₂ and D₃) and cyclin E, in response to PBOX-21 treatment were examined so as to further elucidate the molecular mechanism underlying the cytostatic effects of PBOX-21.

3.2.3.1 PBOX-21 causes a downregulation in the expression levels of CDK2 and CDK4 but has no effect upon the expression levels of CDK6 in 1321N1 cells

It was found that treatment with PBOX-21 up to 48 hours resulted in a decrease in protein expression levels of CDK2 and CDK4 (Figure 3.12 and Figure 3.13 respectively). The levels of CDK2 expression began to decline following 8 hours of treatment with PBOX-21 whilst CDK4 expression was first affected after 12 hours. This pattern in downregulation of both CDK2 and CDK4 kinase activity parallels, and thus may be attributable to, the reduction in their expression following PBOX-21 treatment. This reduction in expression of CDK2 and CDK4 also agrees with the observed decline in protein precipitated by the CDK2 and CDK4 antibodies over the duration of PBOX-21 treatment (Figure 3.9 (b) and 3.10 (b) respectively).

In contrast, treatment with PBOX-21 did not result in a change in expression levels of CDK6 in the 1321N1 cells relative to the vehicle control (0.5% (v/v) ethanol) treated cells (Figures 3.14) suggesting that the observed

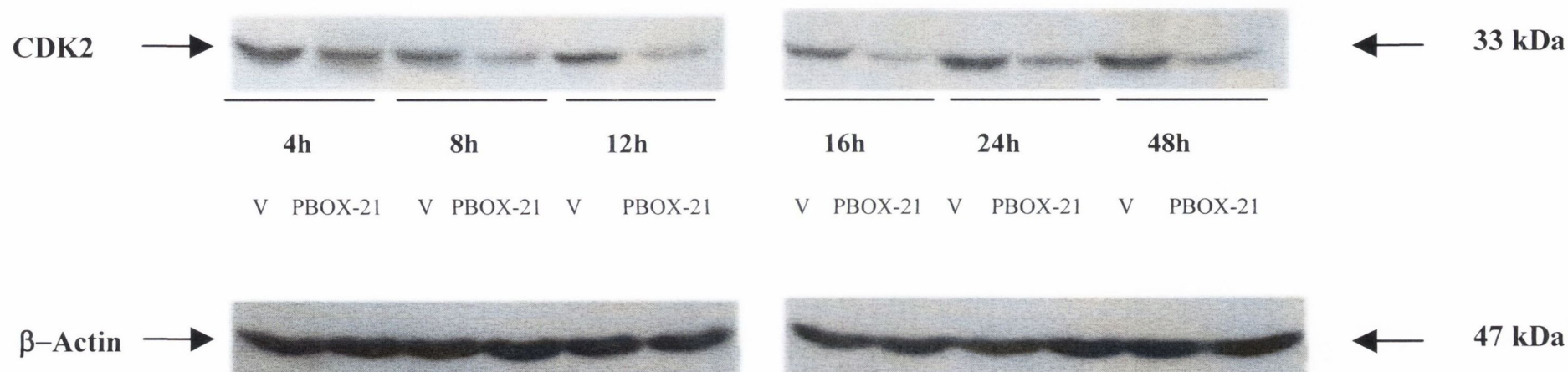


Figure 3.12 PBOX-21 causes a downregulation in the expression levels of CDK2 in 1321N1 cells

Whole cell extracts were prepared from 1321N1 cells as described previously (Section 2.2.5.1) from cells following treatment with either vehicle (0.5% (v/v) ethanol, V) or PBOX-21 (25 μ M) for 4, 8, 12, 16, 24 and 48 hours. The protein (70 μ g) was resolved using SDS-PAGE, transferred onto PVDF and probed with anti-CDK2 antibody. The blots were stripped and re-probed with anti- β -actin antibody. The results shown are representative of 3 separate experiments.

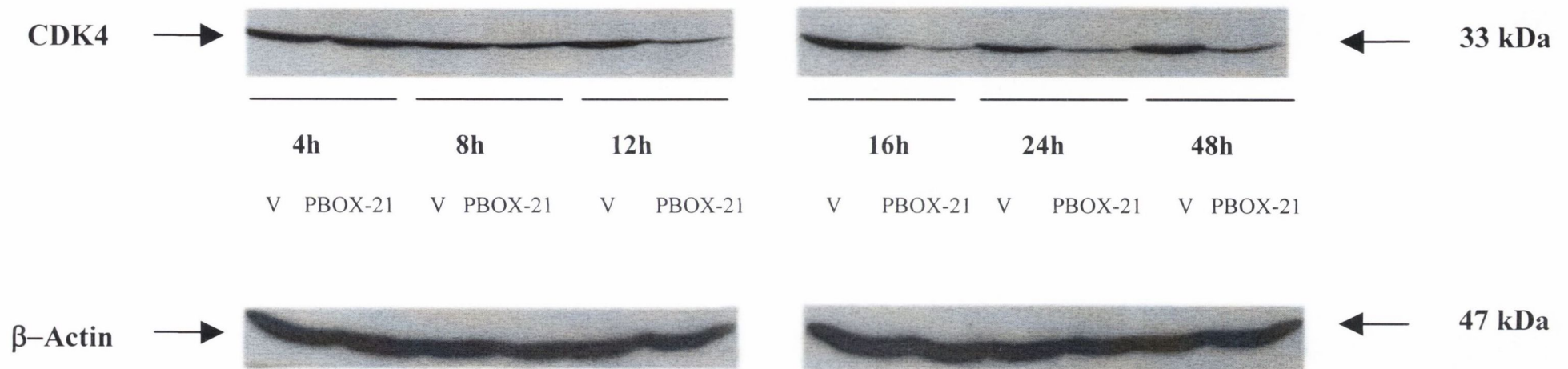


Figure 3.13 PBOX-21 causes a downregulation in the expression levels of CDK4 in 1321N1 cells

Whole cell extracts were prepared from 1321N1 cells as described previously (Section 2.2.5.1) from cells following treatment with either vehicle (0.5% (v/v) ethanol, V) or PBOX-21 (25μM) for 4, 8, 12, 16, 24 and 48 hours. The protein (70μg) was resolved using SDS-PAGE, transferred onto PVDF and probed with anti-CDK4 antibody. The blots were stripped and re-probed with anti-β-actin antibody. The results shown are representative of 3 separate experiments.

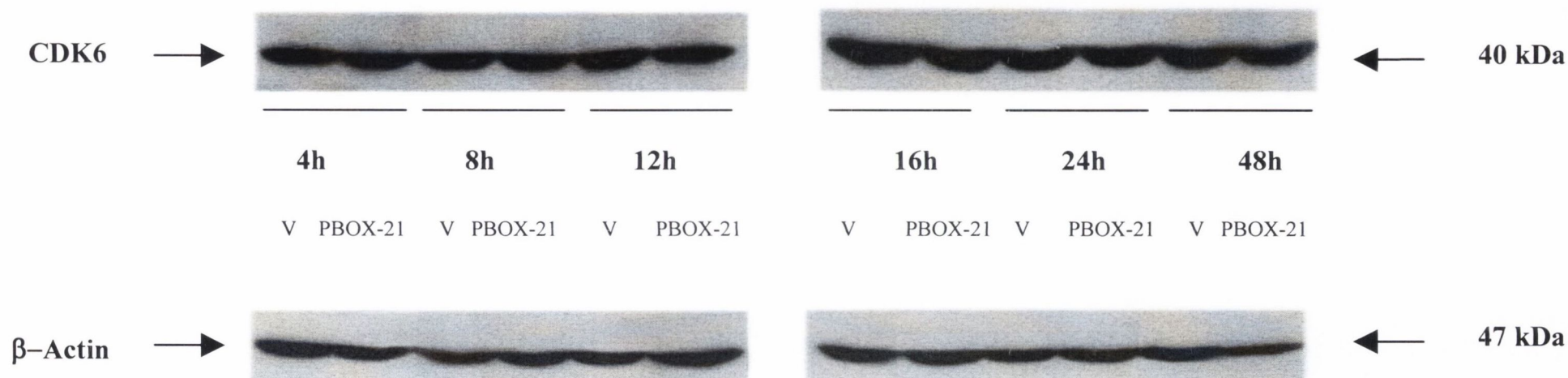


Figure 3.14 PBOX-21 has no effect upon the expression levels of CDK6 in 1321N1 cells

Whole cell extracts were prepared from 1321N1 cells as described previously (Section 2.2.5.1) from cells following treatment with either vehicle (0.5% (v/v) ethanol, V) or PBOX-21 (25 μ M) for 4, 8, 12, 16, 24 and 48 hours. The protein (70 μ g) was resolved using SDS-PAGE, transferred onto PVDF and probed with anti-CDK6 antibody. The blots were stripped and re-probed with anti- β -actin antibody. The results shown are representative of 3 separate experiments.

reduction in CDK6 kinase activity was not a result of a decrease in the CDK6 protein level.

3.2.3.2 Lack of expression of Cyclin D₁ in 1321N1 cells

Cell lysates (70µg) from 1321N1 and MCF-7 cells (as a positive control) were prepared as described in Section 2.2.5.1. Western blot analysis with a monoclonal antibody directed against cyclin D₁ demonstrated the absence of this cyclin in 1321N1 cells (Figure 3.15, Lane A). MCF-7 cells, however, in accordance with previous reports (Skildum *et al.*, 2002), were shown to express cyclin D₁ (Figure 3.15, Lane B), demonstrating the sensitivity and selectivity of the antibody.

3.2.3.3 PBOX-21 causes a downregulation in the expression levels of cyclin D₂ in 1321N1 cells

PBOX-21 was found to decrease the levels of cyclin D₂ relative to the vehicle control (0.5% (v/v) ethanol) treated cells (Figure 3.16). An induction of G1 arrest by PBOX-21 was first observed around 12 hours. No change in expression of cyclin D₂ protein was observed prior to this time, suggesting that cyclin D₂ is not responsible for initiating growth inhibition. Subsequent to this PBOX-21 induced growth inhibition was associated with a downregulation in cyclin D₂ protein levels at both 24 and 48 hours. As this was following the onset of G1 arrest, it was likely to be a secondary response to the cessation of cell cycle progression.

3.2.3.4 PBOX-21 causes a downregulation in the expression levels of cyclin D₃ in 1321N1 cells

Total cell lysates were prepared as described in section 2.2.5.1 and subjected to western blot analysis using a monoclonal antibody directed against cyclin D₃. It was found that following treatment with PBOX-21, a downregulation occurs in the expression levels of cyclin D₃ relative to the vehicle control (0.5% (v/v) ethanol) treated cells (Figure 3.17). This reduction in cyclin D₃ expression was first visible after 8 hours of treatment with PBOX-21 and this downregulation in expression was maintained up to 16 hours of treatment. After this time the levels of expression of cyclin D₃ within the untreated 1321N1 cells

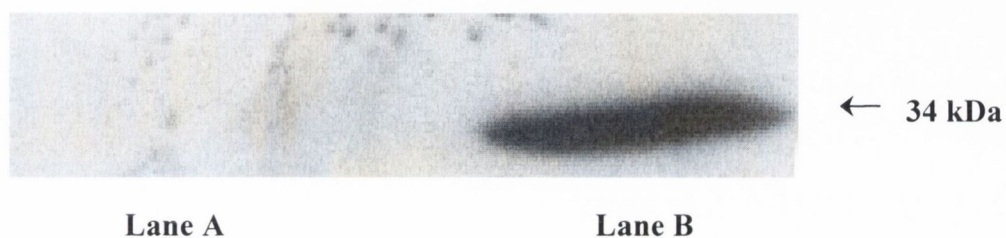


Figure 3.15 Lack of cyclin D₁ expression in the 1321N1 cell line

Whole cell extracts from 1321N1 cells (Lane A) and MCF-7 cells (Lane B) were prepared as described previously (Section 2.2.5.1). The protein (70µg) was resolved using SDS-PAGE, transferred onto PVDF and probed with anti-cyclin D₁ antibody. The results shown are representative of 2 experiments.

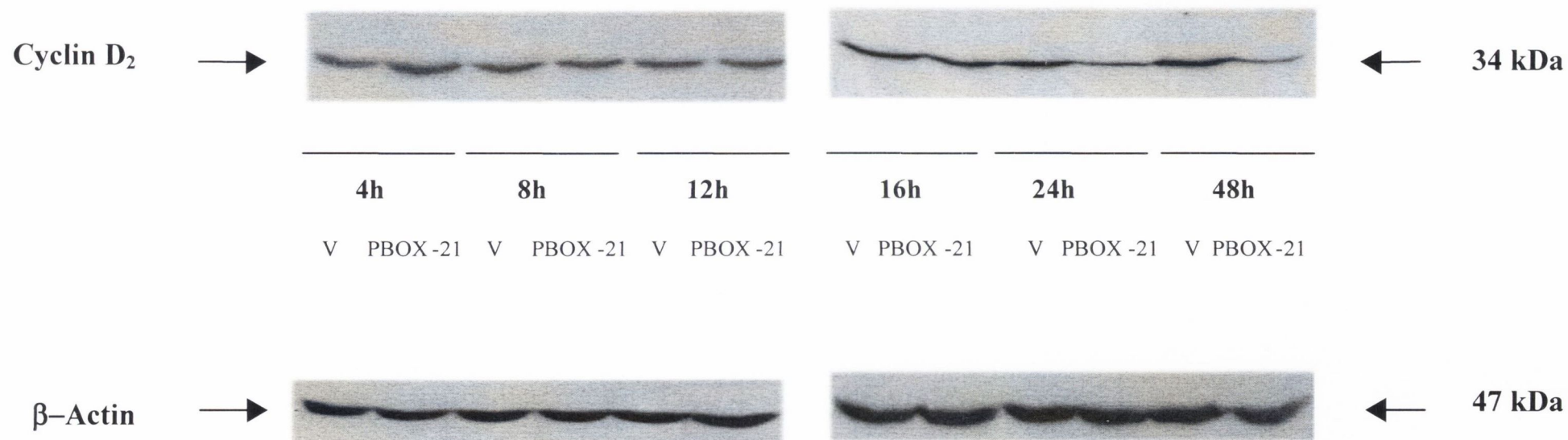


Figure 3.16 PBOX-21 causes a downregulation in the expression levels of cyclin D₂ in 1321N1 cells

Whole cell extracts were prepared from 1321N1 cells as described previously (Section 2.2.5.1) from cells following treatment with either vehicle (0.5% (v/v) ethanol, V) or PBOX-21 (25μM) for 4, 8, 12, 16, 24 and 48 hours. The protein (70μg) was resolved using SDS-PAGE, transferred onto PVDF and probed with anti-cyclin D₂ antibody. The blots were stripped and re-probed with anti-β-actin antibody. The results shown are representative of 3 separate experiments.

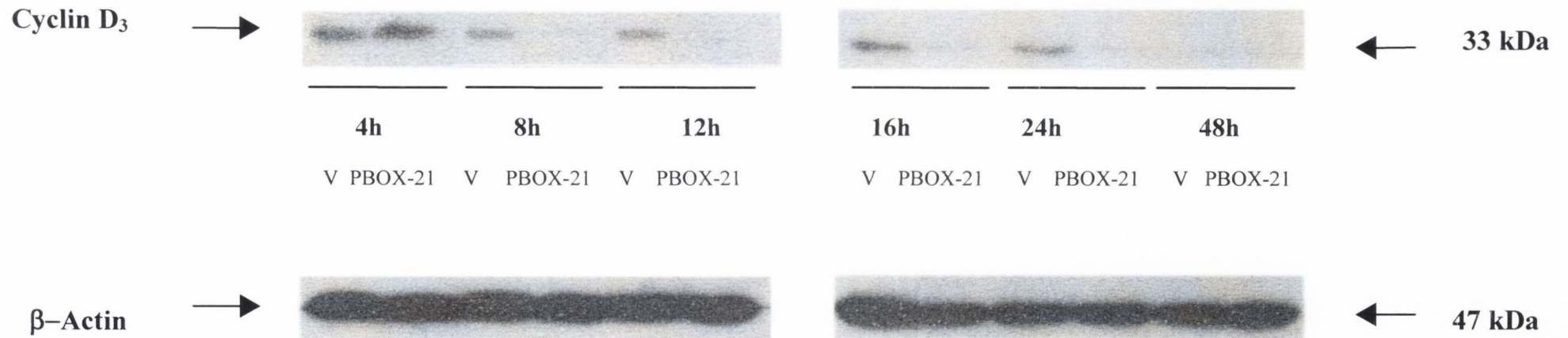


Figure 3.17 PBOX-21 causes a downregulation of cyclin D₃ in 1321N1 cells

Whole cell extracts were prepared from 1321N1 cells as described previously (Section 2.2.5.1) from cells following treatment with either vehicle (0.5% (v/v) ethanol, V) or PBOX-21 (25μM) for 4, 8, 12, 16, 24 and 48 hours. The protein (70μg) was resolved using SDS-PAGE, transferred onto PVDF and probed with anti-cyclin D₃ antibody. The blots were stripped and re-probed with anti-β-actin antibody. The results shown are representative of 3 separate experiments.

began to decline with no cyclin D₃ expression visible at 48 hours. This is possibly due to a depletion in growth factors within the growth medium. PBOX-21 induced G1 arrest was first observed after 12 hours of treatment.

As the downregulation in cyclin D₃ expression began at 8 hours which preceded the observed induction of G1 arrest first seen at 12 hours, it is possible that this reduction in cyclin D₃ could be at least partially responsible for the G1 arrest of the 1321N1 cells. Cyclin D₃ is considered to typically associate with either CDK4 and/or CDK6. Therefore, the observed reduction in cyclin D₃ may be a contributory factor to PBOX-21 downregulation of CDK4 and CDK6 kinase activity.

3.2.3.5 PBOX-21 reduces cyclin D₃-CDK4 and cyclin D₃-CDK6 complex formation

As CDK4 and CDK6 are known to partner the D-type cyclins, and as PBOX-21 has been shown to cause a downregulation in the expression levels of cyclin D₃, we were interested to see if there was an observed reduction in the association between both kinases and cyclin D₃ following treatment with PBOX-21.

CDK4 and CDK6 were immunoprecipitated from 1321N1 cell lysates as described previously (Section 2.2.6.2), following 20 hours of treatment with PBOX-21 as there was a reduction in both CDK4 and CDK6 kinase activities at this time point (Figure 3.10 and 3.11 respectively). The resultant immunoprecipitated protein was resolved by SDS-PAGE and immunoblotted for cyclin D₃. It was found that after 20 hours of treatment with PBOX-21, there was a reduction in the levels of CDK4 and CDK6 bound-cyclin D₃ relative to the vehicle control treated 1321N1 cells (Figures 3.18 and 3.19 respectively). Therefore, PBOX-21 causes a downregulation in the complex formation between CDK4 and CDK6 with cyclin D₃. This was to be expected in the case of the association between CDK4 and cyclin D₃, as PBOX-21 caused a downregulation in the expression of CDK4 and thus results in a reduction in the complex formation with cyclin D₃ relative to the control. PBOX-21 does not, however, have any effect upon the expression of CDK6, and thus the reduction in complex formation with cyclin D₃ is likely to be due entirely to the downregulation of cyclin D₃ expression by PBOX-21. This may explain the reduction in CDK6

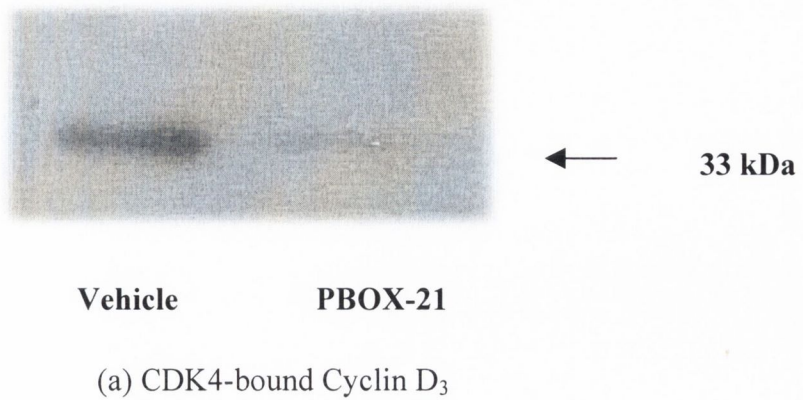


Figure 3.18 PBOX-21 causes a reduction in Cyclin D₃-CDK4 complex formation

Whole cell extracts were prepared as described previously (Section 2.2.6.1) from 1321N1 cells following treatment with either vehicle (0.5% (v/v) ethanol) or PBOX-21 (25 μ M) for 20 hours. The protein (200-500 μ g) was incubated with anti-CDK4 antibody and then incubated with Protein A beads. The immunoprecipitated protein was resolved using SDS-PAGE, transferred to a PVDF membrane and probed with anti-cyclin D₃ antibody (a) and anti-CDK4 antibody (b). The results shown are representative of 2 separate experiments.

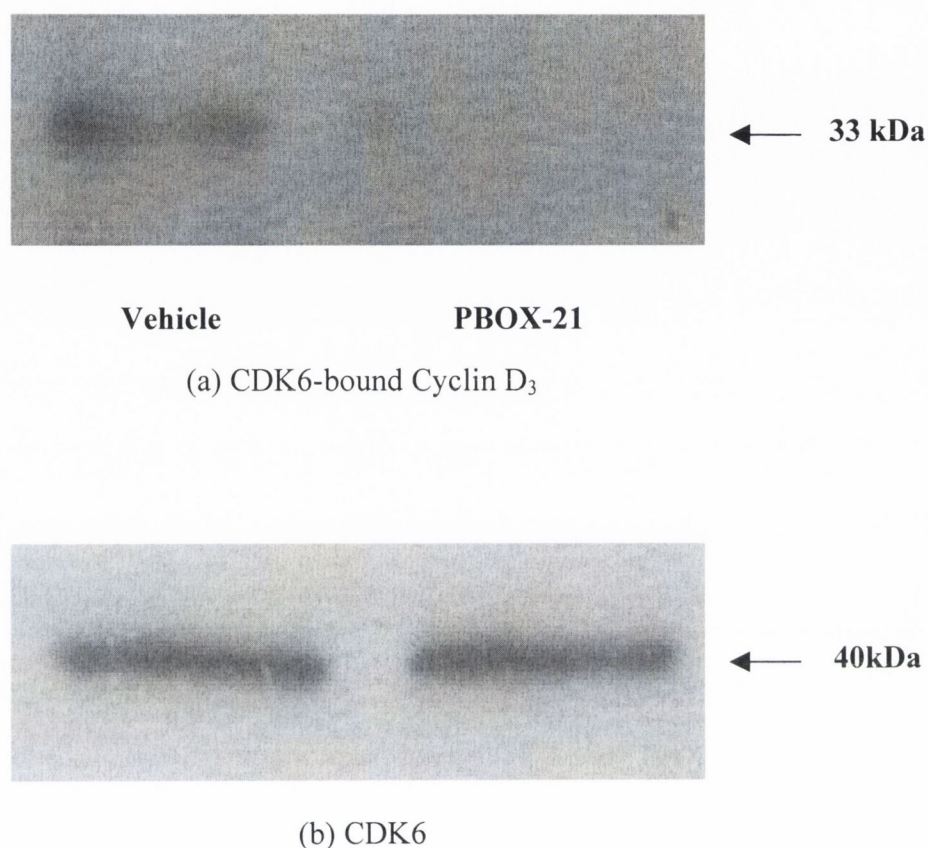


Figure 3.19 PBOX-21 causes a reduction in Cyclin D₃-CDK6 complex formation

Whole cell extracts were prepared as described previously (Section 2.2.6.1) from 1321N1 cells following treatment with either vehicle (0.5% (v/v) ethanol) or PBOX-21 (25 μ M) for 20 hours. The protein (200-500 μ g) was incubated with anti-CDK6 antibody and then incubated with Protein A beads. The immunoprecipitated protein was resolved using SDS-PAGE, transferred to a PVDF membrane and probed with anti-cyclin D₃ antibody (a) and anti-CDK6 antibody (b). The results shown are representative of 2 separate experiments.

kinase activity observed at 20 hours of PBOX-21 treatment.

3.2.3.6 PBOX-21 has no effect upon the expression levels of cyclin E in 1321N1 cells

It was found that treatment with PBOX-21 had no effect upon the expression levels of cyclin E over the time course examined. This suggests that cyclin E is not responsible for mediating the PBOX-21 induced growth arrest of the 1321N1 cells (Figure 3.20). Any change in the activity of the cyclin E CDK partner, CDK2, is therefore not regulated by the expression level of cyclin E.

3.2.3.7 Lack of expression of p16 in 1321N1 cells

In order to investigate other possible causes of the induction of G1 arrest, the expression of some CDK inhibitors was examined by Western blot analysis. The first inhibitor to be analysed was p16, the founder member of the INK4 family, which specifically inhibit cyclin D-associated kinases (Fahraeus *et al.*, 1996).

Cell lysates (70µg) from 1321N1 and SAOS-2 cells (as a positive control) were prepared as described in section 2.2.5.1). Western blot analysis with a monoclonal antibody directed against p16 demonstrated the absence of this CDK inhibitor in 1321N1 cells (Figure 3.21, Lane A). SAOS-2 cells, however, in accordance with previous reports (Cheng *et al.*, 2000), were shown to express p16 (Figure 3.21, Lane B), demonstrating the sensitivity and selectivity of the antibody.

3.2.3.8 PBOX-21 causes an upregulation in the levels of expression of p21 in 1321N1 cells

The second CDK inhibitor to be examined was p21, which is the founder member of the CIP/KIP family of CDK inhibitors. These inhibitors are capable of inhibiting all CDKs, earning them the title of the ‘universal inhibitors’. They, however, preferentially inhibit G1 CDKs (McDonald *et al.*, 2001). It was found that treatment with PBOX-21 caused an upregulation of expression levels of p21 relative to the vehicle control (0.5% (v/v) ethanol) treated 1321N1 cells (Figure 3.22). An initial and maximal induction in expression of p21 was seen after 16 hours of treatment with PBOX-21. The induction in expression of p21 began to decline after 16 hours and up to 24 hours of treatment with PBOX-21.

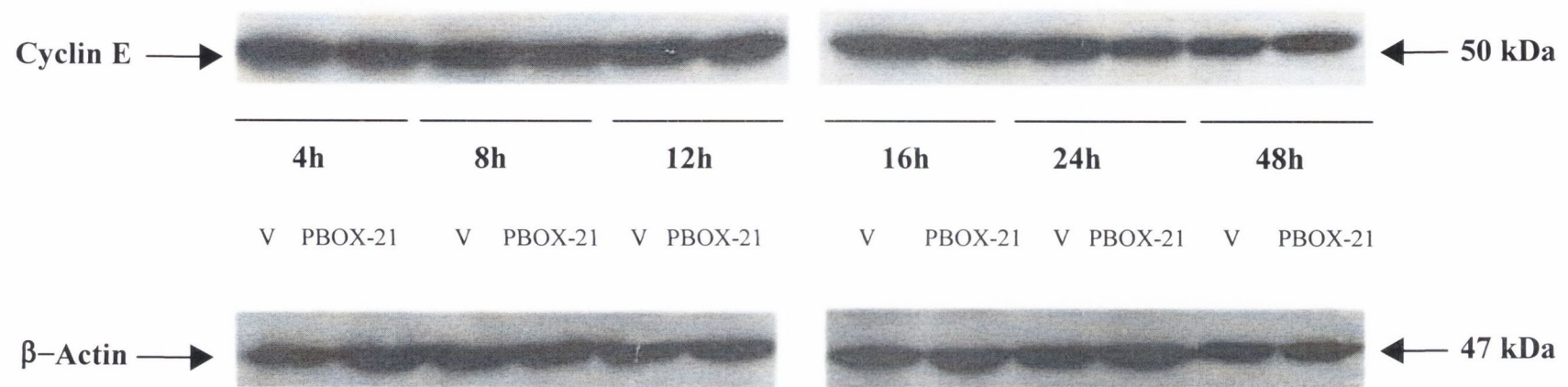


Figure 3.20 PBOX-21 has no effect upon the expression levels of cyclin E in 1321N1 cells

Whole cell extracts were prepared from 1321N1 cells as described previously (Section 2.2.5.1) from cells following treatment with either vehicle (0.5% (v/v) ethanol, V) or PBOX-21 (25 μ M) for 4, 8, 12, 16, 24 and 48 hours. The protein (70 μ g) was resolved using SDS-PAGE, transferred onto PVDF and probed with anti-cyclin E antibody. The blots were stripped and re-probed with anti- β -actin antibody. The results shown are representative of 3 separate experiments.

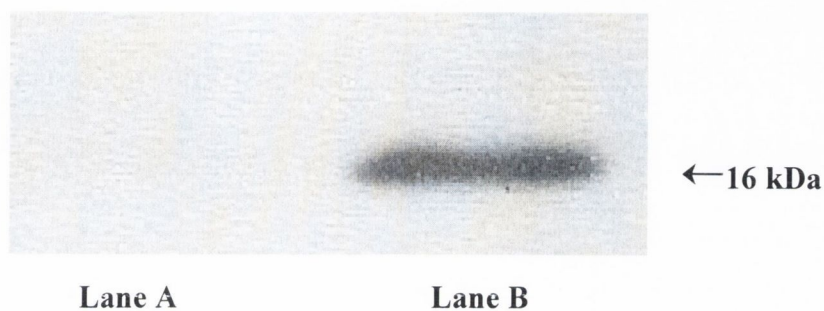


Figure 3.21 Lack of expression of p16 in the 1321N1 cells

Whole cell extracts from 1321N1 cells (Lane A) and SAOS-2 (Lane B) were prepared as described previously (Section 2.2.5.1). The protein (70 μ g) was resolved using SDS-PAGE, transferred onto PVDF and probed with anti-p16 antibody. The results shown are representative of 2 experiments.

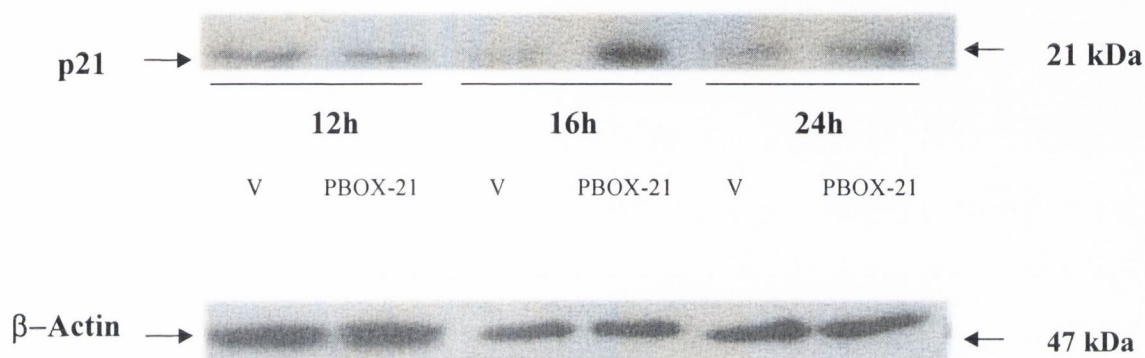


Figure 3.22 PBOX-21 causes an upregulation in the levels of expression of p21 in 1321N1 cells

Whole cell extracts were prepared from 1321N1 cells as described previously (Section 2.2.5.1) from cells following treatment with either vehicle (0.5% (v/v) ethanol, V) or PBOX-21 (25μM) for 12, 16, and 24 hours. The protein (70μg) was resolved using SDS-PAGE, transferred onto PVDF and probed with anti-p21 antibody. The blots were stripped and re-probed with anti-β-actin antibody. The results shown are representative of 2 separate experiments.

This upregulation in p21 expression was seen after the downregulation of CDK2 and CDK4 kinase activity, and following the observed induction of G1 arrest. This would suggest that p21 played no part in the early inhibition of these kinases, but may be involved in maintaining their downregulated activity from 16 hours onwards. The timing of the upregulation in p21 does suggest however, that it may play a part in the observed inhibition of CDK6 kinase activity following PBOX-21 treatment

3.2.3.9 PBOX-21 has no effect upon the expression levels of p53 within 1321N1 cells

p21 expression is known to be under the control of both p53-dependent and p53-independent mechanisms. To examine whether p53 protein expression levels contribute to PBOX-21 mediated induction of p21, the protein levels of p53 were analysed following treatment of 1321N1 cells with PBOX-21. The basal levels of p53 expression within the 1321N1 cells did not change upon treatment with PBOX-21 (Figure 3.23). This result may suggest that PBOX-21 induced p21 independent of p53, but further studies are required to determine fully whether PBOX-21 induced p21 expression is independent or dependent upon p53.

3.2.3.10 PBOX-21 has no effect upon the expression levels of CDC25a within the 1321N1 cells

Tyrosine phosphorylation as well as the binding of various CDK inhibitors can negatively regulate the activity of the CDKs and their subsequent control over cell growth and division. Dephosphorylation of these residues is therefore necessary to achieve maximal kinase activity and cell progression. The molecule responsible for these dephosphorylations during G1 is the phosphatase, CDC25a. It was found that the expression levels of CDC25a did not change following treatment with PBOX-21 (Figure 3.24) suggesting that the observed reduction in CDK2, CDK4 and CDK6 kinase activity was induced by a mechanism that does not employ repression of CDC25a.

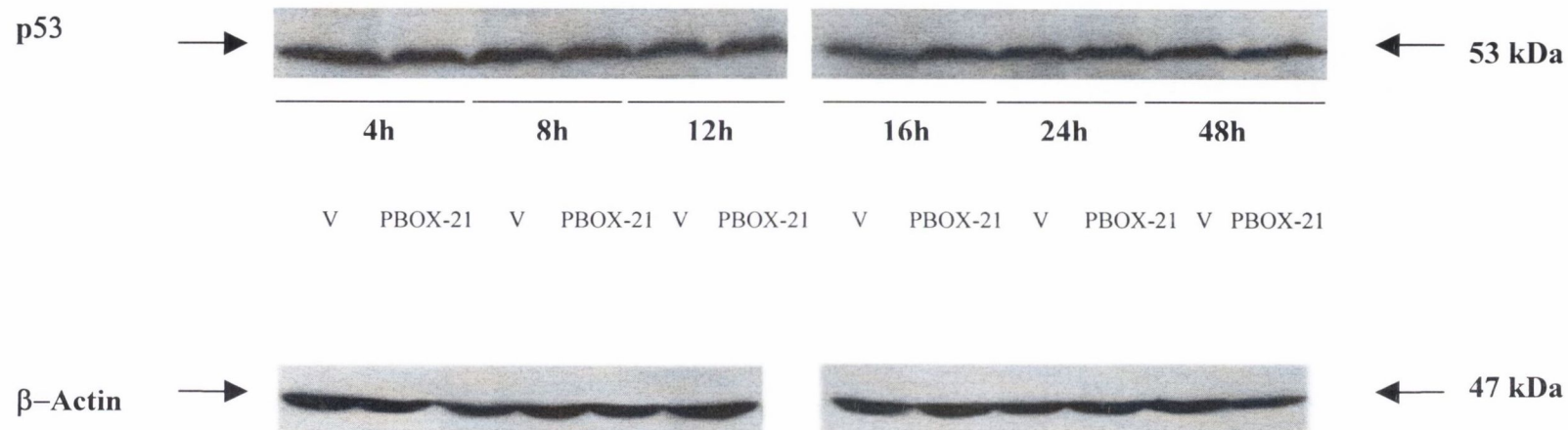


Figure 3.23 PBOX-21 has no effect upon the expression levels of p53 in 1321N1 cells

Whole cell extracts were prepared from 1321N1 cells as described previously (Section 2.2.5.1) from cells following treatment with either vehicle (0.5% (v/v) ethanol, V) or PBOX-21 (25 μ M) for 4, 8, 12, 16, 24 and 48 hours. The protein (70 μ g) was resolved using SDS-PAGE, transferred onto PVDF and probed with anti-p53 antibody. The blots were stripped and re-probed with anti- β -actin antibody. The results shown are representative of 2 separate experiments.

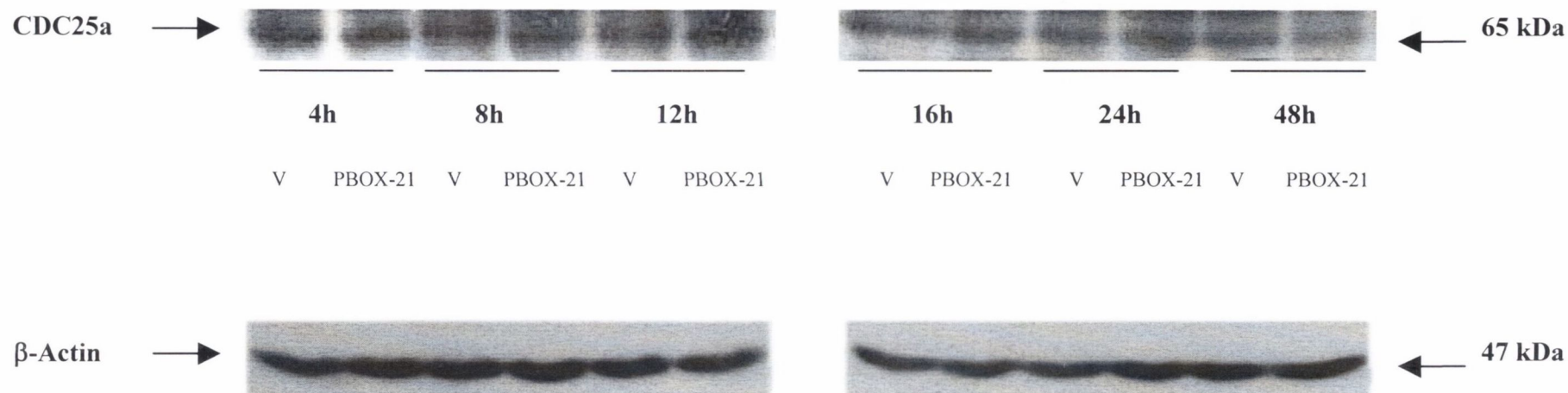


Figure 3.24 PBOX-21 has no effect upon the expression levels of CDC25a in 1321N1 cells

Whole cell extracts were prepared from 1321N1 cells as described previously (Section 2.2.5.1) following treatment with either vehicle (0.5% (v/v) ethanol) or PBOX 21 (25 μ M) after 4, 8, 12, 16, 24 and 48 hours. The protein (70 μ g) was resolved using SDS-PAGE, transferred onto PVDF and probed with anti-CDC25a antibody. The blots were stripped and re-probed with anti- β -actin anti-body. The results shown are representative of 2 experiments.

3.2.4 The effect of PBOX-21 upon the phosphorylation status of Rb

It has been shown that treatment with PBOX-21 resulted in a reduction in the kinase activities of CDK2, CDK4 and CDK6. As the phosphorylation status of the retinoblastoma protein (Rb) is mediated by the cyclin-CDK complexes, it was necessary to determine whether Rb became hypophosphorylated as a result of the reduced kinase activities of CDK2, CDK4 and CDK6. At 0 hours, pRb is mostly present in its slower migrating hyperphosphorylated form. After treatment with PBOX-21 for 24 and 48 hours a clear mobility shift was observed in the Rb protein from the hyperphosphorylated to the hypophosphorylated form which appears as a faster migrating single band (Figure 3.25). Rb is a negative regulator of the cell cycle that acts in the G1 phase and its activity is modulated by its phosphorylation status. These results would indicate that PBOX-21 mediated growth inhibition is associated with suppression of phosphorylation of Rb retaining it in a hypophosphorylated growth-inhibitory state.

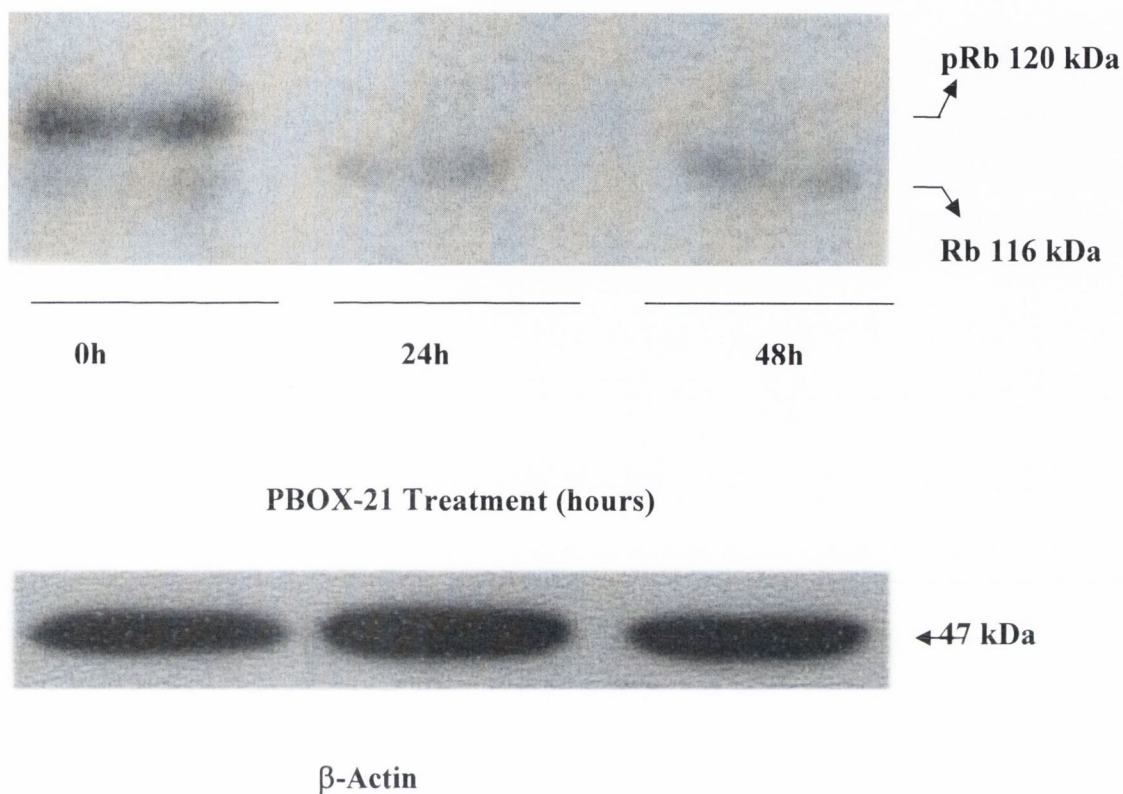


Figure 3.25 PBOX-21 causes hypophosphorylation of Rb

Whole cell extracts were prepared from 1321N1 cells as described previously (Section 2.2.5.1) following treatment with PBOX-21 (25 μ M) for the indicated times. The protein (70 μ g) was resolved using a gradient SDS-PAGE gel, transferred onto PVDF and probed with anti-pRb antibody. The blots were stripped and re-probed with anti- β -actin antibody. The results shown are representative of 2 separate experiments.

3.3 Discussion

This study demonstrates the potent growth inhibitory effects of some PBOX compounds upon both the human 1321N1 astrocytoma and MCF-7 breast carcinoma cell lines. Compounds that exert such inhibitory effects upon cancerous cell growth are candidates for therapeutic use in the treatment of cancer. The validity of any new therapy is more easily established if a sound mechanistic basis can be shown. As such, it was of interest to elucidate the molecular mechanisms underlying these cytostatic effects.

The growth inhibitory effects induced by a representative cytostatic PBOX compound, PBOX-21, was mediated through the induction of cell cycle arrest in the G1 phase, and was not associated with cytotoxic effects as was demonstrated both by LDH release from the cells treated with PBOX-21, and the reversibility of the arrest once the drug was washed away. This indicates a specificity of action of PBOX-21. The induction of G1 arrest is at the expense of cells in both the S and G2/M with cells being drawn equally from both phases into G1.

The PBR does not appear to be involved in the mechanism by which PBOX-21 induced these cytostatic effects. Saturation of the PBR binding sites with the specific PBR ligand, PK 11195, prior to treatment of the cells with PBOX-21 did not prevent the arrest. This conclusion is in agreement with previous reports of a study of C6 glioma (Gorman *et al.*, 1989) and a Chinese hamster lung cell line V79 (Camins *et al.*, 1995).

In order to study the molecular pathway by which PBOX-21 mediates its antiproliferative action upon the 1321N1 cell line, a complete analysis of the cell cycle events in these cells both prior to and consequent of the initial observed G1 arrest at twelve hours was performed. Thus, it was possible to distinguish between induced changes in cell cycle regulating molecules, which are possibly responsible for the growth arrest, from those that are as a result of the cell cycle arrest itself. In so doing, it was found that the activities of CDK2, CDK4 and CDK6 were down-regulated sequentially. CDK2 kinase activity was the first to be affected with a decrease seen as early as 8 hours with CDK4 and CDK6 kinase activities reduced following 12 and 20 hours of treatment with PBOX-21 respectively. These results would suggest that the reduction in CDK2 and CDK4

activity is a mechanism of braking the cell cycle during the early phase of PBOX-21 treatment as CDK2 and CDK4 activities are reduced respectively both prior to and coincident with any changes in cell cycle distribution. The reduction of CDK6 activity, on the other hand would appear to be a consequence of, and a method of maintaining the PBOX-21-induced arrest, rather than a cause.

In order to address the mechanism by which the reduction in kinase activity of these CDKs occur, the expression of the CDKs themselves and the various G1 cyclins and CDK inhibitors was analysed. It was found that PBOX-21 caused a reduction in protein expression of CDK2 and CDK4 in parallel with, and therefore explaining at least in part, their observed decrease in kinase activity. On the other hand, the expression levels of CDK6 remained unaffected by PBOX-21 treatment indicating an alternative mechanism by which this kinase's activity was being modified.

Western blot analysis of the cyclins revealed that PBOX-21 had no effect upon the expression level of the cyclin E. As cyclin E is known to complex with CDK2 late in the G1 phase, this indicated that the inhibition of CDK2 occurred independent of its association with cyclin E. Since cyclin D₁ was not found to be expressed within the 1321N1 cells, we next examined the effect of PBOX-21 on expression levels of cyclins D₂ and D₃. PBOX-21 treatment was found to cause a downregulation in the expression of cyclin D₂, first being seen at 24 hours. As this too is much later than the onset of the arrest, it would seem to imply that this event is a secondary response to the cessation of cell cycle progression. In contrast, progression of the expression level of cyclin D₃ was reduced significantly by PBOX-21, with downregulation occurring after 8 hours of treatment, preceding the arrest and suggesting a role in the mechanism by which G1 arrest is induced. The PBOX-21 induced decrease of cyclin D₃ was maintained up to 16 hours of treatment. It should also be noted that after 16 hours of treatment the levels of cyclin D₃ within the untreated 1321N1 cells also began to decline, with no cyclin D₃ expression visible at 48 hours. This may be due to a depletion within the culture medium of necessary growth factors. The expression of the D-type cyclins was under the control of mitogenic stimuli and once these began to diminish the cyclin may undergo ubiquitin-mediated proteolysis (Diehl *et al.*, 1997; Pagano, 1997). The downregulation of cyclin D₃ may be responsible for the reduction in the kinase activity of CDK6, and may contribute, in

combination with the downregulation of CDK4 expression, to the reduction in CDK4 kinase activity. This would result in a reduction in complex formation between these kinases and cyclin D₃ leading to a decrease in the levels of active cyclin D₃-CDK4 and cyclin D₃-CDK6 complexes with kinase activity. In order to confirm this theory, the association between cyclin D₃ and CDK4 and CDK6 was analysed and revealed a reduction in complex formation between both these kinases and cyclin D₃. Therefore, cyclin D₃ downregulation may be responsible for the maintenance of PBOX-21 mediated G1 arrest via modulation of CDK4 and CDK6 kinase activity. At present we do not know whether the reduction in cyclin D₃ expression occurs through active transcriptional repression or by a post-translational mechanism affecting protein stability of cyclin D₃. It would also be of interest in the future to determine whether enforced expression of cyclin D₃ could abrogate PBOX-21 induced G1 arrest.

Another method by which CDK activity and thus cell cycle progression is modulated is via the expression of the CDK inhibitors. This study looked at the expression of p16. It is the most well documented member of the INK4 CDK inhibitor family that specifically inhibit only the cyclin D-associated kinases (Ruas *et al.*, 1998). We also looked at p21, a member of the Kip/Cip CDK inhibitor family. It was found that p16 was not expressed within the 1321N1 cells. PBOX-21 treatment did however result in an upregulation in the expression of p21, with an initial and maximal induction in expression seen after 16 hours. After this time the induced expression of p21 began to decline up to 24 hours. Again, as this effect is seen following the G1 arrest onset, p21 induction cannot be viewed as a causative factor in the arrest observed, but instead may play a role in its maintenance.

The expression of p21 is known to be under the control of both p53-dependent and independent mechanisms. p21 possesses strong p53 response elements in the p21 gene promoter leading to p53-induced transcription of the p21 protein (Macleod *et al.*, 1995). It is clear, however, that p21 has many roles in cell-cycle control beyond its function as an effector of p53 responses to genotoxic stress and can in fact function independent of p53 stimuli (Macleod *et al.*, 1995; Parker *et al.*, 1995). Thus, the protein expression levels of p53 were examined to assess whether p53 expression contributes to the PBOX-21 mediated induction of p21. p53 expression levels remained unaltered following PBOX-21 treatment and

this may suggest that the induction of p21 by PBOX-21 is independent of p53 control. In future studies, it would be of interest to characterise the effect PBOX-21 mediates upon p21 expression levels within a p53-null astrocytoma cell line.

As well as positive regulation via the association of cyclins, and negative regulation via the binding of inhibitory molecules such as CDK inhibitor, the activity of CDKs is also regulated by its phosphorylation status, which is controlled by the antagonistic action of Wee 1/Myt1 kinases and CDC25a phosphatase (Bartek *et al.*, 2001). Phosphorylation of conserved tyrosine and threonine residues near the ATP binding sites of CDKs is an important mechanism employed to keep CDKs inactive. CDC25a acts to strip the CDKs of these inhibitory phosphorylations and thus allow cell cycle progression (Bartek *et al.*, 2001). CDC25a is assumed to be responsible for CDK2 activation by removing the inhibitory phosphates from Tyr 15 and Thr 14 of CDK2 (Santoni-Rugiu *et al.*, 2000). Reports published recently suggest that pathways targeting CDC25a may underlie the molecular events that account for p53-independent arrest of cells at G1 (Mailand *et al.*, 2000), (Molinari *et al.*, 2000). A decrease in CDC25a expression that leads to persistent inhibitory phosphorylations of CDK2 on Tyr 15/Thr 14 results in G1 arrest. In the present study PBOX-21 had no effect on the CDC25a expression levels. Hence, it would appear that PBOX-21 induced G1 arrest occurs independently of CDC25a repression.

It is well documented that a major subcellular target of the cyclin-CDK complexes is the retinoblastoma protein (Rb) ensuring progression through the cell cycle by maintaining Rb in a hyperphosphorylated state. Growth inhibition is thus often associated with suppression of Rb phosphorylation retaining Rb in an unphosphorylated, growth-inhibitory state. After treatment with PBOX-21 a clear mobility shift was observed in the Rb protein from the hyperphosphorylated to the hypophosphorylated form. This result is consistent with the reduced CDK2, CDK4 and CDK6 activity in PBOX-21 treated cells as Rb is a substrate of these kinases.

In conclusion, our findings demonstrate that PBOX-21 inhibits the growth of 1321N1 and MCF-7 cells by arresting them at the G1 phase of the cell cycle. This G1 arrest does not appear to be mediated through binding to the PBR. The arrest is preceded by a rapid decrease in CDK2 kinase activity and cyclin D₃ expression. The arrest is concomitant with a decrease in CDK4 kinase activity,

indicating that these events are likely to be part of the mechanism responsible for initiating the G1 arrest. The inhibition of the activity of both CDK2 and CDK4 is at least in part due to their early downregulation in protein expression. The decrease in CDK4 activity may also be due to the decreased complex association of CDK4 with cyclin D₃. The later reduction in CDK6 kinase activity and its decreased association with cyclin D₃, as well as the upregulation of p21 and down regulation of cyclin D₂ may be involved in maintaining the arrest. Furthermore, we found that Rb becomes hypophosphorylated in response to PBOX-21 treatment. These results indicate that PBOX-21 exerts its growth inhibitory effects through the modulation of the expression and/or activity of several key G1 regulatory proteins. It will be important in future studies to define the upstream signal transduction pathway mediating PBOX-21 action.

**4 INVESTIGATION INTO THE MOLECULAR
MECHANISM UNDERLYING THE CYTOSTATIC
AND APOPTOTIC EFFECTS OF PBOX-6 IN MCF-
7 CELLS**

4.1 Introduction

Apoptosis is a genetically regulated process that is responsible for normal cell turnover in the body and serves as a counterbalancing force to the cell generating effects of mitosis. Inappropriate cell death, leading to too little or too much death, is responsible for the development of many disorders including autoimmune diseases, neurodegenerative disorders and cancer. This finding has led to a vast expansion in apoptosis research in recent years, with the view to increasing our understanding of this cell suicide mechanism. Elucidation of the molecular components of the cell death pathway will provide an opportunity to target and modulate expression and activity of key components that are deregulated during disease development and which will lead to improved therapeutics for disease treatment.

This laboratory has recently demonstrated in a series of papers that some members of a novel series of pyrrolo-1,5-benzoxazepine (PBOX) compounds potently induce apoptotic cell death in a variety of human cancerous cells derived from the haematopoietic system such as promyelocytic leukaemia HL-60 cells, Jurkat T lymphoma and chronic myeloid leukaemia (CML) cells (Mc Gee *et al.*, 2001; Mc Gee *et al.*, 2002a; Mc Gee *et al.*, 2002b; Zisterer *et al.*, 2000). It has also been shown that PBOX-6, a representative pro-apoptotic member of these PBOX compounds, potently induces apoptosis in the human breast carcinoma MCF-7 cell line (Mc Gee *et al.*, 2002b), the human prostate carcinoma LNCaP cell line and the cervical carcinoma Hela cell line (unpublished results). Thus, the PBOX compounds appear to be subdivided into two groups, those that exert antiproliferative effects (e.g. PBOX-21) as studied in the previous chapter, by arresting cells in the G1 phase of the cell cycle without eliciting cytotoxic effects (Mulligan *et al.*, 2003; Zisterer *et al.*, 1998), and those that can induce apoptotic cell death (e.g. PBOX-6) (Mc Gee *et al.*, 2001; Mc Gee *et al.*, 2002a; Mc Gee *et al.*, 2002b; Zisterer *et al.*, 2000). Thus the apoptotic activity of members of the PBOX series is dependent on their structure (Mc Gee *et al.*, 2001; Mc Gee *et al.*, 2002a; Mc Gee *et al.*, 2002b; Zisterer *et al.*, 2000). The pro-apoptotic members have in general been found to contain a naphthyl side-chain, but further studies are required to determine a clear structure-activity relationship (Figure 1.9).

The pro-apoptotic action of some members of this series of compounds suggests the potential of these drugs as novel anticancer therapeutics in the treatment of both solid tumours and those derived from the haematopoietic system. The validity of any new therapeutic is more easily established if a sound mechanistic basis can be shown. It has previously been demonstrated that the mechanism by which PBOX compounds induce apoptosis does not involve their specific interaction with the mitochondrial peripheral type benzodiazepine receptor (PBR), which they bind with high affinity. This was deduced due to a lack of correlation between their affinities for the receptor and their apoptotic potencies, their high apoptotic activity in PBR-deficient cells such as Jurkat acute lymphoblastic leukaemia cells and their lack of apoptotic induction in PBR-rich rat leydig cells (Zisterer *et al.*, 2000). The involvement of reactive oxygen intermediates (ROIs) in PBOX-6 induced apoptosis has also been ruled out (Mc Gee *et al.*, 2001; Zisterer *et al.*, 2000). Furthermore, it has been shown that pro-apoptotic PBOX compounds do not function as farnesyl protein transferase inhibitors or as topoisomerase inhibitors (Mc Gee PhD thesis 2001).

Part of the mechanism involved in the induction of apoptosis by PBOX-6 has, however, been elucidated. The mitogen activated protein (MAP) kinase family is activated in response to a wide variety of external stress signals such as UV irradiation and chemotherapeutic drugs (Ichijo, 1999). The ability of PBOX-6 to induce activation of MAP kinase signalling pathways was investigated. Activation of the c-jun N-terminal kinase (JNK) signalling pathway (a MAP kinase family member, see Section 1.6.3) has been shown to be essential during PBOX-6 induced apoptosis in CML cells. Treatment of CML K562 cells with PBOX-6 resulted in the early (within minutes) and transient activation of JNK. Furthermore, treatment of K562 cells with the JNK inhibitors, dicumarol or JIP-1, inhibited PBOX-6 induced JNK activity and apoptosis, demonstrating that JNK activation is an essential component of the apoptotic pathway induced by PBOX-6 (Mc Gee *et al.*, 2002a). The importance of JNK in the apoptotic signalling pathway has also been confirmed in a number of other cell lines such as HL-60 (unpublished data), demonstrating that this is not just a cell type-specific effect. In addition, unpublished results also show that PBOX-6 induces the phosphorylation and inactivation of two anti-apoptotic members of the Bcl-2 protein family, Bcl-2 itself and Bcl-XL and that JNK appears to be responsible for the phosphorylation

of these proteins. The consequences of this phosphorylation and the other signalling events prior to and following JNK activation during PBOX-6 induced apoptosis remain to be elucidated.

The identification of the molecular target of the pro-apoptotic PBOX compounds is of the utmost importance if they are to be developed as anti-cancer therapeutics. The microtubule is a primary target for many anticancer agents. A common feature of microtubule targeting agents such as paclitaxel, docetaxel, vinblastine, vincristine, nocodazole and colchicine is that they arrest cells at the G2/M phase, and induce apoptosis of cancer cells (Wang *et al.*, 1999). It has previously been demonstrated that PBOX-6 induces apoptosis in human breast cancer MCF-7 cells (Mc Gee *et al.*, 2002b). Cell cycle analysis of propidium iodide-stained cells treated with PBOX-6 for 48 hours demonstrated the appearance of a pre-G1 apoptotic peak (22% of cells in PBOX-6 treated cells compared to less than 3% in vehicle control cells). The cell cycle phases G1, S and G2/M as well as the pre-G1 apoptotic peak following PBOX-6 treatment were also examined. It was observed that a 48 hour PBOX-6 treatment caused a notable accumulation of cells in the G2/M phase of the cell cycle (38% of PBOX-6 treated cells compared to 15% of vehicle control cells). Since a detailed time course of the changes in the distribution of the cell cycle phases in response to PBOX-6 had not been performed in that study, it remained unclear as to whether the PBOX-6 induced G2/M arrest occurred prior to the onset or concomitant with the observed apoptosis.

It has been shown in numerous studies that apoptotic cell death of MCF-7 cells is preceded by a G2/M arrest following treatment with antimicrotubule agents (Ling *et al.*, 2002; MacCarthy-Morrogh *et al.*, 2000; Vogt *et al.*, 2000; Wu-Wong *et al.*, 2001). Following the completion of the G1 phase the cell enters the S phase during which time the DNA of the cell is precisely duplicated. The cell then enters the second of its gap phases, the G2 phase. The completion of DNA replication leaves the G2 cell with two accurate copies of the entire genome. The cell then undergoes the dramatic upheaval of mitosis (M phase). Progress through these phases of the cell cycle is under the control of a new wave of cyclin/CDK complexes. The expression of cyclin A is induced shortly after cyclin E and is mainly controlled at the transcription level, involving E2F and other transcription factors. Cyclin A binds CDK2 in S phase and it is considered to be responsible for

the progression through the S phase of the cell cycle. During G2/M, cyclin A associates with a new CDK partner, namely CDK1. CDK1 is the principle CDK in operation during the G2/M phase. A second cyclin/CDK complex is also involved in regulation of the progression of the cell cycle through the G2/M phase. In cycling mammalian cells, cyclin B is first synthesized during the S phase and increases in concentration as cells proceed through G2, peaking in early mitosis and dropping after anaphase. *Xenopus*, mammalian cells and other vertebrates contain two closely related, functionally equivalent B-type cyclins called B₁ and B₂. Cyclin B₂ is a non-essential protein that associates with the Golgi apparatus and probably plays a role in Golgi remodelling during mitosis (Brandeis *et al.*, 1998; Jackman *et al.*, 1995). Cyclin B₁ is an essential protein that is thought to be responsible for most of the other actions of CDK1 in the cytoplasm and the nucleus during G2 and mitosis (Brandeis *et al.*, 1998). Mitosis is the most dramatic period of the cell cycle, involving a major reorganisation of virtually all cell components. During mitosis, the chromosomes condense, the nuclear envelope of most cells breaks down, the cytoskeleton reorganises to form the mitotic spindle, and the chromosomes move to opposite poles. The entry into and events of mitosis are triggered by the cyclin B₁/CDK1 complex, which was originally defined as the M phase- or Maturation-promoting factor (MPF) (Masui *et al.*, 1971).

Regulation of cyclin B₁/CDK1 complexes at multiple levels ensures the tight regulation of the timing of mitotic entry. The first level of regulation of entry of the cell into mitosis is the expression of cyclin B₁ itself. This is followed by a series of phosphorylation events, resulting in activation or inhibition of kinase activity. The final layer of regulation of progression into mitosis, is the subcellular localisation of the complexes and their regulators. Cyclin B₁ is initially localised in mammalian cells within the cytoplasm during the S and G2 phase, and translocated to the nucleus at the beginning of mitosis (Pines *et al.*, 1991), where it is required for the majority of its function. As cells enter mitosis, phosphorylation of key components of the subcellular structures causes the complete reorganisation of the architecture of the cell. It is one of the most remarkable features of cell cycle control that a single protein kinase, cyclin B₁/CDK1, is capable of orchestrating these diverse and complex rearrangements that occur in the early stages of mitosis by phosphorylating some of the structural

proteins involved in the mitotic cellular reorganisation. The breakdown of the nuclear envelope occurs following phosphorylation and consequent disassembly of the nuclear lamina by cyclin B₁/CDK1 (Luscher *et al.*, 1991). The cyclin B₁/CDK complex is also believed to be involved in chromosome condensation and is at least partially responsible for the complex microtubule rearrangement and other events that lead to the assembly of the mitotic spindle (Chang *et al.*, 2001; Ookata *et al.*, 1995).

Regulation of the progress through the G2 and M phases is crucial to ensuring that cells are not allowed divide when DNA is damaged or incompletely replicated, or when chromosomes are incorrectly partitioned, resulting in increased genetic damage and insult to genomic integrity. DNA-integrity checkpoints monitor DNA status and prevent inappropriate transitions into S phase and mitosis when problems are detected. The G2 phase DNA damage checkpoint leads to inhibition of the cyclin B₁/CDK1 activity through maintenance of inhibitory phosphorylation of the Tyr15 residue of CDK1. In so doing, the cells remain in the G2 phase and do not progress into mitosis. Cells can also arrest at various stages within mitosis. During mitosis, condensed sister chromatids are attached to the mitotic spindle via their kinetochores during prometaphase and metaphase and pulled to opposite poles as they undergo anaphase. As the mis-segregation of sister chromatids can lead to aneuploidy, this process must be tightly controlled by checkpoints that monitor the completion of critical steps in the pathway. Mitotic exit requires sister chromatid separation, spindle disassembly and cytokinesis. The initiation and coordination of these processes are controlled by ubiquitin-dependent proteolysis of key regulatory proteins. The important step in this destruction is mediated by a multimeric ubiquitin ligase, called anaphase-promoting complex (APC) or cyclosome (Zachariae *et al.*, 1999). Cyclin B degradation is essential for cell cycle progression and CDK1 inactivation at the end of mitosis has been found to be essential for cell cycle progression in all eukaryotes (Murray *et al.*, 1989). Degradation of mitotic cyclins appears to be a universal mechanism for CDK1 inactivation even though other mechanisms also exist.

The spindle assembly checkpoint ensures that cells do not undergo chromosome segregation until all of the chromosomes have properly aligned on the mitotic spindle (Amon, 1999; Gorbsky, 2001). Once the checkpoint is

activated it results in arrest of the cell in mitosis. The cell cycle arrest induced by the spindle assembly checkpoint is the result of negative regulation of APC activity, preventing proteolytic degradation of cyclin B, resulting in sustained CDK1 activity and prevention of mitotic exit. It is by this mechanism that antimicrotubule agents mediate their effects. These agents have broad activity both in solid and in hematologic neoplasias. Microtubules are cytoskeletal components that play a critical role in many cellular processes, such as maintenance of cell shape and polarity, and intracellular transport of vesicles and organelles. During mitosis, microtubules form the bipolar microtubular array known as the mitotic spindle (Zhai *et al.*, 1996). Disruption of microtubule dynamics can lead to the formation of abnormally stable or unstable microtubules (Wilson *et al.*, 1995), thereby preventing the normal dynamic rearrangements of the microtubule network required for cell proliferation, and thus activation of the spindle assembly checkpoint (Amon, 1999; Gorbsky, 2001). One group of agents that target microtubules inhibits microtubule polymerisation, and included in this group are the *vinca* alkaloids, colchicine and nocodazole. Another group of agents promote microtubule polymerisation. Members of this group include the taxanes and the epothiloines. Antimicrotubule agents currently in clinical use for cancer chemotherapy include the taxanes and the *vinca* alkaloids. The taxanes such as paclitaxel (taxol) and docetaxel (taxotere) are effective in the treatment of refractory ovarian cancer, metastatic breast cancer, non-small cell lung cancer and head and neck and bladder carcinomas (Crown *et al.*, 2000; Rowinsky, 1997). The *vinca* alkaloids, typified by vincristine, vinblastine and vinorelbine have importance in the treatment of leukaemia, Hodgkins's disease, non-Hodgkin's lymphomas, testicular cancer, Kaposi's sarcoma, breast cancer small cell lung cancer, and other malignancies (Rowinsky, 1997; van Tellingen *et al.*, 1992)

Studies in both normal and transformed human cells treated with members of either of these two groups of antimicrotubule inhibitors have shown that apoptosis can be initiated rapidly and directly from mitotic arrest (Fan *et al.*, 2001; Hayne *et al.*, 2000; Woods *et al.*, 1995; Zhou *et al.*, 2002a). An important mediator of apoptotic signalling is the JNK subgroup of Mitogen-Activated Protein Kinases (MAPKs) (Chan-Hui *et al.*, 1998). JNKs are activated in response to proinflammatory cytokines and a variety of stressful stimuli, including UV- and γ -irradiation, heat shock, oxidative and osmotic stress, growth factor deprivation,

metabolic inhibitors, and chemotherapeutic drugs (Kyriakis *et al.*, 1996). The JNK pathway is critical for certain forms of stress-induced apoptosis. A role for JNK signalling in the response of cells to antimicrotubule agents has been suggested. JNK activity is increased by antimicrotubule agents in a wide variety of cell types (Lee *et al.*, 1998; Shtil *et al.*, 1999; Wang *et al.*, 1999; Wang *et al.*, 1998), and inhibition of JNK inhibits paclitaxel induced apoptosis (Lee *et al.*, 1998; Wang *et al.*, 1999). Reversible phosphorylation and dephosphorylation are the main mechanisms that directly regulate the functional activity of Bcl-2 and Bcl-XL, with phosphorylation leading to inhibition of their anti-apoptotic function (Blagosklonny, 2001). JNK has been shown to mediate vinblastine-induced phosphorylation of Bcl-2 and Bcl-XL (Fan *et al.*, 2000), consistent with an earlier study that also identified JNK as the kinase responsible for paclitaxel-induced Bcl-2 phosphorylation (Yamamoto *et al.*, 1999). The activation of the JNK pathway may be a stress response to the disruption of microtubule dynamics. A model, proposed by Wang *et al.* (1998), suggests that antimicrotubule agents enter the cell and disrupt the dynamics of microtubule assembly which leads to activation of both Ras and ASK1 via an as yet undefined mechanism. Ras activates the Raf-MAPK kinase (MAPKK)-MAPK signalling cascade, whilst ASK1 itself is a MAPKK. Activation of either Ras and/or ASK1 therefore may lead to activation of JNK. Although, the exact mechanism by which JNK is activated and indeed how it mediates apoptotic cell death is not clearly defined, JNK does appear to play an important role in apoptotic regulation of cancer cells after chemotherapy with antimicrotubule agents.

As discussed above, a common feature of antimicrotubule agents is that they arrest cells at the G2/M phase of the cell cycle and cause an induction in cyclin B1/CDK1 kinase activity. Moreover, they activate JNK and phosphorylate Bcl-2 and Bcl-XL and induce apoptosis in cancerous cell lines. Preliminary results in MCF-7 cells show that PBOX-6 arrests cells in the G2/M phase (Mc Gee *et al.*, 2002b). PBOX-6 has also been shown to activate JNK (Mc Gee *et al.*, 2002a) and phosphorylate Bcl-2 and Bcl-XL in the CML K562 cell line (McGee *et al.*, manuscript submitted). These observations may suggest that PBOX-6 acts as a microtubule targeting drug.

Aims of this chapter

In the present chapter the hypothesis that PBOX-6 acts at a molecular level in a manner similar to antimicrotubule agents was examined. An investigation into whether the G2/M arrest in MCF-7 cells occurs prior to or concomitant with the induction of apoptosis following treatment with PBOX-6 or two representative antimicrotubule agents, paclitaxel and nocodazole, was carried out. In addition, in an attempt to dissect out the molecular mechanism underlying the PBOX-6 induced G2/M arrest, the effect of this compound, and of paclitaxel and nocodazole, on the cell cycle machinery controlling the G2/M phase was determined. The importance of JNK in mediating this G2/M arrest and apoptosis in MCF-7 cells was also examined.

4.2 Results

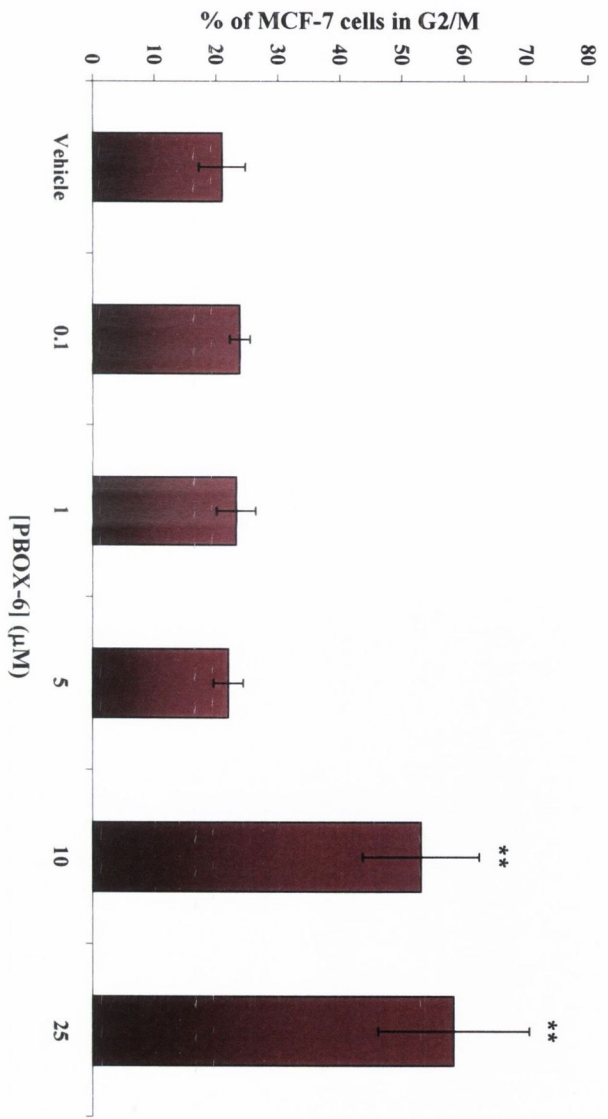
4.2.1 *The effect of PBOX-6, paclitaxel and nocodazole upon the cell cycle*

4.2.1.1 PBOX-6 induces G2/M arrest and apoptosis in MCF-7 cells

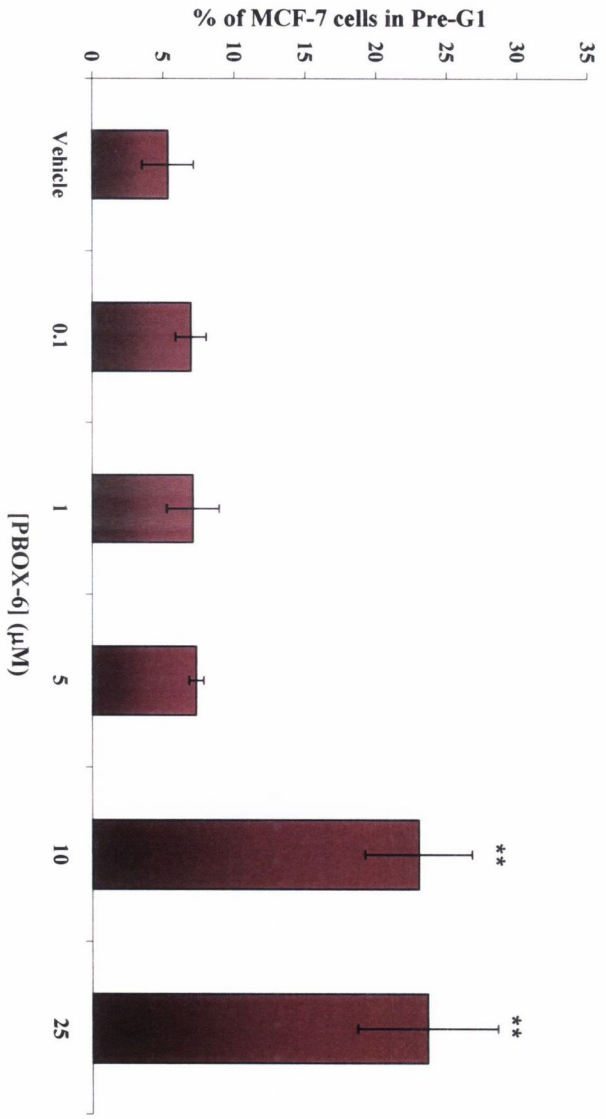
In order to confirm the initial observations by McGee *et al.* (2002) that PBOX-6 induces G2/M arrest and apoptosis within MCF-7 cells, the distribution of MCF-7 cells within the various phases of the cell cycle in response to treatment with increasing doses of PBOX-6 was analysed by flow cytometry. MCF-7 cells were seeded in 25cm² flasks, incubated for 24 hours until the cells were in the log phase of growth and then treated with the indicated concentrations of PBOX-6 for 24 hours. The cells were harvested as described previously (Section 2.2.1) and were prepared and analysed using flow cytometry (Section 2.2.5). It was found that PBOX-6 induced G2/M arrest within the MCF-7 cells (Figure 4.1(a)). However, no significant increase in the number of cells in G2/M over vehicle (0.5% (v/v) ethanol) was observed until a concentration of 10µM PBOX-6 was reached. This concentration of PBOX-6 caused a significant increase of 32% of cells in G2/M relative to the vehicle control. The effect had more or less plateaued following 25µM PBOX-6 treatment. PBOX-6 was also seen to induce apoptosis as determined by the presence of cells in the pre-G1 phase of the cell cycle (Figure 4.1 (b)). However, the range of concentrations with which this effect is seen was again very narrow. There was no significant increase in the number of MCF-7 cells in the pre-G1 phase over vehicle (0.5% (v/v) ethanol) after treatment with 0.1µM to 5µM PBOX-6. The situation changed dramatically following treatment with 10µM PBOX-6, as this dose caused a significant increase of 17% in the number of cells in pre-G1 relative to the vehicle control, whilst treatment with 25µM PBOX-6 did not further increase the percentage of cells in pre-G1. Therefore, PBOX-6 induces G2/M arrest and apoptosis over a very narrow range of concentrations. A concentration of 10µM PBOX-6 was used in all subsequent studies.

Figure 4.1 PBOX-6 induces G2/M arrest and apoptosis in MCF-7 cells

MCF-7 cells were seeded at a density of 1×10^6 in 25cm^2 flasks in 5mls of complete medium and were incubated at 37°C under a humidified atmosphere of 95% O_2 and 5% CO_2 for 24 hours until they were in the exponential phase of growth. The cells were then treated either with vehicle (0.5% (v/v) ethanol) or the range of PBOX-6 concentrations indicated and incubated for 24 hours. The cells were then harvested as described in Section 2.2.1, prepared and analysed using flow cytometry as described in Section 2.2.5. (a) represents the cells in the G2/M phase, whilst (b) represents the cells in the pre-G1 phase. The results represent the mean $\pm$ S.E.M of three separate experiments. Statistical analysis was carried out using the Instat computer program. $**p < 0.01$ with respect to vehicle, Students t-test.



(a)



(b)

4.2.1.2 PBOX-6, paclitaxel and nocodazole induce G2/M arrest and apoptosis in MCF-7 cells in a time-dependent manner

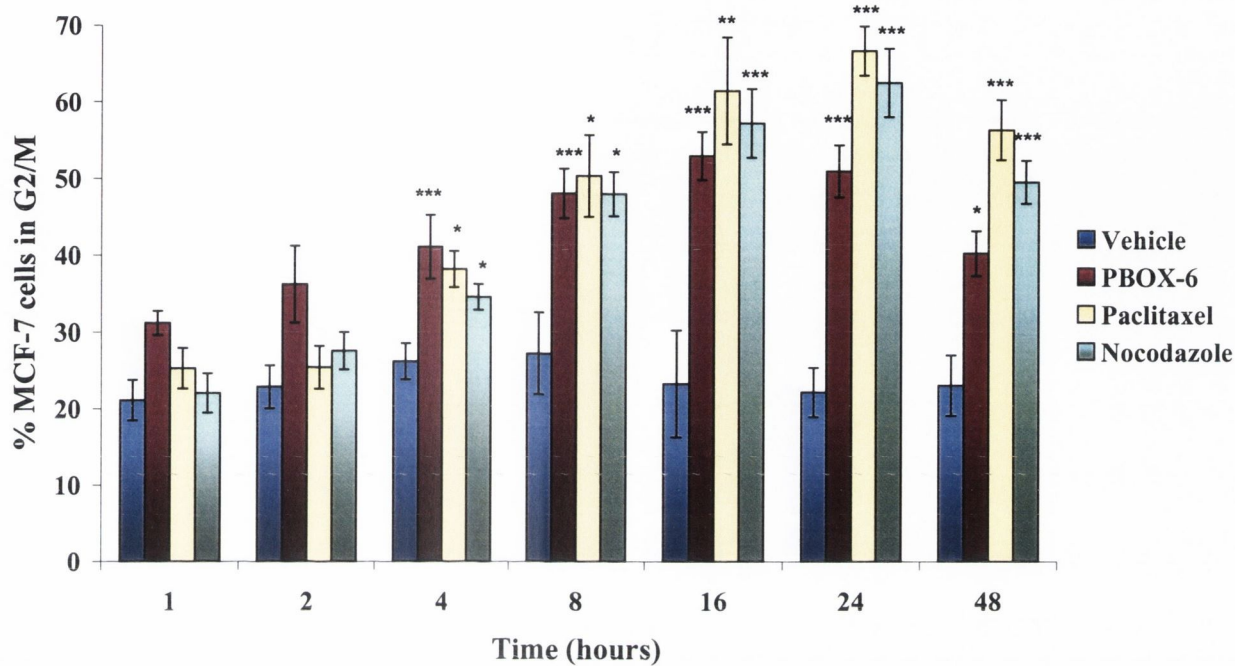
A detailed time course of the changes in distribution of cell cycle phases in response to PBOX-6 treatment has not previously been performed. Preliminary results in MCF-7 cells suggested that PBOX-6 may act as a microtubule targeting drug (Mc Gee *et al.*, 2002a; Mc Gee *et al.*, 2002b). Therefore, MCF-7 cells were treated with two representative antimicrotubule agents, paclitaxel and nocodazole, in order to confirm that PBOX-6 does indeed result in a profile of cell cycle effects similar to that induced by these agents. MCF-7 cells were treated with 10 μ M paclitaxel and nocodazole, as other groups have reported G2/M arrest and apoptosis at this concentration (Wang *et al.*, 1998; Zhou *et al.*, 2002a).

For an analysis of the effect of these compounds over time, the cells were seeded as described above and treated with either vehicle (0.5% (v/v) ethanol) or 10 μ M PBOX-6, paclitaxel or nocodazole for the indicated times. Each of these drugs elicited a time-dependent increase in the number of cells within both the G2/M (Figure 4.2(a)) and pre-G1 (Figure 4.2(b)) phases of the cell cycle. All three compounds induced a significant increase in the number of cells in G2/M over vehicle after 4 hours of treatment, with PBOX-6 having the most significant effect at this time. The G2/M arrest induced by all three compounds was maintained over time up to 48 hours. PBOX-6 treatment achieved a maximum G2/M arrest between 16 and 24 hours at which time there was an approximate 30% increase in the number of cells in G2/M compared to the vehicle. This extent of arrest was comparable to that induced by the two representative antimicrotubule agents, paclitaxel and nocodazole.

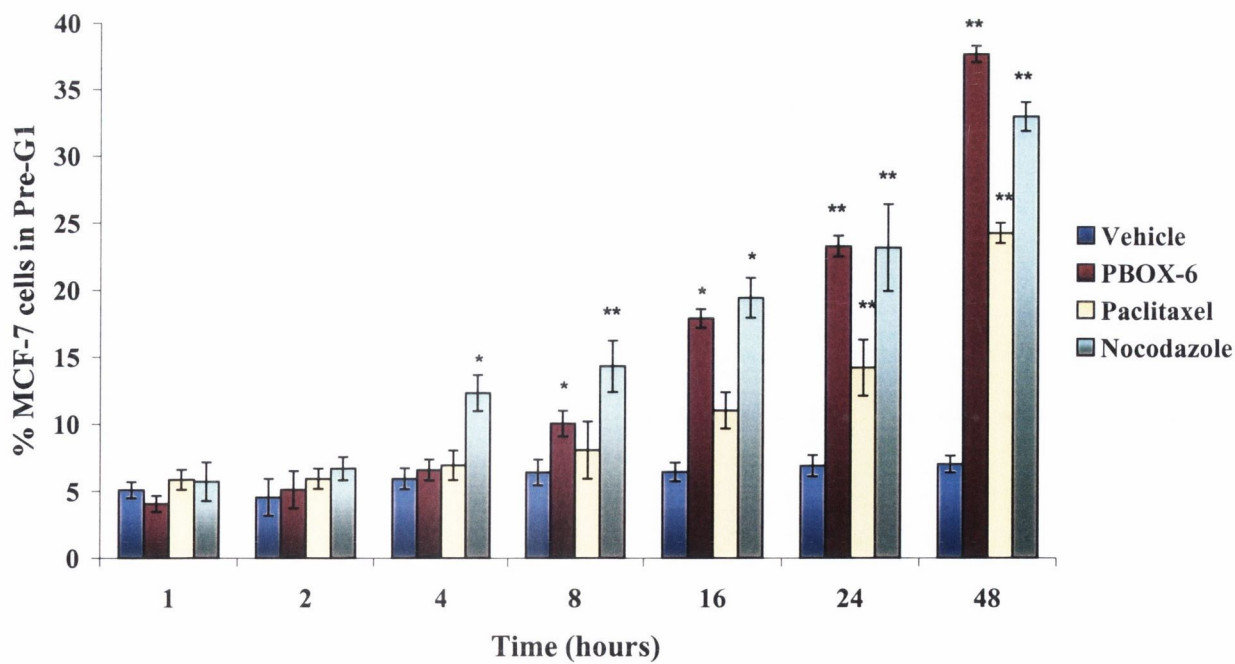
It would appear from the time-course profiles of the distribution of cells, that the G2/M arrest occurs prior to the onset of apoptosis in the case of PBOX-6 and paclitaxel treatment, and concomitant with apoptosis in the case of nocodazole. The onset of a significant increase in the percentage of cells in G2/M arrest at 4 hours of PBOX-6 treatment was followed by an initial significant increase of 4% in the number of cells within the pre-G1 region over vehicle after 8 hours of treatment. This value increased over the duration of PBOX-6 treatment,

Figure 4.2 PBOX-6, paclitaxel and nocodazole induce G2/M arrest and apoptosis in a time-dependent manner.

MCF-7 cells were seeded at a density of 1×10^6 in 25cm^2 flasks in 5mls of complete medium and were incubated at 37°C under a humidified atmosphere of 95% O_2 and 5% CO_2 for 24 hours until they were in the exponential phase of growth. The cells were then treated either with vehicle (0.5% (v/v) ethanol) or $10\mu\text{M}$ PBOX-6, paclitaxel or nocodazole for the indicated times. The cells were then harvested as described in Section 2.2.1, prepared and analysed using flow cytometry as described in Section 2.2.5. (a) represents the cells in the G2/M phase, whilst (b) represents the cells in the pre-G1 phase. The results represent the mean $\pm$ S.E.M of 6 separate experiments. Statistical analysis was carried out using the Instat computer program. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ with respect to vehicle, Students t-test.



(a)



(b)

with an eventual increase of 30% of cells in the pre-G1 region relative to the vehicle control. Nocodazole induced an initial significant increase of 6% after 4 hours of treatment, concomitant with the induction of G2/M arrest. This increased over time to 26% following 48 hours of nocodazole treatment. Paclitaxel, however, did not induce a significant increase of cells in the pre-G1 region until after 24 hours of treatment, eventually reaching 17% after 48 hours of treatment. Therefore, it appears that PBOX-6 elicited an apoptotic response within the MCF-7 cells over time comparable to that induced by nocodazole and somewhat earlier than that induced by paclitaxel.

4.2.1.3 PBOX-6 induces apoptosis in MCF-7 cells as determined by TUNEL staining

In order to confirm that the PBOX-6 induced cell death observed by the increase in MCF-7 cells in the pre-G1 phase of the cell cycle relative to the control was due to apoptotic cell death, TUNEL staining was performed. MCF-7 cells were treated with either vehicle (0.5% (v/v) ethanol) (Figure 4.3 (a)) or 10 μ M PBOX-6 (Figure 4.3 (b)) for 48 hours and subjected to TUNEL staining as described in Section 2.2.9. This confirmed that PBOX-6 induced cell death is associated with DNA fragmentation and thus may be classified as apoptotic cell death.

4.2.1.4 PBOX-6 causes cleavage of PARP in a time-dependent manner

Poly ADP-ribose polymerase (PARP), a regulator of DNA repair, is a common target of activated caspases during apoptotic cell death. Therefore, the cleavage of PARP is a common marker of apoptosis. In addition to the TUNEL staining data, the ability of PBOX-6 to induce apoptosis in MCF-7 cells is further confirmed by the fact that it caused the cleavage of full-length PARP (Figure 4.4). MCF-7 cells were seeded in 75cm² flasks, incubated for 24 hours until they were in the exponential phase of growth, and then treated with either vehicle (0.5% (v/v) ethanol) or 10 μ M PBOX-6 for the indicated times. Total cell lysates were prepared as described in Section 2.2.6.2 and subjected to western blot analysis

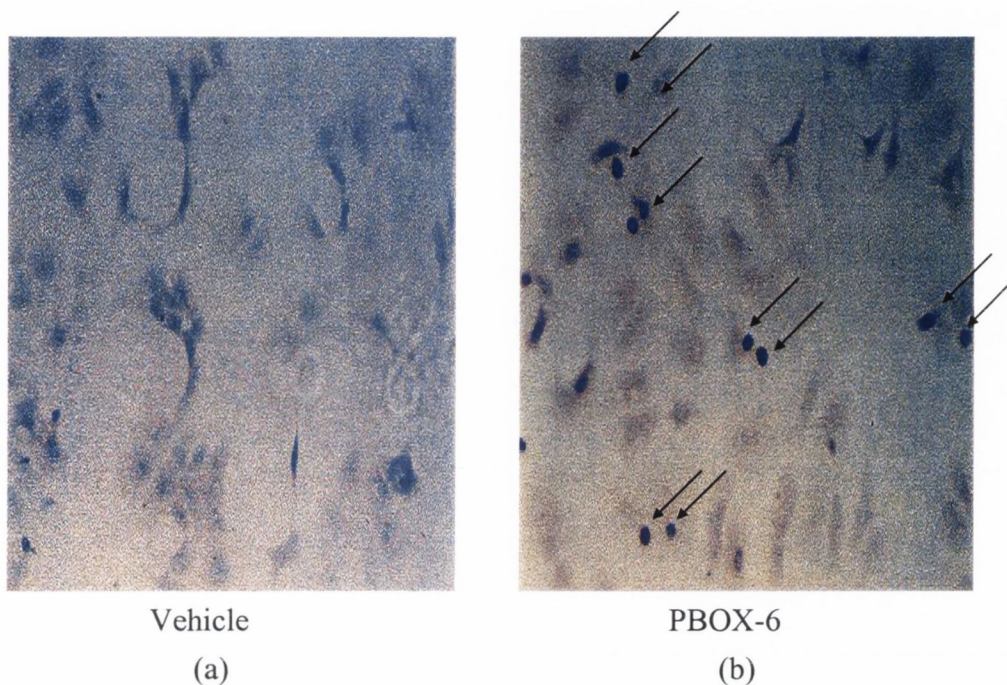


Figure 4.3 PBOX-6 induces apoptotic cell death in MCF-7 cells as demonstrated by TUNEL staining

MCF-7 cells were seeded at a density of 1×10^5 in a volume of 1ml per well of a 24-well plate. Following treatment with either vehicle (0.5% (v/v) ethanol) (a) or 10 μ M PBOX-6 (b) for 48 hours, the cells were washed in PBS, and fixed in 5% formaldehyde in PBS for 15 min at room temperature. Fixed cells were stored dry on slides (Sigma) before processing with the ApopTag Plus Kit (Chemicon) as described in Section 2.2.8. After mounting, slides were analysed by light microscopy under 40X magnification. The arrows indicate the presence of TUNEL-positive cells.

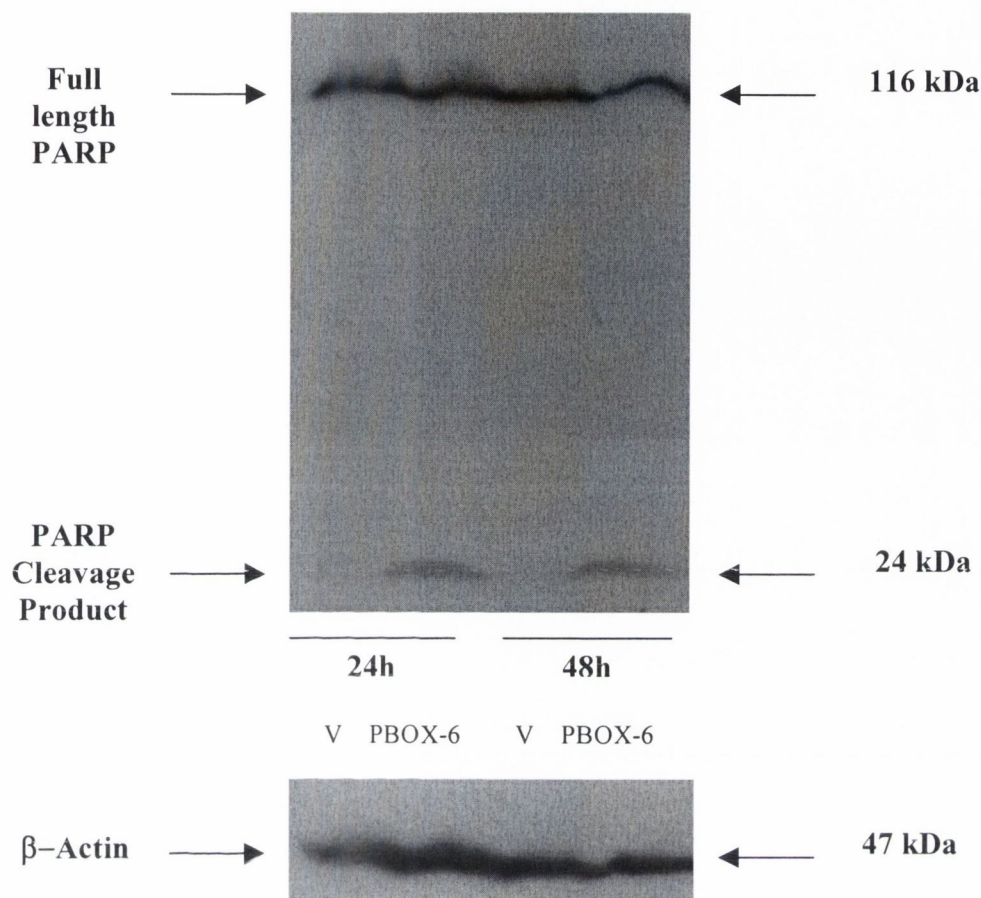


Figure 4.4 PBOX-6 causes cleavage of full-length PARP

Whole cell extracts were prepared as described in Section 2.2.6.1 from MCF-7 cells either treated with vehicle (0.5% (v/v) ethanol) or 10 μ M PBOX-6 for the indicated times. The protein was resolved using SDS-PAGE, transferred onto PVDF and probed with an anti-PARP antibody. The blots were stripped and re-probed with anti- β -actin antibody. The results shown are representative of 2 separate experiments.

using an antibody that recognised the cleavage product of PARP (24 kDa). Full length PARP was cleaved following 24 and 48 hours of PBOX-6 treatment, resulting in the appearance of a 24 kDa cleavage product. This is further evidence that PBOX-6 induced cell death is apoptotic in nature.

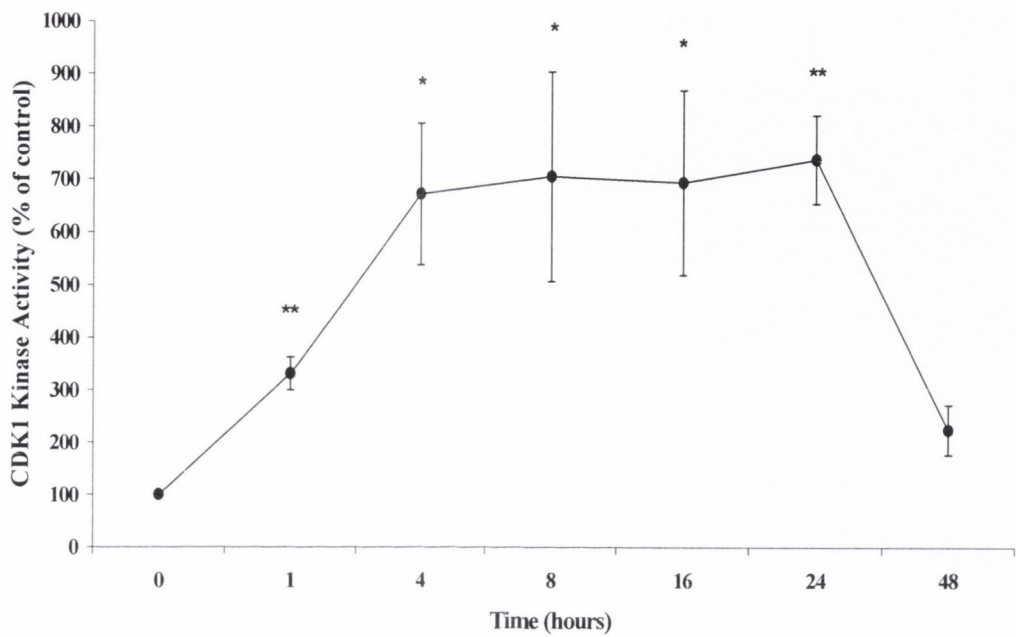
4.2.2 The effect of PBOX-6, paclitaxel and nocodazole upon CDK1 kinase activity

It was necessary to determine what effect PBOX-6 treatment had upon CDK1 kinase activity, as it is responsible, through association with its cyclin partner, cyclin B₁, for driving the cell through G2 and mediating both entrance into and exit out of the M phase of the cell cycle. As previous reports have demonstrated the effect of paclitaxel and nocodazole upon CDK1 kinase activity (Donaldson *et al.*, 1994; King *et al.*, 1994; Wang *et al.*, 1998), treatment with these drugs was used to serve as a control.

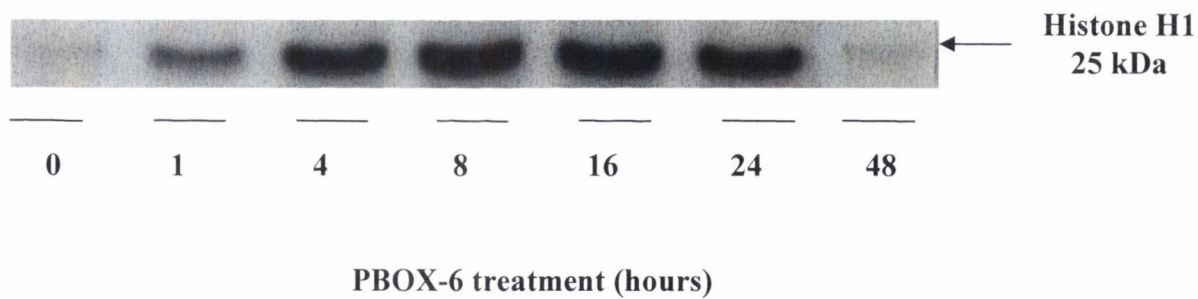
MCF-7 cells were treated with either vehicle (0.5% (v/v) ethanol), 10 μ M PBOX-6, paclitaxel or nocodazole, for the indicated times and the cells were lysed as described previously (Section 2.2.7.1). CDK1 kinase was then immunoprecipitated from the lysate and an *in vitro* kinase assay performed on the immunoprecipitate as described (Sections 2.2.7.2 and 2.2.8 respectively). PBOX-6, paclitaxel and nocodazole treatment was found to cause a time-dependent increase in CDK1 kinase activity relative to the vehicle control (Figure 4.5, 4.6 and 4.7 respectively). The increase in CDK1 kinase activity relative to vehicle control was first observed after 1 hour of treatment with all three compounds. The onset of CDK1 induced kinase activity at 1 hour temporally preceded the onset of G2/M arrest and apoptosis induced by PBOX-6, paclitaxel and nocodazole (See Figure 4.2). CDK1 kinase activity increased steadily over time up to 4 hours following PBOX-6, paclitaxel and nocodazole treatment. This period of increasing CDK1 kinase activity corresponds with the onset of G2/M arrest following treatment with PBOX-6, paclitaxel and nocodazole. The induction in CDK1 activity was maintained up to 24 hours of treatment in each case, corresponding with a maximum G2/M arrest by all three compounds. This comparison of the profile of upregulation of CDK1 kinase activity with the profile

Figure 4.5 PBOX-6 induces an upregulation in CDK1 kinase activity

Whole cell extracts were prepared from MCF-7 cells as described in Section 2.2.6.1 following treatment with either vehicle (0.5% (v/v) ethanol) or 10 μ M PBOX-6 for the indicated times. The protein (200-500 μ g) was immunoprecipitated with anti-CDK1 antibody and Protein A beads and then incubated with Histone H1 substrate and γ^{32} P-ATP and resolved by SDS-PAGE. Densitometry was performed on the resultant bands using Scion Image software with 0 hours vehicle set to 100%. The results represent the mean $\pm$ S.E.M of 3 separate experiments (a). Statistical analysis was carried out using the Instat computer program. * $p < 0.05$, ** $p < 0.01$ with respect to 0 hours vehicle, Students t-test. A representative kinase assay has been included (b).



(a)

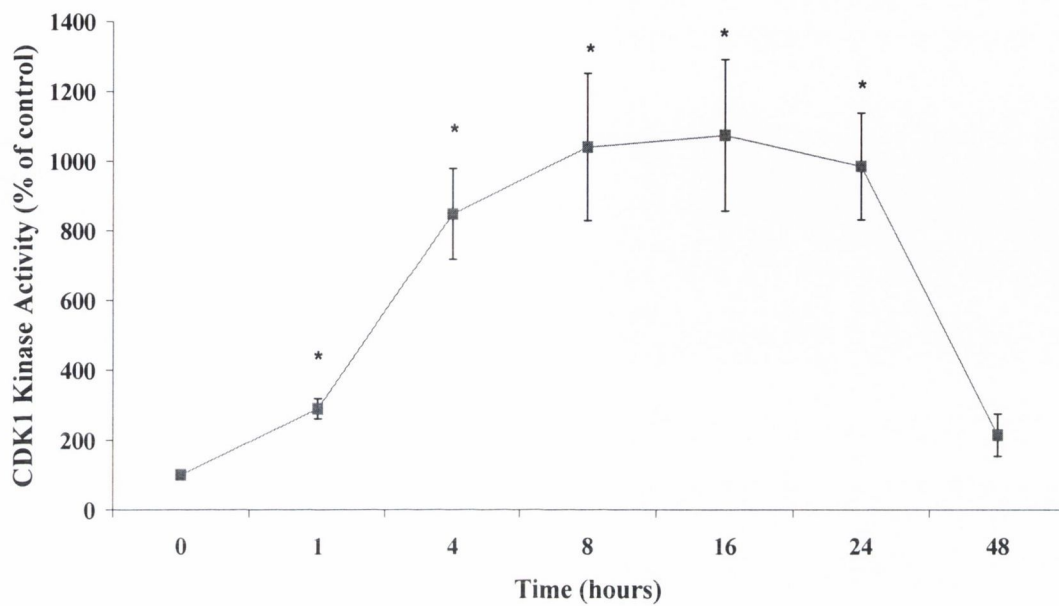


CDK1 Kinase Activity

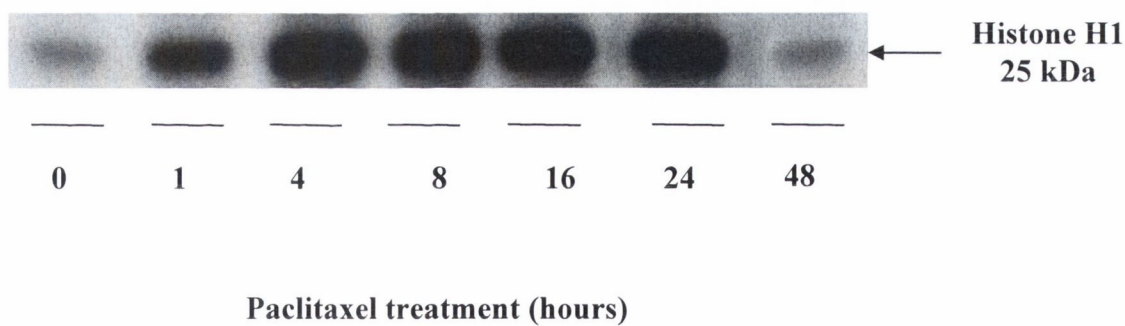
(b)

Figure 4.6 Paclitaxel causes an upregulation in CDK1 kinase activity

Whole cell extracts were prepared from MCF-7 cells as described in Section 2.2.6.1 following treatment with 10 μ M paclitaxel for the indicated times. The protein (200-500 μ g) was immunoprecipitated with anti-CDK1 antibody and Protein A beads and then incubated with Histone H1 substrate and γ^{32} P-ATP and resolved by SDS-PAGE. Densitometry was performed on the resultant bands using Scion Image software with 0 hours set to 100%. The results represent the mean $\pm$ S.E.M of 2 separate experiments (a). Statistical analysis was carried out using the Instat computer program. * $p < 0.05$ with respect to 0 hours, Students t-test. A representative kinase assay has been included (b).



(a)

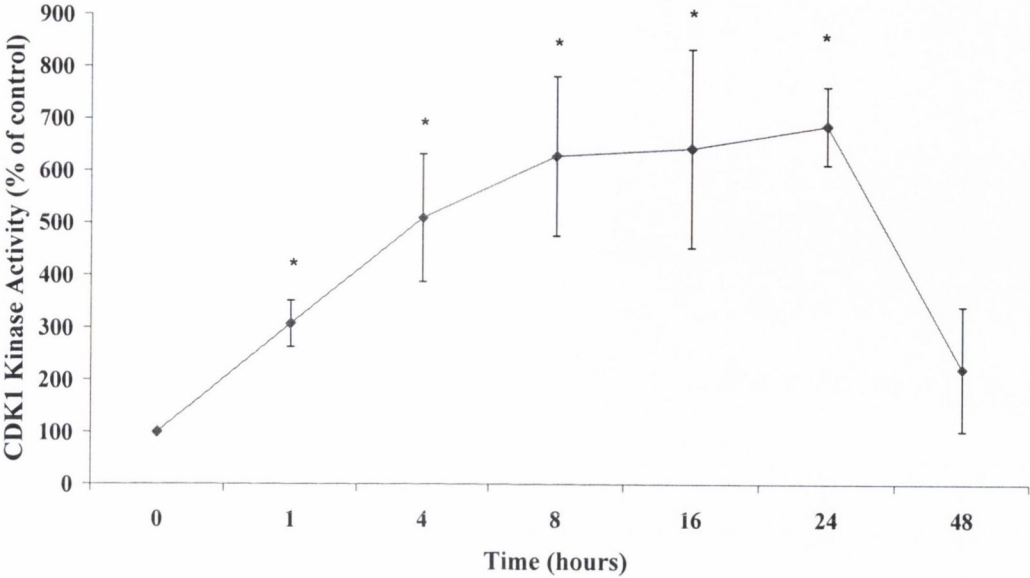


CDK1 Kinase Activity

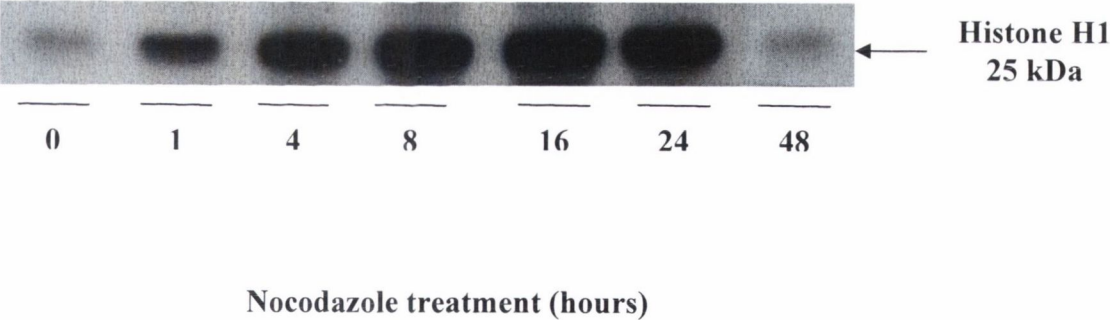
(b)

Figure 4.7 Nocodazole causes an upregulation in CDK1 kinase activity

Whole cell extracts were prepared from MCF-7 cells as described in Section 2.2.6.1 following treatment with 10 μ M nocodazole for the indicated times. The protein (200-500 μ g) was immunoprecipitated with anti-CDK1 antibody and Protein A beads and then incubated with Histone H1 substrate and γ^{32} P-ATP and resolved by SDS-PAGE. Densitometry was performed on the resultant bands using Scion Image software with 0 hours set to 100%. The results represent the mean $\pm$ S.E.M of 2 separate experiments (a). Statistical analysis was carried out using the Instat computer program. * $p < 0.05$ with respect to 0 hours, Students t-test. A representative kinase assay has been included (b).



(a)



CDK1 Kinase Activity

(b)

of G2/M arrest following treatment with PBOX-6, paclitaxel and nocodazole, suggests that the induction in CDK1 may be partially responsible for the cytostatic effects elicited by these compounds.

4.2.3 The effect of PBOX-6, paclitaxel and nocodazole upon the expression levels of key G2/M cell cycle regulatory proteins

PBOX-6, paclitaxel and nocodazole caused an arrest of MCF-7 cells in the G2/M phase of the cell cycle. This was followed by apoptotic cell death in the case of PBOX-6 and paclitaxel treatment and was concomitant with apoptosis following nocodazole treatment. In order to address the mechanism underlying the G2/M arrest and apoptosis induced by these compounds, its effect upon the expression levels of key G2/M cell cycle proteins was examined. As explained in Chapter 3, Section 3.2.2, the expression of cyclin itself serves as one form of regulation of the cyclin/CDK complexes. Therefore, the expression of the cyclins A and B₁ as well as that of CDK1 itself was analysed as these proteins are involved in the regulation of progression through G2/M.

4.2.3.1 PBOX-6, paclitaxel and nocodazole cause a late downregulation in the expression of cyclin A in MCF-7 cells

Total cell lysates were prepared as described (Section 2.2.6.1) and subjected to western blot analysis using an antibody directed against cyclin A. It was found that 10 μ M PBOX-6, paclitaxel and nocodazole had no effect upon cyclin A expression relative to the vehicle control until after 24 hours in the case of paclitaxel treatment (Figure 4.9), and 48 hours following PBOX-6 and nocodazole treatment (Figure 4.8 and 4.9 respectively). At these times, the expression was downregulated, possibly due to the high incidence of apoptosis found at these time-points. As the downregulation in cyclin A occurred well after the onset of both G2/M arrest and apoptosis following treatment with either PBOX-6, paclitaxel or nocodazole, it is unlikely to be a key mediator in either of these events. As the expression level of cyclin A remained unchanged during the PBOX-6, paclitaxel and nocodazole induced upregulation of CDK1 kinase

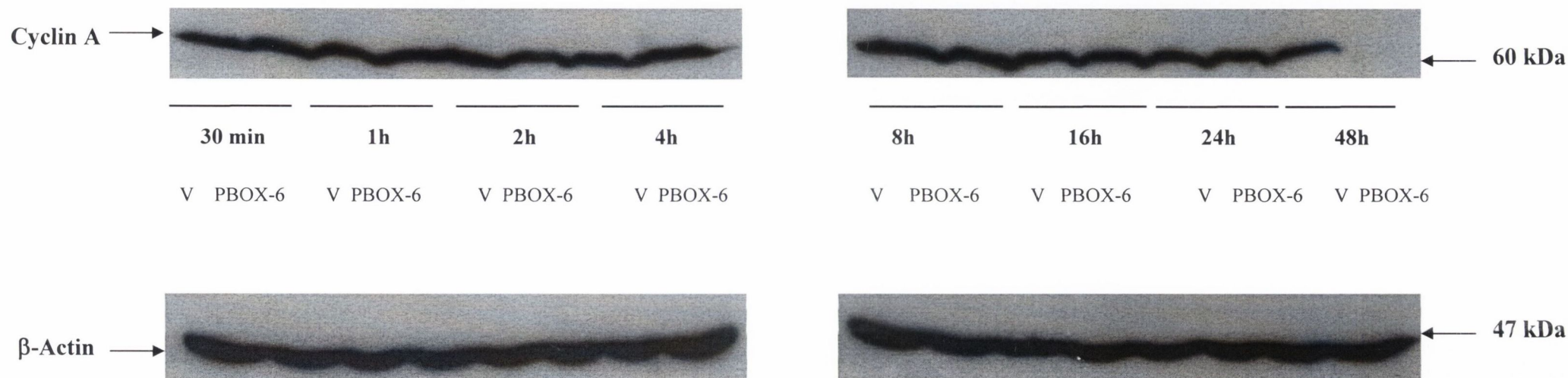


Figure 4.8 PBOX-6 causes a late downregulation in the expression levels of cyclin A

Whole cell extracts were prepared as described in Section 2.2.6.1 from MCF-7 cells treated either with vehicle (0.5% (v/v) ethanol) or 10 μ M PBOX-6 for the indicated times. The protein (50 μ g) was resolved using SDS-PAGE, transferred onto PVDF and probed with anti-cyclin A antibody. The blots were stripped and re-probed with anti- β -actin antibody. The results shown are representative of 4 separate experiments.

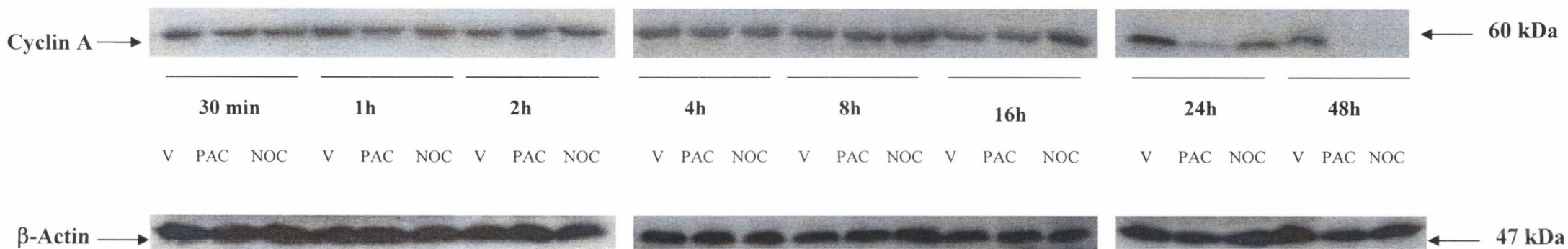


Figure 4.9 Paclitaxel and nocodazole cause a late downregulation in the expression levels of cyclin A

Whole cell extracts were prepared as described in Section 2.2.6.1 from MCF-7 cells treated either with vehicle (0.5% (v/v) DMSO) or 10 μ M paclitaxel or nocodazole for the indicated times. The protein (50 μ g) was resolved using SDS-PAGE, transferred onto PVDF and probed with anti-cyclin A antibody. The blots were stripped and re-probed with anti- β -actin antibody. The results shown are representative of 3 separate experiments.

activity and the subsequent induction of G2/M arrest and apoptosis, it suggested that cyclin A is not responsible for this event .

4.2.3.2 The effect of PBOX-6, paclitaxel and nocodazole upon the expression levels of CDK1 in MCF-7 cells

MCF-7 cell lysates were prepared and subjected to western blotting using an antibody against CDK1 as described in Section 2.2.6. The expression levels of CDK1 remained relatively constant over time compared to the vehicle control following treatment with either PBOX-6, paclitaxel or nocodazole, (Figures 4.10 and 4.11 respectively). However, as was the case in the expression of both cyclin A, the expression of CDK1 decreased following 48 hours PBOX-6 and nocodazole treatment, again possibly due to the apoptosis induced by this time. Interestingly, paclitaxel did not have any effect upon the expression of CDK1 compared to the vehicle control over the time-course examined (Figure 4.11). This may be due to the fact that of the three compounds, paclitaxel induces the least apoptosis (Figure 4.2). Again, as the three compounds did not have any effect upon the expression level of CDK1 in the time-frame of PBOX-6, paclitaxel and nocodazole induced CDK1 kinase activity and subsequent G2/M arrest and apoptosis induction, this would suggest that CDK1 expression was not involved in initiating these events.

4.2.3.3 PBOX-6, paclitaxel and nocodazole alter the expression levels of cyclin B₁

Total cell lysates were subjected to western blotting using an antibody directed against cyclin B₁. PBOX-6, paclitaxel and nocodazole (10 μ M) were found to cause an early upregulation in the expression level of cyclin B₁ (Figures 4.12 and 4.13 respectively). An induction in expression over the vehicle was first seen after 1 hour of PBOX-6 (Figure 4.12) and after 1-2 hours of paclitaxel treatment (Figure 4.13), coincident with the PBOX-6 and paclitaxel induced upregulation of CDK1 kinase activity (Figures 4.5 and 4.6 respectively). Nocodazole elicited a marginally earlier upregulation in cyclin B₁

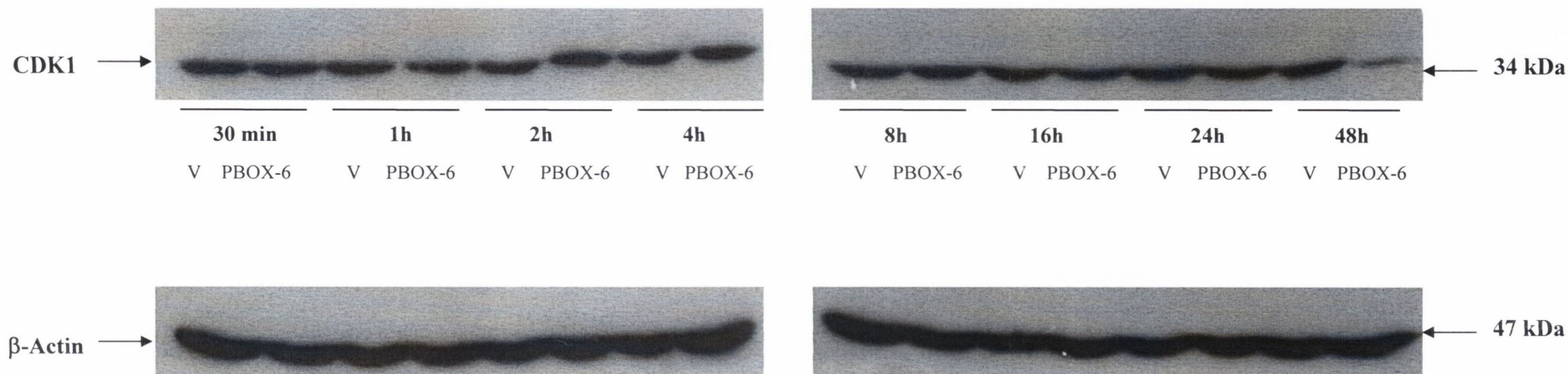


Figure 4.10 PBOX-6 causes a late downregulation in the expression levels of CDK1

Whole cell extracts were prepared as described in Section 2.2.6.1 from MCF-7 cells treated either with vehicle (0.5% (v/v) ethanol) or 10 μ M PBOX-6 for the indicated times. The protein (50 μ g) was resolved using SDS-PAGE, transferred onto PVDF and probed with anti-CDK1 antibody. The blots were stripped and re-probed with anti- β -actin antibody. The results shown are representative of 4 separate experiments.

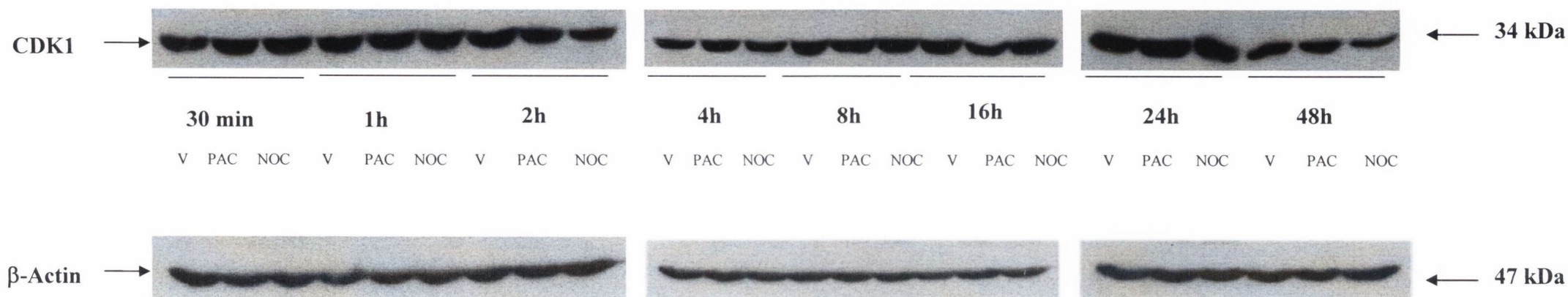


Figure 4.11 Effects of paclitaxel and nocodazole upon the expression levels of CDK1

Whole cell extracts were prepared as described in Section 2.2.6.1 from MCF-7 cells treated either with vehicle (0.5% (v/v) DMSO) or 10 μ M paclitaxel or nocodazole for the indicated times. The protein (50 μ g) was resolved using SDS-PAGE, transferred onto PVDF and probed with anti-CDK1 antibody. The blots were stripped and re-probed with anti- β -actin antibody. The results shown are representative of 3 separate experiments.

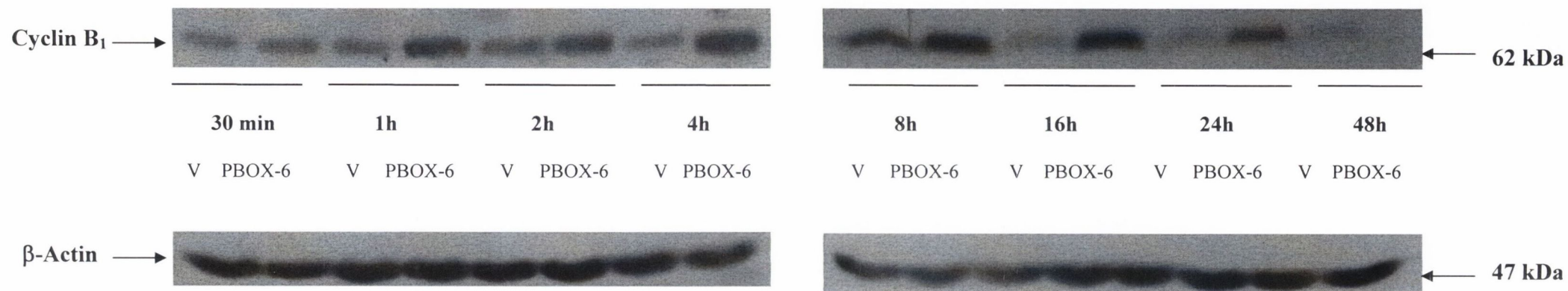


Figure 4.12 PBOX-6 causes an upregulation in the expression levels of cyclin B₁

Whole cell extracts were prepared as described in Section 2.2.6.1 from MCF-7 cells treated either with vehicle (0.5% (v/v) ethanol) or 10 μ M PBOX-6 for the indicated times. The protein (50 μ g) was resolved using SDS-PAGE, transferred onto PVDF and probed with anti-cyclin B₁ antibody. The blots were stripped and re-probed with anti- β -actin antibody. The results shown are representative of 4 separate experiments.

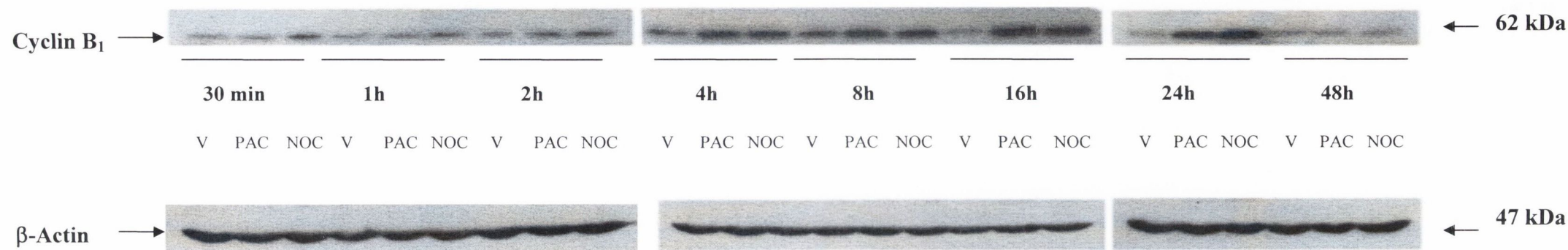


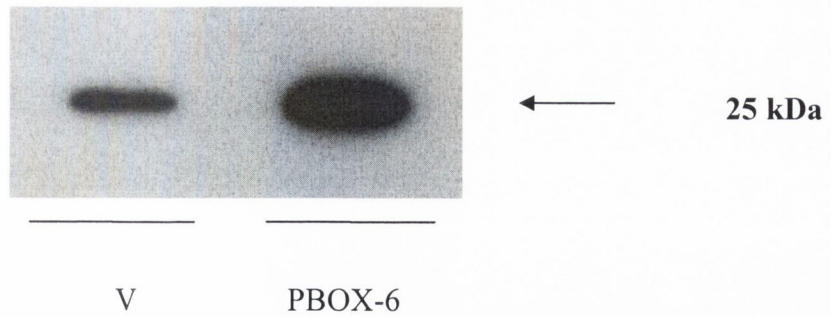
Figure 4.13 Paclitaxel and nocodazole cause an upregulation in the expression levels of cyclin B1

Whole cell extracts were prepared as described in Section 2.2.6.1 from MCF-7 cells treated either with vehicle (0.5% (v/v) DMSO) or 10 μ M paclitaxel or nocodazole for the indicated times. The protein (50 μ g) was resolved using SDS-PAGE, transferred onto PVDF and probed with anti-cyclin B₁ antibody. The blots were stripped and re-probed with anti- β -actin antibody. The results shown are representative of 3 separate experiments.

expression at 30 minutes (Figure 4.13), prior to nocodazole induced CDK1 kinase activity (Figure 4.7). The upregulation in expression of cyclin B₁ relative to the vehicle control was maintained up to 24 hours of PBOX-6, paclitaxel and nocodazole treatment, paralleling the profile of CDK1 kinase activity following treatment with these compounds. After 48 hours of treatment with all three compounds there was a marked downregulation in cyclin B₁ expression, again paralleling the downregulation in CDK1 kinase activity observed following 48 hours of treatment with all three compounds. Again, this may be as a consequence of the apoptosis caused by the compounds. As the upregulation in cyclin B₁ was seen prior to the onset of the G2/M arrest, it could be suggested that cyclin B₁ induced expression, and not that of cyclin A or CDK1, was at least partially responsible for the upregulation of CDK1 kinase activity and subsequent G2/M arrest as elicited by PBOX-6, paclitaxel and nocodazole treatment.

4.2.3.4 PBOX-6 stimulated CDK1 kinase activity is associated with cyclin B₁

In order to confirm that the PBOX-6 induced increase in CDK1 kinase activity was associated with an increased expression of cyclin B₁, MCF-7 cells were treated with either vehicle or 10 μ M PBOX-6 for 16 hours, as CDK1 kinase activity and G2/M arrest are at a maximum at this time. Cyclin B₁ was then immunoprecipitated from the lysate and an *in vitro* kinase assay performed on the immunoprecipitate (as described in Sections 2.2.7.2 and 2.2.8 respectively) using Histone H1 as the substrate. If CDK1 was associated with the immunoprecipitated cyclin B₁, it would phosphorylate the Histone H1 substrate used. It was found that CDK1 kinase activity was associated with the immunoprecipitated cyclin B₁ (Figure 4.14), thus the induction in CDK1 kinase activity may be a consequence of the accumulation of cyclin B₁ following PBOX-6 treatment. As it is known that the entrance to and exit from mitosis is regulated by cyclin B₁/CDK1 kinase activity, and that mitotic arrest is associated with an accumulation of cyclin B₁ and thus an increase in CDK1 kinase activity, these results may suggest that PBOX-6 is arresting the cells specifically in mitosis. In summary, PBOX-6 elicited comparable effects upon MCF-7 cell cycle kinetics, CDK1 kinase activity



(a) Cyclin B₁ associated CDK1 kinase activity



(b) Cyclin B₁

Figure 4.14 PBOX-6 stimulated CDK1 kinase activity is associated with cyclin B₁

Whole cell extracts were prepared from MCF-7 cells as described in Section 2.2.6.1 following treatment with 10 μ M PBOX-6 for 16 hours. The protein (200-500 μ g) was immunoprecipitated with anti-cyclin B₁ antibody and protein A beads and then incubated with Histone H1 substrate and γ^{32} P-ATP and resolved by SDS-PAGE (a). A sample of the immunoprecipitated protein was also resolved using SDS-PAGE, transferred to a PVDF membrane and probed with anti-cyclin B₁ antibody (b). The results shown are representative of 2 separate experiments.

and G2/M cell-cycle regulatory proteins as the two representative antimicrotubule agents, paclitaxel and nocodazole. This suggests that PBOX-6 could potentially be member of this class of compounds.

4.2.3.5 Actinomycin D and cycloheximide inhibit PBOX-6 induced upregulation in the expression levels of cyclin B1 and prevents G2/M arrest

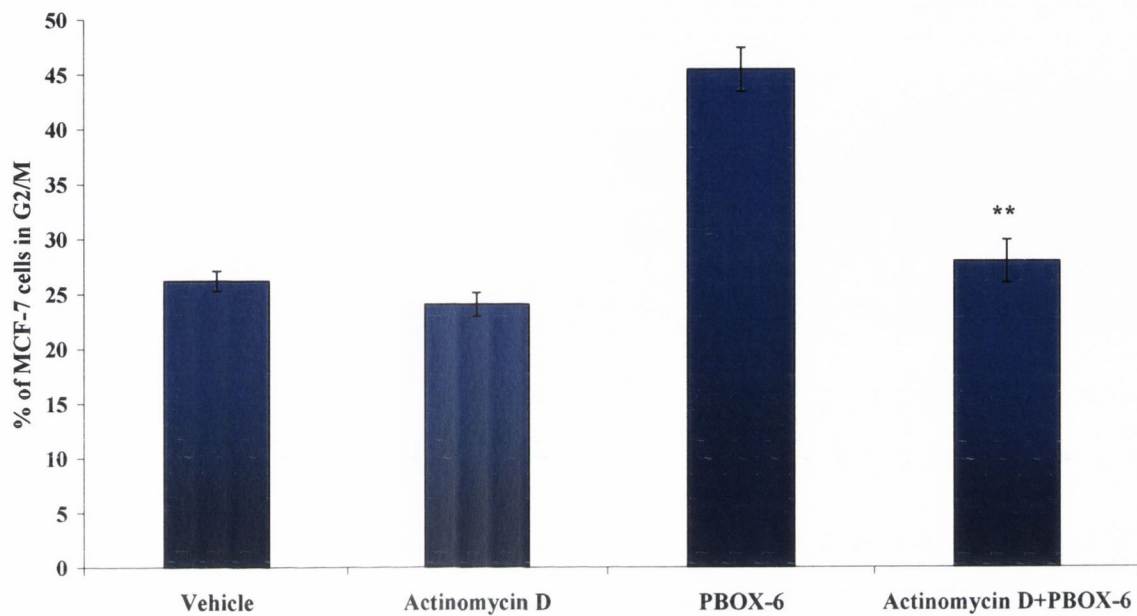
To determine if the early accumulation of cyclin B₁ is important to the mechanism underlying PBOX-6 induced G2/M arrest, MCF-7 cells were pretreated for an hour with the RNA synthesis inhibitor actinomycin D (5μM), and the protein synthesis inhibitor cycloheximide (3.19μM). After this time, the cells were treated for 8 hours with either vehicle (0.5% (v/v) ethanol) or 10μM PBOX-6. Treatment for longer times was not possible as the RNA and protein synthesis inhibitors, by themselves, both resulted in cell death. After the 8 hour treatment, the cells were prepared for both western blotting and flow cytometric analysis. Both actinomycin D and cycloheximide were found to inhibit the upregulation of cyclin B₁ by PBOX-6 (Figures 4.15 (a) and 4.16 (a) respectively). Correspondingly, actinomycin D and cycloheximide also significantly inhibited PBOX-6 induced G2/M arrest (Figures 4.15 (b) and 4.16 (b) respectively). Due to the low incidence of apoptosis after 8 hours of PBOX-6 treatment (6%), a significant inhibition in apoptosis was not observed. Therefore, it would appear that *de novo* synthesis and expression of cyclin B₁, may be at least in part, essential for the initiation of PBOX-6 mediated G2/M arrest.

4.2.4 The effect of PBOX-6 upon JNK kinase activity in MCF-7 cells

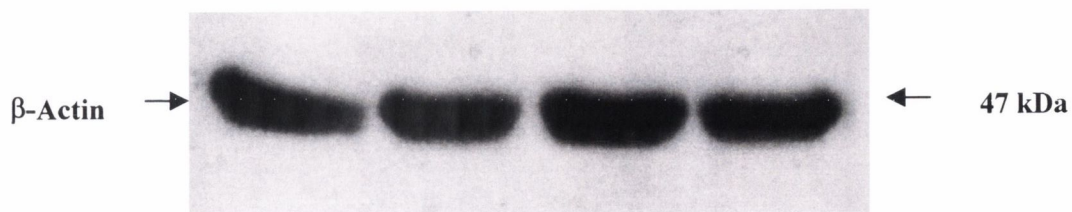
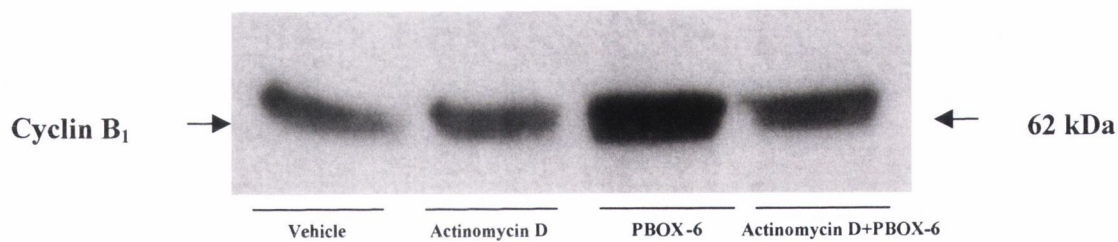
Antimicrotubule agents have been shown to activate JNK in a variety of human cells (Wang *et al.*, 1998). The JNK signalling pathway is known to play an important role in the G2/M transition in mammalian cells (Wright *et al.*, 1999) and respond to various stress-related stimuli resulting in involvement in initiation of apoptosis in many cell types (Green *et al.*, 1997). Mc Gee *et al.* (2002) has previously reported that the JNK signalling pathway is essential during PBOX-6 induced apoptosis in CML and HL-60 cells. Therefore the ability of PBOX-6 to

Figure 4.15 Actinomycin D inhibits PBOX-6 induced upregulation of cyclin B₁ expression levels and G2/M arrest

MCF-7 cells were seeded at a density of 3×10^6 cells in 75cm² flasks in 15mls of complete medium and were incubated at 37°C under a humidified atmosphere of 95% O₂ and 5% CO₂ for 24 hours until they were in the exponential phase of growth. The cells were pretreated with 5µM actinomycin D for 1 hour and then treated either with vehicle (0.5% (v/v) ethanol) or 10µM PBOX-6 for a further 8 hours. The cells were harvested as described in Section 2.2.1, and an aliquot of the cell suspension was prepared and analysed using flow cytometry as described in Section 2.2.5. (a) represents the cells in the G2/M phase of the cell cycle. The results represent the mean $\pm$ S.E.M of 3 separate experiments. Statistical analysis was carried out using the Instat computer program. **p<0.01 with respect to PBOX-6, Students t-test. The remaining cells were lysed as described in Section 2.2.6.1, resolved using SDS-PAGE, transferred onto PVDF and probed with an anti-cyclin B₁ antibody (b). The blots were stripped and re-probed with anti-β-actin antibody. The results shown are representative of 3 separate experiments.



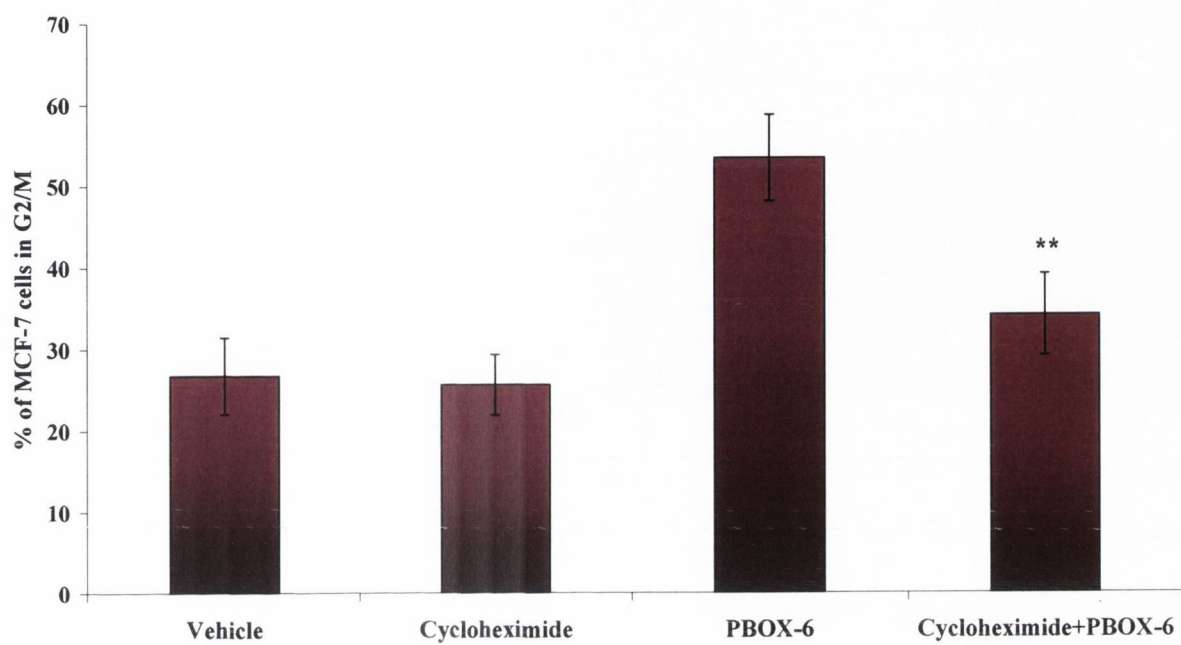
(a)



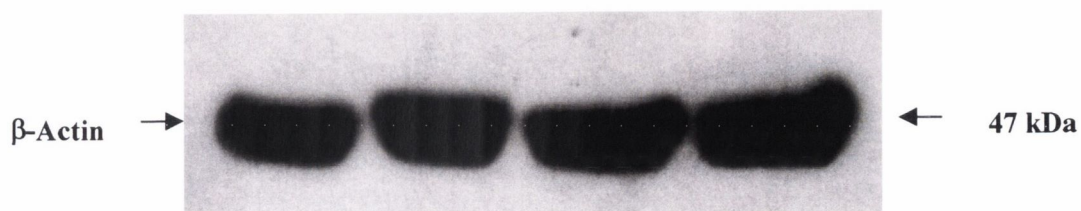
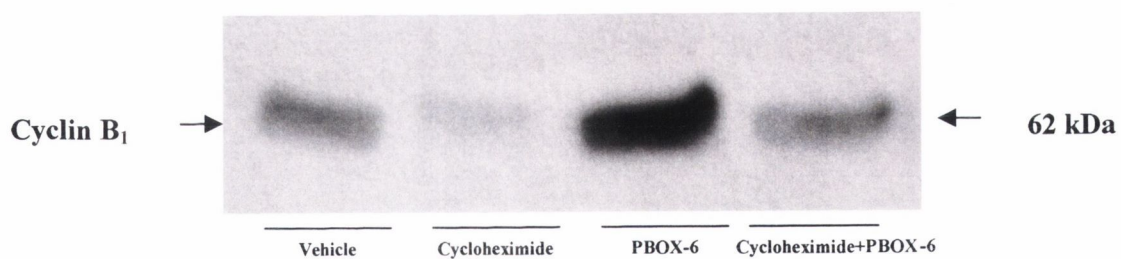
(b)

Figure 4.16 Cycloheximide inhibits PBOX-6 induced upregulation in cyclin B₁ expression levels and G2/M arrest.

MCF-7 cells were seeded at a density of 3×10^6 cells in 75cm² flasks in 15mls of complete medium and were incubated at 37°C under a humidified atmosphere of 95% O₂ and 5% CO₂ for 24 hours until they were in the exponential phase of growth. The cells were pretreated with 3.19µM cycloheximide for 1 hour and then treated either with vehicle (0.5% (v/v) ethanol) or 10µM PBOX-6 for a further 8 hours. The cells were harvested as described in Section 2.2.1, and an aliquot of the cell suspension was prepared and analysed using flow cytometry as described in Section 2.2.5. (a) represents the cells in the G2/M phase of the cell cycle. The results represent the mean $\pm$ S.E.M of 3 separate experiments. Statistical analysis was carried out using the Instat computer program. **p<0.01 with respect to PBOX-6, Students t-test. The remaining cells were lysed as described in Section 2.2.6.1, resolved using SDS-PAGE, transferred onto PVDF and probed with an anti-cyclin B₁ antibody (b). The blots were stripped and re-probed with anti-β-actin antibody. The results shown are representative of 3 separate experiments.



(a)



(b)

4.2.4.1 PBOX-6 induces an upregulation in JNK kinase activity

MCF-7 cells were treated with 10 μ M PBOX-6 for the indicated times and cell lysates were prepared as described in Section 2.2.6.1. JNK was immunoprecipitated from the cell lysates and a kinase assay performed on the immunoprecipitate as described in Sections 2.2.6.2 and 2.2.6.3 respectively. It was found that PBOX-6 induced an upregulation in JNK kinase activity (Figure 4.17). There was a significant induction in JNK kinase activity after 5 minutes of PBOX-6 treatment and the activity steadily rose up to 30 minutes of treatment with PBOX-6 at which point there was an increase in JNK kinase activity of approximately 75%. After this time, the induction in activity was maintained over the remaining period of PBOX-6 treatment. This activation of the JNK kinase pathway was rapid in nature and preceded the accumulation of cyclin B₁ and upregulation of CDK1 kinase activity. Therefore, it may be at least partially responsible for mediating both G2/M arrest and apoptosis.

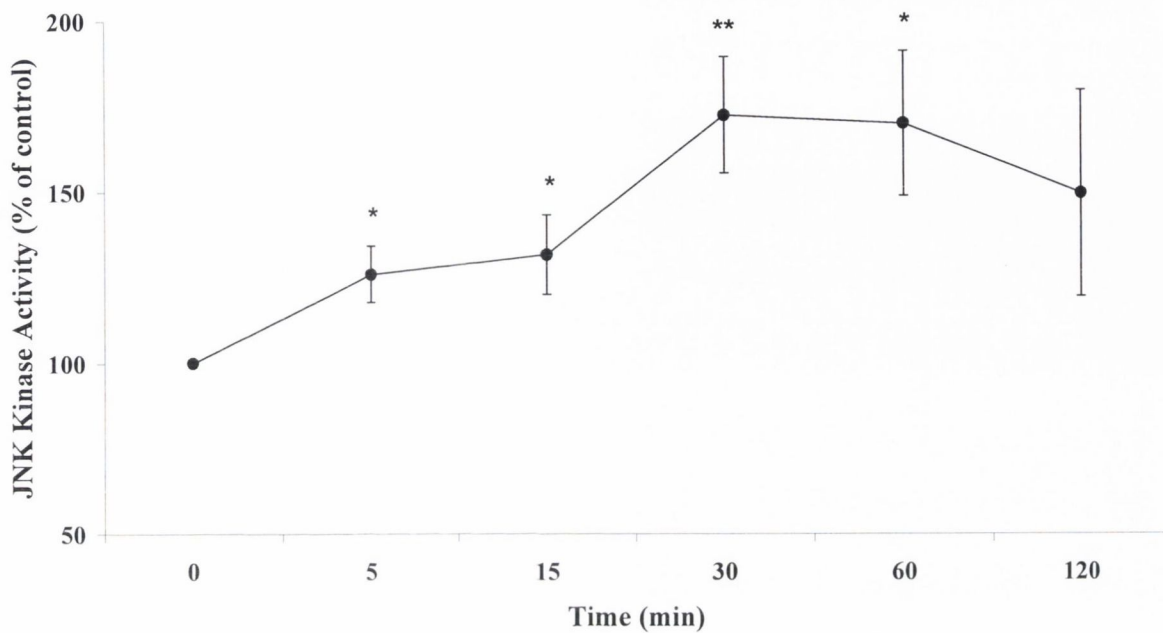
4.2.4.2 The JNK inhibitor, dicumarol, inhibits PBOX-6 induced JNK kinase activity and protects against PBOX-6 induced G2/M arrest and apoptosis

Dicumarol is a coumarin derivative that alters the redox centres of the cell. It has been shown to inhibit activation of JNK kinase in response to a variety of stress stimuli (Krause *et al.*, 2001). It was used in this study to determine the importance of JNK activation in PBOX-6 induced G2/M arrest and apoptosis.

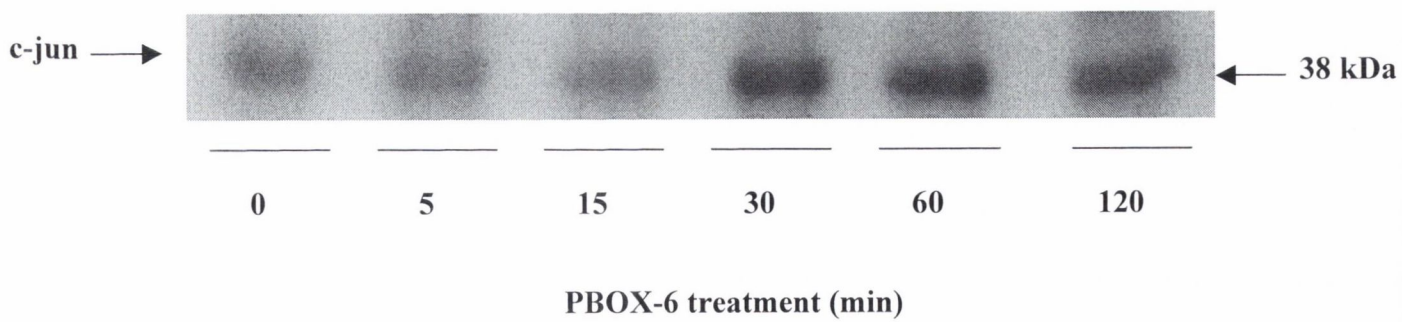
MCF-7 cells were pretreated with dicumarol (200 μ M) for 1 hour prior to treatment with either vehicle or 10 μ M PBOX-6 for a further 1 hour. The cells were then harvested, lysed and JNK was immunoprecipitated from the lysates (Section 2.2.6.1 and 2.2.6.2). A kinase assay was performed on the immunoprecipitate as described in Section 2.2.6.3. This revealed that dicumarol, as well as inhibiting basal levels JNK activity, did indeed inhibit PBOX-6 induced activation of JNK kinase activity, reducing activity to that with dicumarol on its own (Figure 4.18).

Figure 4.17 PBOX-6 induces an upregulation in JNK kinase activity

Whole cell extracts were prepared from MCF-7 cells as described in Section 2.2.6.1 following treatment with 10 μ M PBOX-6 for the indicated times. The protein (200-500 μ g) was immunoprecipitated with anti-JNK1 antibody and Protein A beads and then incubated with c-jun substrate and γ^{32} P-ATP and resolved by SDS-PAGE. Densitometry was performed on the resultant bands using Scion Image software with 0 hours set to 100%. The results represent the mean $\pm$ S.E.M of 3 separate experiments (a). Statistical analysis was carried out using the Instat computer program. * $p < 0.05$, ** $p < 0.01$ with respect to 0 hours, Students t-test. A representative kinase assay has been included (b).



(a)

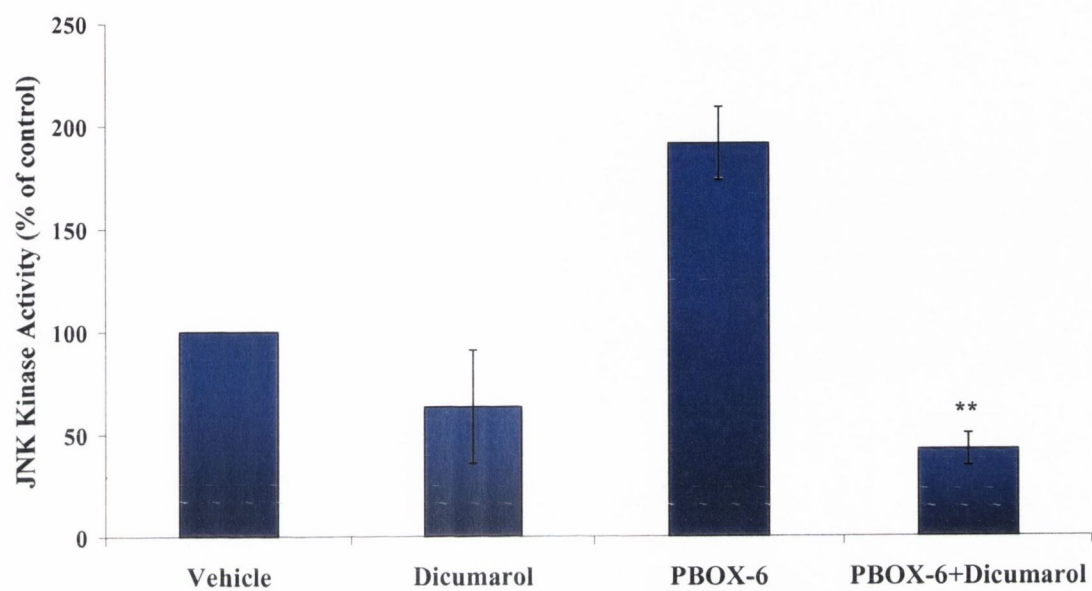


JNK Kinase Activity

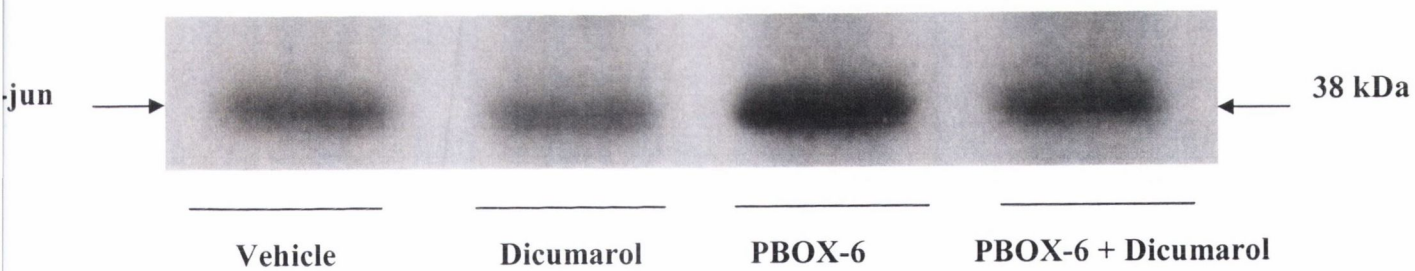
(b)

Figure 4.18 Dicumarol inhibits PBOX-6 induced JNK kinase activity

Whole cell extracts were prepared from MCF-7 cells as described in Section 2.2.6.1. The cells were treated either with vehicle (0.5% (v/v) ethanol), 200 μ M dicumarol or 10 μ M PBOX-6 for 1 hour or a pretreatment of 200 μ M dicumarol for 1 hour prior to treatment with 10 μ M PBOX-6. The protein (200-500 μ g) was immunoprecipitated with anti-JNK1 antibody and Protein A beads and then incubated with c-jun substrate and γ^{32} P-ATP and resolved by SDS-PAGE. Densitometry was performed on the resultant bands using Scion Image software with vehicle set to 100%. The results represent the mean $\pm$ S.E.M of 3 separate experiments (a). Statistical analysis was carried out using the Instat computer program. **p<0.01 with respect to PBOX-6, Students t-test. A representative kinase assay has been included (b).



(a)

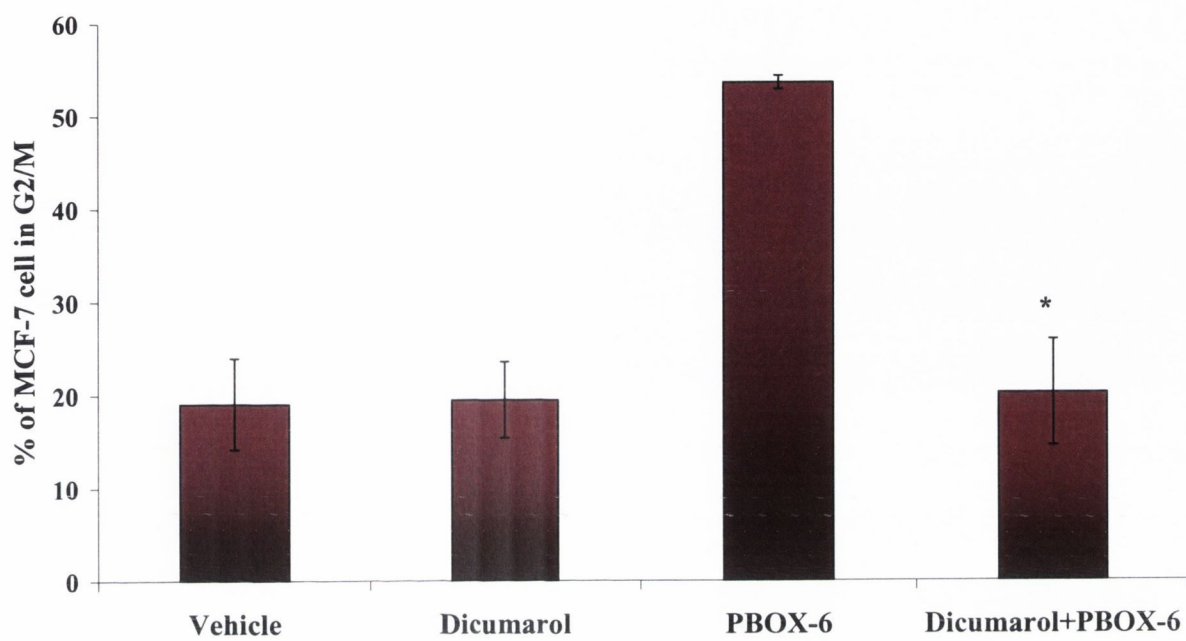


(b)

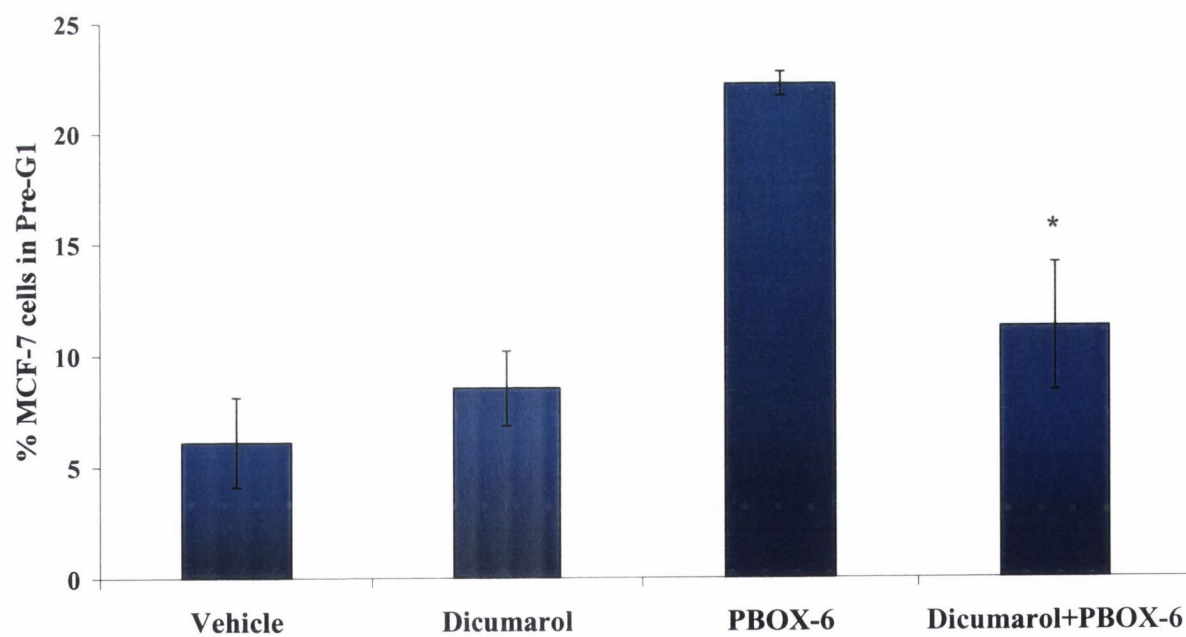
In order to determine if this inhibition of PBOX-6 induced JNK kinase activity inhibited the manifestation of G2/M arrest and apoptosis following PBOX-6 treatment, MCF-7 cells were again pretreated with dicumarol (200 μ M) for 1 hour and subsequently treated with either vehicle (0.5% (v/v) ethanol) or 10 μ M PBOX-6 for 24 hours. The cells were prepared for flow cytometric analyses as described in Section 2.2.5. Dicumarol inhibition of JNK kinase upregulation was found to protect against PBOX-6 induced G2/M arrest and apoptosis (Figure 4.19 (a) and (b) respectively). PBOX-6 induced G2/M arrest was completely inhibited in the dicumarol pretreated cells. Therefore, it would appear that JNK kinase activation is integral to the pathway by which PBOX-6 induces G2/M arrest and apoptosis, and is in agreement with the study performed by McGee *et al.*, (2002) that showed that activation of the JNK kinase signalling pathway was essential during PBOX-6 induced apoptosis in CML cells.

Figure 4.19 Dicumarol protects against PBOX-6 induced G2/M arrest and apoptosis

MCF-7 cells were seeded at a density of 1×10^6 in 25cm² flasks in 5mls of complete medium and were incubated at 37°C under a humidified atmosphere of 95% O₂ and 5% CO₂ for 24 hours until they were in the exponential phase of growth. The cells were then treated for 24 hours with either vehicle (0.5% (v/v) ethanol), 200µM dicumarol or 10µM PBOX-6 or pre-treatment with 200µM dicumarol prior to treatment with 10µM PBOX-6. The cells were then harvested as described in Section 2.2.1, prepared and analysed using flow cytometry as described in Section 2.2.5. The results represent the mean $\pm$ S.E.M of three separate experiments. (a) represents the cells in the G2/M phase of the cell cycle, whilst (b) represents the cells in the pre-G1 phase of the cell cycle. Statistical analysis was carried out using the Instat computer program. * $p < 0.05$ with respect to PBOX-6, Students t-test.



(a)



(b)

4.3 Discussion

The present study found that a potent member of a novel series of pyrrollo-1,5-benzoxazepines, PBOX-6, was capable of inducing G2/M arrest and apoptotic cell death in the MCF-7 human breast carcinoma cell line. This display of potent cytostatic and apoptotic ability by PBOX-6 warranted further investigation into the mechanism by which PBOX-6 mediated its effects, as it may have potential as a novel anticancer agent.

Recently, an ever-increasing number of potentially cytotoxic natural products that interact with microtubules have been isolated (Hamel *et al.*, 1996). Microtubules are cytoskeletal structures assembled from heterodimers of α/β -tubulin. Microtubules are intrinsically dynamic in nature, and it is their dynamic properties that are crucial for them to carry out many of their cellular functions such as reorientation of the microtubule network when cells undergo migration or morphological changes and the dramatic microtubule rearrangement at the onset of mitosis (Joshi, 1998). The rapid microtubule dynamics in mitosis is thought to be critical for both the morphogenesis and activities of the bipolar mitotic spindle, which direct the alignment of chromosomes at the metaphase plate and their final segregation into two daughter cells. Because of this, there is now a tremendous interest in small organic molecules that modulate the dynamics of microtubules primarily as some of the known antimicrotubule agents are useful for chemotherapeutic management of certain kinds of tumours. One group of antimicrotubule agents, such as the taxanes and epothiloines, promote microtubule polymerisation at high concentrations. Another group, including nocodazole, the *vinca* alkaloids and colchicine, inhibit microtubule polymerisation at high concentrations and suppress microtubule dynamics at low concentrations (Wilson *et al.*, 1995). Although these different classes of microtubule inhibitors act differently, they all block cell cycle progression at the prometaphase, thus preventing onset of metaphase-anaphase transition. It is generally believed that these agents cause spindle damage or suppress spindle dynamics, consequently activating the spindle checkpoint to block cells at mitosis via an inappropriate induction of cyclin B₁/CDK1 activity and leading ultimately to apoptotic cell death (Amon, 1999; Blagosklonny *et al.*, 1997; Burke, 2000; Dumontet *et al.*, 1999; Wassmann *et al.*, 2001).

This study found that PBOX-6-induced apoptosis was temporally preceded by the G2/M arrest, suggesting that the arrest was possibly the cause of the apoptosis. MCF-7 cells were also treated with a representative microtubule polymeriser, paclitaxel, and a representative microtubule depolymeriser, nocodazole. The effects of these two compounds upon the cell cycle progression, G2/M cell cycle regulatory molecules and CDK1 kinase activity was determined and compared with that of PBOX-6. A concentration of 10 μ M was used for all three compounds so as to serve as point of reference between the compounds. Paclitaxel and nocodazole also induced a G2/M arrest and apoptosis in the MCF-7 cells, nocodazole and PBOX-6 eliciting the greatest accumulation of cells in the pre-G1 region, whilst paclitaxel on the other hand induced the largest G2/M arrest of the three compounds over time.

In order to confirm that the PBOX-6-induced increase in cells in the pre-G1 region was due to apoptotic cell death, the presence of other characteristic hallmarks of apoptosis were investigated following PBOX-6 treatment. One such biochemical hallmark of apoptosis is internucleosomal DNA cleavage into oligonucleosome-length fragments, and can be determined via TUNEL (Terminal deoxynucleotidyl Transferase mediated bio-dUTP Nick Labelling) staining as it is indicative of nuclease mediated double strand DNA breakage. TUNEL staining of MCF-7 cells showed that PBOX-6 induced cell death was accompanied by DNA fragmentation. Another biochemical hallmark of apoptosis is cleavage of full length PARP as it is a common target of activated proteases called caspases during apoptotic cell death. It was also found that treatment of MCF-7 cells with PBOX-6 resulted in PARP cleavage. Both these sets of data confirm that PBOX-6 induced cell death is apoptotic in nature.

In this study, it was found that treatment of MCF-7 cells with PBOX-6, paclitaxel and nocodazole resulted in an increase in CDK1 kinase activity. This induction was observed rapidly, following 1 hour of treatment with all three compounds, preceding the induction of G2/M arrest, implying its involvement in the initiation of this event. Following PBOX-6 treatment, CDK1 kinase activity increased up to 4 hours, whilst paclitaxel and nocodazole treatment elicited an increasing CDK1 kinase activity up to 8 hours of treatment. After these times, the stimulation in activity was maintained up to 24 hours, corresponding with the

maximum arrest of all three compounds. As the G2/M arrest profiles paralleled the profile of induction of CDK1 activity following treatment with PBOX-6, paclitaxel and nocodazole, this would suggest that the sustained activation of CDK1 kinase activity by these compounds was essential for the cessation of the cell cycle at the G2/M transition. This is in agreement with previous reports in the literature (Donaldson *et al.*, 1994; King *et al.*, 1994).

An upregulation in CDK1 activity has previously been linked to apoptotic cell death. Shi *et al.*, (1994) proposed that premature CDK1 activation may be a general mechanism by which cells induced to undergo apoptosis initiate the disruption of the nucleus. Phosphorylation on Tyr15 of CDK1 by the ErbB2 receptor kinase inhibits CDK1 activation and has been shown to be involved in resistance to paclitaxel-induced apoptosis (Castedo *et al.*, 2002a; Tan *et al.*, 2002; Yu *et al.*, 1998). Sustained activation of CDK1 has been shown to be necessary for induction of apoptosis by the antimicrotubule agent, noscapine (Ye *et al.*, 2001). The activation of CDK1 has also been shown to participate in HIV-1-induced apoptosis, upstream of the p53-dependent mitochondrial permeabilisation step (Castedo *et al.*, 2002b). The next question is how sustained activation of CDK1 leads to apoptosis. Hyperphosphorylation of Bcl-2 has been observed in apoptotic induction by antimicrotubule agents (Haldar *et al.*, 1995; MacCarthy-Morrogh *et al.*, 2000; Yamamoto *et al.*, 1999). Since phosphorylation of Bcl-2, which is thought to negatively regulate its anti-apoptotic effects, can promote apoptotic cell death (Haldar *et al.*, 1995; Yamamoto *et al.*, 1999), CDK1 might be involved in a phospho-regulatory pathway that can promote Bcl-2 phosphorylation and eventually apoptosis.

In order to address the mechanism by which CDK1 kinase activity was being stimulated, the expression levels of both CDK1 itself, and the cyclins known to associate with it, namely cyclin A and cyclin B₁, were next analysed. It was found that treatment with PBOX-6, paclitaxel and nocodazole caused a late downregulation in the expression levels of cyclin A, suggesting that this reduction in expression was a secondary response to the high incidence of apoptosis found after 24 and 48 hours of treatment with these compounds. PBOX-6 and nocodazole also caused a late downregulation in the expression of CDK1 itself, whilst paclitaxel had no effect upon CDK1 expression. Therefore, as there was no alteration in the protein expression of either cyclin A or CDK1 prior to or co-

incident with the increase in CDK1 kinase activity following treatment with PBOX-6, paclitaxel and nocodazole, it can be concluded that neither of these proteins contribute towards the observed induction in CDK1 kinase activity.

Analysis of the expression of cyclin B₁, did however, reveal that there was a marked increase in cyclin B₁ accumulation in response to PBOX-6, paclitaxel and nocodazole treatment. The increase in cyclin B₁ expression following PBOX-6 and paclitaxel treatment, paralleled exactly the increase in CDK1 kinase activity, with an initial induction seen following 1 hour of treatment. Nocodazole treatment resulted in an increase in cyclin B₁ expression after 30 minutes, immediately prior to nocodazole induced CDK1 kinase activity at 1 hour. The upregulation in expression was maintained up to 24 hours of treatment for all three compounds. The expression level was absent entirely after 48 hours, again possibly as a consequence of the apoptosis caused at this time. Immunoprecipitation experiments confirmed that the PBOX-6 induced CDK1 kinase activity was associated with cyclin B₁. Therefore, it would appear that the activation of CDK1 kinase activity in MCF-7 cells in response to PBOX-6, is mediated, at least in part, by a co-incident accumulation of the expression in its CDK partner, cyclin B₁. The expression of cyclin B₁ and G2/M arrest induced by antimicrotubule agents has previously been shown to be abrogated following treatment with the RNA synthesis inhibitor, actinomycin D and the protein synthesis inhibitor, cycloheximide (Ling *et al.*, 1998; Park *et al.*, 2001). Pretreatment of MCF-7 cells with actinomycin D and cycloheximide, inhibited the PBOX-6 induced upregulation in the expression of cyclin B₁. Both of these inhibitors were also found to significantly inhibit PBOX-6 induced G2/M arrest, implying that the *de novo* synthesis and expression of cyclin B₁, may at least in part, be essential for the initiation of PBOX-6 mediated G2/M arrest. This is in agreement with the work presented by Murray *et al.*, (1989) which demonstrated that the translation of cyclin B mRNA leading to cyclin synthesis was necessary and sufficient to induce both the morphological and biochemical events characteristic of mitosis within *Xenopus* embryos. It was not possible to assess the importance of the synthesis and expression of cyclin B₁ to the onset of apoptosis as PBOX-6, paclitaxel and nocodazole only induce a significant apoptotic response after a 24 hour treatment and such a long treatment with cycloheximide and actinomycin D is cytotoxic to the cells.

Activation of the JNK signalling pathway has been mechanistically implicated in the regulation of apoptosis. The role played by JNK is complicated by its ability to also prevent apoptosis (Chen *et al.*, 1998; Meriin *et al.*, 1998; Zanke *et al.*, 1998). Therefore, the exact nature of the involvement of JNK in apoptosis is not at all straightforward and appears to depend on both cell type and the apoptosis-triggering stimuli (Ip *et al.*, 1998; Karin *et al.*, 1997). A role for JNK signalling in the response of cells to antimicrotubule agents has been suggested. JNK activity has been shown to be increased by antimicrotubule agents paclitaxel, vinblastine, vincristine, nocodazole, and colchicine (Lee *et al.*, 1998; Shtil *et al.*, 1999; Wang *et al.*, 1999; Wang *et al.*, 1998), and inhibition of JNK inhibits paclitaxel induced apoptosis (Lee *et al.*, 1998; Wang *et al.*, 1999). Yamamoto *et al.*, (1999) found that the ASK1/JNK pathway is responsible for Bcl-2 phosphorylation at mitosis in paclitaxel-treated Jurkat cells. In addition, Mc Gee *et al.*, (2002a) previously reported that the JNK signalling pathway is essential during PBOX-6 induced apoptosis in K562 and HL-60 cells. PBOX-6 was seen to induce JNK activation within the K562 cells after 15 minutes of treatment. This PBOX-6 induced upregulation of JNK activity peaked following 30-45 minutes of treatment and slowly declined over 8 hours to basal levels. Pretreatment of K562 cells with the JNK inhibitor, dicoumarol, abolished PBOX-6-induced phosphorylation of the JNK substrates, c-Jun and ATF-2, and inhibited the induced apoptosis, suggesting that JNK activation is an essential component of the apoptotic pathway induced by PBOX-6 (Mc Gee *et al.*, 2002a). Therefore the ability of PBOX-6 to activate the JNK pathway and its role in the mediation of PBOX-6 induced G2/M arrest and apoptosis within MCF-7 cells was assessed. It was found that PBOX-6 treatment resulted in a rapid and significant upregulation in JNK kinase activity. The onset of this induction was seen after only 5 minutes of PBOX-6 treatment, earlier than was observed by Mc Gee *et al.*, (2002a). However, similar to the profile observed by Mc Gee *et al.*, (2002a), the upregulation in JNK kinase activity reached a peak at 30 minutes of treatment. Both paclitaxel and nocodazole have previously been reported to activate JNK within MCF-7 cells (Lee *et al.*, 2003; Zhang *et al.*, 2002). The rapid upregulation in JNK kinase activity preceded the increase in CDK1 kinase activity and its associated accumulation of cyclin B₁ expression, possibly implying a role in the mediation of the G2/M arrest and apoptosis induced by PBOX-6. Apoptosis itself

can be considered as a form of stress, hence JNK activation may be a stress response secondary to apoptosis rather than a primary mediator in the apoptotic pathway. However, as this upregulation in JNK activity reached a peak when apoptosis was still minimal, it would suggest that it was not a secondary response to PBOX-6 induced apoptosis.

To determine the importance of this induction in JNK activation in mediating the PBOX-6 induced G2/M arrest and apoptosis, the MCF-7 cells were pretreated with the JNK inhibitor, dicumarol. Dicumarol was shown to completely inhibit the activation of JNK kinase following PBOX-6 treatment and consequently protected against the induction of G2/M arrest and apoptosis by PBOX-6.

In summary, the data presented in this study demonstrated that PBOX-6 is efficacious in suppressing cell growth and inducing apoptotic cell death in the MCF-7 human breast carcinoma cell line. It appeared to do so by inducing a sequential upregulation in JNK activity and cyclin B₁ associated CDK1 kinase activity. The rapid activation of the JNK kinase signalling pathway would appear to be important in the mechanism of PBOX-6 arrest and apoptosis as its inhibition protected against these effects. The results presented in this chapter demonstrated that PBOX-6 also elicited similar effects upon MCF-7 cell cycle kinetics, CDK1 kinase activity and G2/M cell-regulatory proteins to two representative antimicrotubule agents, suggesting that PBOX-6 may be a member of this class of antineoplastic agents.

The clinical applicability of microtubule targeting drugs is hampered by their low aqueous solubility and toxicity to non-cancerous cells. Moreover, although patients have impressive initial responses to these drugs, many of them relapse after treatment because of the development of drug resistance contributed by multifactorial mechanisms, such as overexpression of P-glycoprotein (Gottesman *et al.*, 1993), altered expression of tubulin isotypes (Burkhart *et al.*, 2001) and the presence of tubulin mutations (Giannakakou *et al.*, 1997). Consequently, there is a huge requirement for the development and/or discovery of novel antimicrotubule agents that have similar modes of action to the currently used drugs but with improved biological and pharmacological features, thus underlying the importance of further characterisation of the mechanism of action of PBOX-6.

5 PBOX-6: A NOVEL ANTIMICROTUBULE AGENT?

5.1 Introduction

It was concluded in the previous chapter that PBOX-6 induces G2/M arrest and apoptosis via a molecular mechanism similar to that of two representative antimicrotubule agents, nocodazole and paclitaxel, suggesting that PBOX-6 may be a novel member of this class of compounds. Tubulin-binding agents constitute a large family of compounds that have been used in a wide variety of ways, including acting as herbicides and antiparasitics and in human therapeutics. The first antimicrotubule agent to be used in humans was colchicine, extracted from *colchicum autumnale*, which has been administered to patients with gout since the sixth century AD (Hastie, 1991). The ability of colchicine to block cells in prometaphase/metaphase made it a powerful tool in the study of mitosis (Malkinson, 1982). Tubulin, the building block of microtubules, was first identified as the “colchicine-binding protein” (Little *et al.*, 1988). In the field of antineoplastic chemotherapy, antimicrotubule agents constitute an important class of compounds, with broad activity in solid and in hematologic neoplasias (Crown *et al.*, 2000; Devizzi *et al.*, 1994; Dumontet *et al.*, 1999; Samson *et al.*, 1989). These agents are believed to block cell division by interfering with the function of the mitotic spindle, blocking the cells at the prometaphase/metaphase junction of mitosis (Jordan *et al.*, 1991; Jordan *et al.*, 1993). This makes cytotoxic antimicrotubule agents unique among anticancer drugs, as they target the mitotic spindle rather than DNA. *Vinca* alkaloids, the earliest antimicrotubule agents to be used clinically as antimetotics are classed as microtubule depolymerising agents. At high concentrations, these agents reduce or abolish the microtubule content of cells in culture and prevent polymerisation of purified tubulin *in vitro* (Dumontet *et al.*, 1999; Wilson *et al.*, 1995). Conversely, the taxanes promote the polymerisation of purified tubulin *in vitro* and at high concentrations enhance the fraction of polymerised tubulin in cells, and thus have been classed as microtubule polymerising/stabilising agents (Dumontet *et al.*, 1999; Wilson *et al.*, 1995).

Although antimicrotubule agents such as the *vinca* alkaloids and the taxanes may differ in their gross effect on cellular cytoskeleton in culture, these compounds seem to share a common mechanism of action, namely disruption of microtubule dynamics leading to aberrant formation of the mitotic spindle. The

segregation of the replicated chromosomes is brought about by the mitotic spindle, as it is a complex cytoskeletal machine constructed from microtubules and microtubule associated proteins (MAPs), which serves to pull the daughter chromosomes toward the poles of the spindle and move the poles apart. Prometaphase in animal cells begins abruptly with the breakdown of the nuclear envelope, triggered by cyclin B₁/CDK1 phosphorylation of the nuclear lamina that underlies the nuclear envelope (Luscher *et al.*, 1991). The disassembly of the nuclear envelope allows the microtubules access to the condensed chromosomes. The microtubules of the mitotic spindle attach to the chromosomes via a complex protein structure known as the kinetochore that binds to the centromere of the chromosome (Tanaka, 2002; Van Hooser *et al.*, 2001). Upon attachment, kinetochores move chromosomes along the mitotic spindle towards the poles, and this generates tension (Tanaka, 2002; Van Hooser *et al.*, 2001). A lack of proper chromosome attachment and spindle tension activates the spindle assembly checkpoint and arrests the cell cycle prior to the onset of chromosome segregation during anaphase, until these defects are corrected (Amon, 1999; Wassmann *et al.*, 2001).

Microtubules are intrinsically dynamic polymers and considerable evidence now indicates that the dynamics of mitotic spindle microtubules, not just their presence, are critically required for proper spindle function (Hayden *et al.*, 1990; Skibbens *et al.*, 1993). Microtubules are polymers composed of the protein tubulin, a heterodimer of two related polypeptides, α and β . They are tightly bound together by noncovalent bonds. A microtubule is a stiff, hollow cylindrical structure built from 13 parallel protofilaments composed of alternating subunits of α - and β -tubulin heterodimers. Complex helical proteins are stabilised by multiple subunit contacts that can then elongate rapidly by addition of more subunits. The initial process of assembly of the formative subunits or 'nucleus' is called nucleation. There is a kinetic lag associated with the formation of a microtubule nucleus due to the instability of the individual subunits. This kinetic lag is removed within the cell by the presence of the centrosome, which provides a template for the assembly of the tubulin units and thus acts as a microtubule organising centre. The microtubules are nucleated at their minus end, with the

plus end growing outward from each site of nucleation upon the centrosome to create a microtubule array.

Microtubules are not simple equilibrium polymers. They polymerise and depolymerise by the reversible addition and loss of tubulin dimers at the ends of the microtubules, and so are in unstable equilibrium with the tubulin dimers present in the cell. A compound's ability to abrogate the assembly of tubulin into microtubules can be assessed using a simple spectrophotometry based technique. The assembly of tubulin subunits into microtubule filaments can be followed by measuring the turbidimetry of the solution at 340nm. As the tubulin subunits are assembled into microtubule filaments, the absorbance/turbidimetry of the solution increases accordingly. There are two types of dynamic microtubule behaviour: treadmilling and dynamic instability. Treadmilling or flux, describes the net addition of tubulin at one microtubule end and the net loss of tubulin at the opposite end (Margolis *et al.*, 1978). The other dynamic behaviour, dynamic instability, is a stochastic switching between episodes of prolonged growing and shortening at both ends of the individual microtubules (Mitchison *et al.*, 1984). This phenomenon of dynamic instability is an essential property that makes microtubules some of the most plastic protein polymers in the cell (Mitchison *et al.*, 1984). The two ends of individual microtubules differ both structurally and kinetically, and the rates and durations of growing and shortening at one end, called the plus end, are much more extensive than the rates and durations of growing and shortening at the opposite or minus end (Horio *et al.*, 1986). Although both ends alternately grow or shorten, net growing occurs at the plus end and net shortening at the minus end. It is the simultaneous occurrence of both these actions that gives rise to treadmilling, and it is critical in the polar movement of chromosomes during mitosis (Margolis *et al.*, 1978).

Despite the interaction of tubulin with a large number of drugs that inhibit or promote its assembly into microtubules, a precise definition of drug binding sites on the protein has proved difficult. This is a consequence of the lack of success in crystallising the protein, probably because of its sequence and post-translational heterogeneity, its instability, and its tendency to form oligomers and polymers of highly aberrant morphology in the presence of many of these drugs. Therefore, despite considerable efforts, only three distinct binding sites of antimicrotubule agents have been partially characterised to date, i.e. the binding

sites of paclitaxel (Nogales *et al.*, 1998), vinblastine (Jordan *et al.*, 1986) and colchicine (Serrano *et al.*, 1984; Uppuluri *et al.*, 1993) on tubulin. The recent electron crystallographic determination of a relatively detailed structure for zinc-induced antiparallel tubulin protofilaments has provided insights into the paclitaxel site on these protofilaments, as paclitaxel was used to enhance their stability during data accumulation (Nogales *et al.*, 1998), and provided a scaffold on which to model other drug binding sites (Downing *et al.*, 1998). The ability of a compound to bind to the domains of paclitaxel, colchicine or vinblastine can be determined by inhibition studies, i.e. the ability of the test compound to displace a labelled (fluorescent or radiolabelled) compound from its binding site upon tubulin. The elucidation of the mode of action of these antimicrotubule agents, as well as their interaction with tubulin may lead to the synthesis of potentially more effective and specific anti-cancer drugs.

Aims of this chapter

The results of the last chapter indicated that the molecular mechanism by which PBOX-6 mediated its cytostatic and cytotoxic effects was similar to that elicited by two representative antimicrotubule agents, paclitaxel and nocodazole. This chapter, therefore, aimed to further characterise the mechanism of action of PBOX-6 and attempted to confirm the hypothesis that PBOX-6 is a novel member of the antimicrotubule class of agents. This firstly involved confirmation of PBOX-6 induced arrest as being specific to mitosis. The ability of PBOX-6 to disrupt the microtubule network and to bind tubulin directly was then also assessed. Finally, the ability of PBOX-6 to bind to known binding sites on tubulin was determined.

5.2 Results

5.2.1 The effect of PBOX-6, paclitaxel and nocodazole on the morphological features of MCF-7 cells

Results from the last chapter revealed that treatment of MCF-7 cells with PBOX-6, paclitaxel and nocodazole resulted in a pronounced G2/M arrest. Paclitaxel and nocodazole, as recognised antimicrotubule agents, are known to arrest the cell cycle in early mitosis, i.e. prometaphase/metaphase. PBOX-6 treatment was found to elicit similar effects on cell cycle kinetics as these antimicrotubule agents, and induce hallmarks associated with mitotic arrest such as an induction in cyclin B₁/CDK1 kinase activity. As a result, it was necessary to assess and compare the effects of PBOX-6, paclitaxel and nocodazole on the morphological features of MCF-7 cells, and determine if PBOX-6 does in fact arrest the cell in mitosis.

MCF-7 cells were seeded in a 24 well plate, incubated for 24 hours until the cells were in the exponential phase of growth and then treated for 8 hours with either PBOX-6, paclitaxel or nocodazole, as at this time there was a significant accumulation of cells in the G2/M phase and minimal apoptosis following treatment with all three compounds (See Figure 4.2). Following this time, the cells were centrifuged onto a slide and stained with the RapiDiff kit as described in Section 2.2.9. It was found that the vehicle treated cells were mostly in interphase and exhibited a full and rounded nucleus (Figure 5.1 (a)). Treatment with PBOX-6 and the antimicrotubule agents, paclitaxel and nocodazole, had a distinct effect upon the morphology of the MCF-7 cells compared with the vehicle treated cells. They all showed distinct signs of arrest in the prometaphase stage of mitosis, as the nuclear membrane had disappeared and the chromatin was contorted, as in prometaphase the chromatin twists and folds, eventually forming compacted chromosomes (Figure 5.1 (b), (c) and (d) respectively). Thus, PBOX-6, as well as paclitaxel and nocodazole, did arrest MCF-7 cells in mitosis, specifically during prometaphase, in agreement with the results presented in the last chapter. This confirms PBOX-6 as a mitotic inhibitor and lends further weight to the possibility

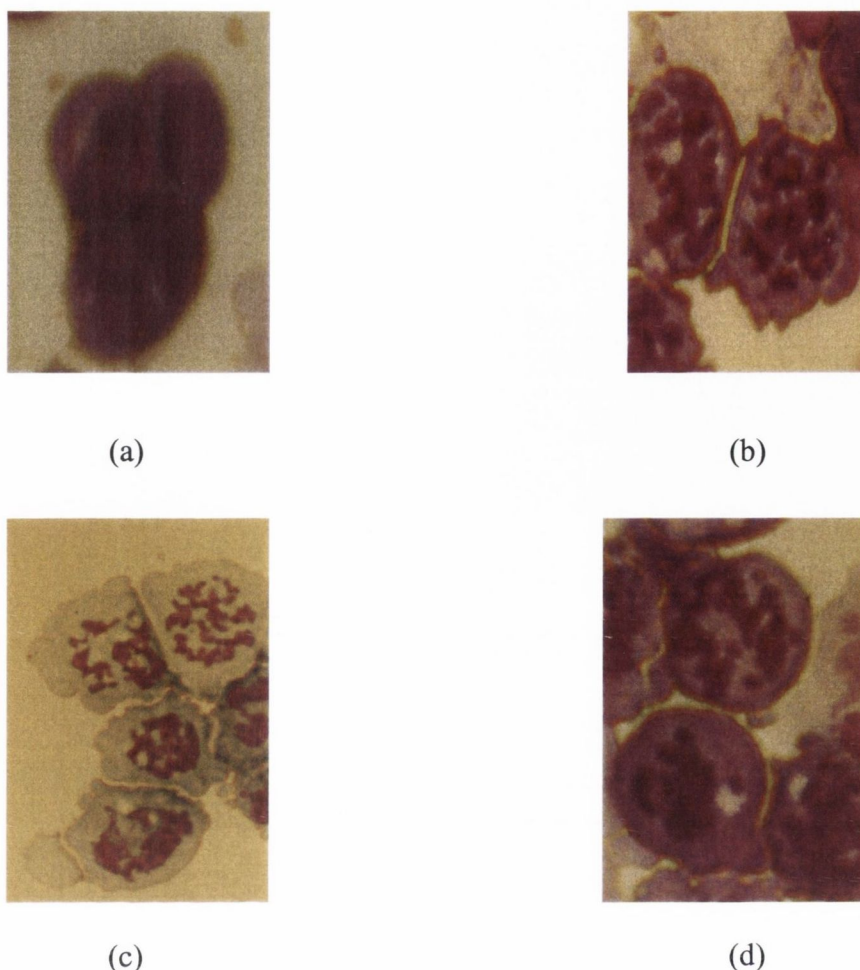


Figure 5.1 PBOX-6 treatment induces morphological features of prometaphase arrest in MCF-7 cells

Microscopic analysis of MCF-7 cells was performed following treatment with either vehicle (0.5% (v/v) ethanol (a)), 10 μ M PBOX-6 (b), 10 μ M nocodazole (c) or 10 μ M paclitaxel (d) for 8 hours. Following treatment, cells were centrifuged onto a glass slide and stained using the Rapi Diff kit as described in Section 2.2.9. The cytoplasm of the cells was stained pink by dipping the slide in eosin Y and the nucleus was stained blue by dipping the slide in methylene blue and visualised under a light microscope (Nikon) at a magnification of 40X. The results shown are representative of 3 separate experiments.

of it being a novel antimicrotubule agent.

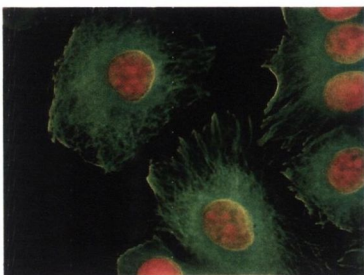
5.2.2 The effect of PBOX-6, paclitaxel and nocodazole upon cellular microtubules

5.2.2.1 PBOX-6 causes disruption of the microtubule network in a dose-dependent manner

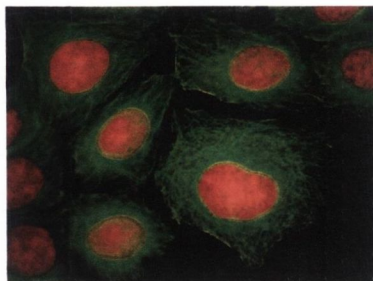
Antimicrotubule agents are known to target the cellular microtubule network, resulting in aberrant formation of the mitotic spindle, subsequent activation of the spindle assembly checkpoint, and resultant apoptotic cell death (Wassmann *et al.*, 2001). It was, therefore, necessary to determine if PBOX-6 had any effect on the organisation of the microtubule network of MCF-7 cells, as the pattern of effects elicited by PBOX-6 upon MCF-7 cells thus far assessed were very similar to that of antimicrotubule agents. MCF-7 cells were seeded in 24 well plates, incubated for 24 hours until in the exponential phase of growth, and then treated with 10 μ M PBOX-6, paclitaxel and nocodazole for 16 hours. The cells were then subjected to indirect immunofluorescence, as described in Section 2.2.10, allowing visualisation of the microtubule network (green immunofluorescence). Control cells (either 0.5% (v/v) ethanol or DMSO) (Figure 5.2 (a) ethanol, Figure 5.2 (e) and (i) DMSO) exhibited normal arrangement and organisation of the microtubule network, with microtubules seen to traverse intricately throughout the cell. The DNA content of the cells was also visualised via immunofluorescence (red), and control cells displayed a normal compact rounded nucleus, devoid of any microtubules. In contrast, treatment with all three compounds was seen to have very definite effects upon the microtubule network relative to control cells. Nocodazole, a known microtubule depolymeriser, caused a complete loss of the normal microtubule network with only a diffuse stain visible throughout the cytoplasm (Figure 5.2 (f)-(h)). Paclitaxel, on the other hand, exerts its effects by stabilising the microtubule network, resulting in a distinctive rigidity of the microtubules causing them to ultimately form microtubule ‘bundles’ as indicated by the arrows in Figure 5.2 (i)-(l). The DNA

Figure 5.2 PBOX-6, paclitaxel and nocodazole disrupt the organisation of MCF-7 cellular microtubule network

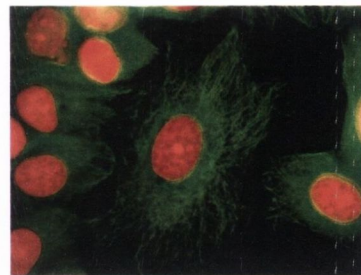
MCF-7 cells were treated with either vehicle (0.5% (v/v) ethanol (a) or DMSO (e) and (f)) or 100nM, 1 μ M or 10 μ M of PBOX-6 ((b), (c) and (d) respectively), nocodazole ((f), (g) and (h) respectively), or paclitaxel ((j), (k) and (l) respectively), for 16 hours. After this time, the medium was carefully removed and the cells fixed in cold methanol. Cells were incubated with monoclonal anti- α -tubulin antibody at room temperature for 1 hour at room temperature, and then incubated with FITC-conjugated anti-mouse secondary antibody for a further hour at room temperature. After washing, the cells were stained briefly with propidium iodide. The organisation of the microtubule network (green) and the cellular DNA (red) was visualised by Nikon PS200 fluorescence microscopy at a magnification of 100X. The results shown are representative of 3 separate experiments.



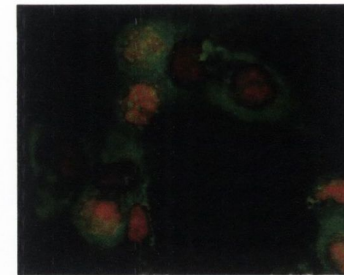
(a) Ethanol



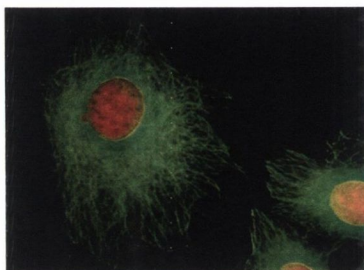
(b) 100nM PBOX-6



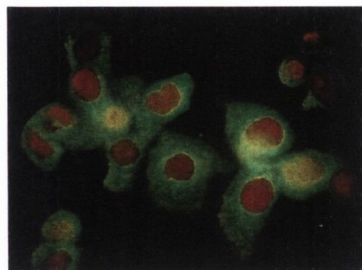
(c) 1μM PBOX-6



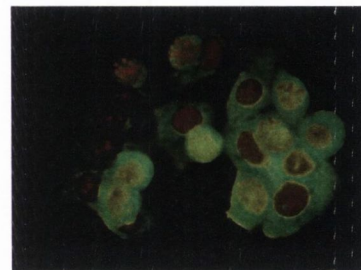
(d) 10μM PBOX-6



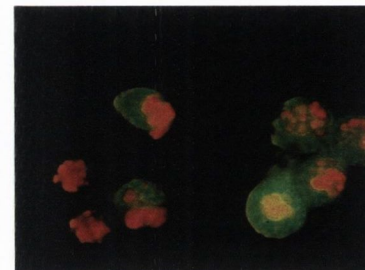
(e) DMSO



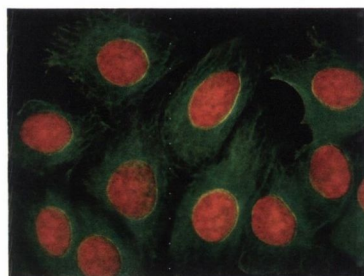
(f) 100nM nocodazole



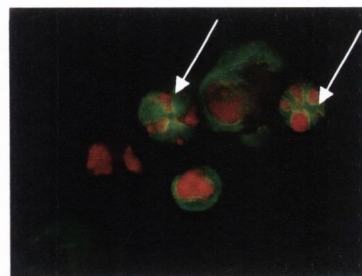
(g) 1μM nocodazole



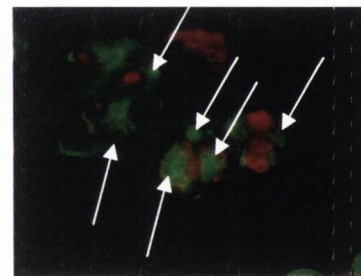
(h) 10μM nocodazole



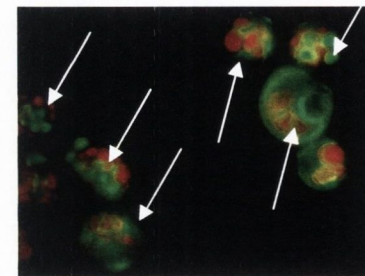
(i) DMSO



(j) 100nM paclitaxel



(k) 1μM paclitaxel



(l) 10μM paclitaxel

following treatment with 10 μ M nocodazole and paclitaxel was condensed and contorted, and the cells were shrunken and rounded up, characteristic and indicative of mitotic arrest.

PBOX-6 appeared to elicit its disruption upon the microtubule network in a manner similar to that of nocodazole as it too resulted only in a diffuse tubulin staining with no definition of structure (Figure 5.2 (d)). This was unlike 10 μ M paclitaxel treatment that results in a defined rigid bundling of the microtubules due to its stabilisation of microtubule polymerisation. The DNA of PBOX-6 treated cells is also condensed and contorted and agrees with the morphology seen in Figure 5.1 (b). Therefore, it would appear that PBOX-6 possibly elicits its effects through depolymerisation of cellular microtubules resulting in deformation of the mitotic spindle.

It is apparent that the effect of PBOX-6 upon cellular microtubules is dose-dependent in nature, as treatment with 100nM and 1 μ M PBOX-6 caused no disruption to the network of MCF-7 microtubules (Figure 5.2 (b) and (c) respectively). This is in agreement with the lack of any appreciable effect of these concentrations of PBOX-6 upon the incidence of G2/M arrest or apoptosis in MCF-7 cells (Figure 4.1 (a) and (b) respectively). Therefore, the effects of PBOX-6 upon the cellular microtubule network would appear to correlate with its effects upon cell cycle progression, suggesting that PBOX-6 induced mitotic arrest and apoptotic cell death may be mediated through its ability to inhibit microtubule assembly.

5.2.2.2 PBOX-21 has no effect upon the microtubule network, whilst a second pro-apoptotic PBOX compound, PBOX-15 disrupts the microtubule network

PBOX-21, a member of the antiproliferative subset of PBOX compounds, elicits a different cytological effect upon MCF-7 cells to that of PBOX-6. It has been shown in Section 3.2.1 that PBOX-21 induced a G1 arrest within the MCF-7 cells without any associated cytotoxicity. PBOX-6 induced mitotic arrest and apoptotic cell death may be mediated through its ability to inhibit microtubule assembly. To test the hypothesis that the differential response of the antiproliferative and pro-apoptotic subsets of PBOX compounds is based upon their ability to disrupt the microtubule network, the effect of PBOX-21, as well as a second more potent pro-apoptotic PBOX compound, PBOX-15 (personal

communication, Dr. M. McGee) on the microtubule network of MCF-7 cells was examined. It was found that, similar to PBOX-6 (10 μ M) (Figure 5.3 (b)), PBOX-15 (1 μ M) (Figure 5.3 (c)) appeared to induce a loss of the microtubule network of the cell. PBOX-21 (25 μ M) (Figure 5.3 (d)) had no effect upon the MCF-7 microtubule network. As such, PBOX-21 may be considered a negative control. This supports the theory that the mechanism of action of the pro-apoptotic PBOX compounds is mediated through inhibition of the microtubule network.

5.2.3 Effect of PBOX-6, PBOX-15, PBOX-21, nocodazole and paclitaxel on tubulin polymerisation in vitro

In order to confirm that PBOX-6 and PBOX-15 interact directly with tubulin causing depolymerisation of microtubules and that PBOX-21 does not interact with microtubules, the effect of PBOX-6, PBOX-15 and PBOX-21 upon tubulin polymerisation *in vitro* was studied, and compared with the effects of nocodazole and paclitaxel. The polymerisation of bovine tubulin in the presence of the compounds was observed at 37°C and monitored by an increase in absorbance at 340nm as described in Section 2.2.11. It was found that PBOX-6 inhibited the polymerisation of tubulin *in vitro* in a dose-dependent manner compared to vehicle control (0.5% (v/v) ethanol) (Figure 5.4 (a)). PBOX-15 was also found to inhibit polymerisation of tubulin *in vitro* (Figure 5.4 (b)). Both concentrations of PBOX-15 (0.2 and 1 μ M) elicited a similar extent of tubulin depolymerisation. The concentrations of PBOX-15 used differ from that of PBOX-6 as it is a more potent inducer of mitotic arrest and apoptosis (personal communication, Dr. Margaret Mc Gee). Nocodazole, a known tubulin depolymeriser, also inhibited the polymerisation of tubulin *in vitro* in a dose-dependent manner (Figure 5.4 (d)), confirming that PBOX-6 and PBOX-15 manifest their cellular effects through depolymerisation of tubulin, similar to nocodazole. Paclitaxel, as a known tubulin polymeriser was seen to

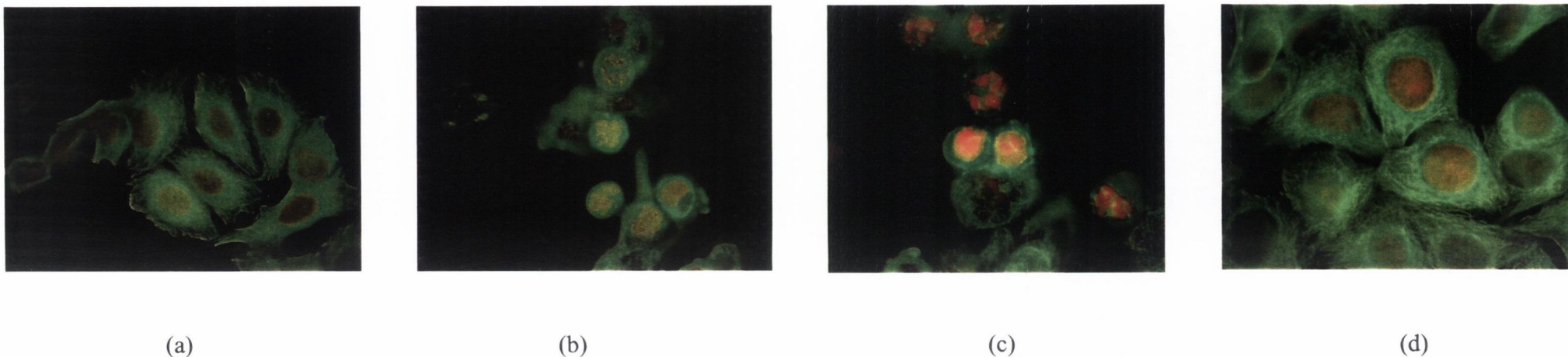
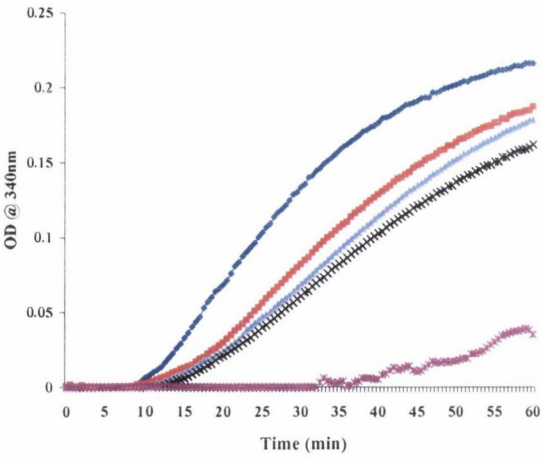


Figure 5.3 Effect of PBOX compounds upon the microtubule network

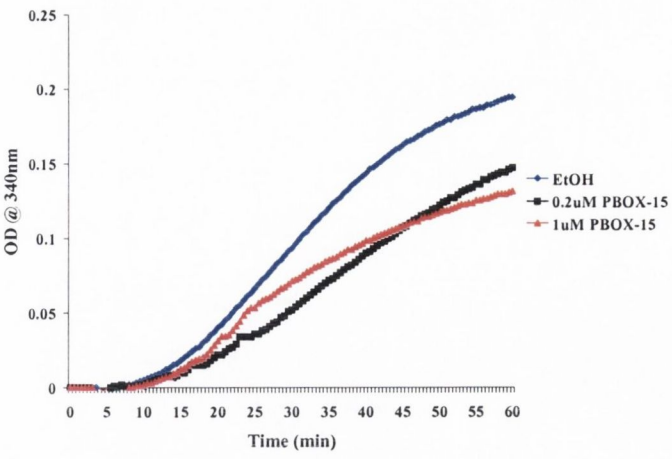
MCF-7 cells were treated with either vehicle (0.5% (v/v) EtOH) (a), 10 μ M PBOX-6 (b) 1 μ M PBOX-15(c) or 25 μ M PBOX-21 (d) for 24 hours. After this time, the cells were fixed in cold methanol, incubated with monoclonal anti- α -tubulin antibody and subsequently FITC-conjugated anti-mouse secondary antibody at room temperature for 1 hour. After washing, the cells were stained briefly with propidium iodide. The organisation of the microtubule network and the cellular DNA was visualised by Nikon PS200 fluorescence microscopy at a magnification of 100X. The results shown are representative of 3 separate experiments.

Figure 5.4 PBOX-6 and PBOX-15 cause a dose-dependent inhibition of polymerisation of tubulin, whilst PBOX-21 has a negligible effect upon polymerisation status of tubulin

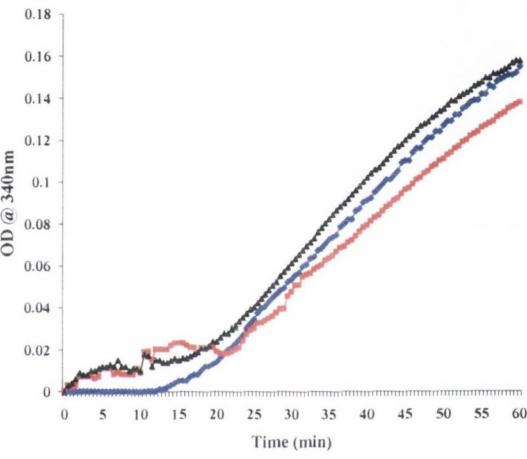
Purified bovine tubulin was incubated at 37°C in the presence of either vehicle (0.5% (v/v) ethanol or DMSO) or the indicated concentrations of PBOX-6 (a), PBOX-15 (b), PBOX-21 (c), nocodazole (d) or paclitaxel (e) as described in Section 2.2.11. Tubulin polymerisation was determined by measuring the increase in absorbance over time at 340 nm. The results shown are representative of three separate experiments.



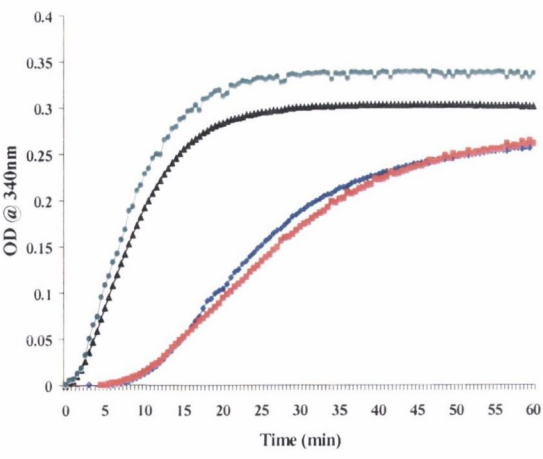
(a)



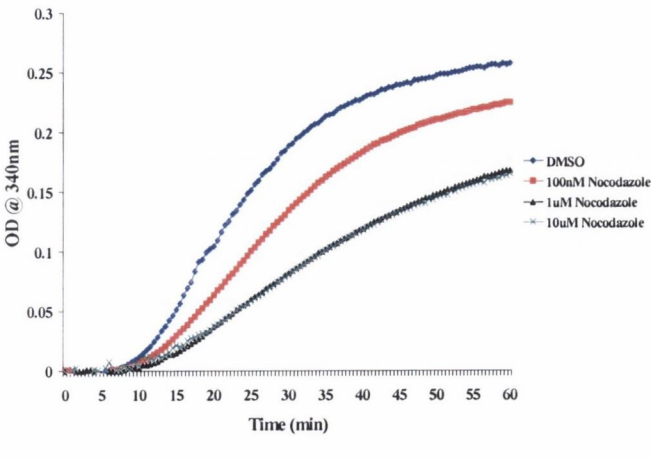
(b)



(c)



(d)



(e)

induce polymerisation of tubulin *in vitro* relative to the vehicle control (0.5% (v/v) DMSO) (Figure 5.4 (e)). In contrast, PBOX-21 was found to have a negligible effect upon the polymerisation of tubulin *in vitro* relative to the vehicle control (0.5% (v/v) EtOH) (Figure 5.4 (c)), in agreement with its lack of effect upon the cellular microtubule network. This again, distinguishes the mechanism of action of the antiproliferative and pro-apoptotic subsets of the PBOX compounds, and suggests tubulin as the molecular target of the pro-apoptotic PBOX members.

It is notable that both 100nM and 1 μ M PBOX-6 inhibited tubulin assembly *in vitro*, yet these concentrations of PBOX-6 did not cause any disruption to the cellular microtubule network as seen in Figure 5.2 (b) and (c) respectively. This may possibly be due to the fact that determination of the effect of these compounds upon tubulin polymerisation was carried out in an *in vitro* cell-free assay with pure tubulin, whilst the visualisation of the microtubule network was carried out *in situ* at a cellular level, and thus the effects of the lower concentrations of PBOX-6 upon tubulin polymerisation may be insufficient to manifest a gross structural change to the microtubule network as determined by indirect immunofluorescence. Also, the *in vitro* assay was carried out in the absence of Microtubule Associated Proteins (MAPs), which when present, are known to alter microtubule assembly dynamics by preventing disassembly.

5.2.4 PBOX-6 binds to a novel site on purified tubulin

As PBOX-6 has been shown to directly bind to and cause depolymerisation of purified tubulin, it was necessary to gain information about the binding site of PBOX-6 on tubulin. To date, only the binding sites of the depolymerisers colchicine (Serrano *et al.*, 1984; Uppuluri *et al.*, 1993) and vinblastine (Rai *et al.*, 1996) on tubulin have been well characterised. Compounds capable of depolymerising tubulin may bind to either of these two sites, or to an as yet uncharacterised novel site on tubulin.

Assessment of the binding of PBOX-6 to the colchicine binding site involved a fluorescence-based assay as described in Section 2.2.12. The assay operated on the basis of size-exclusion chromatography. Purified tubulin, being

the largest molecular component within the assay mixture with a molecular weight of 100,000, eluted first, approximately between fractions 6 and 10. If labelled colchicine was bound to tubulin, the fluorescent signal would be detected within these fractions. If the labelled colchicine had been displaced by a compound which also binds to the colchicine binding site, there would be a reduction in the fluorescent signal present within these fractions. This is due to the fact that fluorescently labelled colchicine has a much smaller molecular weight (789) than tubulin and therefore elutes much later from the column as it is retained in the interstitial volume of the column. As a positive control, both excess (100 μ M) unlabelled colchicine and nocodazole (10 μ M), which is known to bind to the colchicine binding site (Friedman *et al.*, 1978; Uppuluri *et al.*, 1993), displaced the binding of fluorescently-labelled colchicine relative to the vehicle control (0.5% (v/v) DMSO) (Figure 5.5). PBOX-6 at a concentration of 1 μ M and 10 μ M, did not displace the binding of labelled colchicine relative to the PBOX-6 vehicle control (0.5% (v/v) ethanol), therefore suggesting that PBOX-6 does not bind to the colchicine binding site on tubulin (Figure 5.5).

Determination of the ability of PBOX-6 to bind to the vinblastine binding site on tubulin, involved a modification of the technique used to assess binding to the colchicine binding site. Use of a BODIPY fluorescently labelled vinblastine compound supplied by Molecular Probes to determine binding to the vinblastine binding site was found to be untenable as it could not be displaced by an excess concentration of unlabelled vinblastine. Therefore, a [3 H]labelled vinblastine compound was used to determine displacement of vinblastine from its binding site on tubulin. The same size-exclusion principles applied to this assay as to the colchicine binding site assay. As a positive control, it was found that excess (100 μ M) unlabelled vinblastine was able to displace [3 H]vinblastine relative to the vinblastine vehicle control (0.5% (v/v) DMSO), causing a reduction in the radioactive signal in the fractions containing tubulin, i.e. fractions 5-9 (Figure 5.6). In contrast, neither 1 μ M or 10 μ M PBOX-6 caused a reduction of the radioactive signal relative to the PBOX-6 vehicle control (0.5% (v/v) ethanol) in the fractions containing tubulin, suggesting that PBOX-6 did not displace vinblastine from, and so does not bind to, the vinblastine binding site. As such

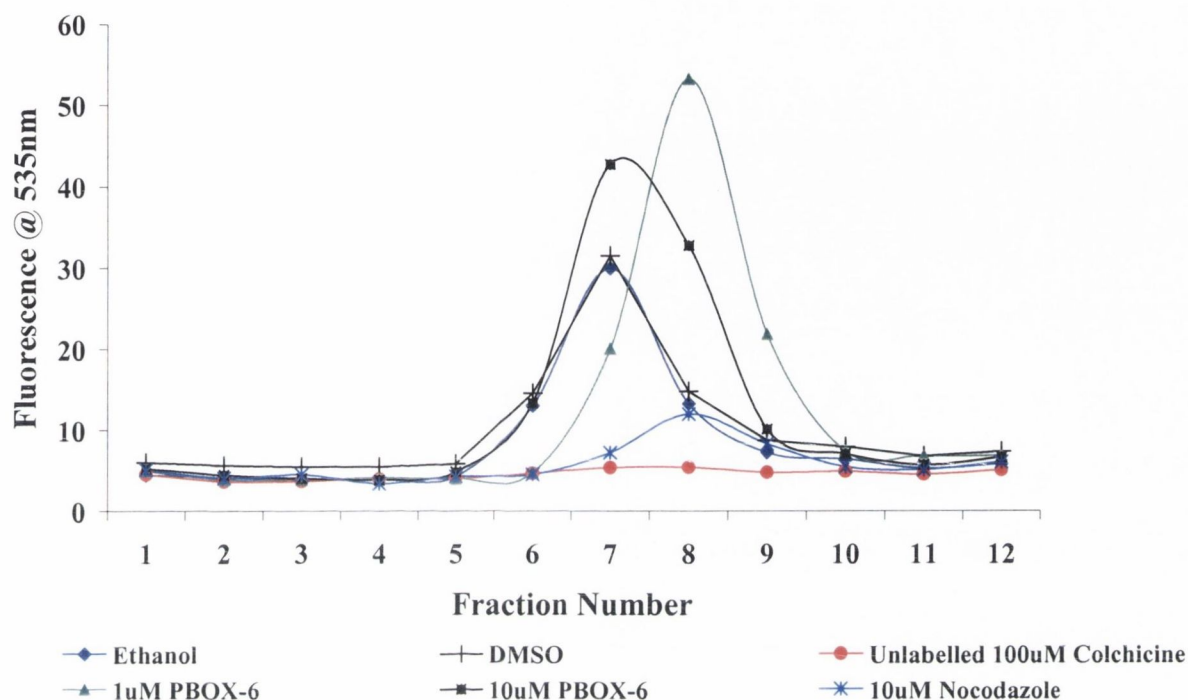


Figure 5.5 PBOX-6 does not bind to the colchicine binding site on purified tubulin

Each reaction mixture contained 1mg/ml >99% purified bovine tubulin, 0.25mM PIPES buffer at pH 6.9, containing 0.05mM GTP, and 0.25mM MgCl₂, and the indicated compounds. The colchicine binding assay was performed as described in Section 2.2.11. Each reaction mixture contained 0.5μM FITC-labelled colchicine and was incubated for 30 minutes at 37°C. The data presented is representative of 2 separate experiments.

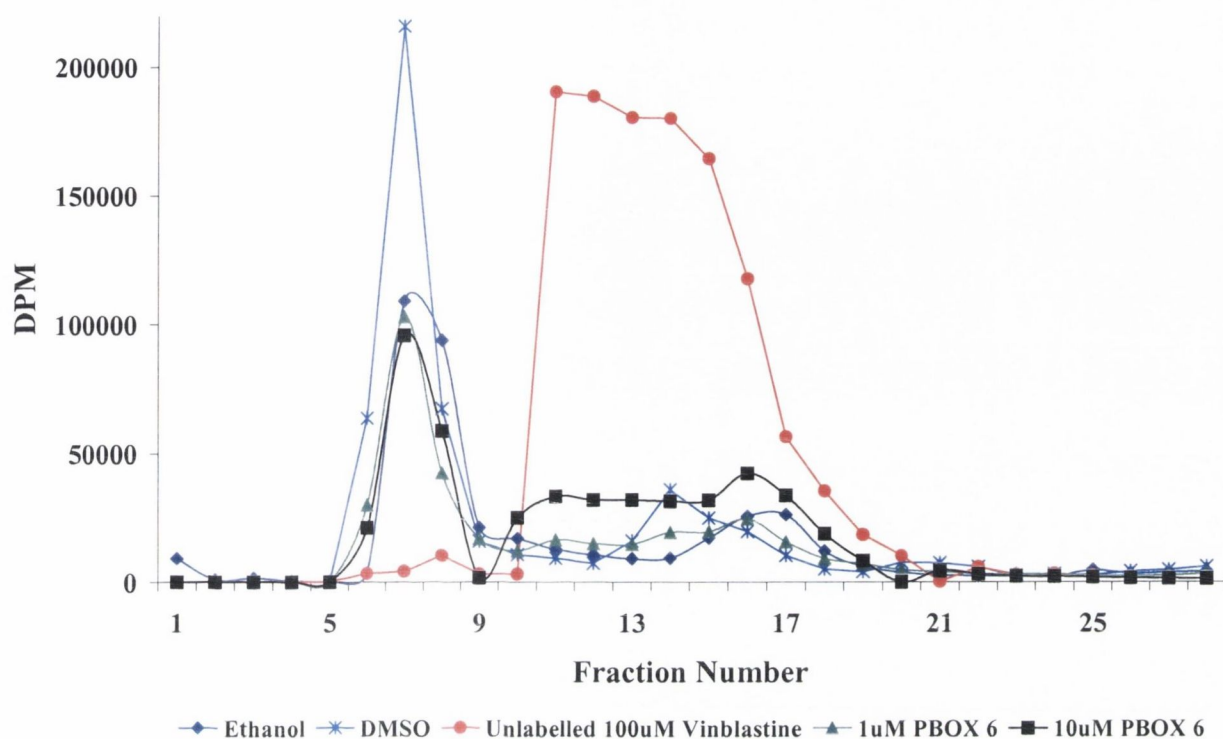


Figure 5.6 PBOX-6 does not bind to the vinblastine binding site on purified tubulin

Each reaction mixture contained 1mg/ml >99% purified bovine tubulin, 0.25mM PIPES buffer at pH 6.9, containing 0.05mM GTP, and 0.25mM MgCl₂, and the indicated compounds. The vinblastine binding assay was performed as described in Section 2.2.12. Each reaction mixture contained 0.5μM [³H]vinblastine and was incubated for 30 minutes at 37°C. The data presented is representative of 2 separate experiments.

any residual [^3H]vinblastine that did not bind tubulin, or [^3H]vinblastine that had been displaced from binding to tubulin by a vinblastine binding site competitor, was seen to elute between fractions 10 and 21. As can be seen, there is a large discrepancy between the counts achieved for the fractions containing both DMSO and unlabelled vinblastine compared with those containing ethanol and PBOX-6. This is possibly due to quenching of the [^3H] signal by ethanol relative to DMSO.

5.3 Discussion

The results presented in the last chapter demonstrated that part of the signalling pathway elicited in MCF-7 cells following treatment with PBOX-6 (10 μ M) was similar to that evoked following treatment with two representative antimicrotubule agents, nocodazole and paclitaxel. This chapter aimed, therefore, to establish whether PBOX-6 could be classed as a novel member of the antimicrotubule family of anti-neoplastic agents.

Treatment with PBOX-6 was shown to cause a significant G2/M arrest of MCF-7 cells (Figure 4.1 (a) and 4.2 (a)). In order to address if this arrest was specific to mitosis, the morphological features of the cells were analysed following treatment with PBOX-6, and the representative antimicrotubule agents, paclitaxel and nocodazole. In each case, the cells displayed marked morphological features that distinguished a mitotic arrest specifically in prometaphase. During prometaphase, the DNA chromatin network which was duplicated in the S period of interphase begins to twist and fold, eventually forming compacted chromosomes. The nuclear envelope breaks down allowing the nucleoplasmic contents to come into contact with the spindle network, as the cell prepares to align its duplicated chromosomes during metaphase for separation by the mitotic spindle during anaphase (Crespo *et al.*, 2001). As antimicrotubule agents have previously been shown to arrest the cell cycle in prometaphase (Li *et al.*, 1999; Woods *et al.*, 1995), this result was further evidence that PBOX-6 may possess antimicrotubule activity.

This claim could only be proven by assessing the ability of PBOX-6 to interact directly with the microtubule network of the cell. Indirect immunofluorescence allowed visualisation of both the microtubule network and the DNA of the cell. This technique allowed detection of morphological changes in the microtubule network, such as alterations in microtubule organisation and arrangement. It also allowed for detection of disruption to the DNA of the cell. It revealed that PBOX-6 does possess antimicrotubule activity, as PBOX-6 treatment disrupted the microtubule network of the cell in a dose-dependent manner. This is in agreement with the dose-dependent effect of PBOX-6 upon the induction of mitotic arrest and apoptosis, and suggests that these effects are a

direct result of the abrogation mediated by PBOX-6 upon the microtubule network of the cell. The disruption to the microtubules following treatment with 10 μ M PBOX-6 appeared similar to that elicited by the known microtubule depolymeriser, nocodazole. Increasing concentrations of nocodazole and other known tubulin depolymerisers are known to kinetically 'cap' the actively growing plus end of microtubules, preventing growth and thus leading ultimately to disassembly of the microtubules (Dumontet *et al.*, 1999). Both PBOX-6 and nocodazole resulted in a dramatic destruction of the microtubule complex network of the cell, relative to the intricate mesh of microtubules witnessed in the vehicle control cells. The effect of PBOX-6 upon the microtubule network was distinct to that elicited by treatment with paclitaxel. Paclitaxel is a known microtubule polymeriser, which with increasing concentration causes an increase in microtubule mass and a consequential distinctive 'bundling' of the microtubules. It would therefore appear that PBOX-6 targets the microtubule network of the cell, causing its dissolution via microtubule depolymerisation. In order to address whether the differential response of the antiproliferative and pro-apoptotic subsets of PBOX compounds is based upon the ability to distort the microtubule network, the effect upon the microtubule network of MCF-7 cells of PBOX-15, a potent member of the pro-apoptotic subset of PBOX compounds, and PBOX-21, a member of the antiproliferative PBOX compounds, was assessed. This revealed that PBOX-15 also disrupted the microtubule network of the cell in a manner similar to PBOX-6. In contrast, PBOX-21 had no effect upon the microtubule network of the cell. This suggests that the ability of a PBOX compound to inhibit microtubule polymerisation determines its ability to induce apoptotic cell death.

The disassembly of the microtubule network following treatment with PBOX-6 and PBOX-15 suggested that they may act as microtubule depolymerising agents. In order to examine this hypothesis, the effect of PBOX-6 and PBOX-15 upon tubulin assembly in a cell-free system *in vitro* was assessed and compared with the effects of nocodazole and paclitaxel. The effect of PBOX-21 upon the assembly of tubulin *in vitro* was also determined as a negative control. These assays revealed that PBOX-6 inhibits the assembly of tubulin in a dose-dependent manner, confirming it as a microtubule depolymeriser. Another potent pro-apoptotic PBOX compound, PBOX-15, was also shown to inhibit the

polymerisation of tubulin. As expected, and in agreement with the literature, nocodazole inhibited and paclitaxel induced tubulin assembly, both in a dose-dependent fashion. PBOX-21 had a negligible effect upon the assembly of tubulin relative to the vehicle control (0.5% (v/v) ethanol), once again suggesting that the differential response of members of the antiproliferative and pro-apoptotic subsets of PBOX compounds depends upon their ability to directly bind to and inhibit the polymerisation of cellular microtubules.

It is notable that both 100nM and 1 μ M PBOX-6 were capable of inhibiting the assembly of tubulin in the cell-free assay system, whilst these concentrations were found to elicit no effect upon either the microtubule network of the cell or the cell cycle progression. Conversely, 100nM paclitaxel exhibited no effect upon tubulin assembly whilst this concentration of paclitaxel was shown to cause dramatic disruption to the microtubule network. These discrepancies between the concentrations of PBOX-6 and paclitaxel that elicit an effect in a cell-free system and cell-based assay are possibly due to the fact that the cell-free system does not provide the ideal intracellular environment present in a cell-based system for appropriate tubulin assembly. The purified tubulin used was devoid of any Microtubule Associated Proteins (MAPs) which are known to regulate microtubule dynamics by stabilising microtubules and preventing disassembly. Therefore, their absence along with other possibly critical intracellular agents, would perhaps serve to overestimate and underestimate the effects of the lower concentrations of PBOX-6 and paclitaxel respectively. Discrepancies between the concentrations of antimicrotubule agents that elicit effects in cell-based and cell-free systems have been widely reported (Jiang *et al.*, 1998a; Jiang *et al.*, 1998b; Ling *et al.*, 2002).

Disruption of cellular microtubule dynamics has long been associated with activation of the spindle assembly checkpoint and resultant mitotic arrest and apoptosis (Amon, 1999; Gorbsky, 2001). Specifically, triggering of the spindle assembly checkpoint is thought to result from sensing of both the attachment of the mitotic spindle to the kinetochores of sister chromatids, and the subsequent tension created as the kinetochores move chromosomes along the mitotic spindle towards the poles (Tanaka, 2002; Van Hooser *et al.*, 2001). The attachment of the chromosomes to the spindle is a dynamic process that when viewed by video

microscopy seems to involve a ‘search and capture’ mechanism in which microtubules nucleated from each of the rapidly separating centrosomes grow outward toward the chromosomes. If the dynamics of the microtubules is compromised, then the basis of ‘seeking and capturing’ the chromosomes has also been compromised, resulting in triggering of the spindle assembly checkpoint which causes mitotic arrest and apoptosis. Disruption of the microtubule dynamics may also give rise to inappropriate separation of the chromosomes and resultant micronucleation. Each of these two outcomes result ultimately in triggering of a cell death mechanism.

Despite the interaction of tubulin with a large number of drugs that inhibit or promote its assembly into microtubules, precise definition of drug binding sites on the protein has not been possible. This is a consequence of the lack of success in crystallising the protein, probably because of its sequence and post-translational heterogeneity, its instability, and its tendency to form oligomers and polymers of highly aberrant morphology in the presence of many of these drugs. The recent electron crystallographic determination of a relatively detailed structure for zinc-induced antiparallel tubulin protofilaments has provided insights into the paclitaxel site on these protofilaments (Nogales *et al.*, 1998), since paclitaxel was used to enhance their stability during data accumulation, and provided a scaffold on which to model other drug sites (Downing *et al.*, 1998). A limitation in such analysis, however, is that drugs that inhibit assembly have limited ability to bind to tubulin polymers containing linear protofilaments.

However, through the use of techniques such as photoaffinity labelling (Bai *et al.*, 1996a; Bai *et al.*, 1996b; Rai *et al.*, 1996) and protein footprinting experiments (Chaudhuri *et al.*, 2000) as well as the crystal structure identified by Nogales *et al.* (1998), specific binding residues and binding domains upon tubulin have been at least partially characterised for paclitaxel, vinblastine and colchicine. *Vinca* alkaloids, rhizoxin, dolastetins and spongistatin react with the domain for vinblastine. Colchicine, nocodazole, podophyllotoxin and curacin A bind to the colchicine domain (Uppuluri *et al.*, 1993). As these are the only two defined binding sites of tubulin depolymerisers, it was necessary to determine if PBOX-6 mediated its antimicrotubule activity via binding to either of these sites. The results from the competitive binding experiments revealed that PBOX-6 did not inhibit binding of either colchicine or vinblastine to tubulin, indicating that

PBOX-6 has its own, novel, binding site upon tubulin. Other antimicrotubule agents such as estramustine (Panda *et al.*, 1997), arsenic trioxide (Ling *et al.*, 2002), naphthopyran (Dell, 1998) and cemadotin (Jordan *et al.*, 1998a) have also been shown to bind to as yet uncharacterised novel sites on tubulin.

In conclusion, the work presented in this chapter provides further insight into the mechanism of action of a member of the pro-apoptotic subset of PBOX compounds, PBOX-6. It confirms that PBOX-6 targets the microtubule network causing depolymerisation of the microtubule network of the cell. This leads to prometaphase arrest, and as demonstrated in the last chapter, this arrest is followed by apoptotic cell death. The induction of arrest and apoptosis as a consequence of depolymerisation of the microtubule network by members of the pro-apoptotic subset of PBOX compounds was further suggested by the ability of a second pro-apoptotic member, PBOX-15 to inhibit tubulin polymerisation causing disruption to the cellular microtubule network. The antiproliferative and non-apoptotic PBOX compound, PBOX-21, did not have any effect upon the assembly of tubulin *in vitro* or upon the microtubule network of the cell. Finally, PBOX-6 was found to bind to a novel site upon tubulin. The ability of PBOX-6 to bind tubulin and cause microtubule depolymerisation, suggests it as a novel candidate for antineoplastic therapy. This is all the more significant, as both the clinical success of, and increasing resistance to antimicrotubule agents in the treatment of a variety of cancers has prompted the search for new agents with a similar mechanism.

6 FUTURE WORK

The cell cycle is a potential target for therapeutic intervention in cancer as cell cycle dysregulation is a hallmark of tumorigenesis. This study has concentrated on determining the potential of a series of pyrrolo-1,5-benzoxazepines (PBOXs) as novel anticancer agents. The PBOX compounds appear to be subdivided into two groups, those that elicit antiproliferative effects by arresting cells in the G1 phase of the cell cycle without eliciting cytotoxic effects (e.g. PBOX-21) and those that can induce apoptotic cell death (e.g. PBOX-6). A fuller understanding of the molecular mechanisms underlying the oncostatic and apoptotic effects of these PBOX compounds is essential to their development as therapeutic agents in the treatment of cancer.

The first part of this study concerned the elucidation of the molecular mechanism underlying the oncostatic effect of PBOX-21, a representative member of the antiproliferative subset of PBOX compounds. The growth inhibitory effects induced by PBOX-21 was mediated through G1 arrest. This arrest was preceded by a rapid decrease in CDK2 kinase activity and cyclin D₃ expression and was concomitant with a decrease in CDK4 kinase activity, indicating that these events are likely to be part of the mechanism responsible for initiating the PBOX-21 induced G1 arrest. The expression of the CDK inhibitor, p21, as well as the kinase activity of CDK6, were upregulated and downregulated respectively following PBOX-21 treatment, but only subsequent to the onset of arrest suggesting a possible role in the maintenance of the arrest.

The effect of PBOX-21 treatment on the protein expression levels of p27, another member of the CIP/KIP family of CDK inhibitors, could be examined. Induction of p27 has previously been shown to be associated with downregulation of cyclin D₃ protein levels and the inhibition of CDK2 kinase activity during G1 arrest (van Oirschot *et al.*, 2001). In addition, in order to determine if downregulation of cyclin D₃ is crucial to the G1 arrest seen in response to PBOX-21 treatment, it would be of interest to determine whether enforced expression of cyclin D₃ could abrogate PBOX-21-induced G1 arrest. This could be achieved in two ways:

- (1) Ectopic expression of cyclin D₃ by transfection of the 1321N1 cells with an expression vector for cyclin D₃, and co-transfection with a plasmid encoding a modified version of the green fluorescent protein (GFP) (Mendelsohn *et al.*, 2002). Using bivariate flow cytometry we could then analyse the cell cycle

distribution of the GFP-positive population, and determine if transfection of the cyclin D₃ expression plasmid resulted in a rescue from the cell cycle arrest that normally occurs in response to PBOX-21 treatment.

(2) It is believed that cyclin D₃ degradation is mediated via the ubiquitin-proteasome pathway. Therefore, pretreatment of the cells with the 26S proteasome inhibitor *N*-carbobenzoxyl-Leu-Leu-Leucinal (ALLN), should result in the prevention of the downregulation of cyclin D₃ (Hu *et al.*, 2002).

CDK2 kinase activity is inhibited *in vivo* by inhibitory phosphorylations upon both the Tyr15 and Thr14, whilst CDK4 and CDK6 kinase activity is inhibited via phosphorylations upon Tyr15. These inhibitory phosphorylations are mediated by Wee1 and Myt1 kinases. Therefore, determination of the kinase activity of Wee1 and Myt1 in response to PBOX-21 treatment would be interesting. It is possible that their kinase activity is induced following treatment with PBOX-21 in order to maintain inhibitory phosphorylations upon the Tyr15 and Thr14 residues. To confirm this possibility, the phosphorylation status of Tyr15 could be investigated via immunoprecipitation with CDK2, CDK4 and CDK6 and western blotting with a phospho-tyrosine antibody (Cangi *et al.*, 2000).

It also remains to be determined fully whether or not the induction of p21 in response to PBOX-21 is dependent or independent of p53 control. This would involve analysis of the p21 protein expression levels in a p53-null astrocytoma cell line following PBOX-21 treatment (Xu *et al.*, 2001). It would be interesting to determine what lies upstream in regulating cyclin D₃ and D₂ expression levels and the kinase activities of CDK2, 4, and 6. For example are the mitogen activated protein kinases (MAPKs) JNK, ERK, and p38 involved? These kinases form a cascade that transmits mitogenic (Blenis, 1993) or differentiating signals (Brondello *et al.*, 1995) within most cell types. Also, the positive effect of c-myc on cell growth is becoming apparent from work with both *Drosophila* and mammalian cells, (Elend *et al.*, 1999). Ectopic expression of the c-myc oncoprotein prevents cell cycle arrest in response to growth-inhibitory signals, differentiation stimuli, or mitogen withdrawal. Moreover, c-myc activation in quiescent cells is sufficient to induce cell cycle entry in the absence of growth factors. Myc is a positive regulator of G1-specific CDKs via downregulation of CDK inhibitors and upregulation of CDC25a activity (Amati *et al.*, 1998).

Therefore, the expression of c-myc may be abrogated by PBOX-21, and an analysis of the expression levels of this protein following treatment with PBOX-21 may identify a potential upstream regulator of the PBOX-21 mediated G1 arrest.

The second part of this study concentrated on elucidating the molecular mechanisms underlying the oncostatic and apoptotic effects of a representative pro-apoptotic PBOX compound, PBOX-6. It was demonstrated that PBOX-6 is efficacious in suppressing cell growth and inducing apoptotic cell death in the MCF-7 human breast carcinoma cell line. PBOX-6 induced G2/M arrest by sustained activation of CDK1 prior to apoptosis induction. In fact, PBOX-6 was shown to elicit similar effects upon MCF-7 cell cycle kinetics, CDK1 kinase activity and G2/M regulatory proteins as two antimicrotubule agents, nocodazole and paclitaxel, suggesting that PBOX-6 may also target the microtubule network of the cell.

The biochemical events downstream of microtubule disruption which lead to apoptosis remain largely unclear (Jordan *et al.*, 1998b; Wang *et al.*, 1999). While the G2/M arrest induced by antimicrotubule agents has been observed to initiate apoptosis (Jordan *et al.*, 1993; Jordan *et al.*, 1996; Ling *et al.*, 2002; Park *et al.*, 2001; Woods *et al.*, 1995; Yu *et al.*, 1998), evidence has also been presented that indicates that the mitotic arrest of the cell cycle may not be the only mechanism to induce apoptosis (Lanni *et al.*, 1997; Lieu *et al.*, 1997; Moos *et al.*, 1998). Thus, the precise molecular mechanism underlying apoptosis and mitotic arrest induced by antimicrotubule agents and the relationship between the two events remain ill-defined. Therefore, the dependency of the observed PBOX-6 induced apoptosis upon the PBOX-6 induced G2/M arrest could be investigated, so as to address whether these two effects of PBOX-6 were intimately related or simply two independent consequences of disrupting the normal physiological balance of microtubule dynamics. This could be achieved in several ways, including:

- (1) Pretreatment of the cells with aphidicolin, a DNA polymerase that arrests cells in the S-phase, before treatment with PBOX-6 (Park *et al.*, 2001). The cells could then be analysed by flow cytometry to determine if arresting the cell in the S-phase prevented the ability of PBOX-6 to induce G2/M arrest and thus apoptosis. To further characterise the effect of

aphidicolin-induced S-phase arrest upon PBOX-6-induced apoptosis, western blot analysis of PARP cleavage as well as TUNEL staining could also be carried out.

- (2) The mitotically arrested state of the MCF-7 cells was associated with an upregulation in activity of CDK1 kinase activity following treatment with PBOX-6. Therefore, this sustained CDK1 activation may be a link between the PBOX-6 induced arrest and apoptosis. To examine this hypothesis, the activity of CDK1 could be inhibited by pretreatment with olomoucine, a purine derivative that inhibits CDKs (Ye *et al.*, 2001), or with another purine derivative that selectively inhibits CDK1, CGP74514A (Dai *et al.*, 2002), prior to treatment with PBOX-6. If these CDK inhibitors achieved inhibition of CDK1 activity, the effect of this upon PBOX-6 induced apoptosis could then be assessed. Alternatively, one could examine whether overexpression of cells with a dominant/negative mutant of CDK1 by transient transfection results in resistance to apoptosis induction by PBOX-6.

Apoptosis is controlled by a complex interplay between regulatory proteins (Lockshin *et al.*, 2001). Bcl-2, an integral membrane oncoprotein, was the first anti-apoptosis gene product discovered (Reed, 1994). Phosphorylation of Bcl-2 was first reported by Alnemri *et al.* (1992) and is considered to abrogate its anti-apoptotic function. Many reports have demonstrated phosphorylation of Bcl-2 following treatment with antimicrotubule agents (Fan *et al.*, 2000; Fan *et al.*, 2001; MacCarthy-Morrogh *et al.*, 2000; Park *et al.*, 2001; Wang *et al.*, 1999; Wu-Wong *et al.*, 2001; Yamamoto *et al.*, 1999). In addition, Bcl-2 has been shown to be phosphorylated by JNK (Fan *et al.*, 2001) and CDK1 (Vantieghem *et al.*, 2002) following treatment with antimicrotubule inhibitors. In this study, PBOX-6 has been shown to upregulate the activity of both JNK and CDK1 within the MCF-7 cells. Therefore, it would be interesting to assess the phosphorylation status of Bcl-2 in MCF-7 cells following PBOX-6 treatment. If Bcl-2 were seen to be phosphorylated following treatment with PBOX-6, the question of which, if either, of JNK or CDK1, was responsible for the phosphorylation could be assessed by pretreatment with the JNK inhibitor, dicumoral, or the CDK1 inhibitor, olomoucine or CGP74514A. Also, to further characterise the ability of either of these kinases to phosphorylate Bcl-2, immunoprecipitation studies could

be used to determine if JNK or CDK1 associates with Bcl-2 following PBOX-6 treatment.

The third part of this study aimed at examining the hypothesis that the molecular target of PBOX-6 is the microtubule network of the cell. Indirect immunofluorescence techniques allow detection of morphological changes in the microtubule network such as alterations in microtubule organisation and arrangement. PBOX-6 treated cells were shown to undergo microtubule disassembly in a manner similar to the known tubulin depolmeriser, nocodazole. Furthermore, a tubulin assembly assay was set up and consistent with the cellular effect, demonstrated that PBOX-6 has a nocodazole-like effect to destabilise tubulin polymerisation *in vitro*.

Despite the interaction of tubulin with a large number of drugs that inhibit or promote its assembly into microtubules, a precise definition of drug binding sites on the protein has proved difficult. This is a consequence of a lack of success in crystallising the protein, its instability and its tendency to form oligomers and polymers of highly aberrant morphology in the presence of many of these drugs. Therefore, only three distinct binding sites of antimicrotubule agents have been characterised to date, i.e. the binding sites of the tubulin polymeriser, paclitaxel (Nogales *et al.*, 1998) and of the tubulin depolymerisers, vinblastine and colchicine (Serrano *et al.*, 1984; Uppuluri *et al.*, 1993). Using competitive binding studies, the present study demonstrated that PBOX-6 does not bind to either the colchicine or the vinblastine binding sites. This would suggest that PBOX-6 binds to an as yet uncharacterised binding site on tubulin. Photoaffinity labelling methods could be used to define the molecular contacts between PBOX-6 and its target tubulin. To identify the site(s) of photoincorporation, the photolabelled tubulin could be subjected to sequential cyanogen bromide and tryptic digestions. The radiolabelled peptides would be purified by reverse phase HPLC and amino acid sequencing studies could identify a peptide(s) containing the major photolabelled band. Such studies have previously been performed in characterising the colchicine (Uppuluri *et al.*, 1993), vinblastine (Rai *et al.*, 1996) and paclitaxel (Rao *et al.*, 1999) binding sites on tubulin.

In order to further characterise the site at which PBOX-6 binds tubulin, the ability of PBOX-6 to displace the binding of GTP to tubulin may be assessed. All known agents that bind in the *vinca* domain, are known to also prevent GTP

nucleotide exchange on tubulin and inhibit the ability of tubulin to hydrolyse GTP (Hamel, 1996). Although PBOX-6 did not inhibit the binding of vinblastine to tubulin, determination of the ability of PBOX-6 to displace GTP and inhibit GTP hydrolysis by tubulin would further qualify this result. GTP displacement may be determined by using size-exclusion chromatography to measure the amount of [^3H]GTP displaced from binding to tubulin following incubation with PBOX-6. In addition, pretreatment of MCF-7 cells with exogenous GTP (250–750 mM) prior to treatment with PBOX-6, would further address whether binding to the exchangeable nucleotide/GTP site upon tubulin by PBOX-6 is involved in mediation of its cytostatic and cytotoxic effects. Determination of the effect of PBOX-6 upon tubulin associated GTPase activity may be achieved by measurement of the release of ^{33}P from [^{33}P] γ -GTP as described by Hamel and Lin (1981) following a tubulin assembly reaction as described in Section 2. 2.11 in the presence of [^{33}P] γ -GTP and PBOX-6.

Multidrug resistance (MDR) is either an innate or acquired resistance to a wide array of chemotherapeutic agents with diverse chemical structures and mechanisms of action. MDR is a serious problem, preventing the successful application of many otherwise efficacious therapeutics in the treatment of cancer. MDR is multifactorial, including the overexpression of the P-glycoprotein drug efflux pump (Aran *et al.*, 1999) and overexpression of MDR-associated protein (MRP) (Cole *et al.*, 1998). The clinical success of the antimicrotubule agents in the treatment of various cancers has prompted the search for new agents with similar mechanisms of action. The search for new antimicrotubule agents has been vigorous with the goal of providing new agents with advantages over the older members of this class of drug, specifically those that can circumvent cellular responses that confer multidrug resistance. As PBOX-6 displays antimicrotubule activity against cell proliferation and viability in the human MCF-7 breast carcinoma cell line, it would be of interest to determine its effect upon a P-glycoprotein-overexpressing MCF-7 cell line and also upon an MRP-expressing MCF-7 cell line (resistance indices, 2.3 and 1.9 respectively) (Ling *et al.*, 2002). If it were found that PBOX-6 were capable of eliciting a cytostatic and cytotoxic effect within either or indeed both of these cell lines, it would suggest that it may be useful for treating drug-refractory patients.

As antimicrotubule agents are believed to mediate their apoptotic effects by activating the spindle assembly checkpoint, it would, therefore, be interesting to assess the effect that PBOX-6 had upon the key proteins involved in mediating the signalling cascade that mediates this checkpoint. In order to determine the spindle checkpoint status, the cellular localisation patterns of the key checkpoint proteins, namely Mad2, Bub1 and BubR1, within mitotically arrested cells following treatment with PBOX-6 could be assessed via selective detachment of the mitotically arrested cells and subsequent indirect immunofluorescence (Hoffman *et al.*, 2001; Skoufias *et al.*, 2001; Zhou *et al.*, 2002b). In particular, it would be interesting to determine which, if any, of these checkpoint proteins associate with the unattached kinetochores following treatment with PBOX-6. Immunofluorescent staining of sister centromeres with anti-human centromere antibodies would allow for visualisation of the kinetochores of sister chromatids (Hoffman *et al.*, 2001; Skoufias *et al.*, 2001; Zhou *et al.*, 2002b). In prophase and prometaphase, when kinetochores are not attached and under tension, the 3F3/2 antibody recognises a phosphoepitope upon the unattached kinetochore. This phosphorylation may be the trigger for activation of the spindle assembly checkpoint. It is thought that dephosphorylation occurs when the kinetochores attach to the spindle, leading to inactivation of the checkpoint (Nicklas, 1997; Stern *et al.*, 2001). The phosphorylation of this kinetochore epitope recognised by the 3F3/2 antibody is believed to be tension-sensitive (Zhou *et al.*, 2002c). Therefore, it would be interesting to determine if this epitope is phosphorylated following treatment with PBOX-6, indicating induction of the spindle assembly checkpoint. This may be achieved by staining with the 3F3/2 antibody of mitotically arrested cells following treatment with PBOX-6.

If this epitope was found to be phosphorylated, the next issue to be addressed would be which kinase is responsible for this phosphorylation event? There is evidence that the Ipl1/Aurora family of protein kinases, play a role in tension-dependent checkpoint signalling (Ditchfield *et al.*, 2003; Giet *et al.*, 1999; Tanaka *et al.*, 2002) and thus a member of this family of kinases may be responsible for the tension-sensitive phosphorylation of the 3F3/2 recognised epitope of unattached kinetochores. Initially, determination of the Aurora kinase activity within the cell following treatment with PBOX-6 could be determined via an *in vitro* kinase assay (Ditchfield *et al.*, 2003). This would reveal if PBOX-6 induced

the activity of the Aurora kinases, suggesting a role of these kinases in the molecular mechanism of PBOX-6. The hypothesis that the Aurora kinase family members may phosphorylate the 3F3/2 recognised epitope of unattached kinetochores may be determined by assessing the extent of staining with the 3F3/2 antibody following PBOX-6 treatment of cells in which the Aurora kinases have been inhibited by pretreatment with a novel selective Aurora kinase inhibitor, ZM447439 (Ditchfield *et al.*, 2003).

Using the current knowledge of structure-activity relationships for activity of PBOX compounds, new compounds should be rationally designed and synthesised for screening *in vitro* for cytostatic and apoptotic potential, in order to optimise structures for potency and determine a clear structure-activity relationship. It would also be of interest to perform molecular modelling studies comparing PBOX compounds to known microtubule targeting agents to determine if there are any pharmacophore similarities between these drugs.

Finally, in order to further assess the ability of PBOX-21 and PBOX-6 to act as novel anticancer agents, their ability to slow tumourgenesis *in vivo* would need to be examined. This may be assessed by determining the ability of PBOX-21 and PBOX-6 to inhibit the growth of murine and/or human tumours implanted into a nude mice model. This *in vivo* xenograft study would involve injecting the mice subcutaneously with a specified amount of either murine or human tumour cells. After the implanted mice grew tumours of 10-15mm³, one group of mice would be injected intraperitoneally with vehicle saline solution, and another group with PBOX-6 daily for 3 weeks. The growth rate of tumours would be monitored by measuring the tumour volumes by calliper measurements (Towle *et al.*, 2001; Ye *et al.*, 1998).

In summary, the current study has elucidated much of the molecular mechanisms underlying the oncostatic and apoptotic activity of PBOX-21 and PBOX-6 respectively. For example, it has identified the molecular target of PBOX-6 as tubulin. Further characterisation of the oncostatic and apoptotic pathways, coupled with *in vivo* experiments will provide further insights into the use of these novel compounds as anticancer therapeutics.

7 GENERAL DISCUSSION

Cell cycle dysregulation is a hallmark of tumorigenesis. Timely and coordinated progression through the cell cycle is controlled by the sequential activation of members of cyclin-dependent kinases (CDKs) at specific intervals throughout the cell cycle (McDonald *et al.*, 2001). As such, attention has been increasingly focused upon the cell cycle as a potential target for therapeutic intervention in several proliferative disorders, including cancer. Disrupted cell cycle control due to aberrant CDK activity has been linked directly to the molecular pathology of cancer (Kamb, 1995; Pavletich, 1999), and has provided the main impetus to the active search for pharmacological inhibitors of these kinases. The first part of this study concerned the elucidation of the molecular mechanism underlying the oncostatic effects manifested by a member of a novel series of pyrrolo,1,5-benzoxazepines (PBOXs), PBOX-21.

A number of PBOX compounds were demonstrated in this study to induce a potent growth inhibitory effect upon both the human 1321N1 astrocytoma and MCF-7 breast carcinoma cell lines. Compounds that exert such inhibitory effects upon cancerous cell growth are candidates for therapeutic use in the treatment of cancer. The growth inhibitory effects induced by a representative cytostatic PBOX compound, PBOX-21, was mediated through the induction of cell cycle arrest in the G1 phase, and was not associated with a significant cytotoxic effect. PBOX compounds are high affinity ligands to the peripheral type benzodiazepine receptor (PBR), however, the arrest did not appear to be mediated through binding to this receptor, as saturation of the PBR binding sites with the specific PBR ligand, PK 11195, prior to treatment of the cells with PBOX-21 did not prevent the arrest. The arrest was preceded by a rapid decrease in CDK2 kinase activity and cyclin D₃ expression, and was concomitant with a decrease in CDK4 kinase activity, indicating that these events are likely to be part of the mechanism responsible for initiating the PBOX-21 induced G1 arrest. The expression of the CDK inhibitor, p21, as well as the kinase activity of CDK6, were upregulated and downregulated respectively following PBOX-21 treatment, but not until subsequent to the onset of arrest suggesting a possible role in the maintenance of the arrest. Furthermore, Rb was found to be hypophosphorylated in response to PBOX-21 treatment. These results indicate that PBOX-21 exerts its growth inhibitory effects through the modulation of the activity of the key G1 regulatory

CDKs, and suggests its potential as a novel CDK inhibitor with possible therapeutic application.

The second part of this study concentrated upon the molecular machinery involved in the mediation of the apoptotic effects of a second PBOX compound, PBOX-6. The PBOX compounds appear to be subdivided into two groups, those that exert antiproliferative effects (e.g. PBOX-21) by arresting cells in the G1 phase of the cell cycle without eliciting cytotoxic effects (Mulligan *et al.*, 2003; Zisterer *et al.*, 1998), and those that can induce apoptotic cell death (e.g. PBOX-6) (Mc Gee *et al.*, 2001; Mc Gee *et al.*, 2002a; Mc Gee *et al.*, 2002b; Zisterer *et al.*, 2000). Thus the apoptotic activity of members of the PBOX series is dependent on their structure (Mc Gee *et al.*, 2001; Mc Gee *et al.*, 2002a; Mc Gee *et al.*, 2002b; Zisterer *et al.*, 2000). The pro-apoptotic members have in general been found to contain a naphthyl side-chain, but further studies are required to determine a clear structure-activity relationship.

Apoptosis represents a universal and exquisitely efficient cellular suicide pathway. As the understanding of its vital role in normal development has deepened, numerous genes have been identified that encode apoptotic regulators, some of which represent familiar oncogenes or tumour-suppressor genes. Because of this convergence between cancer biology and cell death regulation in development, researchers into anticancer therapies now frequently focus on molecular pathways whose endpoint – death – coincides with the goal of successful treatment. A representative member of the pro-apoptotic subset of PBOX compounds, PBOX-6 was chosen for investigation in this study, as it has previously been shown to potently induce apoptosis within a variety of human cancerous cells derived from the haematopoietic system such as promyelocytic leukaemia HL-60 cells, Jurkat T lymphoma and chronic myeloid leukaemia (CML) cells (Mc Gee *et al.*, 2001; Mc Gee *et al.*, 2002a; Mc Gee *et al.*, 2002b; Zisterer *et al.*, 2000), as well as in the human prostate carcinoma LNCaP cell line and the cervical carcinoma HeLa cell line (McGee, unpublished results). This suggests the potential of these drugs as anticancer therapeutics in the treatment of both solid tumours and haematologic neoplasias. This study revealed that PBOX-6 was efficacious in suppressing cell growth and inducing apoptotic cell death in the MCF-7 human breast carcinoma cell line. It appeared to do so by inducing a sequential upregulation in JNK activity and cyclin B₁ associated CDK1 kinase

activity. The rapid activation of the JNK kinase signalling pathway would appear to be important to the mechanism of PBOX-6 arrest and apoptosis as its inhibition protected against these effects. It was also shown that PBOX-6 elicited similar effects upon MCF-7 cell cycle kinetics, CDK1 kinase activity and G2/M cell-regulatory proteins as two representative antimicrotubule agents, nocodazole and paclitaxel, suggesting that PBOX-6 may be a member of this class of anticancer agents. In the field of antineoplastic chemotherapy, tubulin-binding agents constitute an important class of compounds, with broad activity both in solid and in hematologic neoplasias (Crown *et al.*, 2000; Devizzi *et al.*, 1994; Samson *et al.*, 1989).

The third part of this study aimed to further characterise the mechanism of action of PBOX-6 and attempted to confirm the hypothesis that the molecular target of PBOX-6 is tubulin, characterising it as a novel member of the antimicrotubule class of agents. There is a huge requirement for the development and/or discovery of novel antimicrotubule agents that have similar modes of action to the currently used drugs but with improved biological and pharmacological features, thus underlying the importance of further characterisation of the mechanism of action of PBOX-6. This study confirmed that PBOX-6 targets the microtubule network causing depolymerisation of the microtubule network of the cell leading to prometaphase arrest and followed by apoptotic cell death. Depolymerisation of the microtubule network by a second pro-apoptotic PBOX compound, PBOX-15, further suggested that the induction of arrest and apoptosis was as a consequence of a direct effect upon the cellular microtubule skeleton. The antiproliferative and non-apoptotic PBOX compound, PBOX-21, did not have any effect upon the assembly of tubulin *in vitro* or upon the microtubule network of the cell. This would suggest that the molecular target of the pro-apoptotic PBOX compounds is tubulin. The possibility that the mechanism by which PBOX-6 depolymerises the microtubule network may be unique amongst antimicrotubule agents was suggested by the fact that PBOX-6 was found to bind to a novel site upon tubulin. The ability of PBOX-6 to bind tubulin and cause microtubule depolymerisation, suggests it as a novel candidate for antineoplastic therapy. This is all the more significant, as both the clinical success of, and increasing resistance to antimicrotubule agents in the treatment of a variety of cancers has prompted the search for new agents with a similar

mechanism. Thus, there is much effort being directed towards the identification and synthesis of new agents that target the tubulin/microtubule system and display efficacy against drug-refractory carcinomas.

A major problem of cancer therapy is to not kill the normal cells essential for life while killing the great majority of cancer cells. Subtle differences that arise during progression of cancer can provide novel therapies, such as targeting normal cells for protection against chemotoxicity. The increasing understanding gained by applying cellular and molecular biological techniques including expression genetics to detect molecular differences is revealing potential targets, including those related to cell proliferation, apoptosis, and differentiation. The quantitative differences of gene or enzyme expression between normal and tumour cells have provided the basis for drug discovery that can either reversibly target the normal cells or differentially target the tumour cells. Most investigators would agree that the ideal chemotherapeutic agent should be 1) directed at a validated target; 2) potent, preferably active at nanomolar or subnanomolar concentrations; 3) active against drug-resistant cells; and 4) less toxic or ideally not toxic to normal cells. The antimicrotubule agents have been so successful in the treatment of cancer that no one would dispute that tubulin is a valid target. Indeed, given the widespread activity of the taxanes and the utility of the *vincas*, it could be argued that tubulin represents the single best target identified to date (Jordan *et al.*, 1998b). This should not be surprising, since the majority of known antimicrotubule agents are natural products, or derivatives thereof, and have been obtained from many classes of organisms, marine and terrestrial, from Cyanobacteria to mammals. One could argue that tubulin, as a target, has been validated time and again by "Mother Nature" (Hamel, 1996).

There are ongoing efforts to develop even more potent PBOX compounds. Indeed, PBOX-15 has been shown to exert apoptotic activity within the CML K562 cell line at nanomolar concentrations (personal communication, Dr. M. McGee). As stated in the future work section of this study, it is necessary to now determine the effect of PBOX-6 upon a P-glycoprotein-overexpressing MCF-7 cell line and also upon an MRP-expressing MCF-7 cell line (resistance indices, 2.3 and 1.9 respectively) (Ling *et al.*, 2002), so as to determine if it has possible clinical applicability in the treatment of drug-refractory patients. It also remains to

determine the preferential toxicity of PBOX-6, if any, between normal and malignant cells.

In summary, this study has revealed the therapeutic potential of two members of the PBOX compounds, namely PBOX-21 and PBOX-6, in the treatment of human cancers. Part of the mechanism(s) by which these two compounds induce their respective effects has been uncovered. Further characterisation of the molecular pathways involved, coupled with *ex vivo* and *in vivo* experiments, will provide a mechanistic basis to the mode of action of these novel agents and will provide further insight into the use of these novel compounds as anticancer therapeutics.

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Inhibition of G1 cyclin-dependent kinase activity during growth arrest of human astrocytoma cells by the pyrrolo-1,5-benzoxazepine, PBOX-21

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Abstract

The present study examines the molecular mechanisms by which a member of a novel series of pyrrolo-1,5-benzoxazepines, PBOX-21, induces G1 arrest in 1321N1 cells. PBOX-21-induced G1 arrest is preceded by both a decrease in CDK2 kinase activity, which is critical for the G1/S transition, and a downregulation in cyclin D₃ protein expression levels, suggesting that these two events may be crucially involved in the mediation of the cell cycle arrest. The decrease in CDK2 activity may be due to an observed decrease in CDK2 protein levels following PBOX-21 treatment. Coinciding with the arrest is a reduction in the activity of CDK4, due to either the observed PBOX-21 induced downregulation in CDK4 expression, or a reduction in complex formation between cyclin D₃–CDK4 leading to a decrease in the levels of active cyclin D₃–CDK4 complexes with kinase activity. The level of CDK6 activity was also seen to be reduced following PBOX-21 treatment, also possibly due to a reduction in complex formation with cyclin D₃. However, this reduction in CDK6 kinase activity was not seen until after PBOX-21-induced G1 arrest has reached its maximum, and therefore may be viewed as a consequence of, and a method of maintaining the PBOX-21-induced arrest, rather than a cause. Also in parallel with the G1 arrest elicited by PBOX-21 is an upregulation in the universal CDK inhibitor, p21. Furthermore, the retinoblastoma protein (Rb), a substrate of CDK2 and CDK6, whose phosphorylation is necessary for cell cycle progression, becomes hypophosphorylated. These results indicate that PBOX-21 exerts its growth inhibitory effects through the modulation of the expression and activity of several key G1 regulatory proteins.

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Keywords: Cell cycle; G1 arrest; Cyclin-dependent kinase; Pyrrolo-1,5-benzoxazepine; 1321N1

1. Introduction

We have previously shown that some members of a series of novel pyrrolo-1, 5-benzoxazepines (PBOXs) elicit antiproliferative effects upon tumour cells including human 1321N1 astrocytoma cells [1]. These PBOX compounds were found to induce a specific accumulation of cells in the G1 phase of the cell cycle, although the time of onset of the G1 arrest remains unclear. Significantly, the antiproliferative effects of these compounds were found to be entirely due to this G1 arrest as the PBOX compounds were not cytotoxic to the cells.

Cancer has always been attributed to an abnormal proliferation of cells, and agents that interfere with cell

cycle progression may have potential as anticancer therapeutics. The discovery of inhibitors of the cell cycle such as paclitaxel [2] and olomucine [3] has led to rapid advances in the investigation and design of a variety of anti-mitotic compounds that are used clinically or have the potential for development as antiproliferative agents. The cytostatic effects of PBOX compounds in human tumour cell lines indicated the potential of these compounds for use as novel anticancer drugs and prompted us to examine the direct mechanisms responsible for the initiation and/or maintenance of the growth-arrested state. Because cell growth and proliferation are ultimately regulated by a highly conserved set of cell cycle regulatory proteins, a productive approach to the study of the antiproliferative mode of action of the PBOXs is to examine their influence on the regulation of the cell cycle-regulating machinery.

The eukaryotic cell cycle is regulated by the action of the cyclin-dependent kinases (CDKs) and their activating sub-

Abbreviations: PBOX, pyrrolo-1,5-benzoxazepine; CDK, cyclin-dependent kinase; Rb, retinoblastoma protein

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units, the cyclins [4]. In mammalian cells, CDK2 associated with either D- or E-type cyclins, and CDK4 and CDK6 associated with D-type cyclins, regulate G1 progression. Progression from G1 to S requires inactivation of the retinoblastoma protein (Rb) by phosphorylation and the consequent release of a number of factors including the E2F family of transcription factors. These transcription factors then activate transcription of various genes that promote cell cycle progression. Thus the phosphorylation status of Rb is a critical determinant in the execution of the progression of G1 to S.

In the present study, we systematically analysed the changes that occurred in kinase activities, expression levels, interactions and phosphorylation status of the cell cycle regulatory molecules in human 1321N1 cells, in response to treatment with a potent antiproliferative PBOX compound, PBOX-21, and compared them to the timing of onset of the growth arrest process.

2. Materials and methods

2.1. Cell culture

1321N1 cells were obtained from the European Collection of Animal Cell Culture (Salisbury, UK) and were grown in Dulbecco's Modified Essential Medium supplemented with 10% (v/v) Foetal Calf Serum (FCS), 2 mM L-glutamine and 100 µg/ml gentamicin (complete medium) all obtained from Sigma (Poole, Dorset, UK). The pyrrolobenzoxazepine 7-[(diethylcarbamoyloxy)-6-*p*-toyl-pyrrolo[2,1-*d*][1,5]benzoxazepine (PBOX-21) was synthesised as described previously [5]. To ensure exponential cell growth, 1321N1 cell cultures were set up 48 h prior to treatment with either vehicle (0.5% ethanol (v/v)) or PBOX-21 (25 µM) for the indicated times. Cells were harvested and processed for cell growth curve, MTT assays, flow cytometric analysis, immunoprecipitation, Western blotting and kinase assays as described below.

2.2. Determination of cell growth curve

1321N1 cells (2.5×10^6) were seeded in 175 cm² flasks and following 48 h were treated with or without PBOX-21 (25 µM). Viable cell number was determined at the indicated times by counting the trypan blue-excluding cells using hemocytometer.

2.3. MTT assay

Proliferation of 1321N1 cells in response to PBOX-21 was determined using the MTT assay as described previously [6]. Fifty microliters of 5 mg/ml MTT was added to the culture medium of growing cells and incubated for 3 h at 37 °C. The medium was removed and 200 µl of DMSO was

added to each well. The absorbance of the dissolved dye was measured at 540 nm.

2.4. Flow cytometric cell cycle analysis

Flow cytometric analysis was performed as described previously [1] using a Becton Dickinson FACSCalibur flow cytometer. Analysis of data was performed using the computer program Cell Quest.

2.5. Immunoprecipitation and kinase assays

Cell lysates for immunoprecipitation were prepared as previously described [7]. Equal amounts of protein (200–500 µg) were incubated with the appropriate antibody for 3 h at 4 °C. Antibody complexes were recovered on protein A-Sepharose beads (Sigma). The *in vitro* kinase assays were performed as previously described [7] in the presence of either 10 µg of histone H1 (Sigma) or GST-Rb fusion protein (Santa Cruz) and 5 µCi of [γ -³²P]ATP (Amersham International) per reaction. Samples were then analysed on SDS-PAGE followed by autoradiography.

2.6. Western blot analysis

Preparation of cell lysates and Western blot analysis was performed as previously described [8]. A 10% SDS-PAGE gel was used for detection of cyclin E, whilst a 12% SDS-PAGE gel was used for detection of cyclins D₂ and D₃, CDK2, CDK4, CDK6, p21 and β -actin. A 5–20% gradient gel was employed for detection of Rb phosphor-

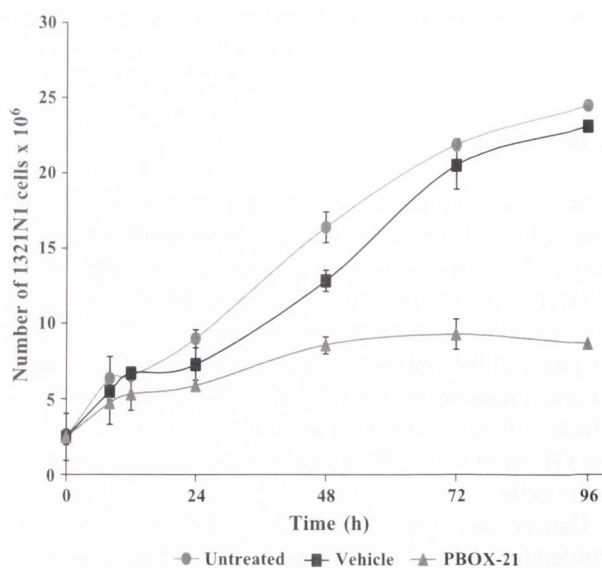


Fig. 1. Growth response of 1321N1 cells to PBOX-21. 1321N1 cells (2.5×10^6) cells were seeded in 175 cm² flasks and either left untreated or treated with PBOX-21 (25 µM). Cell number was counted at the indicated times using a hemocytometer. Results represent the mean $\pm$ S.E.M. of three separate experiments.

ylation status. Antibodies for CDK2, CDK4, CDK6, cyclin D₃, p21 and Rb were obtained from BD-Pharmingen. Antibodies for cyclin D₁ and E were obtained from Oncogene, the antibody for cyclin D₂ was obtained from Calbiochem and the β -Actin antibody was obtained from Sigma.

3. Results

3.1. PBOX-21-treated 1321N1 cells display decreased proliferation and an accumulation in the G1 phase

To assess the inhibitory effect of PBOX-21 on the growth of 1321N1 cells, we determined their growth rate

in the absence and presence of both vehicle (0.5% ethanol (v/v)) and PBOX-21 (25 μ M) (Fig. 1). Proliferation of exponentially growing 1321N1 cells was inhibited following treatment with PBOX-21 with an inhibitory effect seen after 24 h, and the cell number did not increase significantly after 48 h. This indicates that the action of PBOX-21 is exerted rapidly. The vehicle itself inhibited cell proliferation slightly over time (Fig. 1). A lower concentration of ethanol could not be used due to solubility problems with the PBOX compounds. Thus, vehicle controls were included in all subsequent experiments involved in elucidating the mechanism of the growth inhibition initiated by PBOX-21.

To characterise the antiproliferative action of PBOX-21, an analysis of the distribution of cells within the various

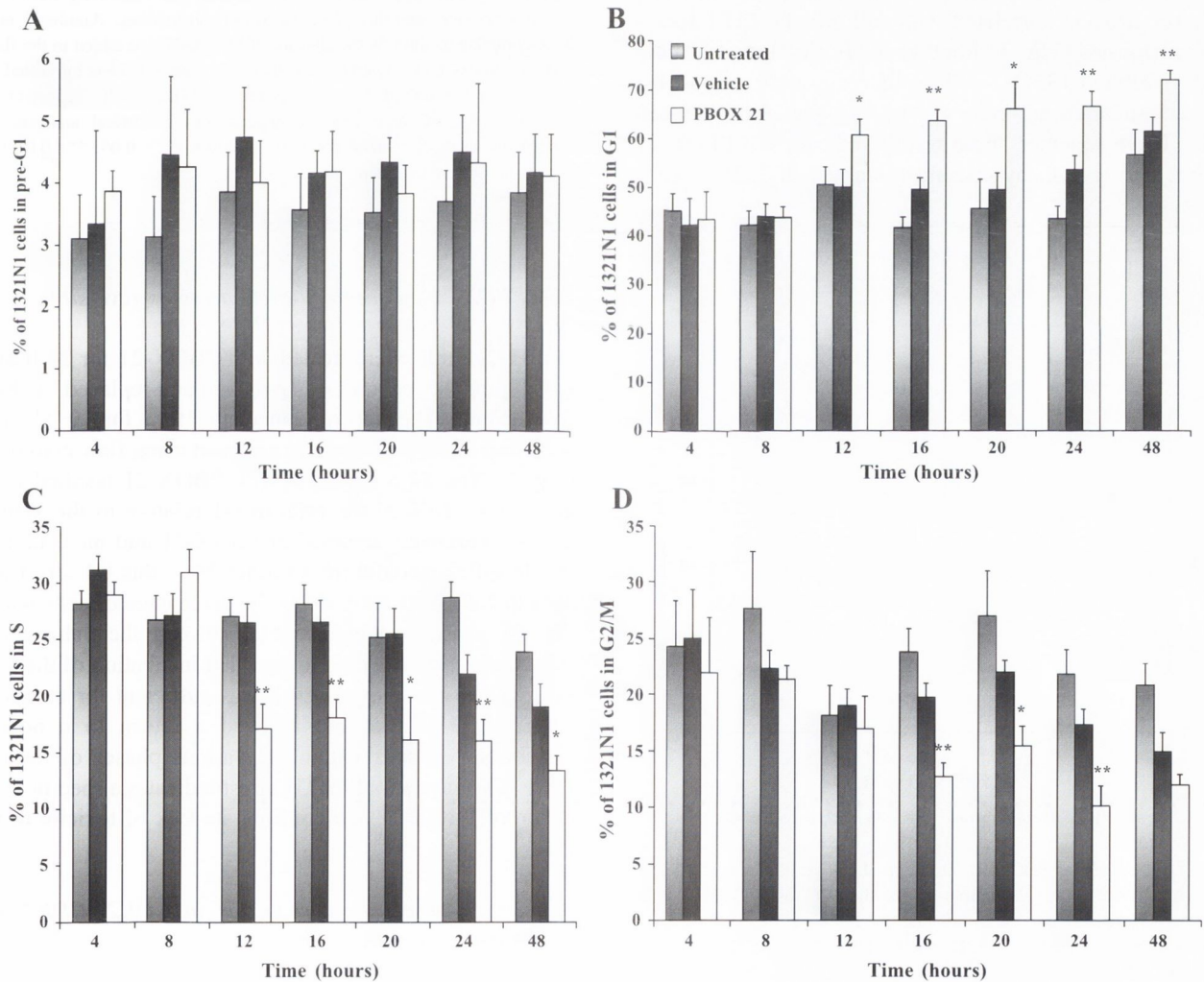


Fig. 2. Changes in cell cycle phase distribution following PBOX-21 treatment. 1321N1 (2.5×10^6) cells were seeded down in 175 cm² flasks and were incubated at 37 °C for 48 h until the cells were in the exponential phase of growth. The cells were then treated with vehicle (0.5% (v/v) ethanol) or PBOX-21 (25 μ M) for the indicated times and analysed by flow cytometry. The cell cycle phases sub-G1, G1, S and G2/M are indicated. Results represent the mean $\pm$ S.E.M. of four separate experiments. Statistical analysis was carried out using the Instat computer program. * $P < 0.05$, ** $P < 0.01$ with respect to vehicle treatment; Student's *t*-test.

phases of the cell cycle was performed by flow cytometry as a function of time following PBOX-21 treatment. No change in cell cycle distribution was apparent during the first 8 h following exposure to PBOX-21 (Fig. 2). A significant increase in the percentage of cells in the G1 phase (Fig. 2B) and concomitant reduction in both S (Fig. 2C) and G2/M (Fig. 2D) was noticeable between 12 and 16 h relative to vehicle-treated cells, with a maximum induction of G1 arrest being observed at 20 h. At 48 h, although a significant G1 arrest was still noticeable in response to PBOX-21 treatment, the vehicle itself (0.5% ethanol (v/v)) also appeared to elicit a small effect, relative to untreated cells, indicating that this arrest is not due to a cell density effect. This is in agreement with the growth curve data (Fig. 1). As can be seen from Fig. 2A, the fraction of cells with a pre-G1 DNA-content (apoptotic/necrotic cells) remained unchanged after treatment with PBOX-21.

To determine if the effect of PBOX-21 on 1321N1 cell cycle progression correlated with cell growth, MTT assays were performed. Fig. 3 shows that PBOX-21 dose-dependently inhibited 1321N1 cell proliferation which paralleled the accumulation of cells in the G1 phase of the cell cycle. Taken together, these results indicate that PBOX-21 inhibits cell growth by causing the arrest of 1321N1 cells in G1.

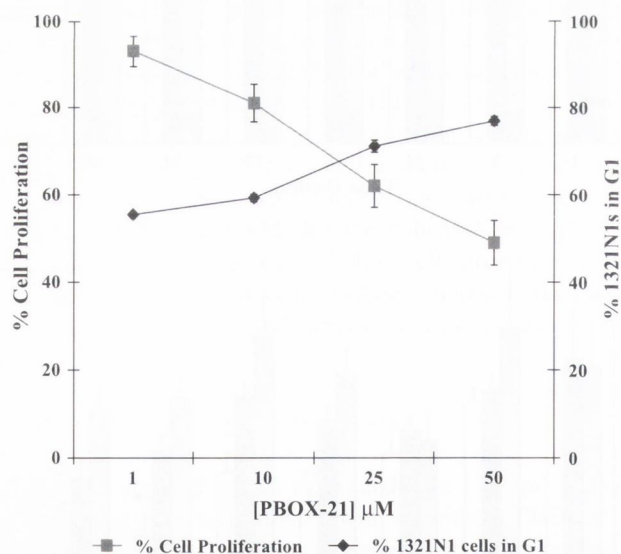


Fig. 3. Dose-dependent PBOX-21 inhibition of cell proliferation of 1321N1 cells parallels the cell cycle inhibition. For the MTT assay 1321N1 cells (0.5×10^5) were seeded in each well of a 96-well plate in 200 μl medium. For flow cytometric analysis, 1321N1 cells (2.5×10^6) were seeded in 175 cm^2 flasks. The cells were left for 48 h, until they were in the exponential phase of growth, after which they were treated with a final concentration of 1, 10, 25 and 50 μM of PBOX-21 and were incubated for a further 48 h. The cell proliferation and cell cycle arrest is expressed as a percentage of the vehicle (0.5% (v/v) EtOH) and was determined by performing an MTT assay and flow cytometry analysis respectively, as described. The results represent the mean $\pm$ S.E.M. of three separate experiments.

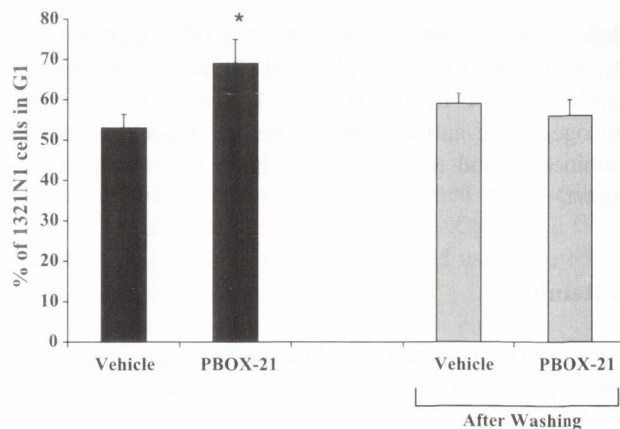


Fig. 4. PBOX-21-induced cell cycle arrest is reversible. 1321N1 (3×10^5) cells were seeded down in 25 cm^2 flasks and incubated for 48 h until the cells were in the exponential phase of growth. The cells were then treated with either vehicle (0.5% (v/v) EtOH) or PBOX-21 (25 μM) and left for 24 h. The cells from vehicle and PBOX-21-treated flasks were harvested and analysed by flow cytometry. The medium in the remaining flasks was carefully removed and the cells washed in fresh medium. Another 5 ml of fresh complete medium in the absence of PBOX-21 was added to the flasks and the cells were incubated for a further 24 h. The cells were harvested and prepared and analysed by flow cytometry. The results represent the mean $\pm$ S.E.M. of three separate experiments. Statistical analysis was carried out using the Instat computer program. * $P < 0.05$, ** $P < 0.01$ with respect to vehicle treatment; Student's *t*-test.

3.2. PBOX-21-induced cell cycle arrest is reversible

1321N1 cells were treated with PBOX-21 for 24 h after which time the medium was removed and replaced by drug-free medium. The cells were incubated for a further 24 h and were harvested, prepared and analysed using flow cytometry (Fig. 4). The 24 h treatment with PBOX-21 resulted in an increase of 16% of the cells in G1 relative to the vehicle control. Following removal of PBOX-21 and incubation in the drug-free medium for a further 24 h, this G1 arrest was seen to have been reversed as the percentage of cells within the G1 phase was comparable between the vehicle and treated samples. It would appear that incubation of the cells in drug-free medium for 24 h was sufficient for transition from the G1 block and allowed a return to a normal distribution of cells within the various phases of the cell cycle. This reversal of the G1 arrest indicates a specificity of action of PBOX-21 and confirms its lack of toxicity to the cells.

3.3. PBOX-21 causes a reduction in CDK2, CDK4 and CDK6 kinase activity

Since CDKs regulate cell cycle progression, we next investigated whether the PBOX-21-induced blockage at the G1 phase of the cell cycle was associated with changes in the activity of various CDKs responsible for mediating G1–S progression, namely CDK2, 4 and 6. PBOX-21

treatment caused a time-dependent reduction in the kinase activity of CDK2 with a reduction of approximately 50% in activity at 20 h (Fig. 5A). The reduction was clearly

apparent at 8 h, which is before any changes in cell cycle distribution could be detected, suggesting that this was a cause of the G1 arrest. PBOX-21 treatment also caused a

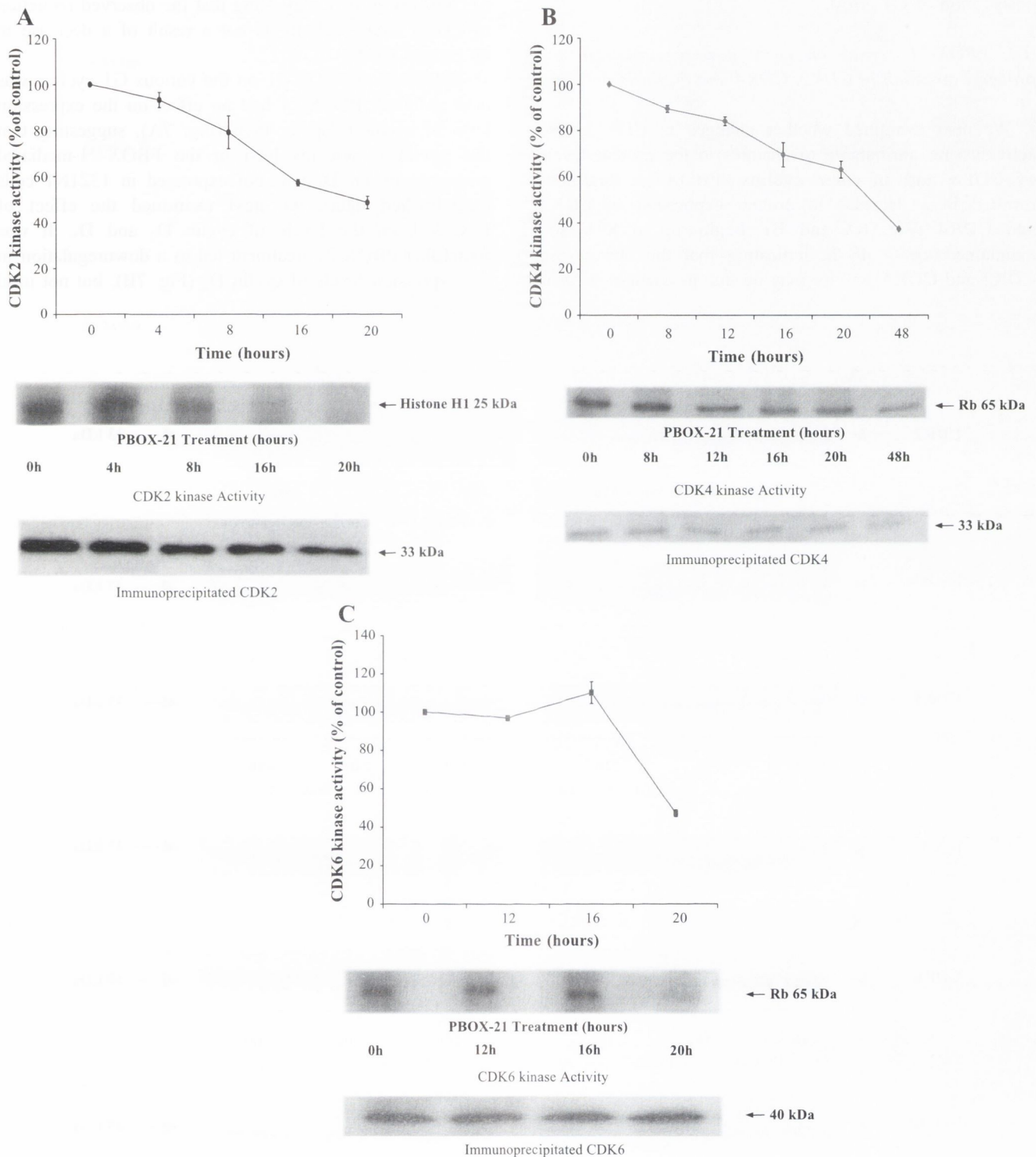
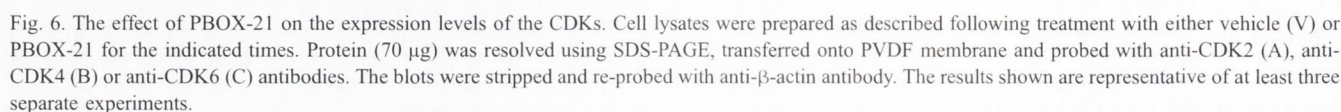


Fig. 5. PBOX-21 causes a time-dependent downregulation in CDK2, CDK4 and CDK6 kinase activity. Whole cell extracts were prepared as described following treatment with PBOX-21 for the indicated times. The protein was immunoprecipitated with either CDK2, CDK4 or CDK6 antibodies and the kinase activity was determined by phosphorylation of Histone H1 or Rb, respectively. For the graph, densitometry was performed on the resultant bands using Scion Image software with 0 h set to 100%. The results shown are the mean $\pm$ S.E.M. of three separate experiments.

3.4. PBOX-21 causes an early downregulation in the protein expression of CDK2, CDK4 and cyclin D₃

protein expression levels. The amount of protein precipitated by the CDK2 and CDK4 antibodies also began to decline with PBOX-21 treatment, in agreement with the observed reduction in their expression levels. PBOX-21 treatment up to 48 h did not change the expression level of CDK6 (Fig. 6C) suggesting that the observed reduction in CDK6 kinase activity is not a result of a decrease in its protein level.

The effect of PBOX-21 on the various G1 cyclins was next analysed. PBOX-21 had no effect on the expression level of cyclin E up to 48 h (Fig. 7A), suggesting that this cyclin is not involved in the PBOX-21-mediated arrest. As cyclin D₁ was not expressed in 1321N1 cells (unpublished data), we next examined the effect of PBOX-21 on the levels of cyclin D₂ and D₃. It was found that PBOX-21 treatment led to a downregulation in the expression levels of cyclin D₂ (Fig. 7B), but not until



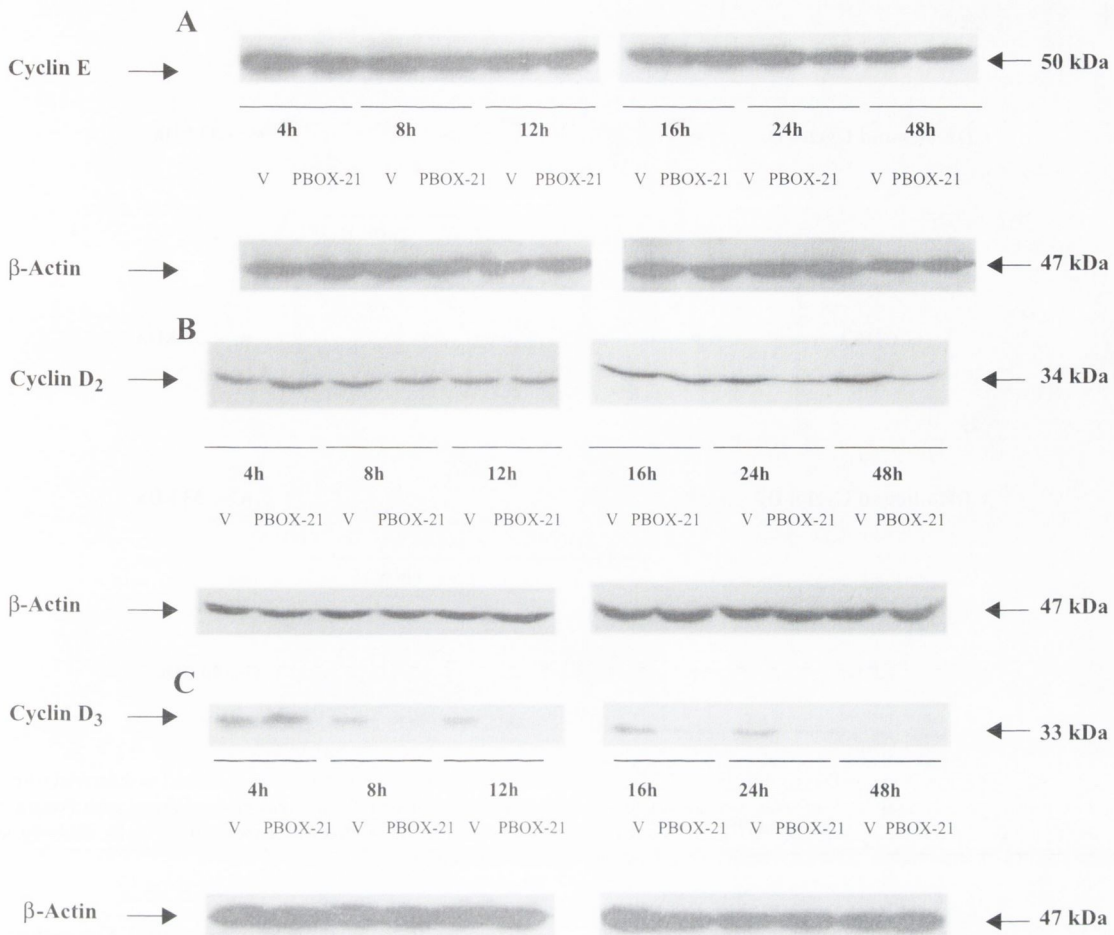


Fig. 7. The effect of PBOX-21 on the expression levels of the G1 cyclins. Cell lysates were prepared as described following treatment with either vehicle (V) or PBOX-21 for the indicated times. Protein (70 μ g) was resolved using SDS-PAGE, transferred onto PVDF membrane and probed with anti-cyclin E (A), anti-cyclin D₂ (B) or anti-cyclin D₃ (C) antibodies. The blots were stripped and re-probed with anti- β -actin antibody. The results shown are representative of at least three separate experiments.

24 h of PBOX-21 treatment, again following the onset of G1 arrest. Further analysis of expression of G1 cyclins showed that the expression level of cyclin D₃ was reduced significantly after treatment with PBOX-21 (Fig. 7C). This downregulation in cyclin D₃ levels was first observed after 8 h of treatment which is prior to the onset of G1 arrest, and around the time of the observed reduction in CDK4 kinase activity. The PBOX-21 induced decrease of cyclin D₃ was maintained up to 16 h of treatment. It should also be noted that after 16 h of treatment, the levels of cyclin D₃ within the untreated 1321N1 cells also began to decline, with no cyclin D₃ expression visible at 48 h. This may be due to a depletion within the culture medium of necessary growth factors. The expression of the D-type cyclins is under the control of mitogenic stimuli and once these begin to diminish the cyclin may undergo ubiquitin-mediated proteolysis [9].

The PBOX-21-induced early downregulation in cyclin D₃ may suggest that it is involved in the mechanism by

which G1 arrest is initiated. Cyclin D₃ is considered to typically associate with either CDK4 and/or CDK6. It was found that at 20 h of PBOX-21 treatment, when both CDK4 and CDK6 kinase activities are seen to be reduced, there was a decrease in the complex formation between both kinases and cyclin D₃ (Fig. 8A and B).

3.5. PBOX-21 causes an upregulation in the protein expression of p21

To further assess how PBOX-21 is mediating its anti-proliferative effect, the expression levels of the CDK inhibitors p16, p21 and p27 were analysed. There were no detectable expression levels of p16 found within the 1321N1 cells and PBOX-21 treatment had no effect upon the expression level of p27 (unpublished data). PBOX-21 did cause a transient upregulation in p21, with an initial induction in expression seen after 16 h of treatment (Fig. 9) after which time the expression declined up to 48 h of PBOX-21 treatment.

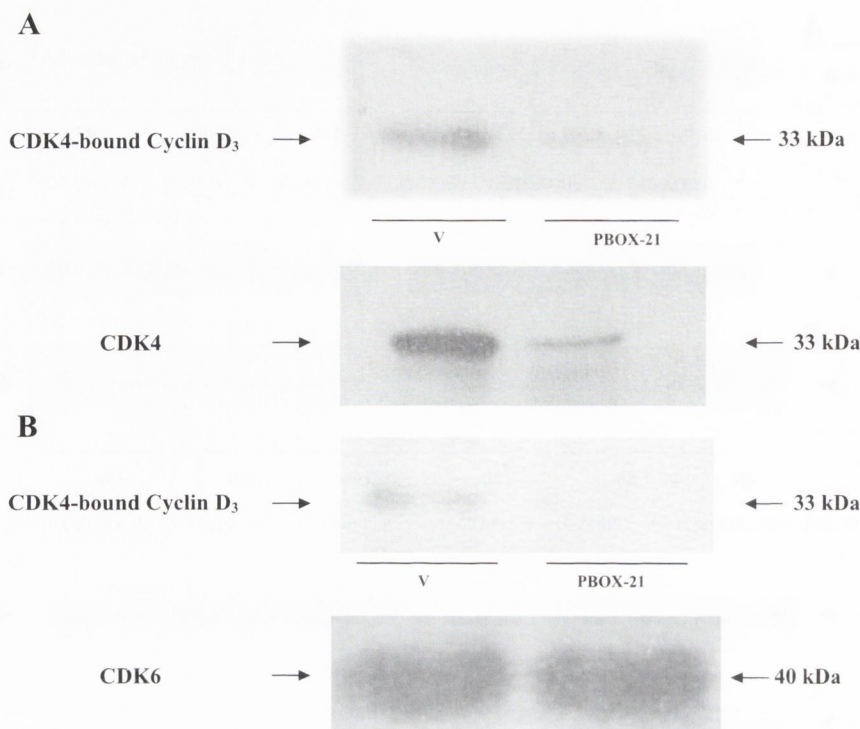


Fig. 8. PBOX-21 causes a reduction in cyclin D₃–CDK4 and cyclin D₃–CDK6 complex formation. Cell lysates were prepared as described with either vehicle or PBOX-21 for 20 h. The protein (200–500 µg) was incubated with anti-CDK4 and anti-CDK6 antibodies and then incubated with Protein A beads. The immunoprecipitated protein was resolved using SDS-PAGE, transferred to a PVDF membrane and probed with either anti-cyclin D₃ antibody, anti-CDK4 or anti-CDK6 antibodies. The results shown are representative of at least two separate experiments.

3.6. PBOX-21 treatment suppresses the phosphorylation of Rb

It has been shown that treatment with PBOX-21 resulted in a sequential reduction in the kinase activities of CDK2, CDK4 and CDK6. As the phosphorylation of Rb is mediated by the cyclin–CDK complexes, it was necessary to determine whether Rb became hypophosphorylated following PBOX-21 treatment. The reduction in mobility of Rb on SDS-PAGE and Western blot analysis is a widely accepted indicator of Rb phosphorylation. In vehicle-treated cells, Rb was found in both hyper- and hypophosphorylated forms which migrated as two bands in the molecular mass range of 115–120 kDa (Fig. 10). PBOX-21 treatment caused a

disappearance of the slow migrating hyperphosphorylated form of Rb.

4. Discussion

This study demonstrates the potent growth inhibitory effects of a representative PBOX compound, PBOX-21 upon the human 1321N1 astrocytoma cell line. The growth inhibitory effects induced by PBOX-21, are mediated through the induction of cell cycle arrest in the G1 phase, and are not associated with cytotoxic effects. The induction of G1 arrest is at the expense of cells in both S and G2/M with cells being drawn equally from both phases into G1.

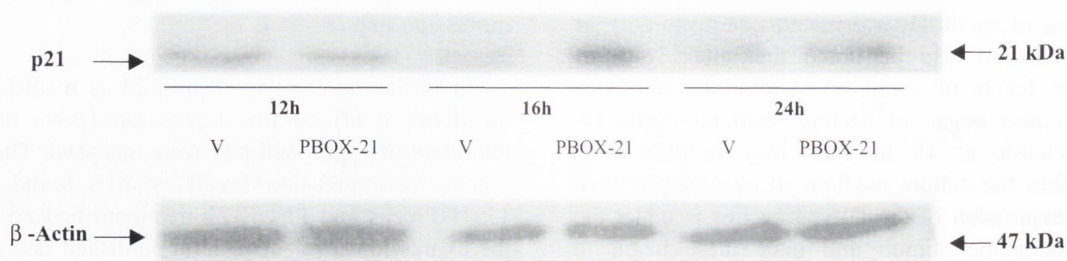


Fig. 9. PBOX-21 causes an upregulation in the expression of p21. Cell lysates were prepared as described with either vehicle or PBOX-21 for the indicated times. Protein (70 µg) was resolved using SDS-PAGE, transferred onto PVDF and probed with anti-p21 antibody. The blots were stripped and re-probed with anti-β-actin antibody. The results shown are representative of at least three separate experiments.

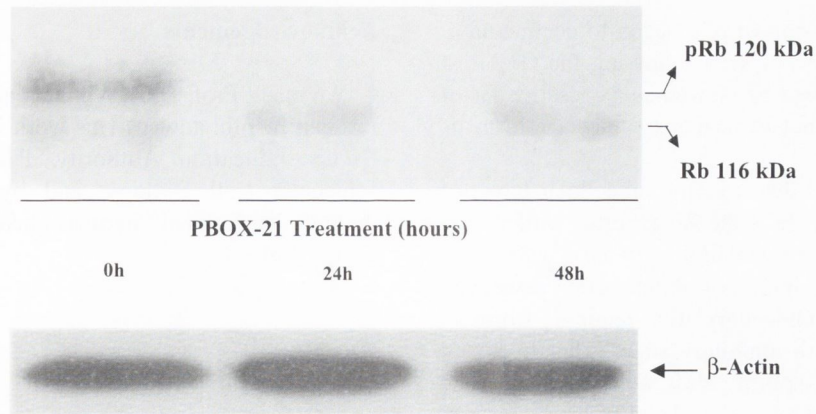


Fig. 10. PBOX-21 reduces the phosphorylation of Rb. Cell lysates were prepared as described with PBOX-21 for the indicated times. The protein (70 μ g) was resolved using a gradient SDS-PAGE gel, transferred onto PVDF and probed with anti-Rb antibody. The blot was stripped and re-probed with anti- β -actin antibody. The results shown are representative of at least two separate experiments.

In order to characterise the molecular pathway by which PBOX-21 mediates its antiproliferative action upon the 1321N1 cell line, a complete analysis of the cell cycle events in these cells both prior to and consequent of the initial observed G1 arrest at 12 h was performed. Thus, it is possible to distinguish between induced changes in cell cycle regulating molecules, which are possibly responsible for the growth arrest, from those that are as a result of the cell cycle arrest itself. In so doing, it was found that the activities of CDK2, CDK4 and CDK6 were downregulated sequentially. CDK2 kinase activity is the first to be affected with a decrease seen as early as 8 h with CDK4 and CDK6 kinase activities reduced following 12 and 20 h of treatment with PBOX-21, respectively. These results would suggest that the reduction in CDK2 and CDK4 activity is a mechanism of breaking the cell cycle during the early phase of PBOX-21 treatment as CDK2 and CDK4 activities are both respectively reduced both prior to and coincident with any changes in cell cycle distribution. The reduction of CDK6 activity, on the other hand, would appear to be a consequence of, and a method of maintaining the PBOX-21-induced arrest, rather than a cause.

In order to address the mechanism by which the reduction in kinase activity of these CDKs occur, the expression of the CDKs themselves and the various G1 cyclins and CDK inhibitors was analysed. It was found that PBOX-21 caused a reduction in protein expression of CDK2 and CDK4 in parallel with, and therefore explaining at least in part, their observed decrease in kinase activity. On the other hand, the expression levels of CDK6 remained unaffected by PBOX-21 treatment indicating an alternative mechanism by which this kinase's activity is being modified.

Western blot analysis of the cyclins revealed that PBOX-21 had no effect upon the expression level of cyclin E. As cyclin E is known to complex with CDK2 late in the G1 phase, this indicates that the inhibition of

CDK2 occurs independent of its association with cyclin E. Cyclin D₁ was not found to be expressed within the 1321N1 cells, whilst cyclin D₂ expression was seen to be downregulated following 24 h of PBOX-21 treatment, again indicating this to be a secondary response to the cessation of cell cycle progression. In contrast, progression of the expression level of cyclin D₃ is reduced significantly, with downregulation occurring after 8 h of treatment, preceding the arrest and suggesting a role in the mechanism by which G1 arrest is induced. The downregulation of cyclin D₃ may be responsible in part for the reduction in the kinase activities of CDK4 and CDK6, due to a reduction in complex formation between these kinases and cyclin D₃ leading to a decrease in the levels of active cyclin D₃-CDK4 and cyclin D₃-CDK6 complexes with kinase activity. In order to confirm this theory, the association between cyclin D₃ and CDK4 and CDK6 was analysed and revealed a reduction in complex formation between both these kinases and cyclin D₃. Therefore, cyclin D₃ downregulation may be responsible for the maintenance of PBOX-21-mediated G1 arrest via modulation of CDK4 and CDK6 kinase activity. At present, we do not know whether the reduction in cyclin D₃ expression occurs through active transcriptional repression or by a post-translational mechanism affecting protein stability of cyclin D₃. It would also be of interest in the future to determine whether enforced expression of cyclin D₃ could abrogate PBOX-21-induced G1 arrest.

Another method by which CDK activity and thus cell cycle progression is modulated is via the expression of the CDK inhibitors. This study looked at the expression of p16, p27 and p21. It was found that p16 was not expressed within the 1321N1 cells and that PBOX-21 did not result in a change in expression in p27 nor in a redistribution of its association with the various CDKs (unpublished data). PBOX-21 treatment did however result in a transient upregulation in the expression of p21, with an initial and maximal induction in expression seen after 16 h. After this

time, the induced expression of p21 began to decline up to 48 h. Again, as this effect is seen following the G1 arrest onset, p21 induction cannot be viewed as a causative factor in the arrest observed, but instead may play a role in its maintenance.

It is well-documented that a major subcellular target of the cyclin-CDK complexes is the Rb ensuring progression through the cell cycle by maintaining Rb in a hyperphosphorylated state. Growth inhibition is thus often associated with suppression of Rb phosphorylation retaining Rb in an unphosphorylated, growth inhibitory state. After treatment with PBOX-21, a clear mobility shift was observed in the Rb protein from the hyperphosphorylated to the hypophosphorylated form. This result is consistent with the reduced CDK2, CDK4 and CDK6 activity in PBOX-21-treated cells as Rb is a substrate of these kinases.

In conclusion, our findings demonstrate that PBOX-21 inhibits the growth of 1321N1 cells by arresting them at the G1 phase of the cell cycle, and the arrest is preceded by a rapid decrease in CDK2 kinase activity and cyclin D₃ expression. The arrest is concomitant with a decrease in CDK4 kinase activity, indicating that these events are likely to be part of the mechanism responsible for initiating the G1 arrest. The inhibition of the activity of both CDK2 and CDK4 is at least in part due to their early downregulation in protein expression. The decrease in CDK4 activity might also be due to the decreased complex association of CDK4 with cyclin D₃. The later reduction in CDK6 kinase activity and its decreased association with cyclin D₃, as well as the upregulation of p21 and downregulation of cyclin D₂ may be involved in maintaining the arrest. Furthermore, we found that Rb becomes hypophosphorylated in response to PBOX-21 treatment. These results indicate that PBOX-21 exerts its growth inhibitory effects through the modulation of the expression and/or activity of several key G1 regulatory proteins. It will be important in our future studies to define the upstream signal transduction pathway mediating PBOX-21 action.

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