

**Mechanisms of fostriecin, AGM-1470 and Taxol  
disruption of cell cycle progression and cell activation.**

**By**

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**B.Sc. (Southern Connecticut State University) 1991**

**A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF  
THE REQUIREMENTS FOR THE DEGREE OF  
DOCTOR OF PHILOSOPHY**

**in**

**THE FACULTY OF GRADUATE STUDIES  
(Department of Medicine – Experimental Medicine)**

**We accept this thesis as conforming to the required standard**

**THE UNIVERSITY OF BRITISH COLUMBIA**

**March 1999**

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Date April 14, 1999

Mechanisms of fostriecin, AGM-1470 and Taxol disruption of cell cycle  
progression and cell activation.

**ABSTRACT**

Signal transduction pathways regulating cell growth and differentiation consist largely of protein kinases that act within restricted phases of the cell cycle prior to a checkpoint and are also necessary for monitoring the integrity of DNA, DNA replication, and cell proliferation versus differentiation. Aberrations in these processes can often lead to uncontrolled growth and de-differentiation, and ultimately a pathological state. To gain insight into the particular signalling proteins that are responsible for these aberrations that lead to a diseased state, this study focused on three potential therapeutic agents, fostriecin, AGM-1470 and Taxol, that have demonstrated anti-neoplastic, anti-arthritic and anti-angiogenic activity, and that mediate their effects at different phases of the cell cycle. Because of fostriecin's growth inhibitory effect on proliferating cells, its mechanism of action was investigated with BHK-21 cells, as well as the immortalized cell lines Jurkat, HL-60, CEM and U937. It was observed to accelerate entry into mitosis based on premature chromosome condensation (PCC) and nuclear lamina breakdown. These events also transpired in the presence of the DNA replication inhibitor aphidicolin and the DNA damaging agent VM-26. Mitotic induction and abrogation of the DNA replication and damage G2 phase checkpoint following fostriecin incubation occurred although fostriecin inhibited p34<sup>cdc2</sup> kinase activity and endogenous histone H1 hyperphosphorylation. Mitotic induction was also observed in the presence of fostriecin at the restrictive temperature in FT210 cells that contain a temperature-sensitive lesion in the *cdc2* gene. I conclude that fostriecin acts within the S and G2 phases of the cell cycle (no effect was observed in G1 synchronized cells) by accelerating cells into a mitotic catastrophe while down-regulating p34<sup>cdc2</sup> kinase, perhaps by inhibiting protein phosphatase 2A. An agent affecting G1 phase progression, AGM-1470, was studied in HUVEC, a primary endothelial cell line that has been used to gain insight into angiogenesis mechanisms. AGM-1470 was shown to inhibit HUVEC proliferation while in G1 phase. This was probably due to its inhibition of protein

kinase C (PKC), which must occur in G1 phase in HUVEC for entry into S phase. AGM-1470 inhibition of DNA synthesis occurred if cells were incubated for only 15 minutes followed by re-incubation in drug-free medium. Cyclin-dependent kinase 4 (Cdk4) activity was also inhibited and may have contributed to the G1 block. To determine if AGM-1470 inhibited mitogen-activated protein (MAP) kinase family members, and because AGM-1470 has demonstrated anti-arthritis efficacy *in vivo*, further studies were conducted in neutrophils as cell activation has been correlated with MAP kinase activity. Taxol was concurrently studied in this system as it has been reported to affect G1 phase-associated signalling events, including MAP kinase activation, in non-differentiated cells. Furthermore, Taxol is currently undergoing pre-clinical studies as an anti-arthritis and pseudogout agent. Taxol failed to inhibit PKC in response to calcium pyrophosphate dihydrate (CPPD) crystals, f-Met-Leu-Phe (fMLP) or phorbol myristate acetate (PMA), yet it reduced neutrophil activation by each of these agents. Taxol inhibited MAP kinase activation only in response to CPPD crystals. In contrast to Taxol, AGM-1470 inhibited activation of PKC in neutrophils in response to CPPD crystals, fMLP or PMA, but inhibited neutrophil activation only in response to fMLP or PMA. AGM-1470 did not block MAP kinase activation in response to CPPD crystals, but as noted for neutrophil activation, inhibited MAP kinase activation by fMLP or PMA. In view of these findings, I conclude that there appears to be an alternate-signalling pathway for neutrophil activation that proceeds through MAP kinase. Wortmannin, a PI 3-kinase inhibitor known to inhibit neutrophil activation when pretreated with CPPD or fMLP, failed to inhibit PMA induced chemiluminescence and activation of p70 S6 kinase. This further provides evidence for the hypothesis that AGM-1470 regulates the MAP kinase pathway and that MAP kinase is utilized by stimulated neutrophils during oxidative response.

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## LIST OF ABBREVIATIONS

|                   |  |
|-------------------|--|
| A595              | spectrophotometric absorbance at 595 nm                                |
| ADP               | adenosine diphosphate  |
| ALP               | alkaline phosphatase   |
| APC               | anaphase-promoting complex or aphidicolin (Figure 17)                  |
| APS               | ammonium persulfate  |
| ATP               | adenosine 5'-triphosphate disodium salt                                |
| AUC               | area under curve   |
| BCIP              | 5-bromo-1-chloro-3 indoyl phosphate                                    |
| bFGF              | basic fibroblast growth factor   |
| BSA               | bovine serum albumin   |
| C°                | degrees celsius  |
| C5a               | the fifth component of the complement                                  |
| Cdk               | cyclin-dependent kinase  |
| CFA               | complete Freund's adjuvant   |
| Ci                | Curie, $2.22 \times 10^{12}$ disintegrations per minute                |
| CIA               | collagen-induced arthritis   |
| CIP               | cyclin inhibitory protein (p21 <sup>CIP1</sup> , p21 <sup>Waf1</sup> ) |
| Ck1               | casein kinase 1  |
| CkII              | casein kinase 2  |
| CDKI              | cyclin-dependent kinase inhibitor                                      |
| cPKCs             | conventional PKC (Ca <sup>2+</sup> -dependent)                         |
| cpm               | counts per minute  |
| CPPD              | calcium pyrophosphate dihydrate  |
| DAG               | diacylglycerol   |
| dH <sub>2</sub> O | distilled water  |
| DNA               | deoxyribonucleic acid  |
| DMF               | N,N-dimethyl formamide   |
| DMSO              | dimethyl sulfoxide   |

|                  |   |
|------------------|---|
| DO               | diolein (DAG homolog; containing C8 fatty acid side chains) |
| DTT              | dithiothreitol  |
| ECL              | enhanced chemiluminescence                                  |
| ECM              | extracellular matrix  |
| EDTA             | ethylene diamine tetraacetate disodium salt                 |
| EGTA             | ethylene bis (oxyethylenitrilo) tetraacetic acid            |
| ERK              | extracellular signal-regulated kinase                       |
| ERK1-CT          | C terminal domain of ERK1 (peptide)                         |
| FPLC             | fast protein liquid chromatography                          |
| fMLP             | formyl-Met-Leu-Phe  |
| g                | gram  |
| GAP              | GTPase activating protein                                   |
| GDP              | guanosine diphosphate                                       |
| GMCSF            | granulocyte/macrophage-colony-stimulating factor            |
| GSK              | glycogen synthase kinase                                    |
| GST              | glutathione S-transferase                                   |
| GTP              | guanine triphosphate  |
| GVBD             | germinal vesicle breakdown                                  |
| x g              | times the force of gravity                                  |
| h                | hour  |
| HBSS             | HEPES buffered Hank's balanced salt solution                |
| HRP              | horseradish peroxidase                                      |
| HUVEC            | human umbilical vascular endothelial cells                  |
| IC <sub>50</sub> | 50% inhibitory concentration                                |
| Ig               | immunoglobulin  |
| IGF-1            | insulin-like growth factor-1                                |
| IgG              | immunoglobulin G  |
| IL-8             | interleukin-8   |
| IP-3             | inositol-1,4,5-trisphosphate                                |
| IR               | insulin receptor  |

|                  |   |
|------------------|---|
| IRS-1            | insulin receptor substrate-1  |
| IP               | immunoprecipitation, immunoprecipitate  |
| kDa              | kilodalton, a measure of molecular mass   |
| l                | litre   |
| LC <sub>50</sub> | lethal concentration for 50% of animals   |
| LTB <sub>4</sub> | leukotriene B <sub>4</sub>  |
| M                | moles/litre   |
| mM               | millimolar  |
| μ                | micron (10 <sup>-6</sup> meters)  |
| mA               | milli Amps  |
| mAb              | monoclonal antibody   |
| MAP              | mitogen-activated protein (not abbreviated for microtubule-associated protein)      |
| MAP-2            | mitogen-associated protein-2 (not abbreviated for microtubule-associated protein-2) |
| MAPK             | MAP kinase  |
| MBP              | myelin basic protein  |
| MBP-NT           | N terminal peptide of MBP   |
| MEK              | MAP kinase kinase or ERK kinase   |
| MEKK             | MEK kinase  |
| MetAP-2          | methionine aminopeptidase-2   |
| μg               | microgram   |
| mg               | milligram (10 <sup>-3</sup> gram)   |
| min              | minute  |
| μl               | microlitre  |
| ml               | millilitre  |
| MSUM             | monosodium urate monohydrate  |
| NADPH            | nicotinamide adenine dinucleotide phosphate (reduced form)                          |
| NaF              | sodium fluoride   |
| NBT              | nitro blue tetrazolium  |

|                     |   |
|---------------------|---|
| nPKCs               | novel PKC (Ca <sup>2+</sup> independent)  |
| nM                  | nanomolar (10 <sup>-9</sup> molar)  |
| nm                  | nanometres (10 <sup>-9</sup> meters)  |
| NP-40               | Nonidet P-40  |
| o/n                 | overnight   |
| p42 <sup>ERK2</sup> | the 42-kDa MAP kinase encoded by ERK2 gene, also referred to as p42 <sup>mapk</sup> |
| p44 <sup>ERK1</sup> | the 44-kDa MAP kinase encoded by ERK1 gene, also referred to as p44 <sup>mapk</sup> |
| P70s6k              | 70 kDa ribosomal protein S6 kinase  |
| PA                  | phosphatidic acid   |
| PAF                 | platelet-activating factor  |
| PAGE                | polyacrylamide gel electrophoresis  |
| PCC                 | premature chromosome condensation   |
| PCNA                | proliferating cell nuclear antigen  |
| PD                  | phospholipid  |
| PDGF                | platelet-derived growth factor  |
| PD/DO               | phosphatidylserine and diolein  |
| PIP <sub>2</sub>    | inositol 4,5-bisphosphate   |
| PI 3-K              | phosphatidylinositol 3-phosphate kinase   |
| PKA                 | cyclic AMP-dependent protein kinase   |
| PKC                 | protein kinase C  |
| PKB                 | protein kinase B (c-Akt, Rac kinase)  |
| PKI                 | cAMP-dependent protein kinase inhibitor peptide                                     |
| PKM                 | protein kinase M  |
| PLC                 | phospholipase C   |
| PMA                 | phorbol 12-myristate 13-acetate   |
| PMSF                | phenyl methylsulphonyl fluoride   |
| PP-                 | protein phosphatase (i.e., PP-2A, PP-1)   |
| PS                  | phosphatidylserine  |

|               |   |
|---------------|---|
| PTPase        | protein tyrosine phosphatase                          |
| PVDF          | polyvinylidene difluoride membrane                    |
| RA            | rheumatoid arthritis                                  |
| rpm           | revolutions per minute                                |
| rsk           | ribosomal S6 kinase                                   |
| SAPK          | stress-activated protein kinase                       |
| SBTI          | soybean trypsin inhibitor                             |
| SDS           | sodium dodecyl sulfate                                |
| SDS-PAGE      | sodium dodecyl sulfate polymerase gel electrophoresis |
| sec           | second  |
| Ser           | serine  |
| SH2           | src homology domain 2                                 |
| SH3           | src homology domain 3                                 |
| SOS           | son of sevenless (guanine nucleotide exchange factor) |
| TBS           | Tris-buffered saline                                  |
| TBST          | Tris-buffered saline with Tween-20                    |
| Thr           | threonine   |
| TNF- $\alpha$ | tumor necrosis factor- $\alpha$                       |
| Topo II       | topoisomerase II                                      |
| TOP           | target of rapamycin                                   |
| Tyr           | tyrosine (amino acid)                                 |
| UV            | ultraviolet   |
| v             | volume  |
| VEGF          | vascular endothelial growth factor                    |
| w             | weight  |

## ACKNOWLEDGEMENTS

The greatest thing in the world is not whether we stand, as in what direction we are moving... We must sail some times with the wind and sometimes against it – but we must sail, and not drift, or lie at anchor. *Oliver Holmes*

For those whom I recognize here, this is an appropriate quotation for the journey that I have taken to accomplish this recent endeavor, for certainly it was not the destination that merits my accomplishment, but the journey that unfolded before me. Before recognizing those who played an important role academically in my pursuit of the Doctorate Degree in Medicine, I wish to acknowledge those who had the greatest influence on me, those who helped me to sustain my values, perseverance and focus, particularly during those periods, at the beginning, and towards the end, when a great number of forces seemed to be working against me. My greatest thanks go out to my family. First my wife, Rachael, who never wavered, and always continued to love me and remind me of what, was truly important. She not only sacrificed a great deal so that I might finish this leg of my life while tackling the many other challenges that I had undertaken, but she also contributed to this text in typing a great deal of it, and by her assistance in the lab those many late nights. I am also aware of the continuous thoughts and caring that was extended to me by my Father and Valerie, my sister, Jennifer and her husband, Roy, who were a constant source of encouragement. And to my late mother who wrote to me many years ago:

Life has many challenges, some that are welcomed, and some that I would rather refuse, but all have created me,

Also to my wife's family, whose constant prayers and thoughts truly made a difference, including Mr. Blain, who has been an example to me, who I respect dearly for his contributions daily to everyone that is around him, and for the strength that he now shows during his latest struggle.

Dr. Steven Pelech, my Ph.D. supervisor, was more than my academic mentor, but is my friend, and has always been there to listen to me, and criticize me when I really needed it. He took me on when I was about to give up, and recognized my abilities and strengths before I did, and made sure that I sought to develop these. I could not have done this without him. I will always have the utmost respect for him, and I look forward to the years

ahead that we may collaborate. I also wish to recognize the many others whom made an impact on me, and contributed in so many ways to this accomplishment. They are: Dr. Norman Wong and his assistant, Anita, the Vanderveers, John Jackson, Drs. John Gilliard, Zuhan Hu, Helen Burt, Peter Winocour, Salam Kadim, Samir Mounir, Christopher Penny, Donald Sanger, David Kaplan, and Peter Vaktor, and Dean Hardwick.

This thesis was also based upon the collaborations with many scientists who also taught me a great deal, including Drs. Michel Roberge, Helen Burt, Giorgio Attardo, Terry Bowlin, Bill Salh, Frank Jirik, Salam Kadhin, John Th'ng, Zuhan Hu, Serge Lamothe, and John Jackson, John-Marc Demuys, and Danny Leung. Enormous support and patience went into the development of this thesis, for which I also wish to thank Drs. Roger Brownsey, Vince Duronio, Michael Gold, David Litchfield, Karl Riabowol, Robert Kay, Robert McMaster, and of course, Steven Pelech.

The order in which I presented my acknowledgements by no means indicates the weight by which I measure the efforts the many individuals had so thoughtfully made. For those that are not mentioned here, let it be known that I frequently think of all those who have played a part in my life, and that I recognize that I am a composition of all of my experiences, which have been so much more meaningful because of the many people who have selflessly come in-and-out of my life to offer a part of themselves, and they have made all of the difference. Thank you.

1. Signal transduction and therapeuticsCell signal networking

Regulated cell growth and differentiation is a consequence of the operation of a communication network that responds to physiological stimuli. It can be initiated by an interaction between an extracellular mediator such as a growth factor, hormone or chemoattractant, and its membrane bound receptor. The response is conveyed via discrete biochemical reactions of finite duration, ultimately controlling transcription of both immediate and delayed early-response genes. Binding of extracellular effectors to their respective receptors stimulates progression through the G1 phase of the cell cycle, and similar signal transduction cascades are utilized for both cellular growth and differentiation (Fantl *et al.*, 1993; Karin, 1994). The decision for a cell to progress through the cell cycle or to arrest in G1 and proceed through differentiation, involves genes whose expression is delayed relative to immediate nuclear responses by growth factors (Johnson and Vaillancourt, 1994; Collins *et al.*, 1994). The signal transduction cascades regulating these responses often utilize sequential protein kinase reactions. Ligand binding to the extracellular domain of receptor tyrosine kinases is commonly followed by receptor dimerization, stimulation of protein-tyrosine kinase activity and autophosphorylation, which determines the selectivity of the ligand-recruited signalling pathway. Specific seven transmembrane receptors coupled to heterotrimeric G proteins can also stimulate protein kinase cascades, inducing sequential phosphorylation reactions converging in the nucleus to phosphorylate and regulate the activity of transcription factors, as well as proteins that regulate the cell cycle checkpoints such as the cyclin-dependent kinases and tumor suppressor proteins. Aberrant expression or mutations in these growth factor receptors, or of the downstream signalling proteins, can either lead to uncontrolled proliferation or defects in essential developmental and differentiation processes. Further, because there is a plethora of messages from mitogens, hormones, cytokines, ions and other molecules, these other signal pathways must be coordinated precisely via feedback and feedforward loops as

well as pathway crosstalk. Aberrations in these processes can lead to uncontrolled growth and attenuated differentiation.

### Receptor signalling

Receptors for growth factors such as epidermal growth factor (EGF), colony stimulating factor (CSF-1), platelet derived growth factor (PDGF), fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) undergo autophosphorylation at tyrosine residues following ligand binding and subsequent dimerization of receptors (Fantl *et al.*, 1993). Some tyrosine autophosphorylation sites act as high-affinity binding sites for regions on secondary signalling molecules known as Src-homology 2 (SH2) domain sequences. It is a consequence of the sequences containing the tyrosine phosphorylated residue on the activated receptor and the precise structure of the SH2 domain that dictates the specificity of interaction with a particular secondary messenger and the receptor (Moran *et al.*, 1990). Examples of such SH2 containing signalling messenger proteins include phospholipase C $\gamma$  (PLC $\gamma$ ), the p85 subunit of phosphatidylinositol (PI) 3-kinase, Shc, growth receptor binding protein 2 (Grb2), and the p21<sup>ras</sup>-GTPase activating protein (GAP); (Moran *et al.*, 1990; Anderson *et al.*, 1990; Simon *et al.*, 1993). These proteins act as adapters or regulatory subunits to eventually elicit activation of downstream kinases. For example, PLC $\gamma$ , PI 3-kinase and GAP have important functions in signal transduction pathways. Activation of GAP enhances GTPase activity of Ras and is therefore likely to be involved in the regulation of Ras action, while PI 3-kinase activation results in the production of the 3'-phosphorylated secondary messengers that are implicated in cell survival and differentiation (Fry, 1994; Rameh *et al.*, 1995). Activation of PLC $\gamma$  leads to the generation of inositol trisphosphate, and diacylglycerol (DAG), which in turn mobilize intracellular calcium and activate protein kinase C (PKC).

PKC isoforms are ubiquitous signal transducers and have been demonstrated to be essential for numerous physiological conditions including angiogenesis and neoplasia and have been implicated in insulin signalling (Rotenberg and Weinstein, 1991; Cheatham and Kahn, 1995; Kent *et al.*, 1995). For example, activators of PKC were shown to exert cell-cycle dependent growth regulatory effects on human vascular endothelial cells (HUVEC)

(Zhou *et al.*, 1993), and the activation of PKC in HUVEC in the absence of an increase of intracellular calcium indicates that one or more Ca<sup>2+</sup>-independent PKC isoenzymes are both necessary and sufficient for human endothelial cell proliferation (Kent *et al.*, 1995). Further angiogenic implications of these PKC results will be discussed later. GAP stimulates the GTPase activity of Ras, and Grb2 is an adapter protein that binds Sos, which catalyzes the dissociation of GDP from Ras, thereby allowing GTP to bind to and activate Ras (Johnson and Vailancourt, 1994). The literature is replete with evidence for Ras and other G proteins regulating cell proliferation and differentiation pathways, many of which control mitogen transmitting signals through the activity of mitogen-activated protein kinase (MAPK). Both loss-of-function and activating mutations, and mutations causing constitutive activation of G protein-coupled seven transmembrane domain receptors are implicated in a variety of diseases including diabetes insipidus, severe neonatal hyperparathyroidism and retinitis pigmentosa, amongst many others (Coughlin, 1994).

#### Cell cycle control

Cell growth (proliferation) and differentiation are two fundamental phases of multicellular existence. The phenomenon of uncontrolled proliferation, which may result in neoplasia, or cancer, is thought to result from a block in the ability of cells to undergo differentiation and/or apoptosis. The cell cycle is divided into four phases; the periods associated with DNA synthesis (i.e. S phase), and mitosis (i.e. M phase), are separated by gaps referred to as G1 (preceding S phase) and G2 (between S and M phases). At the transition of each gap phase are the biochemical pathways referred to as checkpoints that monitor the integrity of the DNA prior to progressing out of the respective gap phases. Checkpoints prevent the specific events associated with cellular growth such as DNA replication, nuclear envelope breakdown, spindle formation and chromosome condensation and segregation. Aberrations in the checkpoints and growth suppressor genes would permit the cells to progress through the cell cycle and divide with DNA lesions and incomplete DNA replication. This could result in uncontrolled proliferation or neoplasia.

An elaborate signal transduction system of signalling pathways and feedback loops exist that ensures each event is performed correctly and in proper sequence. As previously

mentioned, specific receptor associated transduction pathways connect the cell with extracellular signals, such as mitogens, which bind to their specific receptors and initiate a cascade of events that culminates in the expression of proteins. This includes regulatory subunits of kinases called cyclins which form a diverse family of proteins whose level oscillate during the cell cycle, and are required for the activation of cyclin-dependent kinases (Cdks) (Pines, 1993). Cdks undergo transient activation at various stages of the cell cycle and are believed to directly trigger the major transitions of the cell cycle. Several classes of cyclins have been discovered (cyclin A to H) (Lees, 1995). Cyclin B binds mainly to Cdc2 (Cdk1) during late G2 and mitosis to form a complex that has been implicated in the initiation of mitosis (O'Connor *et al.*, 1997; Poon *et al.*, 1997). Cyclins A and E bind to Cdk2 during late G1 and S phase, and these complexes are suspected to be involved in progression past the 'Start' or 'Restriction Point' in the initiation and maintenance of DNA replication (Lees, 1995; Zwicker *et al.*, 1995). The first Cdk activated after mammalian cells are released from a quiescent state is composed of cyclin D and either Cdk4, or Cdk6 depending on the cell type (Lavoie *et al.*, 1996). Cyclin D/Cdk4 (Cdk6) is the first link between differentiation and cell growth as pRb is a substrate for the cyclin D/Cdk4 complex, and phosphorylation inactivates the inhibitory activities of pRb, thus promoting proliferation (Kato *et al.*, 1993; Johnson, 1995). Also, pRb (and p107 and p130) target the transcription factor E2F that regulates the early immediate genes such as *c-myc* and *B-myb*, as well as genes that encode enzymes required for DNA synthesis. pRb negatively regulates transcription by binding E2F, holding it in an inactive state (Johnson and Schneider-Broussard, 1998).

Del Bino *et al.* (1994) have suggested that there are distinct differences between proliferating and differentiating cells with respect to the sensitivity of DNA damage detection and apoptosis trigger mechanisms. Proliferating cells have a more sensitive mechanism for DNA damage detection coupled with DNA lesion dependent feedback mechanisms that delays cell cycle progression during which time DNA repair mechanisms are initiated. If the damage is extensive or repair is unsuccessful, apoptosis may be triggered through c-Myc (Evan *et al.*, 1992). Cells that are differentiating and have DNA damage, thus delaying cell division, are less sensitive to apoptosis triggering mechanisms.

This may be a result of the down regulation of c-Myc during differentiation (Cayne *et al.*, 1987).

#### Cell cycle perturbations and cancer

A fundamental characteristic of malignant tumors is the abnormal regulation of their proliferating cells. A possible approach to developing effective therapy would be to exploit this biochemical feature by designing chemotherapeutic strategies based on detailed knowledge of the regulatory mechanisms that constitute the nature of the effects that result in uncontrolled proliferation. This approach to developing chemotherapeutic agents becomes more accessible as the details of these mechanisms are elucidated. The genetic changes that are observed in malignant tumors present to the researcher a broad spectrum of potential applications. For instance, defects in the events that control the cell cycle may render the cell more sensitive to cytotoxic drugs versus a normal cell or a differentiating cell. Proliferating and differentiating cells respond to physiological variations in conditions such as concentrations of nutrients, growth factors, chemoattractants, hormones, metabolites, physiological stress and DNA damage. Perturbations in these conditions can lead to a weakness in the control of cellular proliferation. Kahn *et al.* (1994) have proposed that this same weakness of control that exists in the proliferating cell could increase its vulnerability to various pharmacologically induced stresses, therefore pushing the cell into a 'region of state space', or cell cycle block from which they cannot recover. An example of such a strategy would be to utilize an agent such as an inhibitor of a kinase or phosphatase that affects (positively or negatively) the regulatory factor that prevents the cell from entering a vulnerable phase of the cell cycle, such as the phases preceding the cell cycle checkpoints, at S phase and mitosis. The agent would naturally be targeted to that regulator in the cancer cell versus the normal cell. Given that cellular differentiation plays an essential role in physiology, and the regulatory processes are similar between proliferating cells and differentiating cells, differing often only in how a biochemical signal is utilized, understanding the regulatory mechanisms of both are important in the development of potential antitumour agents. A therapy may be developed based on the differences between proliferating and differentiating cells, and that effectors of both

processes could potentially be utilized synergistically. For instance, I am presently investigating the signalling mechanisms that are mediating survival in human gliomas. I have found that the Rac-MEKK-JNK pathway, which is usually utilized by normal astrocytes in differentiation processes, or apoptosis depending on the integrity of the DNA, is actually utilized by all glioma cell lines investigated as a survival pathway. Therefore, because of aberrations in some signalling proteins upstream and/or downstream of this pathway, gliomas utilize the Rac-MEKK-JNK pathway to mediate survival. Determining where the bifurcation occurs between differentiation and survival in these cells will shed light on putative targets for glioma therapy that could prove to be glioma specific.

Since cancer is generally initiated as a result of deregulated cellular proliferation, understanding the mechanisms that induce cell cycle progression, as well as determining the mechanisms of drugs that inhibit cellular proliferation, can lead to the finding of possible targets for cancer therapy. In this thesis I have investigated the mechanisms of three putative anti-cancer compounds presently under clinical trials, and determined the effects of these drugs on the activities of the aforementioned kinases including Cdk1, MAP kinase, p70<sup>S6K</sup> and PKC, given their roles in mitogenesis and cell cycle regulation.

#### Angiogenesis signalling

A cellular process that affects the proliferation of tissue vascularization and leads to rapid expansion of a tumour population and metastasis is angiogenesis. Tumour growth is partly angiogenesis-dependent. Tumours can induce capillary growth in the vicinity of the tumor cell population for their nourishment, and the resulting neovascularization permits the shedding of cells from the primary tumour. Angiogenesis is also implicated in inflammatory arthritis and diabetic retinopathy (Maione and Sharpe, 1990). A number of mediators of angiogenesis have been identified and are known to be released from the tumour and include several growth factors such as bFGF, PDGF, VEGF, angiogenin, pleiotrophin and IL-8 (Nicosia *et al.*, 1994). These growth factors signal the endothelial cells to become chemotactic and proliferative through their respective tyrosine kinase receptors. Endothelial cells, which form the inner lining of blood vessels, play a crucial role in angiogenesis by degrading the basement membrane, initiated by growth factors and

interaction with the extracellular matrix (E.C.M.). The crucial link to malignant tumors is that solid tumors are angiogenesis-dependent as there is a prevascular and a neovascular phase of tumor growth, with metastasis resulting from the latter. The endothelial cells migrate through the vessel forming capillary sprouts and proliferating to develop new vessels. This process is involved in other 'neovascularization diseases' including rheumatoid arthritis, diabetic retinopathy and psoriasis (Folkman, 1975; Nicosia *et al.*, 1994). Recently, it has been shown that inhibitors of angiogenesis are also effective inhibitors of arthritis. These inhibitors include compounds that inhibit indirectly the activation of cyclin D/Cdk4, and direct inhibitors of secondary messengers of receptor tyrosine kinases including PKC and PLC $\gamma$ . For instance, it has been demonstrated that the Fumagillin analog angiogenesis inhibitor, AGM-1470, inhibits the activation of the pRb kinases Cdk2 and Cdk1 (Abe *et al.*, 1994). As mediators of neovascularization within these diseases are now being discovered to be similar in mechanism, angiogenesis signal transduction studies with endothelial cells would also be beneficial for gaining insight into the signalling processes in arthritis and diabetes.

Angiogenesis inhibitors are being developed primarily for anticancer therapy; however, many of these new compounds may be therapeutic for the neovascularization that accompanies chronic inflammatory diseases, such as arthritis and psoriasis. For instance, the first angiogenesis inhibitor was found in cartilage (Brem and Folkman, 1975). It was characterized as an inhibitor of mammalian collagenase (Moses *et al.*, 1990), and was demonstrated to inhibit angiogenesis *in vivo* and capillary endothelial proliferation and migration *in vitro* (Moses *et al.*, 1990). Other examples include minocycline, which is a semisynthetic tetracycline antimicrobial that inhibits collagenase activity in synovial fluid of patients with rheumatoid arthritis (Greenwald *et al.*, 1987) and inhibits angiogenesis in the cornea as effectively as a cortisone-heparin pellet (Tamargo *et al.*, 1991), and medroxyprogesterone, that inhibits collagenolysis and tumour-induced angiogenesis (Gross *et al.*, 1981). Also, inhibitors currently being designed for anti-cancer therapy generally have little or no toxicity. It would therefore be fruitful to study the mediating pathways in these diseases in parallel with the aid of inhibitory agents, and the assemblage of information on the effects of the inhibitors on signalling in these diseases may result in

further insight into novel targets for therapeutic development in angiogenesis related diseases.

### Neutrophil activation

The inflammatory disease known as acute pseudogout arises from the deposition of calcium pyrophosphate dihydrate crystals (CPPD) in the synovial joints of humans (McCarthy, 1985). Both monoclinic and triclinic (T) crystalline forms of CPPD are able to activate neutrophils in the joint, a process that is thought to be a key factor in the pathophysiology of the disease (McCarthy, 1985; Winternitz *et al.*, 1996; Burt and Jackson, 1994). CPPD crystals have been shown to absorb synovial protein components including small amounts of IgG (synovial fluid is an ultrafiltrate of plasma). *In vitro*, both uncoated and plasma- or synovial fluid-coated crystals have been shown to induce neutrophil activation, including superoxide anion production and cytosomal enzyme release (Kozin *et al.*, 1979; Nagase *et al.*, 1989; Burt and Jackson, 1993).

The primary function of neutrophils is the identification, migration to, and destruction of microbial pathogens, hence protecting the host from infections. At the onset of phagocytosis, antimicrobial enzyme systems including  $O_2^-/H_2O_2$  generating NADPH oxidase, myeloperoxidase, proteases, and hydrolases are activated and delivered into phagocytotic vesicles in high concentrations (Unkeless *et al.*, 1992; Edwards, 1995; Bokosh, 1995). When activated by chemoattractants such as fMLP (N-formyl-Met-Leu-Phe), C5a, PAF, LTB<sub>4</sub> and IL-8, neutrophils undergo responses including cytoskeletal rearrangement, exocytosis and a respiratory burst catalyzed by NADPH oxidase (Ukeless *et al.*, 1992; Bokoch, 1995). The activation of neutrophils by chemoattractants proceeds via intracellular signalling pathways that include many of the components identified in particulate-activated and PMA-induced neutrophils. What has thus far been learned about the events causing the activation of neutrophils indicates that activation is mediated by receptors and G proteins, and is associated with a marked increase in protein phosphorylation (Edwards, 1995). Phospholipase C is rapidly activated resulting in the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) to form inositol triphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). IP<sub>3</sub> acts to mobilize  $Ca^{2+}$  from intracellular stores and

DAG acts in conjunction with  $\text{Ca}^{2+}$  to activate various isoforms of PKC. PKC has been demonstrated to phosphorylate a component of the NADPH oxidase complex leading to its activation (Jones, 1994) and generation of superoxide anions. Chemoattractants have been shown to stimulate phospholipase  $\text{A}_2$  ( $\text{PLA}_2$ ) and PLD, but it has not been known whether these are downstream events resulting from PKC activation and  $\text{Ca}^{2+}$  mobilization or events regulated by distinct pathways (Cook and Wakelam, 1992; Bokoch, 1995).  $\text{PLA}_2$  has been suggested to play a role in regulating the production and release of  $\text{O}_2^-$  by neutrophils (Forehand *et al.*, 1993). Inhibition of  $\text{PLA}_2$  specifically blocks fMLP-induced superoxide production (Koike *et al.*, 1992). PLD catalyzes the hydrolysis of phosphatidylcholine to generate phosphatidic acid (PA) plus the choline headgroup. PA may act as a secondary messenger, or be further metabolized to DAG, which can activate PKC (Lopez *et al.*, 1995). Evidence that fMLP activates PLD through G protein activation without affecting PLC activity is well-established (Edwards, 1995; Bokoch, 1995). Stimulation of the respiratory burst is unaffected by inhibition of PKC or PLD activities, but both are required for a sustained respiratory burst (Watson *et al.*, 1994). Although PLD acts downstream of PLC, and both provide DAG for PKC, it is believed that PLD activation provides DAG to sustain rather than initiate PKC activation (McPhail *et al.*, 1995). PKC has been demonstrated to induce events producing MAP kinase activation (Worthen *et al.*, 1994; Krump *et al.*, 1997; Jackson *et al.*, 1997a), together with neutrophil chemiluminescence, superoxide anion generation and degranulation (Jackson *et al.*, 1997a).

## 2. Protein kinases

Multicellular eukaryotic organisms are subject to complex interactions of differentiation and proliferation events that occur in a highly ordered manner. These events can be initiated in response to an extracellular signal. Frequently, these signals ultimately regulate or modulate gene expression. This 'signal transduction' occurs through the interaction of different cellular components that activate target key metabolic proteins, or modulate gene expression at both the transcriptional and translational levels. Most components of these signal transduction pathways are proteins whose activity is altered by either binding to primary or secondary messengers, subunit composition, covalent

modification and subcellular localization. Protein phosphorylation is the major mechanism of reversible covalent modification. Further, altered protein phosphorylation is the fundamental route of hormone modulation of cellular processes. The majority of covalent modifications observed are on serine or threonine residues, and the Ser/Thr protein kinases are themselves substrates for other protein kinases, or can modulate their own activity via autophosphorylation.

### PKC

PKC represents a large gene family of isoenzymes responsible for the phosphorylation of many intracellular, cytoskeletal, nuclear and plasma-associated proteins (Nishizuka, 1992; Malviya and Black, 1993). Its isoforms differ remarkably in their structure and expression in different tissues, mode of activation and in substrate specificity (Hug and Sarre, 1993; Newton, 1995). The classical PKC activity was discovered by Nishizuka's group as a histone kinase that may be activated by  $\text{Ca}^{2+}$  and phospholipids, proteolysis or phorbol esters (Nishizuka, 1988, 1995). PKC isoforms are single polypeptides consisting of an N-terminal regulatory region (~20-40 kDa) and a C-terminal catalytic domain (~45 kDa) (Newton, 1995). The regulatory and catalytic regions are separated by a hinge region, which upon membrane binding, becomes proteolytically labile (Newton, 1993), and the proteolytically generated kinase domain, also termed PKM, remains constitutively active since it is freed of pseudosubstrate inhibition (James *et al.*, 1992; Hug and Sarre, 1993; Newton, 1995; Nishizuka, 1995). Thus far, cDNAs coding for PKC isoforms have been divided into two major groups: the Group A  $\text{Ca}^{2+}$ -dependent, or conventional PKCs (cPKCs), and the Group B  $\text{Ca}^{2+}$ -independent PKCs, which include novel and atypical PKCs (nPKCs and aPKCs) (Nishizuka, 1988; Huang, 1990). Group A, or conventional isoforms such as PKC- $\alpha$ , - $\beta$ , - $\gamma$  are  $\text{Ca}^{2+}$ -dependent as well as dependent on phospholipid and DAG (Kikkawa and Nishizuka, 1986; Hug and Sarre, 1993). Following stimulation of tyrosine kinase-linked or G protein-linked receptors, DAG and  $\text{IP}_3$  are generated by the hydrolysis of  $\text{PIP}_2$  by PLC.  $\text{IP}_3$  then mobilizes  $\text{Ca}^{2+}$  from calcisome vesicles in the endoplasmic reticulum, subsequently causing translocation of cPKCs to the

plasma membrane for activation by DAG (Nishizuka *et al.*, 1992; Malviya and Block, 1993; Nishizuka, 1995). DAG is rapidly degraded following generation by PLC, but a second phase of elevation occurs that is responsible for sustained or repetitive activation of PKC (Nishizuka, 1995). PKC can be activated independent of membrane translocation (Lu *et al.*, 1994), probably due to DAG-stimulated activation of pre-existing membrane-associated PKC or as a result of the phosphorylation state of the enzyme. Group B, or novel PKC isoenzymes ( $\delta$ ,  $\epsilon$ ,  $\eta$ ,  $\theta$ ) differ from that of group A in that they do not contain the region of conserved amino acids conferring the  $\text{Ca}^{2+}$ -dependence of cPKCs (Huang, 1990; Newton, 1995). Proteolysis of PKC can result in the generation of  $\text{Ca}^{2+}$ -independent kinase, PKM, as mentioned earlier by the  $\text{Ca}^{2+}$ -activated proteases, calpains I and II (Kishimoto *et al.*, 1989; Savart *et al.*, 1992). Novel PKCs  $\delta$ ,  $\epsilon$ ,  $\eta$  and  $\theta$  do not respond to calcium, but require DAG, and the atypical PKCs  $\zeta$  and  $\lambda$  are not activated by either calcium or DAG, but can be selectively activated by the products of PI 3-kinase; phosphatidylinositol (3,4) biphosphate and phosphatidylinositol (3,4,5) triphosphate (Toker *et al.*, 1994). In fact, PKC $\zeta$  has been shown to be negatively regulated by treatment with wortmannin, resulting in the subsequent inhibition of the Ras-Raf-Mek1-MAP pathway (Sontag *et al.*, 1997). PI 3-kinase is a heterodimer lipid kinase composed of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit, and plays a role in a variety of cellular functions including mitogenic signalling, cell transformation, inhibition of apoptosis and regulation of actin and integrin functions (Carpenter and Cantley, 1996).

The importance of PKC lies in the fact that it is an early signal transduction enzyme that responds to extracellular signals through second messengers such as  $\text{Ca}^{2+}$  and phospholipids and proteolysis, and it represents a direct receptor for tumor promoting phorbol esters. With its broad substrate specificity, it is an enzyme that allows for great divergence in signal transduction immediately following second messenger binding. Newly synthesized PKC associates with the insoluble fraction of cells (Borner *et al.*, 1989). It is processed to a mature cytosolic form via three functionally distinct phosphorylations: transphosphorylation at the activation loop to render the kinase catalytically competent; autophosphorylation at the C terminus that stabilizes the present conformation; and a second autophosphorylation that releases the protein into the cytoplasm (Newton *et al.*, 1995).

This conformation remains inactive since the N terminal pseudosubstrate occupies the C terminal substrate binding domain. Membrane translocation is mediated by DAG binding to the C1 domain (C1-C4 domains, defined starting from the N-terminus of the protein) and phosphatidylserine at the C2 domain (Orr and Newton, 1992). It is the high affinity binding of both domains that results in the release of the pseudosubstrate and maximal activation of the enzyme. Calcium only acts to increase the affinity of the conventional PKC isoenzymes for the acidic lipids (Newton *et al.*, 1994). Therefore, PKC is regulated by phosphorylation, which regulates the subcellular localization and the active site, and by second messengers that control membrane translocation and reduce pseudosubstrate exposure.

Several lines of evidence couple PKC to an important role in Raf-1 activation, but the precise mechanism is unknown (Cacace *et al.*, 1996; Ueda *et al.*, 1996). Ras has been shown to interact with the regulatory domain of PKC $\zeta$ , and the association *in vivo* is stimulated with PDGF (Larrodera *et al.*, 1990). PKC has been shown to couple Ras activation to the Raf protein kinase during mitogenic signal transduction (discussed in Cacace *et al.*, 1996). PKC has also been shown to synergize in the activation of the MAP kinase pathway in rat aorta smooth muscle cells in response to angiotensin II (Malarkey *et al.*, 1996) and colonic epithelial cells in response to phorbol ester (Perletti *et al.*, 1995).

Work done by Zhou *et al.* (1993) with human umbilical vein endothelial cells demonstrated that activation of PKC during the early G1 phase potentiated growth-factor induced DNA synthesis, whereas activators of PKC completely inhibited the initiation of DNA synthesis in HUVEC cells when applied in late G1 or after entry into S phase. This cell cycle-dependent regulation mediated by PKC was found to be associated with either the induction or inhibition of pRb phosphorylation, and Cdk2-dependent histone H1 phosphotransferase activity which usually results following pRb phosphorylation by Cdk4 in early G1, and mediates the G1 to S phase transition (Sherr, 1993). PKC activation in response to phorbol ester has also been demonstrated to inhibit apoptosis in response to daunorubicin treatment (Mansat *et al.*, 1997) and to inhibit cellular proliferation in capillary endothelial, NIH3T3 and intestinal epithelial cells by modulating Cip/Kip family

cyclin-dependent kinase inhibitors and the pR protein (Li *et al.*, 1996; Frey *et al.*, 1997).

It is believed that in addition to different PKC isoenzymes being expressed differentially in different cells and tissues, each member of the PKC family plays a unique role in physiological processes. The role of PKC in numerous pathological processes strongly suggests that selective PKC inhibitors may have a broad range of therapeutic applications in the control of PKC-related disorders (Blobe *et al.*, 1994; Hu, 1996). Inappropriate activation of PKC isoenzymes has been shown to be involved in an array of diseases including cancer, inflammation, viral infections, CNS disorders including Alzheimers disease, cardiovascular malfunctions, angiogenesis and vascular complications including diabetes and insulin resistance (Shiba *et al.*, 1993; Bradshaw *et al.*, 1993; Grunicke *et al.*, 1994; Kellerer and Hering, 1995; Hu *et al.*, 1996). Intensive efforts are underway in the development of selective PKC inhibitors for use as therapeutic agents (Hu, 1996), and these new compounds have allowed researchers to probe PKC function in various diseases and to re-evaluate the role of selective PKC isoenzymes in pathological processes. This is invaluable now as earlier studies were conducted with potentially non-kinase-selective PKC inhibitors including staurosporine.

#### p70 S6 kinase

p70<sup>S6K</sup> is involved in mitogenesis, and it has been shown to be involved with progression through G1 phase, although its expression is upregulated at the end of mitosis, in addition to throughout G1 phase (Chou and Blenis, 1996). For instance, translation of specific messages such as those encoding elongation factors eIF1A and eIF2, the translational apparatus including ribosomal proteins S3, S6, S14 and S24, or the insulin-like growth factor II, appear to be regulated by the p70<sup>S6K</sup> pathway cycle (Ferrari and Thomas, 1994; Jefferies *et al.*, 1997). These proteins have been describes as being necessary for progression through the G1 phase of the cell (Jefferies *et al.*, 1997). This Ser/Thr kinase was originally identified as the enzyme that phosphorylates the S6 protein of the 40S ribosomal subunit *in vivo*, which has been found to enhance the translation of mRNAs containing 5'-polypyrimidine tracts, and is likely to play a role in growth-related

control of translation (Frost *et al.*, 1995; Pullen and Thomas, 1997). This family of mRNA transcripts has been shown to encode for a number of components of and insulin-like growth factor II (IGF-II) (Brown and Stuart, 1996). The production of these ribosomes and initiation factors are required for efficient transit through the G1 phase of the cell cycle (Edelmann *et al.*, 1996).

The regulation of p70<sup>S6K</sup> is distinct from Rsk, a related S6 kinase that serves in the Ras-dependent MAP kinase signalling pathway. Also, unlike Rsk, p70<sup>S6K</sup> is regulated by the immunosuppressant rapamycin, which is a highly potent inhibitor of p70<sup>S6K</sup> phosphorylation and activation by all stimuli. Rapamycin is therefore widely utilized as a tool with which to define the cellular function of the S6 kinase. In mammals, the intracellular target for rapamycin is the binding protein FKBP12, which forms a complex with a protein kinase identified as FRAP (or mTOR, target of rapamycin), and also shares sequence homology with PI 3-kinase (von Manteuffel *et al.*, 1997). Although FRAP kinase is required for activation of p70<sup>S6K</sup>, it does not appear to phosphorylate it directly. The mechanism by which FRAP regulates p70<sup>S6K</sup> has not as yet been elucidated (von Manteuffel *et al.*, 1997). p70<sup>S6K</sup> is also sensitive to the PI 3-kinase inhibitors wortmannin and LY294002, implicating PI 3-kinase in the signalling pathway regulating p70<sup>S6K</sup>.

Activation of p70<sup>S6K</sup> requires multisite phosphorylation by several protein kinases, at least one of which is proline directed. However, despite the knowledge of the phosphorylation sites, not all of the S6 kinase kinases have been identified. Activation of p70<sup>S6K</sup> requires hierarchical phosphorylation at multiple sites, culminating with the phosphorylation of Thr-229 in the catalytic domain, which is exposed for phosphotransferase action only after the previous phosphorylation events have occurred. An analogous site to the p70<sup>S6K</sup> Thr-229 site in PKC, Thr-308, has been shown to be phosphorylated by the phosphoinositide-dependent protein kinase 1 (PDK1) (Alessi *et al.*, 1997; Shaw *et al.*, 1997; Cohen *et al.*, 1997). Thomas and associates demonstrated that PDK1, a protein kinase that is regulated by phosphatidylinositol (3,4,5) trisphosphate at a concentration ten times less than that which is required for PKB (Alessi *et al.*, 1997), phosphorylates and activates directly, p70<sup>S6K</sup> at the Thr-229. They have proposed a model based on these results and the previous literature regarding the regulation of p70<sup>S6K</sup> (Pullen

*et al.*, 1998). Activation appears to be first initiated by the phosphorylation of the Ser/Thr residues (Ser-411, Ser-418, Thr-421, and Ser-424) in the autoinhibitory domain that facilitates the phosphorylation at Thr-389 by disrupting the interaction of the C- and N-termini of p70S6K, thereby allowing phosphorylation of the Thr-229 by PDK1. Thr-389 itself may be phosphorylated by mTOR which can be regulated by treatment with rapamycin or wortmannin, but direct evidence for this hypothesis is not yet available (Pullen *et al.*, 1998). Activation of p70<sup>S6K</sup> may occur through either PKC-dependent or independent pathways (Cheatham *et al.*, 1994; Han *et al.*, 1995).

### MAP kinases

MAP kinases are a family of Ser/Thr protein kinases activated as an early response to many stimuli including growth factors, cytokines, integrin-matrix association or exposure to cellular stresses. Extracellularly regulated protein kinases (ERK) 1 and 2 are MAP kinases that have been linked to cell cycle progression through G1 phase in response to mitogens. Stimulation of MAP kinase requires dual phosphorylation on threonine and tyrosine residues (Thr-183 and Tyr-185 in ERK2), that is catalyzed by the dual specificity kinases of the Mek family. MEK1 is regulated by a MEK kinase (MEKK3) and/or Raf kinase (Cobb and Goldsmith, 1995), p67<sup>PAK</sup> (Frost *et al.*, 1997; Tang *et al.*, 1997) as well as some non-conventional PKC isoforms (Toker *et al.*, 1994; Laio *et al.*, 1997). Additionally, MAP kinase can be differentially regulated in a Ras-dependent versus independent manner (Ablas *et al.*, 1996; Clark and Hynes, 1996; Ueda *et al.*, 1996) or via different heterotrimeric G proteins depending on cell type (Gardner *et al.*, 1993; Crespo *et al.*, 1994; Clark and Hynes, 1996; Chen *et al.*, 1996; Denhardt, 1996). Therefore, MAP kinases are thought to be key intermediate proteins functioning in a variety of signal transduction networks.

The activation of ERK1 and ERK2 is achieved by direct phosphorylation onto the Thr-Glu-Tyr (TEY) residues, which are located just before the conserved kinase catalytic domain VIII Ala-Pro-Glu motif. The *in vivo* phosphorylation of the TEY site is catalyzed by the MAP kinase kinases of the MEK (MAP kinase/Erk kinase) isoforms MEK1 and MEK3 that appear to be the only physiological regulators of the ERK's. Raf1 is one of at

least six distinct protein kinases that have been reported to directly phosphorylate and activate MEK1 *in vitro*, including Mos, MEKK, PKC, p67<sup>PAK</sup> and an insulin-activated MAP kinase kinase kinase (Ueda *et al.*, 1996; Pelech and Charest, 1995; Frost *et al.*, 1996, 1997). MEK1 has been shown to be the best substrate for Raf1. Through the N-terminal non-catalytic CR1 domain that is conserved within the 68-kDa RafA and 95-kDa RafB isoforms, Raf1 binds the monomeric G protein Ras in its effector binding regions (Shirouzu *et al.*, 1994).

The activation of PKC downstream of PLC is one of the routes in which ERK2 is activated by the seven transmembrane serpentine receptor such as M1 and M2 acetylcholine muscarinic receptors (Blenis *et al.*, 1993). The  $\alpha$ - and  $\beta\gamma$ - subunits of several G proteins can stimulate  $\beta$  isoforms of PLC to catalyze the PI 4,5-bisphosphate breakdown and the induction of PKC. Phorbol ester de-regulation of PKC attenuates ERK activation stimulated by the M1 receptor (Winitz *et al.*, 1993). p67<sup>PAK</sup> is a homolog of yeast Ste20 and a kinase that by analogy might be expected to recruit a MAP kinase-dependent pathway in response to activation by the Ras-related G proteins of the Rho family, Rac and Cdc42. Recently, Cobb and associates, and others, have demonstrated in angiogenesis models, that p67<sup>PAK</sup> directly phosphorylates and stimulates MEK1 activity in response to integrin signalling (Ueda, *et al.*, 1996; Frost *et al.*, 1996, 1997; Tang *et al.*, 1997; Xu *et al.*, 1997).

MAP kinases phosphorylate a variety of proteins *in vitro* including Rsk1, MAP kinase-activated protein kinase (MAPKAPK-2, or Rsk2), c-Fos, PHAS-1, PLA<sub>2</sub>, c-Jun and p62<sup>TCF</sup> (Blenis, 1993; Pelech and Charest, 1995). Furthermore, agonists of MAP kinase and neutrophil activation are mediated through various receptors, secondary messengers and other kinases, and similarly, activation of a down-stream target subsequent to MAP kinase activation is also regulated by the integration of other signals from MAP kinase-dependent and independent pathways.

### Cyclin-dependent kinases

A number of cyclins and cyclin-cdk complexes have been identified and their roles in cell proliferation, differentiation and apoptosis have recently been elucidated. Cyclin D1, D2 and D3 have been demonstrated to associate with Cdk4 and Cdk6 and function during G1 phase prior to activation of cyclin E/Cdk2 by regulating the activity of the pRb protein (Sherr, 1993). This complex also associates with PCNA, p32, p27, p15 and p16 cdk inhibitors (CDKIs). Cyclin E is expressed after cyclin D, and it complexes with Cdk2 to control the progression of G1 phase cells into S phase and is regulated by association with the p21<sup>CIP1</sup> inhibitory protein, which is controlled by p53, and p27. Although C, F and G type cyclins have been identified, their roles in the cell cycle have not been fully elucidated. Cyclin H has recently been demonstrated to have a role in all phases of the cell cycle, but most particularly associates with Cdk7 (MO15), to form CAK, Cdk2 activating kinase, which can regulate the phosphorylation of Thr-160 of Cdk2 and the C-terminus of RNA polymerase II (Dunphy, 1994; Solomon, 1994).

The cell cycle is initiated following a response to mitogenic stimuli. For instance, the MAP kinase cascades have been demonstrated to regulate transcription factors such as SRF and Jun, which are responsible for the transcription of cdks (Grana and Reddy, 1995), the cdk-activating tyrosine phosphatase Cdc25A, which can be activated via Raf (Galaktionov *et al.*, 1995), the CDKIs, and regulators of apoptosis (Chou and Blenis, 1995; Zwicker *et al.*, 1995). The MAP kinase and related pathways convey mitogenic signals in response to activation of both tyrosine kinase receptors and G protein-coupled receptors. Mitogens are required for the mammalian cell cycle prior to G1 phase, up to a specific period designated the 'restriction point'. Cells are committed to complete DNA replication, even in the absence of growth factors (Dasso and Newport, 1990; Massaue and Roberts, 1995) so long as cells are provided with necessary survival factors (Pardee, 1989). After cells are committed to DNA replication, they are no longer sensitive to mitogenic signals (Grana and Reddy, 1995).

The cdk-cyclin complex that most closely links the regulation of the restriction point (START) to mitogen stimulation are the D-type cyclins and Cdk4, and in some cells Cdk6 (Tsai *et al.*, 1993). Cyclin D1 (PRAD1), which is encoded by the CCND1 gene on

chromosome 11q13, has been implicated in breast, gastric and esophageal carcinomas as a result of amplification of the 11q13 region that corresponds to the CCND1 gene (Hunter and Pines, 1994; and references within). D-type cyclin expression is stimulated by growth factors in quiescent cells, and once growth factors are removed, their levels drop immediately. The Cdk4/cyclin D complex functions in part by regulating the tumor suppressor protein, Rb, which is hypophosphorylated throughout G1 phase, thus arresting cells in G1, and phosphorylation of Rb by this complex relieves this inhibition (Johnson, 1995; Kato *et al.*, 1995). Therefore, an important role of Cdk4 kinase is to inactivate pRb to allow cells to progress towards S phase (Musgrove *et al.*, 1994). The hypophosphorylated form of pRb is bound to the E2F-1 transcription factor and DP (DP-1 or DP-2), which is a factor responsible for the binding E2F-1 to DNA. Phosphorylation of RB releases the E2F-1-DP heterodimer to activate the transcription of genes required for the progression of S phase (Resnitzky *et al.*, 1994). pRb is dephosphorylated by protein-Ser/Thr phosphatase 1 (PP-1), which is also responsible for the dephosphorylation and deregulation of Cdc25 during mitosis (Randall *et al.*, 1994) and allows for its binding to the E2F-1-DP complex. This pathway is also regulated by cyclin dependent kinase inhibitors p15<sup>INK4/MTS2</sup>, p16<sup>INK4/MTS1</sup>, p18 and p19, as well as p27<sup>KIP1</sup> that are sequestered by cyclinD/Cdk4 or Cdk6. These inhibitors reduce Cdk4 activity by binding in competition with cyclin D (Grana and Reddy, 1995). Although the extracellular signals that negatively regulate these CDKIs are presently unknown, it has been demonstrated that Rb partially suppresses p16<sup>INK4</sup> expression (Li *et al.*, 1994), indicating the existence of a feedback loop that ensures that Cdk4 activity is repressed once Rb has been inactivated via its phosphorylation.

Similar to cyclin D, cyclin E has a rapid turnover with its expression peaking in late G1 following the cyclin D peak and proteolysis. Cyclin E has affinity for p33<sup>cdk2</sup>, and this complex regulates S phase commitment. After the cells have entered S phase, cyclin E is degraded and Cdk2 then associates with cyclin A, which mediates the progression of S phase (Randall and Reisinger, 1994). p21<sup>CIP1</sup> binds to and inhibits a variety of cyclin-cdk complexes including cyclin E/Cdk2 and cyclin A/Cdk2. p21 also inhibits S phase progression through binding and inactivation of DNA replication machinery including

PCNA (proliferating cell nuclear antigen), which is the processivity subunit of DNA polymerase- $\delta$  (Hunter and Pines, 1994).

Given its role in suppressing Cdk4 activity, p16 has been implicated as a tumor suppressor (Hunter and Pines, 1994). The proto-oncoproteins, c-Myc and Ras, have been implicated in the link between mitogenic signalling and G1 progression (Daksis *et al.*, 1994). Continuous expression of *c-myc* downregulates cyclin D<sub>1</sub> expression (Philip *et al.*, 1994). *C-myc* expression is stimulated by E2F-1 following release from Rb. Therefore, cyclin D1/Cdk4 phosphorylation of Rb increases the expression of *c-myc* further, which in turn abrogates cyclin D and participates with E2F-1 in inducing the expression of cyclins E and A (Daksis *et al.*, 1994) which are the next cyclins in the circuit regulating progression of the cell cycle. De-regulation of the mediators of progression through G1 phase, for example at restriction point, may allow cell growth and division to become insensitive to mitogens and extracellular mediators, therefore causing cells to bypass the S phase checkpoint and hence accumulate genetic changes that contributes to the development of tumour cells. This supports the suggestion that deregulation of the Cdk4-cyclin D complex that controls progression to S phase, renders the progression of the cell cycle less dependent on growth factors and contributes to oncogenesis. Direct evidence for this has been reported in cyclin D1 gene knockout experiments where it has been shown that cyclin D1 knockout in DT40 lymphoma B-cells resulted in specific alterations in the ability of the cells to transit through the cell cycle (Lahti *et al.*, 1997), and cyclin D1-deficient mice were observed to have small eyes with thin retinas, and reduced level of retinal cell proliferation (Ma *et al.*, 1998). Although cyclin D1 expression has thus far been demonstrated to be indispensable in normal cells for cell cycle progression, cyclin D1 has been shown to be dispensable in pRb gene-deficient cells, which signal independently of Cdk4 activity, similar to tumour cells with mutations in their pRb or cyclin D genes (Lukas *et al.*, 1995).

### Cdk1

The state of DNA replication and integrity is monitored by proteins that also participate in mitotic induction by regulating the transcriptional activity of genes necessary for mitosis. Experimental evidence thus far indicates that cells contain mechanisms that coordinate the initiation of mitosis with the completion of DNA synthesis, or S phase (Dasso and Newport, 1990). For example, Rao and Johnson (1970) demonstrated that when S phase cells were fused to cells in G2 phase, the nucleus of the G2 cells is delayed from entering mitosis until the S phase cells had replicated all of its DNA. The onset of mitosis is coupled closely to the activation of the M-phase promoting factor (MPF - originally designated as the maturation promotion factor), a heterodimeric complex consisting of a B-type cyclin and the protein kinase Cdk1 (Cdc2) (Dunphy, 1994). Mitosis can be subdivided into three transitions that involve cyclin B (Randall *et al.*, 1994). In the initial stage, posttranslational modification to the cyclin B/Cdk1 complex occurs to activate the kinase and initiate prophase. Anaphase is initiated following cyclin B proteolysis in the second transition, a process which is ubiquitin-dependent (Draetta *et al.*, 1989; Glotzer *et al.*, 1991). The last transition is that associated with the turning off of the cyclin B degradation system, which is mediated by a transcriptional mechanism. The activity of G1 phase cyclins is required for the inactivation of the deregulation process to allow for the accumulation of cyclin B for the next mitotic cycle (Randall *et al.*, 1994).

In all cell types, cyclin B must accumulate to a threshold in order for mitosis to occur. After this threshold is reached, new protein synthesis is not required. Thereafter is a period during which both Cdc2 and cyclin B are present but the kinase remains inactive, implying the existence of regulators of Cdk1 activation (Murray and Kirschner, 1989). Although the translocation of cyclin B/Cdc2 into the nucleus just prior to the onset of prophase is well documented, the role of its nuclear translocation in controlling activation and/or action of the kinase is not established. For instance, Cdc25, the activating tyrosine phosphatase, is predominantly nuclear prior to mitosis, but has been observed as nuclear or cytoplasmic during the remainder of interphase, depending upon the experimental system (Dunphy, 1994). Therefore, it is not understood whether the regulated translocation of Cdc25 is a feature of mitotic control.

Binding of cyclin B is not sufficient for activation of Cdk1. Additional phosphotransferase reactions mediate the activity of the complex, where negative acting phosphorylation of Thr-14 and Tyr-15 on the p34<sup>cdc2</sup> protein prevents the premature activation, and phosphorylation of Thr-161 has been shown to be necessary for its activity, providing targets for feedback controls (Solomon, 1994). Phosphorylation on the Thr-14 and Tyr-15 residues, which overlap the ATP-binding site on Cdk1 in the conserved kinase subdomain I, maintains Cdk1 in a catalytically inactive state, and subsequent dephosphorylation of the Tyr-15 residue by the Cdc25 phosphatase correlates with its activation (Berry and Gould, 1996). In *S. pombe*, the regulatory phosphorylation of Tyr-15 is mediated by the tyrosine kinase Wee1, or related Mik-1 (Randall *et al.*, 1994). Mutation of *wee1* in *S. pombe* causes these cells to undergo premature mitosis (Berry and Gould, 1996), and double mutants of *wee1* and *mik1* produces premature mitosis that is lethal for *S. pombe* (Lungren *et al.*, 1991). Yeast Wee1 is tightly regulated through phosphorylation by the kinase Nim1 (Nasmyth, 1993). Phosphorylation of Wee1 at its C-terminus causes a lower activity, and N-terminal domain phosphorylation completely shuts off Wee1 (Murray, 1992; McGowan and Russell, 1995). This process is reversed by a PP2A-like, okadaic acid-sensitive phosphatase. Cdc25 is a substrate for Cdk1 (Draetta *et al.*, 1989) and of the ME (MPM-2 epitope) kinase. ME is an epitope for the MPM-2 monoclonal antibody that recognizes a myriad of mitotic phosphoproteins. It is possible, therefore, that the Cdk1, ME and perhaps other kinases all contribute to the activation of Cdc25. Protein phosphatase 1 (PP-1), which is inhibited during mitosis, can inactivate Cdc25 by dephosphorylating the Thr residue that is necessary for activation, which is also positively regulated by Cdk1 phosphorylation. This inhibition is presumably by mitotic phosphorylation as it has been determined that phosphorylation events play an important role in regulating Cdc25 and Wee1 (Izumi *et al.*, 1992). PP-1 may be regulated by inhibitor 2, which increases in activity during mitosis (Brautigan *et al.*, 1990). PP-2A has also been demonstrated to inhibit Cdc25 *in vitro* (Izumi *et al.*, 1992), and inhibition of this phosphatase by okadaic acid causes premature chromosome condensation and entry into mitosis in *Xenopus* extracts (Felix *et al.*, 1990). The basis by which phosphorylation mediates the function of Wee1 or Cdc25 is presently not known, but it has been suggested

that Cdc25 phosphorylation can enhance the recognition of Cdk1 or relieve the inhibitory effects of the N-terminal domain. The converse may be true for Wee1 (Dunphy, 1994). The event initiating the Cdc25 and Wee1 regulatory loops could be the inactivation of the phosphatase, which keeps the Cdc25 phosphatase inactive and the Wee1 kinase active during S phase. Inactivation of the phosphatase during G2 phase would lead concomitantly to the inhibition of the Wee1 kinase and activation of the Cdc25 phosphatase, subsequently inducing tyrosine dephosphorylation of Cdc2 and its activation.

### 3. Cell cycle checkpoints

#### Checkpoints

The events associated with cell cycle progression are strictly ordered and regulated for cell integrity to be maintained (Murray, 1992; Li, 1993). While this is intuitively obvious, the dependency of later cell cycle events on the completion of earlier events is not readily observable during the normal cycle. For example, DNA must be free of lesions before it is segregated or replicated prior to a cell's commitment to enter mitosis. The events leading to the entrance into mitosis are readily observable, but are particularly evident only when the earlier events are experimentally perturbed. This may arise when cells treated with inhibitors of DNA synthesis delay the onset of mitosis until replication is complete, or when cells treated with DNA damaging agents prior to the G2 phase and mitotic transition delay the onset of S phase or of mitosis until DNA repair is completed. These events are carefully controlled and their order is maintained by the monitoring of signalling events, such as the aforementioned protein phosphorylation signal transduction pathways. The completion of earlier events is necessary for cell progression and may involve the phosphorylation or dephosphorylation of additional proteins that are enzymatically affected by their degree of phosphorylation, and is necessary for the initiation of later events. This substrate-product relationship guarantees a strict sequential ordering of events. Additionally, feedback systems that monitor the completion of earlier events, such as the integrity of the DNA, impose controls on cell cycle mediating enzymes, further ensuring the readiness of the cell prior to further progression through the cell cycle.

Mutants and chemicals have been found that allows late events to happen without the completion of earlier ones.

These feedback controls have been demonstrated with the use of cell lines containing lesions at specific sites in genes that represent these checkpoint enzymes, and chemical inhibitors, where it has been observed that the subsequent events have occurred without the completion of the earlier, now abrogated one. These feedback mechanisms are referred to as checkpoints, and they regulate the transitions between G1 and S phase, and G2 and M phase, as well as during spindle formation. Cell cycle events are often thought to be regulated by a series of biochemical pathways, the initiation of which requires the completion of the regulators of these upstream pathways. Such regulators monitor DNA replication integrity at S and G2 phase. A major property of malignant cells is the accumulation of genetic abnormalities. There is increasing evidence that defects in the checkpoints participate in cancer by allowing the accumulation and propagation of genetic abnormalities (Hartwell, 1992). To date, little is known of how checkpoints operate or of the nature of the differences between the checkpoints of normal and cancer cells. It has recently been proposed that inhibitors of certain checkpoints may preferentially kill cancer cells (Andreassen and Margolis, 1992; Murray, 1992; Hunter and Pines, 1994; Roberge *et al.*, 1994), and hence may be potential chemotherapeutic agents.

Checkpoints have presently been demonstrated as being regulated by the cyclin-dependent kinases and their obligate regulating partners, the cyclins. The cyclin-Cdk complexes are formed and activated at various stages of the cell cycle. Although some of the Cdk proteins may be constitutively expressed, such as Cdk1 (p34<sup>cdc2</sup>), the cyclins are expressed at specific points of the cell cycle. Progression through the cell cycle is then controlled by the particular cyclin expressed, and the post translational modification of the cyclin/Cdk complex (i.e., phosphorylation), leading to the phosphorylation of specific substrates that mediate this progression. Therefore, cdk/cyclin complexes and their mediators are a part of a series of feedback loops ensuring completion of earlier events and proper ordering of subsequent events during the cell cycle.

A mitotic entry checkpoint exists at the G2 phase/prophase interface that prevents the entry of cells into mitosis when DNA is not completely replicated or damaged. This

checkpoint is important in neoplasia, for if this checkpoint is not effective, then the cell is allowed to enter mitosis and divide prematurely with lesions in the DNA that may lead to uncontrolled proliferation. Although the existence of the mitotic checkpoint (or G2 checkpoint as it will be referred to in this thesis) has been demonstrated, very little is known about its components or how they operate in mammalian cells. The investigations of these checkpoints have consisted of three approaches: a genetic one, predominantly utilizing the yeast *Schizosaccharomyces pombe*, a pharmacological, and a biochemical one. The pharmacological approach has been one that disrupts checkpoint functions in mammalian cells. The biochemical approach is one that relies on existing immunological, enzymological, and microbiological technologies to detect and isolate the protein activities in extracts from vertebrate cells that modulate entry into mitosis.

The original approach in the search for the mediators of the checkpoints and their mechanisms has been to seek out mutants that are defective in checkpoint control. This has best been applied to organisms that are amenable to high levels of gene transfection or infectability, and genetic manipulations, notably the fission yeast *Schizosaccharomyces pombe*. Several genes have been identified that overcome the mitotic checkpoint when mutated, deleted or overexpressed. These genes include cell cycle regulators that accelerate the rate of entry into mitosis of cycling cells in addition to overcoming the checkpoint. These genes are composed of known cell cycle regulators including the major mitotic inducer Cdk1 (Enoch, 1990; Gabrielli *et al.*, 1993; Desai *et al.*, 1995; Guo *et al.*, 1995; Guadagno and Newport, 1996) which is found in all organisms, and the mitotic inducer NIMA which is present in *Aspergillus nidulans* (Osmani, 1988). Cdk1 is negatively regulated by phosphorylation at Tyr-15 in *S. pombe* (Enoch, 1991; Stueland *et al.*, 1993), and at Thr-14 and Tyr-15 in human cells (Moria *et al.*, 1989; Pondaven *et al.*, 1990; Solomon, 1994). The checkpoint probably acts through this event, since mutations to non-phosphorylatable residues at these positions overcome the checkpoint (Th'ng *et al.*, 1992; Enoch, 1991; Carr *et al.*, 1995). Other checkpoint mediators include Wee1 and Cdc25 (Lundgren *et al.*, 1991; Solomon, 1993). Loss of *wee1* or overexpression of *cdc25* prevents checkpoint function (Enoch, 1991; Solomon, 1993). Therefore, checkpoint control could operate by downregulating Cdc25 or upregulating Wee1. Other genes

overcome the checkpoint but do not accelerate entry into mitosis of cycling cells. Examples of these are the rad genes; *rad 9, 17, 24* in *S. cerevisiae*, and *rad 1, 3, 9, 17, 21* in *S. pombe*. These gene products appear to be dispensable for normal cell cycle progression, although they are required to signal the presence of unrepliated or damaged DNA to the mediators of entry into mitosis (Nasmyth, 1993; Strueland, 1993). Their mechanism of action at this time is not fully understood. Other genes include those that overcome the checkpoint and result in a defect in DNA replication. These include *cdc18* and *cut5* from *S. pombe* that are required for S phase, and *pim1* which is the mammalian RCC1 homologue identified in baby hamster kidney cells (BHK-21) that is not required for S phase (Nishitani, 1991; Handell and Weintraub, 1992; Roberge, 1990). The functions of these genes have not been elucidated, but RCC1 has been demonstrated to monitor the integrity of the DNA, and is an example of an upstream mediator of checkpoints (Nishitani *et al.*, 1991). RCC1 is a 45-kDa protein that has demonstrated DNA binding activity and is thought to have a role at both the G1/S and mitotic checkpoints. RCC1 has been suggested to be required for the synthesis of G1 cyclins, and consequently for the activation of the G1-Cyclin/Cdk complex which is responsible for the progression to S phase (D'Urso *et al.*, 1990). During S and G2 phase, RCC1 regulates entry into mitosis by preventing the dephosphorylation and subsequent activation of Cdk1 (Nishimoto *et al.*, 1991). Nishimoto's laboratory has speculated that this abrogation of Cdk1 (Cdc2/cyclin B complex) activation is mediated through the inhibition of synthesis of a Cdk1-specific phosphatase, such as Cdc25, and hence resembles the treatment of cells with the phosphatase 2A inhibitors okadaic acid and calyculin A (Chambers *et al.*, 1993).

These gene products can be envisioned as representing three modules of the checkpoint: the signal, such as from RCC1 or other proteins that monitor DNA integrity and mediate the transcription and translation of those enzymes necessary for the further progression of the cell cycle, the transducers, and the target proteins that responds to the compilation of these signals. It is, however, not yet possible to order these gene products into meaningful biochemical pathways as some mutations in *S. pombe* have been demonstrated to affect only the response to incomplete DNA replication and others only to DNA damage, implying that the pathways that make up the checkpoint circuitry consist of

a complicated series of branches and nodes. These circuits ultimately act in concert to mediate progression through the cell cycle, eventually to mitosis and cell division once all of the provisions for this event are satisfactory such that the cell can divide with a completely replicated genome and without lesions in the DNA that can generate malignant cells.

A concern for those who are actively investigating these checkpoints via utilization of organisms such as yeast, that are more amenable to genetic manipulations, is that these putative components may not correlate with those in mammalian cells. Although Cdk1 has been demonstrated as being the major mitotic inducer in mammalian cells (Li and Deshaies, 1993) and that the modulation of Cdk1 phosphorylation participates in checkpoint control in *S. pombe*, a similar analysis in *S. cerevisiae* has demonstrated that the phosphorylation of equivalent inhibitory amino acid residues is not required for the checkpoint and full activation of Cdk1 does not overcome checkpoint block (Booher and Beach, 1987; Amon *et al.*, 1992; Nasmyth, 1993; Strueland 1993). This indicates that the checkpoint either regulates Cdk1 by another mechanism, or regulates another mitotic inducer, or downstream regulator of Cdk1.

#### Analyzing checkpoint control

The pharmacological approach, in combination with biochemical analyses, identifies chemicals that overcome a checkpoint and is subsequently utilized in elucidating its target(s). As mentioned earlier, entry into mitosis is dependent upon the completion of previous cell cycle events, such as the replication and repair of DNA. Cells treated with inhibitors of DNA synthesis, or with DNA damaging agents induce a G2 block, subsequently inhibiting the cell from progressing into mitosis. It is generally accepted that this arrest is necessary for the cells to complete replication or repair of the DNA before commitment into mitosis (O'Connor *et al.*, 1993). A majority of the existing chemotherapeutic agents are DNA-damaging agents or DNA-chelators and induce G2 arrest (Yarbro, 1992; O'Connor *et al.*, 1993). Further, the cytotoxic activity of these agents strongly correlates with their ability to cause a G2 arrest (Konopa, 1988). The major mitotic controller, Cdk1, is negatively regulated in cells arrested in G2 phase following

treatment with these compounds (Lock and Ross, 1990; Lock, 1992; Tsao *et al.*, 1992; O'Connor *et al.*, 1992, 1993). This evidence further substantiates the role of Cdk1 kinase as a primary mediator of mitosis. It has recently been suggested that inhibitors of checkpoints may be valuable chemotherapeutic agents, because of their ability to further weaken the checkpoints of cancer cells while having little effect on the checkpoints of normal cells (Murray, 1992; Roberge *et al.*, 1994). This selective killing of cancer cells is logically dependent upon the selectivity of the agent for the mediator of the checkpoint. Ideally, this mediator should have some redundancy, thereby reducing toxicity of the agent, as essential functions may still proceed in normal cells that utilize the same enzyme. To date, only a limited number of checkpoint inhibitors have been identified as indicated by their ability to induce entry into mitosis in cells arrested with damaged or unreplicated DNA. These include caffeine, the protein kinase inhibitors 2-aminopurine, 6-dimethylaminopurine and butyrolactone (Andreasen and Margolis, 1991; Kitagawa *et al.*, 1993) and the protein phosphatase inhibitors calyculin A, tautomycin and okadaic acid (Bush *et al.*, 1978; Schlegel and Pardee, 1986; Downes *et al.*, 1990; Yamashita *et al.*, 1990; Felix *et al.*, 1990; Steinman *et al.*, 1991; Picard *et al.*, 1991; Hartwell *et al.*, 1992; Andreason and Margolis, 1992; Tosuji *et al.*, 1992; Sugiyama *et al.*, 1996). None of these agents are presently used as anti-cancer agents because of their toxicity or species selectivity.

Caffeine is a purine analogue that at high concentrations (2-10 mM) in the cell incubation medium can overcome the checkpoint activated by DNA-damaging agents such as X-rays, UV light, and alkylating agents, and potentiates their killing effects in most cell lines including human cells (Lau *et al.*, 1982). However, caffeine overcomes the checkpoint activated by unreplicated DNA only in rodent cells, without an effect on human cell lines (Schlegel *et al.*, 1986; Downes *et al.*, 1990) and accelerates entry into mitosis in cycling cells.

Okadaic acid, a polyether fatty acid, is a specific inhibitor of protein-Ser/Thr phosphatases 1 and 2A (Cohen *et al.*, 1990; Schonthal and Feramisco, 1993; Roberge *et al.*, 1994). It overcomes the checkpoint activated by unreplicated DNA (Yamashita *et al.*, 1990; Ghosh *et al.*, 1992) and causes activation of Cdk1 (Yamashita *et al.*, 1990; Pickard

*et al.*, 1991). Calyculin A also inhibits protein phosphatase 1 and 2A, but with slightly different specificity, overcoming the checkpoint activated by unreplicated DNA in sea urchin eggs (Tosuji *et al.*, 1992). Investigations of these phosphatase inhibitors using standard biochemical techniques have revealed that cellular extracts contain protein phosphatases that negatively regulate, or antagonize, Cdk1 activity. Such a Cdk1 regulator is Cdc25, which is activated by phosphorylation (Pines and Hunter, 1991; Gautier *et al.*, 1991). Further, it has been demonstrated that the major enzyme in *Xenopus* egg extracts that dephosphorylates and inactivates Cdc25 is inhibited by okadaic acid (Clarke *et al.*, 1991). Interestingly, a type 2A protein phosphatase was found to be the major enzyme in vertebrate cell extracts that dephosphorylates the proteins phosphorylated by Cdk1 (Gautier *et al.*, 1991; Yusada *et al.*, 1991). Thus, a type 2A protein phosphatase could play a role in the control of entry into mitosis by controlling the activation of Cdk1, or by opposing the action of Cdk1 by dephosphorylating its substrates. Studies with okadaic acid and calyculin A indicate that PP-2A, and perhaps PP-1, are required for the checkpoint. Work presented in chapter 3 investigates the roles of a potential phosphatase inhibitor, fostriecin, on the G2/M phase checkpoint, and a mediator of this checkpoint, Cdk1.

To develop a checkpoint inhibitor with high anti-tumour therapeutic efficacy, it would be highly desirable to exploit the differences between normal and cancer cells. There is increasing evidence that cancer cells have defects in checkpoint control that may participate in neoplasia. Tumour cells exhibit a large number of chromosomal lesions that result in a greater number of cells arresting at metaphase 1 hour after X-ray irradiation compared with normal cells (Parshad *et al.*, 1985), indicating that they have a weakened checkpoint. If weakened checkpoints are a common property of cancer cells, then this property might be exploited for cancer therapy. The laboratories of Murray and O'Connor (Murray *et al.*, 1992; O'Connor *et al.*, 1993) have suggested that inhibitors of checkpoint control may further weaken the checkpoints of cancer cells but have little effect on those of normal cells, thus leading to more selective killing of tumour cells. Therefore, a better understanding of the biochemical mechanisms of checkpoint control is relevant not only to the understanding of normal cells, but also to the deviations in cellular signal transduction in the tumour cell, as well as the mechanism of action of anti-tumour drugs.

More effective cancer therapy may be achieved by combining a drug that causes cell cycle arrest with a drug that inhibits checkpoints and overcomes the arrest. In this manner, important cell cycle events may become uncoordinated. Many of the widely used drugs for cancer therapy are not intrinsically cytotoxic, but rather cytostatic: they bring about cell cycle arrest and this arrest is reversible upon short incubation times. For instance, by inducing cell cycle arrest with a more of the commonly used chemotherapeutic agents, such as the nitrogen mustards including mechlorethamine, nitrosoureas such as carmustine, topoisomerase II poisons such as etoposide, intercalators such as dactinomycin, and other drugs such as cisplatin, thiotepa, bleomycin and many more (Liu, 1989; Frosina and Rossi, 1992; Cummings and Smyth, 1993), initial treatment with a more followed by treatment with a checkpoint inhibitor that force the cells into a lethal mitotic state, a significantly higher rate of cell death may result.

Mutation, deletion, or overexpression of certain genes, disrupt the checkpoint that allow cells with unrepliated or damaged DNA to enter mitosis (Murrey, 1992; Li and Deshaies, 1993). As discussed earlier, the proteins translated from these genes are believed to participate in a regulatory mechanism that can sense incompletely replicated or damaged DNA and convey this message to protein phosphatases and kinases that regulate the kinase activity of Cdk1. A checkpoint inhibitor may potentiate the effect of these agents by accelerating mitosis or reversing G2 block, leaving insufficient time for G2 repair of the damaged DNA (Yarbo, 1992). Fostriecin is of particular interest, because it is the only checkpoint inhibitor identified so far that is itself a potential antitumour drug. It may be very efficient in combination with a chemotherapeutic agent that damages DNA or inhibits DNA replication. Therefore, investigation of fostriecin would help in the assessment of checkpoint inhibitors as potential anti-tumour drugs, and further elucidate its mechanism and cellular effects such that they may be exploited in the development of other checkpoint inhibitors, particularly from the investigational standpoint of combinatorial chemistry.

During mitosis, the nucleus undergoes major morphological changes that are required for the segregation of replicated chromosomes and later cellular division. The most apparent change is the dissolution of the nuclear lamina and the condensation of interphase chromatin into metaphase chromatin. The mechanism of chromosome

condensation and the signal transduction events that initiate and control this process remains to be elucidated. In the laboratory of Bradbury (Bradbury *et al.*, 1974, 1983), it was observed in *Physarum polycephalum*, that chromosome condensation correlated with histone H1 hyperphosphorylation. Immediately prior to histone H1 phosphorylation in early G2 phase, growth associated histone H1 kinase, which corresponds to Cdk1, was observed to undergo a 15- to 20-fold increase in activity (Bradbury *et al.*, 1974). Although in *P. polycephalum* histone H1 reached a phosphorylation state of 24 phosphates at metaphase, the onset of histone H1 hyperphosphorylation in conjunction with chromosome condensation was observed in mammalian cells where phosphorylation increased from 2 phosphates at G2 phase, to 6 at mitosis (Gurley *et al.*, 1975;). p34<sup>cdc2</sup>/cyclin B (Cdk1) and p33<sup>cdk2</sup>/cyclin A have been demonstrated to phosphorylate histones H1A and H1B *in vitro* at the sites phosphorylated *in vivo* at metaphase (Guo *et al.*, 1995). These and other related findings (Mueller *et al.*, 1985; Norbury and Nurse, 1992) point to a central role of Cdk1 in the initiation of chromosome condensation associated with entry into mitosis. Histone H3 has also been demonstrated to be hyperphosphorylated during chromosome condensation, in particular, during metaphase (Gurley *et al.*, 1975). It has been proposed that the phosphorylation of the Ser-10 amino acid residue participates in the late stage of chromosome condensation (Bradbury, 1992). This site is not phosphorylated by any of the cyclin-dependent kinases (Guo *et al.*, 1995). The degree of histone phosphorylation is a result of a careful balance between the kinases activated following progression through the checkpoint, and ubiquitous phosphatases (or those activated in a cell cycle-dependent manner). The protein phosphatase 2A (PP-2A) subtype PP-2A<sub>1</sub> is the major phosphatase that dephosphorylates many of the substrates for the cyclin-dependent kinases, including histone H1 (Ferrigno *et al.*, 1993). Treatment of cells with the protein phosphatase 1 and PP-2A inhibitors okadaic acid and calyculin A, also promotes histone H1 phosphorylation (Paulson *et al.*, 1994), and induce cells to undergo an acceleration into mitosis based on morphological observations including histone phosphorylation and chromosome condensation (Yamashita *et al.*, 1990; Tosuji *et al.*, 1992).

#### 4. Chromatin and nuclear structure and histone modification at the onset of mitosis

##### Lamins and chromosome morphology

As a cell progresses through its cell cycle, increases in its DNA content, alterations in the DNA morphology and variations in transcriptional activity occur, along with continual changes in its nuclear size. This form of regulation demonstrates that nuclear components such as the lamina, the nuclear membrane and chromatin are dynamic, with the most dramatic changes in these components occurring at mitosis. At the initiation of mitosis, RNA transcription is halted, chromatin condenses, and the nuclear lamina depolymerizes (Newport and Forbes, 1987; Yasuda *et al.*, 1987; Craig, 1996). The molecular processes regulating the nuclear structure during mitosis may also control S phase-associated processes such as nuclear membrane and lamina growth, and chromatin organization. Therefore, a molecular understanding of nuclear rearrangements during mitosis could provide insight into the nuclear dynamics, and biochemical processes of interphase cells.

The filamentous lamin proteins underlie the nuclear envelope and are thought to play a crucial role in nuclear stability and chromatin organization and transcription (Newport and Forbes, 1987). Based on biochemical properties and structure, lamins are designated as types A, B<sub>1</sub>, B<sub>2</sub>, and C. During metaphase, depolymerization of the nuclear lamina occurs, similar to the other intermediate filament classes of cytoskeletal proteins, such as the spindle microtubules, resulting in soluble A and C lamins, but not the membrane vesicle-associated Lamin B (Newport and Forbes, 1987; Nigg, 1996). Isoprenylation, carboxyl methylation and phosphorylation have been observed as the only lamin modifications. Isoprenylation mediates association of the lamins with the nuclear membrane (Krohne *et al.*, 1989). Phosphorylation and carboxyl methylation have been demonstrated to mediate variations in lamina polymerization initiated at the onset of mitosis (Ottaviano and Gerace, 1985; Burke and Gerace, 1986; Chelsky *et al.*, 1989; Luscher *et al.*, 1991). In demonstrating that phosphorylation of the lamins was responsible for the depolymerization of the lamina at mitosis, Gerace and associates found that the level of lamin phosphorylation increased 4- to 7-fold above that in interphase cells,

followed by dephosphorylation of lamin at telophase (Ottaviano and Gerace, 1985; Burke and Gerace, 1986). Mitosis-associated phosphorylated serine residues have been identified, and these charge-altering modifications are thought to control the monomer-polymer equilibrium regulating stability versus breakdown at mitosis. Further, it is the energy stored in the lamin following mitosis-associated phosphorylation that is utilized to drive nuclear envelope formation at the end of mitosis, where it is the chromosomes that act as the template for its assembly in a DNA sequence nonspecific manner (Newport and Forbes, 1987).

Investigations are ongoing to identify the upstream signalling processes responsible for interphase and mitotic specific phosphorylation of the lamins. Cdk1 specific sites have been identified and mapped to SP/TP motifs that flank the  $\alpha$ -helical rod domain of lamin (Peter *et al.*, 1990; Ward and Kirschner, 1990). It was demonstrated through mutational analysis that phosphorylation of the Cdk1 sites was required for nuclear lamina breakdown (Peter *et al.*, 1990; Hennekes *et al.*, 1993). In conjunction with the observation that lamin B<sub>2</sub> does not dissolve during mitosis, it has been demonstrated that mitotic phosphorylation of lamin B<sub>2</sub> is itself not sufficient to induce nuclear lamina disassembly (Luscher *et al.*, 1991). An additional event such as carboxyl methylation appeared to be required. PKC sites have been identified and are located within the C-terminal end domain between the central  $\alpha$ -helical rod and the nuclear localization signal, and these sites have been demonstrated to be phosphorylated by PKC *in vitro*, as well in response to PMA *in vivo* (Hennekes *et al.*, 1993). Although phosphorylation of lamin B<sub>2</sub> was not responsible for disassembly of the lamin tail-to-tail polymers, its phosphorylation was demonstrated to inhibit nuclear transport, implicating the role of PKC and lamin transport as a method of rearrangements in the structure of the nuclear lamina and a means of relaying signals, including hormones, from the cell surface to the nucleus. Cdk1 has also been demonstrated to phosphorylate the microtubule-associated protein-4 (MAP-4), one of the MAPs responsible for promoting the polymerization of tubulin into microtubules. MAP-4 is considered the major factor in determining the stability of the microtubules during the cell cycle, such as in the formation of the mitotic spindle at the onset of mitosis. The

inactivation of MAP-4 following Cdk1 phosphorylation was suggested to cause disassembly of the interphase microtubule network at the end of G2 (Aizawa *et al.*, 1991).

### Chromosome condensation

Virtually all DNA in the eukaryotic genome is packed into nucleosomes, the fundamental unit of chromosomes. A nucleosome contains  $195 \pm 5$  base pairs of DNA, the histone tetramer (H3,H4)<sub>2</sub> and two pairs of the dimer (H2A, H2B) and one H1, which seals off the two turns of the DNA that coils around the histone octamer (Yasuda, *et al.*, 1987). The 195 base pairs of the nucleosome corresponds to the double-stranded fragments observed in agarose gels following electrophoresis and staining of DNA extracted from apoptotic cells (Wylie *et al.*, 1994). These fragments are a result of the activation of endogenous endonucleases which cleave the DNA between the nucleosome prior to chromosome condensation and formation of apoptotic bodies (Wylie *et al.*, 1984; Gavriale *et al.*, 1992). During the eukaryotic cell cycle there is a progressive decondensation of chromosomes, which reaches its maximum at the beginning of S phase. On the completion of S phase, the chromatin recondenses 1000- to 10,000-fold by metaphase as compared to G1 phase (Igo-Kemenes *et al.*, 1982). At the S/G2 phase boundary, there exists a regulatory mechanism (the G2 checkpoint) that recognizes the completion of S phase and triggers the cascade of events leading to the onset of mitosis. While this mechanism still remains to be completely determined, there is ample evidence that reversible phosphorylation and dephosphorylation of the proteins that regulate the transition of the cell cycle play an important role, and the nuclear proteins and DNA conformational processes that are altered at the onset of mitosis, are also regulated via phosphotransferase reactions (Lamb *et al.*, 1991; Murray, 1992). Cells blocked in G2 do not initiate this cascade of events. This regulatory mechanism is crucial in cellular proliferation, since the activity of chromosome DNA is significantly repressed by chromosome condensation, which is logical as during mitosis the cell is on the verge of dividing its duplicated DNA. If this cascade leading to chromosome condensation were to occur during interphase, the cells would die as a result of repressed macromolecular synthesis. Considering the immense

condensation required during the onset of mitosis, it is of interest to determine how histone modifications correlate with this process, and what cell cycle regulators are responsible.

In the process of determining the mechanisms that control the process of chromosome condensation, attention must be focused on the reversible changes that affect chromosomal morphology. Although topoisomerases are responsible for catalyzing changes in topology of chromatin, they do not account for the highly organized structure of chromosomes. Histones, along with ubiquitin, are the major structural chromosomal proteins, and have the most highly conserved sequences in nature. The reasons behind this are more understandable when one considers the implication that these common residues in ubiquitin, histones H3, H4 and the central non-polar domains of histones H2A and H2B, and the basic terminal regions of H1 are essential to their functions on chromosomes (Nickel and Davie, 1989; Th'ng *et al.*, 1992). The core histones have well defined domains. Histone H2A and H2B consist of three domains; a very basic flexible N-terminal domain of 30 residues, a central structural domain of 80-90 residues, and a basic C-terminal tail where ubiquitination occurs (Mueller *et al.*, 1985a). Histones H3 and H4 each have two domains; a lysine-rich basic N-terminal domain, which is subject to heavy phosphorylation on histone H3, and a central and C-terminal domain. It has been shown that acetylation of the N-terminal domain of histone H4 *in vitro* can suppress the binding of H4 to DNA (Turner, 1989). Histone H1 contains three well-defined domains: basic flexible C- and N-terminal regions, and a central apolar globular region that has been determined to be the binding site to the nucleosomes (Yasuda *et al.*, 1987). The terminal regions are probably involved in stabilizing the 34 nm supercoil of the nucleosome, generating a higher-order chromatin structure, and these regions, particularly the C-terminus, are subject to extensive, reversible phosphorylation that modulates their interaction with the DNA (Bradbury *et al.*, 1983). Although the histone sequences are conserved, they are subject to reversible modifications that alter the character of the residues and relax this sequence conservation for functional requirements. The reversible modifications are of three types. Ubiquitination involves the covalent attachment of the 89 amino acid globular ubiquitin protein through the amino group of a lysine to give a bifurcated ubiquitinated H2A and H2B (Mueller *et al.*, 1985a). Acetylation of the lysine of histones H2A, H2B, H3 and H4

convert a basic lysine to an uncharged  $\epsilon$ -N-acetyl lysine, a process that has its greatest function during interphase. Finally, phosphorylation of histones H1 and H3 converts serine and threonine residues to negatively residues, and phosphorylation of these histones and of H2A have been implicated with the onset of mitosis (Th'ng *et al.*, 1992). The existence of mitosis-inducing factors initiating mitosis and chromosome condensation conjointly came from studies by Johnson and Rao (1970), who reported that the fusion of interphase cells with metaphase cells resulted in the condensation of interphase chromatin into discrete chromosomes, a process termed 'premature chromosome condensation' (PCC).

There is a striking correlation between the condensation of chromosomes during the onset of mitosis and the phosphorylation of histones H1 and H3 (Mueller *et al.*, 1985b). Early detailed studies of histone H1 phosphorylation and H1 kinase activity through the precise nuclear-division cycle of the macroplasmidium *Physarum polycephalum* led to the first proposal that an increase in histone H1 kinase activity both initiated and moderated the mitotic process, and that the phenomenon of chromosome condensation required H1 phosphorylation (Mueller *et al.*, 1985a,b). Cell cycle analysis of histone H1 kinase activity demonstrated a 15-fold increase in activity from G2 phase to mitosis (Bradbury *et al.*, 1974). This histone H1 kinase has been identified as Cdk1. Utilizing *P. polycephalum*, it was revealed that the level of phosphorylation increases from 9 to 16 phosphates per molecule at early S phase, to 15 to 24 phosphates at prophase, and continues up to 20 to 24 at metaphase (Mueller *et al.*, 1985). Similar correlations were subsequently observed between H1 phosphorylation and progression through G2 phase in HeLa cells, CHO cells, and rat hematoma cells (Hanks *et al.*, 1983; Th'ng *et al.*, 1992).

Evidence for histone H1 and H3 hyperphosphorylation having a role in chromosome condensation came from studies of premature chromosome condensation. The tsBN2 cell line, isolated as a DNA mutant from nitrosoguanidine-treated BHK-21 cells by FUDR selection, contains a temperature-sensitive lesion in the RCC1 gene, a defect that controls the condensation of chromosomes (Nishimoto *et al.*, 1981; Nishitani *et al.*, 1991). At the non-permissive temperature (39.5°C.), DNA replication ceases rapidly, and cells synchronized at the G1/S boundary became arrested in mid-S phase when released at the same temperature. Initiation of DNA replication is blocked, but elongation of nascent

chains is not inhibited (Eilen *et al.*, 1980). It has subsequently been demonstrated that the loss of RCC1 overrides the control mechanism that ensures the initiation of mitosis only after the completion of DNA replication (Hartwell and Weinert, 1989). This is accomplished biochemically in S and G2 phases by preventing the activation of Cdk1 until the completion of DNA replication, a process which requires the dephosphorylation of the enzyme when complexed to cyclin B (Atherton-Fessler *et al.*, 1993). Nishimoto's laboratory (Nishitani *et al.*, 1991) has found that the loss of RCC1 function induces the synthesis of Cdk1 phosphatases. Experiments with this cell line demonstrated that at the permissive temperature (33°C) incorporation of radioactive orthophosphate following a G1/S block into histone H1 was very low, and histone H3 phosphorylation was not detectable. Histone H1 phosphorylation subsequently occurred throughout S phase, peaking at metaphase, immediately followed by a rapid dephosphorylation. However, histone H3 was phosphorylated only during mitosis, also peaking at metaphase (Hanks *et al.*, 1983). At the non-permissive temperature, histone H1 was heavily phosphorylated during all time points three hours post release at this temperature. Histone H3 was phosphorylated only at the time of appearance of premature chromosome condensation, approximately two hours following the temperature shift (Nishimoto *et al.*, 1991). Perhaps the phosphorylation of these histones is only necessary for the initiation of chromosome condensation, but not to maintain that morphology. DNA synthesis inhibitors such as hydroxyurea and aphidicolin did not effect the phosphorylation of histones H1 and H3 at the non-permissive temperature, indicating that the phosphorylation of these proteins at this temperature is not a result of rapid transit through the cell cycle, and further, that there is a close correlation between the phosphorylation of histones and the induction of chromosome condensation (Nishimoto *et al.*, 1987).

Another observation of mitotic-related phosphorylation of histones and the role of ubiquitination came from studies carried out on the temperature sensitive mutant FT210 cells derived from the mouse mammary carcinoma cell line, FM3A (Th'ng *et al.*, 1992). At the non-permissive temperature the cells arrest at early G2 phase and it was observed that histones H2A and H2B are ubiquitinated, but histone H1 was not phosphorylated. Upon reducing the temperature of the FT210 cells to the permissive one, histone phosphorylation

was observed, and cell progressed towards metaphase. Evidence indicates that the temperature sensitive lesion in FT210 cells involves the histone H1 kinase (Cdk1), linking its cell cycle role to mitotic initiation and possible chromosome condensation. It is instructive to note that at the non-permissive temperature, the synthesis of nucleic acid and protein were not affected. Thus, it can be suggested that the defect in the Cdk1 at this temperature prevented the mitotic phosphorylation of histone H1. As to whether the cell cycle block and repression of chromosome condensation in these studies are directly linked, has yet to be determined.

##### 5. Crystal activation of neutrophils

Little is known about the sequence of events that govern the level or duration of the neutrophil derived inflammation associated with crystal-induced arthritis. An accumulation of neutrophils in the synovial joints of patients suffering with particulate-induced arthritic diseases has been reported (McCarthy *et al.*, 1985; Nagase *et al.*, 1989; Winternitz *et al.*, 1995), indicating that migration of neutrophils into the joints occurs under the direction of unknown chemotactic factors. Two candidates for initiating this migration are tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and granulocyte-macrophage colony stimulating factor (GM-CSF). TNF and GM-CSF are known to be priming agents in neutrophils (Yuo *et al.*, 1994; Hallet and Lloyds, 1995) and it has been shown that preincubation of neutrophils with these agents results in approximately 100% amplification of crystal induced oxidase activation and degranulation (communication by Dr. Helen Burt). TNF- $\alpha$  and GM-CSF are also potent modulators of programmed cell death (apoptosis) with TNF inducing or accelerating apoptosis in neutrophils (Takeda *et al.*, 1993), whereas GM-CSF is known to prevent apoptosis in a variety of cells (Brach *et al.*, 1992). Neutrophil apoptosis and subsequent phagocytosis by macrophages within the synovial joint is thought to mediate the safe (non-inflammatory) removal of neutrophils from the joint (Firestein *et al.*, 1995). These data indicate that crystal induced cytokine/growth factor generation within the joint may modulate the infiltration of neutrophils into the joint, the level of neutrophil inflammatory response to crystals and the

duration of the neutrophil lifetime within the joint (via modulation of apoptosis). It is interesting to note that GM-CSF activates both p70<sup>S6K</sup> and MAP kinase (ERK1 and ERK2) and inhibits apoptosis (Raines *et al.*, 1992; Vietor *et al.*, 1993). Such a negative correlation between p70<sup>S6K</sup> activation and apoptosis has been previously reported for the interaction of cisplatin with T cells (Shi *et al.*, 1995). Given that CPPD crystals also activate these enzymes, and cells are known to accumulate in the diseased joint, it is tempting to speculate that CPPD activation of p70<sup>S6K</sup> inhibits neutrophil apoptosis (like GM-CSF) and leads to neutrophil accumulation within the joint, resulting in the extended inflammatory responses often encountered with this disease. Since apoptosis is associated with the down regulation of certain mediators of the cell cycle that regulate progression through G1 phase, such as p53, *c-myc*, Bcl-2, or pRb (McCarthy *et al.*, 1994; Meikrantz and Schlegel, 1995; Sala *et al.*, 1996), the suggestion that p70<sup>S6K</sup> may suppress apoptosis is reasonable, since p70<sup>S6K</sup> has been implicated in modulating diverse processes such as protein translation, gene expression and protein degradation that leads cells through G1 phase (Chung *et al.*, 1992; Massagué *et al.*, 1995; Brown *et al.*, 1995; Chou and Blenis, 1996). Potentiating cell cycle mediators in late G1 will delay apoptosis (Meikrantz and Schlegel, 1995). The importance of p70<sup>S6K</sup> in cell survival in cells other than neutrophils has been questioned (Thomas *et al.*, 1997). For instance, growth factor-dependent survival of rodent fibroblasts requires PI 3-kinase, but is independent of p70<sup>S6K</sup> activity (Shield *et al.*, 1996; Yao *et al.*, 1996).

It has been demonstrated that activation of neutrophils is wortmannin- and LY294002-sensitive, indicating that PI 3-kinase is a regulator of crystal induced neutrophil activation pathways, contributing to the activation of neutrophil respiratory burst, degranulation and superoxide release (Jackson *et al.*, 1997b). Azuma *et al.* (1993) have demonstrated that activation of the neutrophil respiratory burst is PKC-independent, and that the role of PKC in superoxide generation following crystal incubation is controversial as inhibitors of PKC have been ineffective in abrogating this action (Naccache *et al.*, 1991; Onello *et al.*, 1991). Further, we have demonstrated in Dr. Helen Burt's laboratory that neutrophil pretreatment with the specific PKC inhibitor Compound 3 is ineffective in inhibiting crystal induced neutrophil activation (data not presented here). Given that I have

ascertained two pathways which are activated in neutrophils in response to crystal incubation that diverge before p70<sup>S6K</sup>, I propose that activation of the PKC and PI 3-kinase pathways each contribute to the arthritic condition in addition to the role of PI 3-kinase in the normal inflammatory response. As the activation of p70<sup>S6K</sup> is completely independent of PI 3-kinase in this system, its role in crystal induced arthritis is unclear. Namely, the PI 3-kinase-PKB and PKC pathways mediate the events leading to activation of neutrophils, and the PKC pathway may also govern, through the activation of p70<sup>S6K</sup>, the duration of the neutrophil lifetime by modulating the expression of apoptosis regulating proteins similar to GM-CSF (Matute-Bello *et al.*, 1997; Sweeney *et al.*, 1997). In support of this hypothesis, it was demonstrated that both MSUM (monosodium urate monohydrate) and CPPD crystals inhibit neutrophil apoptosis (Jackson *et al.*, 1997b). Therefore, the reported delineation of the PI 3-kinase and PKC pathways, and the significance of the robust and transient PKC-dependent activation of p70<sup>S6K</sup> in response to crystal incubation, carries strong physiological significance and warrants further investigation.

The inflammatory disease known as acute pseudogout arises from the deposition of calcium pyrophosphate dihydrate crystals (CPPD) in the synovial joints of humans (McCarthy, 1985). Both monoclinic (M) and triclinic (T) crystalline forms of CPPD are able to activate neutrophils in the joint, a process that is thought to be a key factor in the pathophysiology of the disease (McCarthy, 1985; Burt and Jackson, 1993; Winternitz *et al.*, 1995). CPPD crystals have been shown to absorb synovial protein components including small amounts of IgG (synovial fluid is an ultrafiltrate of plasma). *In vitro*, both uncoated and plasma- or synovial fluid-coated crystals have been shown to induce neutrophil activation including superoxide anion production and degranulation (Kozin *et al.*, 1979; Nagase *et al.*, 1989; Burt and Jackson, 1993). The effect of plasma precoating of CPPD(T) is to amplify neutrophil activation levels relative to uncoated crystals probably via the adsorption of opsonizing proteins such as IgG and complement components (Burt and Jackson, 1993). These adsorbed proteins may bind to specific receptors on the neutrophil plasma membrane leading to the activation of signalling pathways within the neutrophil common to the pathways activated by agents such as opsonized microbial pathogens, the main target of neutrophils (Edwards, 1995). The signalling mechanisms and responses to

crystal activation of neutrophils are therefore paramount in determining precisely how crystals induce the inflammatory response, and hence what the molecular mechanisms are that lead to acute pseudogout.

The biochemical mechanism by which either uncoated or plasma coated CPPD crystals activate neutrophils is poorly understood. Burt *et al.* (1992) have illustrated crystal-induced protein-tyrosine phosphorylation in neutrophils implicating the role of protein-tyrosine kinases in the regulation of neutrophil activation. Additionally, recent investigations have revealed a crystal-induced activation of G proteins, the generation of inositol trisphosphate, increases in intracellular calcium concentrations, and PKC activation (Bosner *et al.*, 1989; Christianson, 1990; Liang *et al.*, 1990; Thelen *et al.*, 1994). Jackson *et al.* (1997b) also identified PI-3 kinase as having a pivotal role in the activation of neutrophils in response to crystal incubation. Two specific inhibitors of PI-3 kinase, wortmannin and LY294002, were shown to completely suppress plasma coated CPPD crystal-induced neutrophil activation at concentrations lower than the known IC<sub>50</sub> of these inhibitors for PI-3 kinase. The downstream targets of PI-3 kinase activation are obscure. However, the involvement of PI-3 kinase activation in chemoattractant stimulated neutrophils has been previously reported (Arcaro and Wymann, 1993; Okada *et al.*, 1994). Therefore, despite our understanding the causative events leading to the development of acute pseudogout, little is known about the cellular biochemical mechanisms that trigger the attack of gout.

The addition of mitogens to quiescent mammalian cells leads to the induction of the signalling cascade resulting in multiple phosphorylation of the 40S ribosomal protein S6. p70<sup>S6K</sup> phosphorylation is implicated in the activation of selected protein synthesis, cell growth and progression from G1 to S phase (Ferrari and Thomas, 1994). p70<sup>S6K</sup> is activated by complex serine and threonine phosphorylation events that are sensitive to rapamycin (Han *et al.*, 1995), and these signalling events lie on a distinct pathway from that of the MAP kinases ERK1, ERK2 and Rsk. Activation of p70<sup>S6K</sup> occurs through either PKC-dependent or -independent pathways (Proud *et al.*, 1994; Chung *et al.* 1995; Han *et al.*, 1995). Wortmannin and LY294002 have been shown to prevent the activation of p70<sup>S6K</sup> by insulin and EGF, and expression of constitutively active PI 3-kinase leads to

p70<sup>S6K</sup> activity (Petritsch *et al.*, 1995; Hart *et al.*, 1995) in mammalian cells, thus implicating PI 3-kinase as being a modulator of the PKC-independent pathway. Given that incubation of neutrophils with plasma-coated CPPD crystals can activate PI 3-kinase and that p70<sup>S6K</sup> has been shown to act downstream of PI 3-kinase, we investigated whether incubation with plasma-coated crystals can activate p70<sup>S6K</sup> in neutrophils. I felt this investigation merited further consideration, since crystals induce the stimulation of the synthesis of low molecular weight glycoproteins referred to as crystal-induced chemoattractant factor which has been implicated in angiogenesis-related diseases (Phelps, 1969).

The interaction of monosodium urate monohydrate (MSUM) or calcium pyrophosphate dihydrate (CPPD) crystals with neutrophils in the synovial joint of humans gives rise to the inflammation associated with acute gouty arthritis. Recently it has been reported that opsonized CPPD crystals lead to activation of a number of protein kinases in neutrophils (Jackson *et al.*, 1997). These kinases included the MAP kinases, ERK1 and ERK2, and PI 3-kinase. Inhibition of PI 3-kinase with wortmannin or LY294002 (both specific inhibitors of PI 3-kinase) reduced crystal induced neutrophil oxidase activation and degranulation responses. However, the individual roles of MAP kinase or p70<sup>S6K</sup> or the integrated roles of PI 3-kinase, MAP kinase and p70<sup>S6K</sup> on neutrophil responses to crystals are unknown.

#### 6. Therapeutic inhibitors of the cell cycle used in this investigation

Alkylating agents, topoisomerase inhibitors and X-irradiation all damage DNA, and anti-metabolites disrupt nucleotide pools leading to inhibition of DNA synthesis, and blocking of cells at S phase. G2 arrest caused by a number of anti-tumour agents is associated with extensive chromosomal damage including DNA breaks and gaps. Chromosome damage induced by agents including amsacrine or etoposide will lead to G2 arrest and reduction of cyclin A and Cdk1 levels resulting in the resetting of the cell cycle to G1 with the ploidy being tetraploid. Some anticancer agents have been shown to induce p53-dependent apoptosis, a G1 phase regulated event, leading to cell death which is characterized by mitotic-like morphological features. In addition to antitumour agents

affecting different phases of the cell cycle, or inducing blocks at different phases, cancer cells are also sensitive to cell cycle perturbations. Variations in cell cycle-mediated cell death are exemplified in cells deficient in the p21 checkpoint due to inactivating *p53* mutations. Some chemotherapeutic drugs apparently induce an uncoupling between mitosis and S phase after the initial DNA damage, and instead of undergoing cell cycle arrest, these cells continue to undergo continued rounds of DNA synthesis, culminating in polyploidy and apoptosis. Angiogenesis related diseases require that endothelial cells, for instance at the periphery of the tumour, or in the synovium, undergo proliferation, therefore, activating their cell cycle signalling pathways. The process by which these cells are induced to undergo proliferation in response to angiogenic inducers (such as bFGF, or VEGF) are not well understood. Given that this proliferation is not itself tumorigenic indicates that there is a distinction between how proliferation can be angiogenic versus tumorigenic, which can potentially be exploited in developing agents with reduced toxicity and greater specificity.

Fostriecin is a product of a beer fermenting actinomycete characterized as *Streptomyces pulveraceus* subspecies *fostreus* (ATCC 31906). It has a polyene lactone phosphate structure which possesses *in vitro* and *in vivo* antitumour activity (Jackson *et al.*, 1985; Mamber *et al.*, 1986). Like many other antitumour drugs such as the podophylotoxins, fostriecin was presumed to exert its antitumour effects through inhibition of topoisomerase II (Charron and Hancock, 1990). Although fostriecin does indeed inhibit purified topoisomerase II with an  $IC_{50}$  of 40  $\mu$ M (Boritki *et al.*, 1988), it does not inhibit topoisomerase II in crude cellular extracts (Frosina *et al.*, 1992). During the course of this thesis work, it was demonstrated that fostriecin is a potent inhibitor *in vitro* of protein-Ser/Thr phosphatase 2A (PP-2A) and, to a lesser extent, protein-Ser/Thr phosphatase 1 (PP-1), but not of protein-Tyr phosphatases (Roberge *et al.*, 1994). Fostriecin inhibits PP-2A with an  $IC_{50}$  of 40 nM, 1000-fold lower than topoisomerase II, making PP-2A a likely *in vivo* substrate for fostriecin. The phosphatase activity was inhibited without inducing a DNA cleavable complex, which is apparent with VM-26 or etoposide, and therefore has been suggested to induce a G2 block by inhibiting topoisomerase II activity before a cleavable complex can be formed. Such a mechanism of checkpoint regulation is novel as

DNA damage may not result from inhibition of this enzyme, whereas DNA replication may be affected. In short, fostriecin induces a cell cycle block without inducing DNA damage, and may therefore affect some signal transduction mediator of the G2/M checkpoint.

AGM-1470, O-(chloro-acetylcarbamoyl)fumagillol, is a synthetic analogue of fumagillin which is at least 50 times more effective as an angiogenesis inhibitor than the parent compound (Ingber *et al.*, 1990). It has been demonstrated to inhibit endothelial cells *in vitro* and angiogenesis in organ culture and tumour-induced neovascularization in mice. AGM-1470 has demonstrated unique specificity (IC<sub>50</sub> 34 pM, Antoine *et al.*, 1994) in that it inhibits a cell cycle control pathway that is active in normal cells, but one which is bypassed, or altered, in transformed cells, including transformed endothelial cells. It is not known what reaction or cellular parameter was altered at the concentration used by Antoine and associates. It is a potent inhibitor of endothelial cell proliferation and prevents entry into the G1 phase of the cell cycle. AGM-1470 has also been investigated and characterized as a new treatment option for rheumatoid arthritis (Oliver *et al.*, 1994, 1995; Firestein, 1995). AGM-1470 has recently been demonstrated to inhibit rat adjuvant and collagen-induced arthritis, two animal models of rheumatoid arthritis (Oliver *et al.*, 1994, 1995; Peacock *et al.*, 1992, 1995). AGM-1470 is being considered as a new treatment option for rheumatoid arthritis, but its mechanism of action in this angiogenesis-dependent disease is not yet established. To gain some insight into the mechanism of action of AGM-1470 and a greater understanding of the signalling events in neutrophils leading to oxidative and degranulation responses, we utilized the neutrophil system induced with the agonists CPPD crystals, fMLP, and PMA. The effects of AGM-1470 on agonist-induced PKC and MAP kinase activation were addressed, and we examined the effects on neutrophil activation based on chemiluminescence and superoxide anion generation. We found a PI 3-kinase-independent correlation between MAP kinase and neutrophil activation.

Taxol is an anti-tumour agent isolated from *Taxus brevifolia* (Pacific Yew) which is thought to exert its activity by binding to microtubules, rendering them resistant to depolymerization (Parness and Horwitz, 1981; Manfredi *et al.*, 1982). The resulting non-functional microtubules would induce a late G2 and M phase block of the cell cycle.

Alternative mechanisms by which Taxol may be acting have recently become apparent, which implicate effects on the cell cycle of proliferating cells. Taxol has been shown to interact with c-Mos which phosphorylates tubulin *in vitro* and co-localizes with tubulin and microtubules, resulting in the modification of the microtubule network and formation of the mitotic spindle apparatus (Zhou *et al.*, 1991; Carboni *et al.*, 1993). Taxol has also been found to induce macrophages to secrete TNF- $\alpha$ , similar to the effect of lipopolysaccharide (LPS) (Bogdan and Ding, 1992), and has been suggested to affect a signal transduction pathway shared by LPS. Taxol shares the ability of LPS to induce tyrosine phosphorylation of MAP kinase (Ding *et al.*, 1993; Rezka *et al.*, 1995) as well as inhibit the interaction of MAP kinase with tubulin and microtubules *in vitro* (Nishio *et al.*, 1995). In addition to reversing the effects of colchicine on tyrosine phosphorylation and superoxide anion generation in neutrophils (Roberge *et al.*, 1996), Taxol has also been shown to be effective as a potential anti-arthritic agent and inhibit angiogenesis related diseases (Firestein, 1999; Oliver *et al.*, 1994; communication by Dr. Helen Burt).

## 7. Thesis objectives

It is the objective of this thesis to investigate the roles of intracellular transducing proteins such as mitogen-activated protein (MAP) kinases and mediators, and cyclin-dependent kinases in signalling pathways following stimulation of the growth factor receptor tyrosine kinases. Focus was on those proteins that play a role in the signal transduction pathways that affect cellular proliferation, inflammation and neovascularization, and are suspected to be common to all cells. Consideration was placed on the knowledge that these proteins may contain different feedback loops depending upon the cell system being analyzed, and that their activities may be regulated by different extracellular receptor ligands, or mediated by different isoforms or enzymes of the same pathway. These proteins include Cdk1 (p34<sup>cdc2</sup>/cyclin B complex), PKC, p70<sup>S6K</sup> and MAP kinases (ERK1 and ERK2). Further, the role in regulation of the aforementioned pathways of PKB and PI 3-kinases are also studied because of their ubiquitous function in cell signalling in response to ligand interaction with receptor tyrosine kinases (Downward, 1998; Alessi and Cohen, 1998). Emphasis was placed on ascertaining how these molecules

play a role in the disease state and within the signalling pathways of interest by reproducing the conditions that stimulate these pathways. Studies of the subsequent regulation of the enzymes following treatment of the cells with compounds that inhibit either the specific enzymes or their respective pathways were undertaken. These compounds were utilized to probe the disease state versus the normal state of the signalling pathway being investigated for the purpose of defining potential biochemical targets for therapeutic intervention for these diseases. Further, these agents are already under investigation in the clinic for therapeutic efficacy, such as fostriecin for cancer, Taxol for cancer, angiogenesis and arthritis, and AGM-1470 for angiogenesis and arthritis. Fostriecin, AGM-1470, and Taxol, have been demonstrated to affect cell cycle proliferation and have been proposed to regulate 'different' phases of the cell cycle. Compounds already known to be biochemical effectors of the diseases were also utilized in this study, such as the PI 3-kinase inhibitors wortmannin and LY29004, which effect insulin and arthritic signalling through de-regulation of PI 3-kinase. The PKC inhibitor compound 3 perturbs proliferation and insulin, arthritis and angiogenesis signalling pathways. The FRAP inhibitor rapamycin affects inflammation, arthritis and insulin signalling. Given the possibilities of utilizing effectors of proliferation and differentiation either synergistically, or efficaciously alone as therapeutic agents for cancer, angiogenesis and angiogenesis-related diseases such as arthritis or diabetes, I hoped to gain further insight into potential biomarkers, or targets, for either therapeutic screening or diagnostics and an enhanced understanding of the role of these pathways in the diseased state. Laboratories often ascribe the actions of a drug to a single physiological pathway when investigating its mechanism, without consideration of the other widespread actions of the drug. This is usually not challenged until another investigator utilizes the same knowledge to study how mediators of that same signalling pathway affect another system or disease state. Therefore, this study will focus on the pathways that may lead to several potential diseases, instead of one, in order to learn more of how effectors of signal transduction can be exploited as effective therapeutics agents.

This study has been divided into three sections to distinguish between discrete signal transduction pathways that were modulated by the therapeutic agents investigated here. Although the diseases that these agents are being investigated for in the clinic

overlap to some extent, emphasis has been placed on an agent in each of the sections because of the particular pathway that is affected by the agent. Chapter 3 investigated the affects of fostriecin on human tumour cell lines, and cells with temperature-sensitive lesions, that are in the S and G2 phases, or mitosis. Chapter 4 characterizes the anti-angiogenesis agent AGM-1470 in endothelial cells, where G1 phase-associated signalling is emphasized. Chapter 5 focuses on the angiogenesis-related signalling in primary human neutrophils, targeting therefore, quiescent cells with the chemotherapeutic agent that is presently being investigated as a potential anti-arthritic treatment, Taxol. AGM-1470 is also studied in this system because of its anti-angiogenic properties. The aim of this thesis is to identify pathways that each therapeutic compound affects in order to gain insight into their mechanism, in addition to signalling events associated with cancer, angiogenesis, and the angiogenesis-related diseases, such as arthritis. Such findings will assist in the discovery of targets for the development of novel therapeutic leads.

## EXPERIMENTAL METHODS

### 1. Materials

|   |                         |
|---|-------------------------|
| Acetic Acid (CH <sub>3</sub> COOH)                                  | Fisher Scientific       |
| Acrylamide  | Fisher Sci./ICN         |
| Adenosine 5'-triphosphate disodium salt                             | Sigma                   |
| Amido black 10B   | ICN                     |
| Alkaline phosphatase  | Boehringer Mannheim     |
| Ammonium bicarbonate (NH <sub>4</sub> HCO <sub>3</sub> )            | BDH                     |
| Ammonium persulfate   | Fisher Scientific       |
| Ammonium sulfate ([NH <sub>4</sub> ] <sub>2</sub> SO <sub>4</sub> ) | Fisher Scientific       |
| Aphidicolin   | Sigma                   |
| Aprotinin   | Sigma                   |
| Attachment Factor   | Cell Systems            |
| Bis-acrylamide  | Fisher Scientific       |
| N,N'-Methylene bis-acrylamide                                       | Fisher Scientific       |
| Bovine serum albumin  | Sigma                   |
| Brilliant Blue G  | Sigma                   |
| 5-Bromo-4-chloro-3 indoyl phosphate (BCIP)                          | Sigma                   |
| 1-Butanol   | Fisher Scientific       |
| iso-Butanol   | Fisher Scientific       |
| Benzamidine   | ICN                     |
| Bisbenzamide  | Sigma                   |
| β-Glycerophosphate  | ICN                     |
| β-Methyl aspartic acid  | Sigma                   |
| Calcium chloride (CaCl <sub>2</sub> )                               | Sigma                   |
| Camptothecin  | Calbiochem              |
| Centricon tubes (10 and 30)   | Amicon                  |
| Colcemid  | Sigma                   |
| Coomassie Brilliant Blue R  | EM Science              |
| Complete medium   | Clonetics Corp.         |
| Counting scintillant  | Amersham                |
| α-Chymotrypsin  | Sigma                   |
| α-casein, dephosphorylated  | Sigma                   |
| Denatured alcohol   | Fisher Scientific       |
| Dextran T500  | Pharmacia LKB           |
| N,N-dimethyl fomamide (DMF)   | Sigma/Fisher Scientific |
| Dimethyl sulfoxide (DMSO)   | Fisher Scientific       |
| Dithiothreitol (DTT)  | BDH                     |
| Diolein   | Sigma                   |
| Enhanced chemiluminescence kit                                      | Amersham                |
| Ethylene bis (oxyethylenenitrilo) tetraacetic acid (EGTA)           | Fisher Scientific       |
| Ficoll paque  | Pharmacia               |

|  |                            |
|--|----------------------------|
| Fostriecin   | NCI                        |
| Glacial acetic acid  | Fisher Scientific          |
| Glutathione  | Sigma                      |
| Glutathione cross-linked 4% beaded agarose                             | Sigma                      |
| Glycerol   | Anachemia                  |
| Glycine  | ICN/Sigma/FS               |
| Histone III-S (histone H1)   | Sigma                      |
| HUVEC cells  | Clonetics Corp.            |
| Hydrochloric Acid  | Fisher Scientific          |
| N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES)        | Sigma                      |
| N-Lauryl sarcosine   | Sigma                      |
| Lauryl sulfate (dodecyl lithium sulphate)                              | Sigma                      |
| Leupeptin  | Sigma/ICN                  |
| Magnesium chloride (MgCl <sub>2</sub> -6H <sub>2</sub> O)              | Fisher Scientific          |
| Matrigel <sup>®</sup>  | Collabor. Biomed. Products |
| 2-Mercaptoethanol  | Bio Rad                    |
| Methanol   | Fisher Scientific/BDH      |
| MES (2-[N-Morpholino]ethanesulfonic acid)                              | Sigma                      |
| MonoQ column   | Pharmacia                  |
| MOPS 3-[N-Morpholino]propanesulfonic acid                              | Sigma/ICN                  |
| Myelin basic protein   | Kinetek/Sigma              |
| Nitro blue tetrazolium (NBT)   | Sigma                      |
| Nonident P-40  | BDH                        |
| Phenyl methylsulfonyl fluoride (PMSF)                                  | Sigma                      |
| p-nitrophenyl phosphate  | Sigma                      |
| p81 phosphocellulose filter paper                                      | Whatmann                   |
| ortho-Phosphoric acid (H <sub>3</sub> PO <sub>4</sub> )                | Fisher Scientific          |
| Phosphate-buffered saline (PBS)  | Gibco                      |
| 15-ml polypropylene tubes  | Corning, N. Y.             |
| 50-ml polypropylene tubes  | Corning, N. Y.             |
| Potassium dihydrogen orthophosphate (KH <sub>2</sub> PO <sub>4</sub> ) | BDH                        |
| Ponceau S concentrate  | Sigma                      |
| PKI-cAMP-dependent protein kinase inhibitor peptide                    | Sigma                      |
| PMA (phorbol 12-myristate 13-acetate)                                  | Sigma                      |
| Prestained SDS-PAGE standards (Low Mr range)                           | UBI/BioRad                 |
| Protein A Sepharose CL-4B  | Pharmacia                  |
| Proteinase K   | Pharmacia                  |
| PVDF membrane  | Millipore/Dupont           |
| Resource Q resin   | Pharmacia                  |
| Sodium azide   | Fisher Scientific          |
| Sodium bicarbonate (NaHCO <sub>3</sub> )                               | Fisher Scientific          |
| Sodium chloride (NaCl)   | Fisher Scientific          |
| Sodium dodecyl sulfate (SDS)   | Fisher Scientific          |

|   |                       |
|---|-----------------------|
| Sodium fluoride (NaF)   | BDH/Fisher Scientific |
| di-Sodium hydrogen orthophosphate ( $\text{Na}_2\text{HPO}_4$ ) | BDH                   |
| Sodium hydroxide  | Fisher Scientific     |
| Sodium orthovanadate ( $\text{Na}_3\text{VO}_4$ )               | Fisher Scientific     |
| Soybean trypsin inhibitor (SBTI)                                | Sigma                 |
| TEMED (N,N,N',N'-tetramethylethylenediamine)                    | Fisher Scientific     |
| Trichloroacetic acid (TCA)                                      | Sigma                 |
| Trifluoroacetic acid (TFA)                                      | Applied biosystems    |
| Triton X-100  | BDS/Fisher Scientific |
| Tween-20  | Fisher Scientific     |
| 3mm filter paper  | Whatmann              |
| Zymolase  | Calbiochem            |

## 2. Cell preparation and treatment

### i Cell culture

BHK-21, FM3A, and the human carcinoma cells were grown as monolayers in DMEM supplemented with 7.5% fetal bovine serum and antibiotics at 37°C in humidified 10% CO<sub>2</sub>. Different concentrations of fostriecin (NSC-339638, lot 94528) (125 µM - 1.2 mM) were added to cell cultures from a fresh stock solution in PBS at time 0 together with 0.5 mg/ml colcemid (from a 0.5 mg/ml stock in ethanol). Fostriecin was obtained as vials containing 25 mg of drug with 3.9 mg ascorbic acid from Mr. Carl Huntley, Drug Management and Authorization Section, National Cancer Institute. Floating and attached cells were collected by trypsinization, fixed, stained with the fluorescent DNA dye *bisbenzimidazole* and examined under the light microscope and photographed for determination of the mitotic index. The slides were coded and scrambled so that the number of mitotic cells and their DNA morphological characteristics were determined without knowledge of the treatment. At least 10 random fields were selected containing at least 40 cells per field. Nuclear lamins were detected by indirect immunofluorescence except that FITC-conjugated goat anti rabbit IgG was used as the secondary antibody.

To block cells at G2 phase, VM-26 and camptothecin were utilized. VM-26 was diluted from a 5 mM DMSO stock in fresh media immediately prior to experimenting. Camptothecin diluted from a 0.5 mM stock in DMSO, or the equivalent volume of DMSO, was added to BHK-21 cell cultures at time 0. After 1 hour, the medium was discarded and the camptothecin was removed by washing the cultures twice in fresh medium. Cells were collected and processed for microscopy at the initial time of treatment ( $t = 5$  hours) and at 1 and 2 hours following treatment.

All cells were synchronized using a double block method. Logarithmically growing cells were first subjected to serum deprivation (0.25% fetal bovine serum in DMEM) for 16 hours followed by a 12 hour treatment with 25 µg/ml aphidicolin in fresh media containing 10% fetal bovine serum to induce a cell cycle block at the G1/S boundary. Cells were released from the aphidicolin block by washing with fresh medium and then immediately treated with 375 µM fostriecin, or not treated. All cultures were incubated with 0.5 µg/ml

colcemid to trap accumulated mitotic cells when indicated in the figures. Approximately  $4 \times 10^6$  cells were harvested by trypsinization and then centrifuged at  $800 \times g$  and washed twice with PBS prior to fixing and staining with *bisbenzamide*. Chromosome morphology was determined with fluorescence microscopy and a minimum of 10 fields containing at least 40 cells per field were selected for determining the percentage of mitotic cells as characterized in Figure 2A and B.

Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics Corp. (San Diego, CA) and were maintained at passage 2 - 8 in a subconfluent state unless frozen at  $-80^\circ\text{C}$  in 10% DMSO. Cells were grown in complete medium containing 2% FCS and  $1 \mu\text{g/ml}$  bFGF in 150 mm flasks or 96 well plates coated with Attachment Factor (a defined fibronectin-based matrix supplied by Clonetics Corp.), collagen or Matrigel<sup>®</sup>. This particular Matrigel<sup>®</sup> was growth factor reduced (0-0.1  $\mu\text{g/ml}$  bFGF,  $>0.5 \text{ ng/ml}$  EGF, 5  $\text{ng/ml}$  IGF,  $<5 \text{ pg/ml}$  PDGF,  $<0.2 \text{ ng/ml}$  NGF, 1.7  $\text{ng/ml}$  TGF $\beta$ ). Cells were maintained at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$ .

FT210 cells were routinely maintained at  $32^\circ\text{C}$  at a density of approximately  $5 \times 10^5$  cells/cm<sup>2</sup> in RPMI-1640 supplemented with 10% fetal calf serum. Cells were synchronized by incubation in isoleucine-deficient RPMI-1640 medium supplemented with 10% heat-inactivated, dialyzed calf serum for 15 hours, followed by incubation in regular medium containing 2.5  $\mu\text{g/ml}$  aphidicolin for 9 hours to arrest the cells at the G1/S boundary. For G2 arrest, the G1/S arrested cells were released into regular medium at  $39^\circ\text{C}$  or in medium containing 100  $\text{ng/ml}$  staurosporine at  $32^\circ\text{C}$  for 18 hours. For mitotic arrest, cells were released from G1/S arrest into regular medium containing 50  $\text{ng/ml}$  nocodazole for 18 hours at  $32^\circ\text{C}$ .

Primary human hepatocytes grown on Matrigel in 24 well plates were obtained from Cedra Corp. (Austin, TX). The solid matrix was removed from cells upon receipt as described in the included instructions. Cells were incubated in 600  $\mu\text{l}$  Complete Nutrient Culture (CM5300, Cedra Corp., Austin, TX) supplemented with 5% fetal bovine serum (buffered at pH 7.4 with 2.2  $\text{g/l}$   $\text{NaHCO}_3$ , 100 U/ml penicillin, 100  $\mu\text{g/ml}$  streptomycin, and 5  $\text{ng/ml}$  dexamethasone). The medium was prepared without the phenol red pH indicator so no interference with metabolism or HPLC analysis would occur. The hepatocytes were

incubated with 50  $\mu$ M AGM-1470 for specified times between 2 min and 4 hours at 37°C. Following the addition of 300  $\mu$ l of acetonitrile (to stop the reaction), extracellular medium aliquots (900  $\mu$ l) were analyzed following solid phase extraction by HPLC analysis as described below. After trypsinization of hepatocytes, cells were lysed, fractionated and extracted for HPLC analysis, as described below.

ii. Neutrophil preparation

Neutrophils were prepared from freshly collected, human, citrated whole blood by dextran sedimentation and Ficoll Hypaque density centrifugation. Four hundred ml of blood were mixed with 80 ml of 4% dextran T500 in HBSS and allowed to settle for 1 hour. Plasma was collected continuously and 5 ml applied to 5 ml Ficoll Paque (Pharmacia) in 15-ml polypropylene tubes. Following centrifugation at 500 x g for 30 min, the neutrophil pellets were washed free of erythrocytes by 20 sec of hypotonic shock in ddH<sub>2</sub>O. Neutrophils were resuspended in HBSS, kept on ice and used for experiments within 3 hours. Neutrophils prepared under these conditions yielded cell suspensions that contained over 95% neutrophils with over 90% cell viability (assessed by trypan blue exclusion).

iii. Crystal preparation

Plasma-opsonized crystals were used in all studies involving CPPD(T) crystal-incubation. CPPD(T) (triclinic) crystals were prepared and characterized as previously reported (Burt and Jackson, 1987). Opsonization of crystals was carried out using 50% heparinized plasma and HBSS (Hanks buffered salt solution, pH 7.4) at 37°C for 30 min immediately prior to experiments. Twenty five mg of CPPD(T) crystals were weighed into 1.5-ml Eppendorf tubes followed by 0.5 ml of 50% fresh human plasma. The tubes were capped and tumbled end-over-end at 30 rpm for 30 min at 37°C. Tubes were then centrifuged at 1000 x g, and washed in HBSS and centrifuged.

#### iv Incubation of neutrophils with agonists and inhibitors

Stock solutions of 28 mM Taxol, 20 mM AGM-1470, 1 mM PMA, 1 mM fMLP, 100 mM compound 3, and 10 mM wortmannin in dimethylsulphoxide (DMSO) were freshly prepared before each experiment. Equal volumes of solutions were added to neutrophils at  $5 \times 10^6$  cells/ml under mild vortexing conditions to achieve concentrations of 28  $\mu$ M Taxol, 0.5  $\mu$ M PMA, 1  $\mu$ M fMLP, 10 nM Compound 3, 10 nM wortmannin and 300 nM - 50  $\mu$ M AGM-1470. Except during chemiluminescence analysis, all experiments with AGM-1470 were performed with 5  $\mu$ M, and the stock solutions were diluted with HBSS to give the appropriate concentrations. The final DMSO concentration never exceeded 1%, which was shown to have no effect on control cell responses (Jackson *et al.*, 1997a).

### 3. General protein biochemistry techniques

#### i. Preparation of extracts

##### a. Cell lysis and homogenization

Cells treated as described in text were washed in cold lysis buffer (20 mM HEPES, pH 7.5, 0.34 M sucrose, 2 mM 2-mercaptoethanol, 100  $\mu$ M sodium vanadate, 0.1  $\mu$ M sodium fluoride, 60 mM  $\beta$ -glycerolphosphate, 1 mM diisopropylfluorophosphate, 10  $\mu$ g/ml each of aprotinin and leupeptin, 1  $\mu$ M pepstatin, 10  $\mu$ g/ml soybean trypsin inhibitor). Tubes were sonicated at 4°C for 30 sec using a micro-sonic probe sonicator. The homogenate was centrifuged at 4°C for 10 min at 8000 x g. The membrane bound protein fraction was obtained by solubilizing the resulting pellet in cold lysis buffer containing 1% Triton-X 100 followed by sonication and centrifugation as mentioned earlier. The crystal-cell pellet was frozen in liquid nitrogen and stored at -70 °C.

##### b. Neutrophil extracts

Neutrophils treated as described above were washed in cold lysis buffer (2 mM HEPES, pH 7.5, 0.34 M sucrose, 2 mM  $\beta$ -mercaptoethanol, 100  $\mu$ M sodium vanadate, 0.1  $\mu$ M sodium fluoride, 60 mM  $\beta$ -glycerolphosphate, 1% Triton X-100, 1  $\mu$ M pepstatin, 10  $\mu$ g/ml each of aprotinin, leupeptin, and soybean trypsin inhibitor). The homogenates were

centrifuged at 4°C for 10 min at 8000 x g. Homogenates were immediately frozen in liquid nitrogen and stored at -70°C.

c. Preparation and extraction of microsomal fractions

Hepatocytes were collected from the wells following trypsinization with 2 x 500 µl of trypsin. Cells were washed 2 x with buffer H containing 10 mM HEPES-NaOH (pH 7.4), 10 mM KCl and 1.5 mM MgCl<sub>2</sub>, followed by centrifugation at 3000 x g for 5 min. Cells were resuspended in 2 ml of buffer H and subjected to sonication (2 x 20 sec, 4°C). The soluble fraction was obtained following centrifugation at 105,000 x g for 30 min. The pellet was resuspended in buffer H containing 0.2% SDS and 1% NP-40, vortexed for 10 min and centrifuged again at 105,000 x g for 30 min at 4°C. The supernate was collected and represents the particulate fraction. Both fractions were pooled, and together represent the intracellular fraction.

Solid phase extraction (SPE) of the extracellular and intracellular fractions was performed with 1cc C<sub>18</sub> Sep-Pak Vac SPE cartridges (Waters, WAT054960) conditioned with 2 x 1 ml methanol and 2 x 1 ml dH<sub>2</sub>O. Fractions were loaded onto the columns with a 0.5 ml/min flow under vacuum and washed 2 x with 15% acetonitrile/water. Cartridges were dried for 2 minutes and drug was eluted with 500 µl acetonitrile. Samples were maintained at 4°C for HPLC analysis unless stored at -70°C.

d. Histone phosphorylation and acid-solubilization

To examine endogenous histone phosphorylation, cells were grown and treated as discussed in the figure legends, and <sup>32</sup>P-orthophosphate (Amersham) was then added to a concentration of 25 µCi/ml and the cells were incubated for the duration specified in the figure legends, or the period of fostriecin treatment. To extract the histones, unattached cells were collected by centrifugation and washed, together with the attached cells that were harvested by trypsinization, in ice cold PBS before lysis in nuclei isolation buffer (250 mM sucrose, 1 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 1% (v/v) Triton X-100, 10 mM Tris-HCl, pH 8.0) containing 10 mM NaF, 50 mM iodoacetamide, 50 mM NaHSO<sub>3</sub> and 2 mM PMSF. The nuclear pellets were collected by centrifugation at 10,000 x g for 1 min at 4°C, and the

supernatant was discarded. To extract total histones, the nuclear pellet was extracted twice with 0.4 N H<sub>3</sub>SO<sub>4</sub> and the solubilized proteins were precipitated with 20% TCA. The precipitate was then washed twice with acetone, dried under vacuum and solubilized in acid-urea sample buffer (5% glacial acetic acid, 8 M urea, 5% glycerol).

#### ii. Protein quantitation

The protein content of the soluble and membrane extracts was determined using the method of Bradford (1976).

#### iii. Partial purification of proteins

Extracts were fractionated by FPLC on a 1-ml MonoQ anion exchange column. Columns were equilibrated before and after use with 2 ml of 2.0 M NaCl, and washed with 10 ml of MonoQ buffer (25 mM β-glycerolphosphate, 10 mM MOPS, pH 7.2, 5 mM EGTA, 2 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, and 2 mM orthovanadate). All buffers were filtered with a 0.22 μM filter before use.

Samples (2 mg) were prepared as described and diluted to 2 mg/ml in MonoQ buffer and filtered through a 0.22 μ filter. The samples were loaded onto a 2.0 ml injection loop and applied directly onto the column. A standard elution program was utilized for all proteins fractionated: the protein was applied onto the column in 2.2 ml of MonoQ buffer, eluted in a 10 ml 0 - 0.8 M NaCl gradient, and 0.25 ml fractions were collected. Column fractions that were not immediately assayed by peptide kinase assays or analyzed by SDS-PAGE were immediately covered and frozen at -70°C.

#### iv. Gel electrophoresis

##### a. SDS-PAGE gels

SDS-polyacrylamide gel electrophoresis of the crude and MonoQ-fractionated rat tissues was performed on 1.5 mm thick gels with the buffer system described by Laemmli (1970). A 10% separating gel and a 4% stacking gel were used. Samples were boiled for 5 min in the presence of 5x concentrated SDS-sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 0.01% bromophenol blue, 10% mercaptoethanol, and 20% glycerol) and electrophoresed

for 18 h at 10 mA, or until the dye front reached the bottom of the gel. In the case of Mek1 autophosphorylation and in-gel MAP kinase and PKC assays, the dye front was allowed to elute off sufficiently to allow for excess ATP to move from the gel.

b. Urea gel electrophoresis

Phosphorylated histones were resolved in 25 x 19 x 0.15-cm acid-urea gels (12% acrylamide, 0.8% bisacrylamide, 5% acetic acid, and 8 M urea) with 5% acetic acid as the running buffer after an overnight pre-electrophoresis. The gels were then stained with Coomassie blue and dried before autoradiography.

v. Staining SDS-PAGE gels

a. Coomassie staining

Gels were immersed in Coomassie stain (0.1% Coomassie brilliant blue R, 50% methanol, 10% acetic acid (w/v/v)) for approximately 1 hour, and then soaked in 40% methanol/10% acetic acid until destain was sufficient to allow for band viewing.

b. Amido black staining

Membranes were immersed in 0.1 % amido black/45% methanol/10% acetic acid (v/v/v/v) for 15 min at room temperature with rotation. Destaining occurred in 5% methanol/10% acetic acid (v/v/v) solution until bands were visible and background was eliminated.

vi. Immunoblot analysis of SDS-PAGE gels

SDS-PAGE of the neutrophil lysates was performed on 1.5 mm thick gels. An 11% separating gel and a 4% stacking gel were used. Samples were boiled for 5 min in the presence of 5 x concentrated SDS-sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 0.01% bromophenol blue, 10%  $\beta$ -mercaptoethanol, and 20% glycerol) and electrophoresed for approximately 12 hours at 10 mA. Subsequently, the separating gel was soaked in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 10 min and then sandwiched with a nitrocellulose membrane. Proteins were transferred for 3 hour at 300

mA. The membrane was blocked with TBS (Tris buffered saline, 20 mM Tris-HCl, pH 7.4, 0.25 M NaCl) containing 5% bovine serum albumin (BSA) for 3 hour at room temperature, and then washed three times in TBS containing 0.05% Tween (TTBS) for 15 min. The membranes were incubated overnight at room temperature with primary antibody (MAP kinase: anti-ERK1-CT, UBI; PKC: anti-PKC mAb, Kinetek Pharmaceuticals Inc.; anti-PKC $\delta$ , Gibco, BRL; p70<sup>S6K</sup>: anti-S6-NT, UBI). Membranes were then washed with TTBS three times before a 1 hour incubation at room temperature with secondary antibody (goat anti-mouse IgG coupled to horse radish peroxidase in 2.5% skim milk-TTBS for blots with anti-PKC-catalytic subunit mAb; goat anti-rabbit IgG coupled to horse radish peroxidase in TTBS for blots probed with anti-S6-NT antibodies; goat anti-rabbit IgG coupled to alkaline phosphatase in TTBS for blots probed with anti-PKC $\delta$  and anti-ERK1-CT antibodies). The membranes were then rinsed three times in TTBS and once in TBS before incubation with 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) colour development solution (mixture of 3% NBT in 1 ml 70% DMF and 1.5% BCIP in 1 ml 100% DMSF before being added to 100 ml of 0.1 M NaHCO<sub>3</sub>, 10 mM MgCl<sub>2</sub>, pH 9.8) for detection of alkaline phosphatase-conjugated secondary antibodies. For detection of horse radish peroxidase conjugated secondary antibodies, membranes were washed as described previously, and enhanced chemiluminescence (ECL) was employed as the detection procedure.

a. Alkaline phosphatase (ALP)-conjugated secondary antibody

The membrane was briefly washed in AP buffer (0.1 M Tris-base, 0.1 M NaCl, 5 mM MgCl<sub>2</sub>, pH 9.5), and then developed in 50 ml AP buffer containing 340  $\mu$ l NBT (50 mg/ml 70% DMF) and 170  $\mu$ l of BCIP (50 mg/ml in 100% DMF). The colour reaction was left to proceed until the signal of interest was of sufficient intensity and was terminated by washing the membrane in dH<sub>2</sub>O, followed by air drying.

b. Horseradish peroxidase-conjugated secondary antibody (ECL)

The membrane were incubated with the ECL reagent for exactly 60 sec with gentle agitation. The membrane was gently blotted to remove excess reagent and carefully

wrapped in plastic wrap, avoiding bubble and wrinkles. The membrane was exposed to film for 10 sec to 2 min depending on the intensity of the band of interest, and background.

vii. Stripping and reprobing Western blots

For ECL blots, membranes were stripped with stripping buffer A (100 mM 2-ME, 2% SDS (w/v), 62.5 mM Tris-HCl (pH 6.7) at 55°C for 20 min with agitation. The membranes were subsequently washed with TBST and then reblocked overnight. For ALP blots, membranes were re-probed by washing with TBS (pH 2.5) for 10 min, followed by two 5 min washes in TBS (pH 7.5). Membranes were subsequently re-probed with primary antibody without blocking.

viii. Immunoprecipitation

Protein A-Sepharose CL4B was swollen for 30 min in 3% NETF (3% NP-40, 100 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, (pH 7.4), 50 mM NaF). The Protein A-beads were pelleted by centrifugation in a Baxter Biofuge Lab Top centrifuge at 5000 rpm for 1 min, washed twice in 3% NETF, and resuspended with an equal volume of 3% NETF to yield a 1:1 suspension.

An appropriate volume of extract, as indicated in the Results Section, was diluted with an equal volume of 6% NETF, and the final volume was brought to 500  $\mu$ l with 3% NETF in an Eppendorf tube. The mixture was precleared with 10  $\mu$ l Protein-A and 2  $\mu$ l preimmune serum and incubated for 15 min at 0°C with rotation. The mixture was separated with centrifugation at 15000 rpm and was charged with the appropriate volume of antibody, as indicated in the Results Section. Following 60 min incubation with agitation at 0°C, 40  $\mu$ l of the Protein-A slurry were added, and incubation was allowed to proceed for an additional 3 h at 4°C with rotation. The immunoprecipitates were washed extensively with 800  $\mu$ l 6% NETF twice, followed by a wash with 800  $\mu$ l of 0% NETF (NETF buffer without NP-40). The IP was washed an additional time with 1 ml KII buffer (12.5 mM  $\beta$ -glycerophosphate, 12.5 mM MOPS, (pH 7.2), 5 mM EGTA, 7.5 mM MgCl<sub>2</sub>, 50 mM NaF, 0.25 mM DTT) for kinase assays and resuspended to the appropriate volume in KII buffer. For IPs intended to be directly loaded onto the gel, the IP was diluted to 48  $\mu$ l

with 0% NETF and 12  $\mu$ l of 5 x loading buffer (250 mM Tris-HCl, (pH 6.8), 10% SDS, 25% glycerol, 0.7 M  $\beta$ -mercaptoethanol, 0.02 % bromophenol blue), boiled at 95  $^{\circ}$ C for 5 min and loaded directly onto the gel.

#### ix. Protein kinase assays

##### a. Assays on MonoQ fractions

MAP Kinase, PKC and p70<sup>S6K</sup> in the Mono Q fractions (5  $\mu$ l aliquots) were assayed at 30 $^{\circ}$ C with 1 mg/ml of myelin basic protein (MBP), histone H1 and the S6-10 peptide respectively, in assay buffer: 20 mM MOPS (pH 7.2), 25 mM  $\beta$ -glycerolphosphate, 5 mM EGTA, 2 mM ethylene diamine tetraacetic acid (EDTA), 20 mM MgCl<sub>2</sub>, 2 mM sodium orthovanadate, 1 mM dithiothreitol, 500nM cAMP-dependent protein kinase inhibitor peptide (Sigma) and 5  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP ( $\approx$  2000 c.p.m./pmol) in a final volume of 25  $\mu$ l. The reactions were allowed to proceed for 10 min after which 20  $\mu$ l were spotted onto a 1.5 x 2 cm<sup>2</sup> P-81 phosphocellulose filter paper. The paper was extensively washed with 1% phosphoric acid with 10 changes, after which the adsorbed radioactivity was quantified by liquid scintillation counting in a Packard TriCarb 4530 counter.

Five hundred micrograms of total protein from extract supernatants were diluted into immunoprecipitation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 5% glycerol, 10 mM (sodium fluoride, 5 mM EGTA, 1 mM EDTA, 30 mM  $\beta$ -glycerolphosphate) and the solutions were incubated with the appropriate antibodies (MAP kinase: ERK1-CT, UBI; PKC: anti-PKC mAb to the catalytic subunit, Kinetek Pharmaceuticals Inc.; p70<sup>S6K</sup>: anti-S6-NT, UBI) for 4 hours at 4 $^{\circ}$ C. Proteins A-agarose beads (Sigma) which had been preincubated in cold (4 $^{\circ}$ C) immunoprecipitation buffer, were added and the samples were further incubated for 1 hour at 4 $^{\circ}$ C. The beads were washed twice with immunoprecipitation buffer, and twice with KII buffer (12.5 mM MOPS, pH 7.5, 12 mM  $\beta$ -glycerolphosphate, 5 mM EGTA, 7.5 mM MgCl<sub>2</sub>, 50 mM NaF, 5 mM glycerolphosphate, and 0.25 mM DTT). The beads were resuspended in KII buffer supplemented with 10 mM MgCl<sub>2</sub> and 5% glycerol and the reactions were initiated with 10  $\mu$ l of 250 $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP ( $\approx$  2000 c.p.m./pmol) in a final volume of 40  $\mu$ l and incubated for 20 min at 30 $^{\circ}$ . The reactions were halted with 5 x SDS-

loading buffer, boiled for 5 min and loaded onto a SDS-PAGE gel. Following transfer of protein as described above, the membrane was immunoblotted for the appropriate protein, exposed to film and the bands representing ERK 1 protein were excised and subjected to Cerenkov counting. Assay conditions for the other kinases are described below.

PKC was assayed from MonoQ fractions (5  $\mu$ l) with and without phospholipid and diolein (PD/DO) and  $\text{Ca}^{2+}$ . Briefly, for assays with (PD/DG), 5  $\mu$ l of the MonoQ fraction were mixed with 5  $\mu$ l of histone H1 cocktail (1.6 mg/ml H1 histone with 25  $\mu$ M PKI in assay dilution buffer [25  $\mu$ M  $\beta$ -glycerophosphate, 20 mM MOPS, pH 7.2, 5 mM EGTA, 2 mM EDTA, 20 mM  $\text{MgCl}_2$ , 2 mM  $\text{NaVO}_4$ , pH 7.2, 0.25 mM DTT, 5  $\mu$ M  $\beta$ -methyldiphenylacetic acid, pH 7.2]), 5  $\mu$ l buffer A (25  $\mu$ M  $\beta$ -glycerophosphate, 10 mM MOPS, pH 7.2, 2.0 mM EGTA, 2 mM EDTA, 0.25 mM DTT) mixed with 50 mM  $\text{MgCl}_2$  and 22.5 mM  $\text{CaCl}_2$  (final concentration) and 5  $\mu$ l of buffer B (buffer A mixed with 300  $\mu$ g/ml phosphatidylserine). For reactions without PD/DG, 5  $\mu$ l of the enzyme fraction were mixed with 5  $\mu$ l of histone H1 cocktail, 5  $\mu$ l buffer A mixed with 50 mM  $\text{MgCl}_2$  (final concentration), and 5  $\mu$ l buffer A only. Each reaction was initiated with 5  $\mu$ l [ $\gamma$ - $^{32}$ P]ATP (a 10 ml stock contained 9.55 ml assay dilution buffer, 250  $\mu$ l 10 mM ATP and 200  $\mu$ l of 2 mCi [ $\gamma$ - $^{32}$ P]ATP). The reactions were allowed to proceed for 10 min at 30°C after which 20  $\mu$ l were spotted onto a P-81 phosphocellulose filter paper. The paper was extensively washed with 1% phosphoric acid and counted for radioactive phosphate incorporation. Phosphatidylserine was prepared by placing 150  $\mu$ l of 10 mg/ml phosphatidylserine in 100-mm disposable culture tubes, and evaporated under a stream of nitrogen. Later, 5 ml of buffer A were added and the solution was sonicated for 5 min on ice at a 40% setting.

p70<sup>S6K</sup> in the MonoQ fractions (5  $\mu$ l aliquots) was assayed at 30°C with 1 mg/ml of the S6-10 peptide (AKRRRLSSLRASTSKSESSQK), MBP-NT peptide (AAQKRPSQRTKYLA) (specific for S6 kinase and PKC), or myelin basic protein (MBP) in assay buffer with the 20  $\mu$ M of the PKC inhibitor, Compound 3, and 5  $\mu$ l [ $\gamma$ - $^{32}$ P]ATP (~2000 cpm/pmol) in a final volume of 25  $\mu$ l. The reactions were allowed to proceed for 10 min after which 20  $\mu$ l were spotted onto a 1.5 x 2 cm<sup>2</sup> P-81 phosphocellulose filter paper.

PI 3-kinase was immunoprecipitated from neutrophil lysates (500 µg total protein, BCA assay, Pierce) with 0.5 µg anti p-85 antibody (Santa Cruz Laboratories) for 3 hr at 4°C with rotation and collected with 25 µl of protein A-conjugated Sepharose. The beads were then washed twice with lysis buffer and 3 times with 10 mM Tris-HCl, pH 7.4. Phosphatidylinositol (PI) (10 µg) was sonicated briefly in 30 mM Hepes, pH 7.4, then mixed with the beads and incubated for 10 min on ice. The reaction was started by adding 10 µCi [ $\gamma$ -<sup>32</sup>P] ATP in 40 µl kinase buffer (30 mM Hepes, pH 7.4, 30 mM MgCl<sub>2</sub>, 200 µM adenosine, 50 µM ATP) and incubating for 15 min at room temperature. One hundred µl of 1 N HCl were added to stop the reaction and PI 3-phosphate. (PI-3-P) was extracted using 200 µl of chloroform:methanol (1:1). PI-3-P was resolved from residual [ $\gamma$ -<sup>32</sup>P] ATP in the organic layer by thin layer chromatography on oxalate treated silica gel 60 plates (E. Merck) using chloroform:methanol:water:concentrated NH<sub>4</sub>OH (45:35:7.5:2.5). PI 3-kinase activity was determined by scintillation counting of the PI-3-P zone on the chromatogram after autoradiography.

b. Radioactive IP assays:

The beads were washed and resuspended in assay buffer supplemented with 10 mM MgCl<sub>2</sub> and 5% glycerol. The reaction containing 15 µl of the appropriate peptide cocktail in assay buffer and 5 µl of the enzyme fraction was started with 10 µl [ $\gamma$ -<sup>32</sup>P]ATP (~2000 cpm/pmol) in a final volume of 40 µl and incubated for 30 min at 30°C. The reactions were terminated with 5x SDS-loading buffer, boiled for 5 min and loaded onto an SDS-PAGE gel. Following transfer of protein, the membrane was immunoblotted for the appropriate protein, exposed to film, and the protein was subjected to Cerenkov counting.

c. S6-10 peptide, MBP-NT, MBP and PKC cocktails:

One mg/ml of the S6-10 peptide (AKRRRLSSLRASTSKSESSQK), MBP-NT peptide (AAQKRPSQRTKYLA), (specific for S6 kinase and PKC) and 5 mg/ml of MBP was prepared in assay buffer. IPs with anti-PKC  $\alpha/\beta$  antibodies were assayed with histone H1 (Histone III-S, Sigma), which was diluted to 5 mg/ml in assay buffer.

d. Cdk1 purification and activity determination

BHK-21 cells were synchronized in S phase by serum deprivation followed by 12 hour treatment with 25  $\mu\text{g/ml}$  of aphidicolin in fresh medium. Synchronization of cells was verified by flow cytometric analysis. Greater than 80% of the cells were blocked at S phase in the aphidicolin block (data not shown). The cells were released from S phase for 6 hours by washing with fresh medium prior to further treatment. Cells were blocked in G2 by 2 hour treatment with 25  $\mu\text{M}$  VM-26 following the 6 hour release from the aphidicolin induced S phase block. To prepare the nuclear cell extract, cells were collected by 2 x washing with 0.01 mM EDTA in phosphate-buffered saline and centrifuged for 10 min at 800 x g. The supernatant was removed and the cells were washed twice with 10 ml of TBS. Following the final centrifugation, cells were lysed by adding 100  $\mu\text{l}$  per  $2 \times 10^6$  cells of buffer containing 40 mM HEPES pH 7.4, 1 % Triton X-100, 250 mM NaCl, 15 mM  $\text{MgCl}_2$ , 80 mM 2-glycerophosphate, 40 mM p-nitrophenyl phosphate, 1.0 mM sodium orthovanadate, 0.1 mM okadaic acid, 10  $\mu\text{g/ml}$  each of aprotinin, leupeptin, and pepstatin and 0.5 mM PMSF, kept on ice for 10 min and then centrifuged at 12000 x g for 15 min at 4°C. A sample of 100  $\mu\text{g}$  of protein (determined with a Bio-Rad protein assay kit) was mixed with 40  $\mu\text{l}$  of 1 mg/ml  $\text{p13}^{\text{suc1}}$  beads and KII buffer (pH 7.2) containing 12.5 mM MOPS, 12.5 mM 2-glycerophosphate, 5 mM EGTA, 75 mM  $\text{MgCl}_2$  and 0.5 mM NaF, to a final volume of 400  $\mu\text{l}$ . The mixture was incubated with agitation for 30 min at 4°C and then centrifuged at 500 x g for 30 sec. The beads were washed 3x with KII buffer and suspended in 20  $\mu\text{l}$  of the buffer and 2x Laemmli SDS loading buffer. Samples were boiled (>90°C) for 3 min. SDS-PAGE was performed with 20  $\mu\text{l}$  samples using 10% polyacrylamide gels (0.8% bisacrylamide). One mm thick slab gels were electrophoresed overnight at 60 V (tracking dye migration approximately 14 cm). For Western blotting, protein was transferred to nitrocellulose overnight at 40 V. Visualization of immunoreactive protein was analyzed using a chemiluminescent method (Amersham ECL) according to the manufacturer's instructions. The primary antibody: anti-Cdc2 (Th'ng *et al.*, 1992) was concentrated to 2  $\mu\text{g/ml}$ .

x. Topoisomerase II assays

The catenation activity of topoisomerase II, (purified from yeast) was used to assess the topoisomerase II inhibitory effect of fostriecin. This method was developed based on modifications of methods described earlier (Minocha and Long, 1984; Ishimi *et al.*, 1992). The restriction endonuclease XhoI (10 units) and VM-26 (5  $\mu$ M) were used as controls in these experiments. The reaction mixture consisted of 50 mM Tris HCl, pH 8.0, 120 mM KCl, 10 mM MgCl<sub>2</sub>, 0.5 mM DTT, 30  $\mu$ g/ml BSA, 0.5 mM ATP, 0.1  $\mu$ g kinetoplast DNA. The reaction was initiated following the addition of purified yeast topoisomerase II to give a final volume of 20  $\mu$ l, and was incubated at 37°C for 30 min. The reaction was stopped by the addition of 10  $\mu$ l of 0.5 M EDTA and 0.3% SDS. The reaction mixture was mixed with 4  $\mu$ l of 6 x DNA loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol in water) and was electrophoresed in 1.2% agarose with 0.9% TBE (10 mM Tris borate, pH 8.0, 25 mM EDTA) at 100 V for approximately 2 hours. DNA was visualized by staining with ethidium bromide by immersing the gel in electrophoresis buffer containing 0.5  $\mu$ g/ml ethidium bromide for 30 min at room temperature followed by a 20 min wash in 1 mM MgSO<sub>4</sub>, also at room temperature.

xi. Pulsed-field gel electrophoresis

Cells treated as described in the figures were quickly trypsinized and centrifuged at 800 x g for 10 min and resuspended in TBE at 6 x 10<sup>5</sup> cells/100  $\mu$ l (each lane of the gel was inserted with a plug containing DNA from an equivalent of 2.5 x 10<sup>5</sup> cells). To 1 ml of the cell suspension were added 0.5 ml of preboiled 1.5% low melting agarose (LMA) (1.5 g/100 ml in 0.5 M TBE), kept at 37°C, and the mixture was pipetted up-and-down carefully for 6 repetitions. The mixtures were placed in 35 mm petri dishes and put on ice. Lysis solution (0.5 M EDTA, pH 7.4, 0.9% sarcosyl, and 0.1% SDS in 10 mM Tris HCl, pH 8.0) was added in excess to the hardened mixture followed by 100  $\mu$ g/ml proteinase K, and the dishes were agitated slowly at 37°C for 2 days with a single change of the lysis buffer. The digested samples were washed 3 x in 500  $\mu$ l TBE (4°C) containing PMSF. Plugs were cut to fit the size of the agarose gel wells and stored in TBE at 4°C. Pulsed-field gel electrophoresis was conducted with the digested samples and a yeast lambda ladder

(BJ1368) in a 100 ml 1% agarose gel with 1 x TBE at approximately 14°C. The pulse times consisted of 70 seconds for 10 hours followed by 120 sec for 9 hours at 100 V. Following electrophoresis, the gels were visualized with ethidium bromide.

BJ2168 strain yeast was grown to OD<sub>600</sub> 1.0 in 5 ml of YPD. Cells were transferred to 1.5-ml Eppendorf tubes and centrifuged at 12,000 rpm for 10 seconds and resuspended in 1 ml 10 mM Tris HCl (pH 7.5) with 50 mM EDTA and washed 2 x in the same buffer followed by a final resuspension in 0.15 ml Tris-EDTA solution with 1 µl of a 20 mg/ml zymolase stock solution (in 10 mM PBS). The mixture was placed in a 42°C heating block for 30 sec. Two hundred fifty µl of low melting point agarose at 42°C were added and mixed and the mixture was placed on ice and the solid was overlaid with LET buffer (10 mM Tris HCl, pH 7.5 and 0.5 M EDTA) and incubated for 8 hours. The solid was transferred to 35 mm Petri dishes with 400 µl NDS buffer (0.5 M EDTA, 1% N-laurylsarcosine, 2 mg/ml proteinase K, and 10 mM Tris HCl, pH 7.5) and incubated overnight at 50°C. NDS was then removed and the plugs were dialyzed 4 times (1 hour each) in 2 to 4 ml EDTA/Tris solution.

#### xii. Analysis of AGM-1470 by HPLC

Samples were analysed by HPLC using a Thermo Separations Products SpectaSYSTEM liquid chromatograph equipped with a P4000 pump system. AS3000 autosampler (maintained at 20°C) and a UV3000 photodiode array detector. Reversed-phase chromatography was performed with a DeltaPak C18 150 x 2.1 mm 5 µm column (waters), and elution was performed at 0.5 ml/min with 2 mM ammonium acetate (pH 5.0 buffer) and a linear gradient (25 min) of acetonitrile from 5% to 30%, followed by an increase to 50% of acetonitrile for 10 more minutes, and maintained at 50% between 35 and 40 min, followed by a decline to 5% of acetonitrile between 40 and 50 min. The column was allowed to equilibrate for 5 minutes before the next injection. Absorbance was monitored at 193 nm and 50 µl of sample was injected each time.

#### xiii. [<sup>3</sup>H]Thymidine incorporation

The effects of AGM-1470 on the proliferation of HUVEC were evaluated by [<sup>3</sup>H]thymidine incorporation into the DNA, which occurs during S phase of the cell cycle.

After 24 - 48 hours of culture, subconfluent cultures of HUVEC were trypsinized, washed, and 2500 - 3000 cells per well were plated in 96-well microtiter plates precoated with Attachment Factor at 37°C for 30 min and rinsed with medium. Following drug incubation as outlined in the figure legends, the cells were incubated with 2.5 µCi of [methyl-<sup>3</sup>H]thymidine (specific activity, 5 Ci/mmol) for 8 or 16 hours at 37°C and then harvested onto filters, dried overnight at room temperature for 2 hours at 37°C, and counted using a liquid scintillation counter (Wallac). Each experimental condition was performed in triplicate, and incorporated radioactivity was expressed in cpm as a percentage relative to cells not treated with drug but incubated with [<sup>3</sup>H]thymidine for the same period. Results shown are the mean +/- the standard deviation of triplicate samples.

#### 4. Microscopy

##### i. Fluorescence microscopy for chromosome morphology

Attached cells were removed following trypsinization and mixed with the floating cells following centrifugation of the medium and fixed with 3.7% formaldehyde for 10 min on ice. The fixed cells were pelleted by centrifugation at 800 x g for 5 min and resuspended in PBS containing 1 µg/ml of the fluorescent DNA stain Hoechst 33258, or bisbenzamide. Following 2 - 5 min staining, the cells were pelleted again and resuspended in 10 µl Hanker Yates reagent containing 90% glycerol. The cells were mounted on glass slides and viewed through a Zeiss Standard microscope equipped with an epifluorescence unit. Photographs were taken on 35 mm Kodak Technical Pan #2415 film at 100 ASA. Chromosome condensation was assessed by counting the percentage of cells in mitosis. Slides were coded and scrambled so that mitotic cells can be counted blindly, and 150 - 300 cells were counted in different fields in each slide.

##### ii. Lamin immunocytochemistry

Rabbit antiserum against BHK Lamins A, B and C (provided by Dr. R. Hancock, Laval University Cancer Research Center, Hotel Dieu Hospital, Quebec) was used at a dilution of 1:1000. Cells were grown on polylysine-coated coverslips and

immunocytochemistry was performed using FITC-conjugated goat anti-rabbit IgG secondary antibody (Tago Inc., Burlingame, CA) with the microscopy equipment described above. The cells were also counterstained with Hoechst 33258 for visualization of the chromosomes.

## 5. Neutrophil activation

### i. Chemiluminescence

Chemiluminescence studies were performed at a cell concentration of  $5 \times 10^6$  cells/ml in HBSS with plasma-opsonized CPPD crystals (50 mg/ml), fMLP (1  $\mu$ M) or PMA (0.5  $\mu$ M). In all experiments 0.5 ml of cells were added to 25 mg of CPPD, 5  $\mu$ l of 1 mM fMLP or 2.5  $\mu$ l of 1 mM PMA in 1.5-ml capped Eppendorf tubes. To the tubes were added 10  $\mu$ l of luminol dissolved in 25% DMSO in HBSS to give a final concentration of 1 mM, and the samples were mixed to initiate neutrophil activation by one of the three agonists indicated. Chemiluminescence was monitored using the LKB Luminometer (Model 1250) at 37°C, with shaking immediately prior to measurements to resuspend the crystals. Control tubes contained cells, AGM-1470 (5  $\mu$ M), and luminol (agonists absent).

### ii Superoxide anion generation

Superoxide anion concentrations were measured using the superoxide dismutase inhibitable reduction of cytochrome c assay (Bobior and Cohen, 1983). Twenty five mg of plasma-opsonized CPPD crystals (50 mg/ml), fMLP (1  $\mu$ M) or PMA (0.5  $\mu$ M) were added to 0.5 ml of cells at 37°C and ferricytochrome c (horse heart, type 3, Sigma) (final concentration 1.2 mg/ml) and the cells were activated by shaking the capped tubes. At appropriate times (described in the figures) tubes were centrifuged at 10,000 x g for 10 sec and the supernatant was collected for spectrophotometric analysis at an average of 550 nm. Readings were taken at 540, 550 and 560 nm, and the peak height was calculated by subtracting the average of the 540 and 560 nm readings from the reading at 550 nm. This procedure corrects for baseline differences between different samples. The change in absorbance resulting from the reduction of cytochrome c was calculated by subtracting a

blank value from the corresponding incubated sample value. Control tubes were set up under the same conditions with the inclusion of superoxide dismutase at 600 units/ml. The measure of  $O_2^-$  generated in the whole reaction is calculated from the following formula :

$$O_2^- \text{ (nmol)} = \text{ABS}_{550(\text{mean; O.D.})} \times 0.5 \text{ ml (incubation mixture volume (ml))} \times 47.4$$

CHAPTER 3

INHIBITION OF THE G2/M CHECKPOINT AND INDUCTION  
OF MITOTIC ACCELERATION THROUGH S AND G2 PHASES  
AS A RESULT OF FOSTRIECIN TREATMENT

## RESULTS

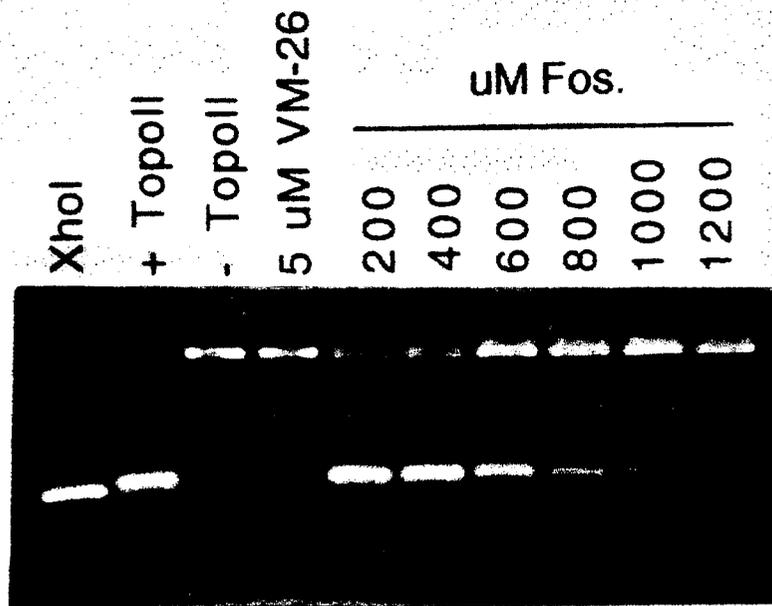
### *Fostriecin induces chromosome condensation in a concentration dependent manner*

At the onset of mitosis, the nucleus begins to re-arrange in preparation for the separation of sister chromatids. The most obvious feature of this cellular transformation is the condensation of interphase chromatin into metaphase chromosomes. The processes of chromosome condensation remain to be elucidated at the molecular level. In various systems, including mammalian cells, chromosome condensation has been correlated with the increase of histone H1 phosphorylation through G2 phase of the cell cycle, reaching hyperphosphorylation at metaphase (Bradbury *et al.*, 1972, 1974; Gurley *et al.*, 1978; Franks, 1983). Antitumour drugs that target topoisomerases induce a G2 phase arrest, probably as a result of the induced DNA lesions, and hence do not undergo the mitotic morphological changes (Liu, 1990). Fostriecin is an antitumour drug that inhibits topoisomerase II *in vitro*, without inducing DNA breaks (Boritzki *et al.*, 1988). My initial goal was therefore to determine if fostriecin induces a G2 phase block, and elucidate the mechanism of this block with the intention of gaining further insight into the mechanism of mitotic induction, in comparison to that of therapeutic G2 checkpoint inhibitors.

To determine if any effects on the cell cycle resulting from fostriecin incubation in cycling cells were due to an anti-topoisomerase II inhibitory effect of the drug, the effects of fostriecin at various concentrations on the activity of topoisomerase II were examined. Inhibition of topoisomerase II was assessed by monitoring the ability of purified topoisomerase II to resolve decatenated (knotted) kinetoplast DNA which was observed in an agarose gel as a band migration from the well (Figure 1). DNA that was catenated remained in the well (well 3; DNA without topoisomerase II), whereas resolved DNA resulting from the presence of restriction endonuclease or topoisomerase II migrated (wells 1 and 2; DNA cleaved by endonuclease XhoI, and the presence to topoisomerase II, respectively). Lane 4 demonstrates the effect of the topoisomerase II inhibitor, VM-26, used here as a positive control, which inhibits topoisomerase II by stabilizing the DNA-topoisomerase II cleavable complex (Minocha and Long, 1984; Ross *et al.*, 1984). No kinetoplast DNA was observed to migrate in the presence of VM-26 (lane 4), whereas

topoisomerase II was effective in resolving the catenated DNA in the presence of fostriecin up to 1 mM. A limited inhibition of topoisomerase II was observed at 400  $\mu$ M (lane 6). These results were reproduced with similar observations on at least four different occasions (not shown here). Morphological and biochemical effects of fostriecin throughout this study were conducted typically with 125  $\mu$ M, but never exceeding 375  $\mu$ M. Therefore, the effects that were observed throughout this chapter most likely were not the result of inhibition of topoisomerase II.

The onset of mitosis is normally characterized morphologically by the rounding up of cells, chromosome condensation, and nuclear lamina breakdown. We examined the effects of fostriecin (NSC-339638, ICRF-193, CI-920) on entry into mitosis in asynchronously growing Baby Hamster Kidney (BHK-21) cells for up to 2 hours. To prevent cells from exiting mitosis, we also added 0.5  $\mu$ g/ml colcemid, an inhibitor of microtubule polymerization (Rieder and Palazzo, 1992). Following treatment, cells were trypsinized, fixed for microscopic examination and stained with the fluorescent DNA dye *bisbenzamide*. Fostriecin treated cells were observed to round up and detach from the culture plate within 30 min of treatment, with at least 80% rounding up following 2 hours of fostriecin treatment at 125  $\mu$ M. During drug treatment (125  $\mu$ M), chromosome condensation appeared normal after shorter periods of incubation (< 2 hour) (Figure 2). Figure 3 shows that BHK cells treated with fostriecin and colcemid entered mitosis earlier than cells treated with colcemid alone, and this effect was dose-dependent, with half-maximal at a concentration of about 125  $\mu$ M. The basis for the mitotic characterization was solely based on the observed chromosome condensation and cell rounding. Cells were scored as mitotic should their chromosome morphology be similar to that illustrated in Figure 2, panels A and B. Panels C and D illustrate the morphology representative of PCC, and were scored as such, as outlined in the discussion below. This data implies that the onset of chromosome condensation was 'premature', and in fact, the induction of these mitotic criteria was accelerated. This was most apparent at 375  $\mu$ M where after 1 hour of treatment, the number of cells having undergone normal chromosome condensation was about 9-fold greater than those treated with colcemid alone. Following 2 hours of

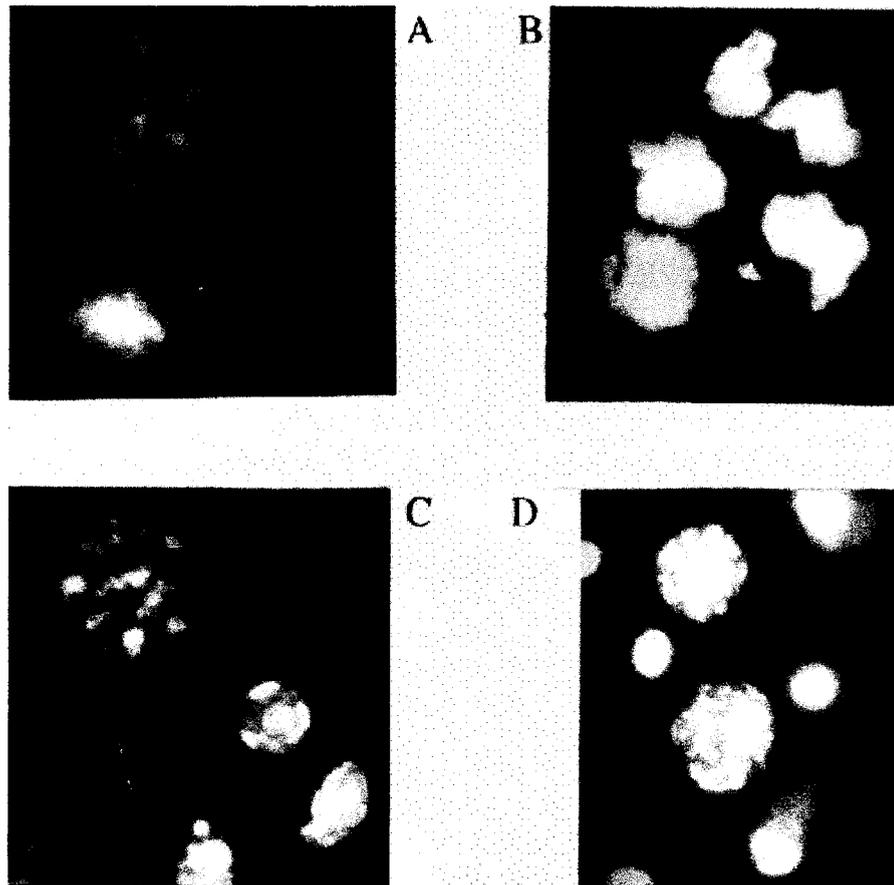


**Figure 1.** Inhibition of purified yeast topo II decatenation activity by fostriecin *in vitro*. Purified yeast topo II was incubated with kinetoplast DNA for 30 min at 37°C with or without drugs as indicated above the figure (fostriecin but not VM-26 treatment included yeast topo II). Catenated and decatenated DNA were separated by electrophoresis through 1% agarose. The upper band corresponds to catenated DNA, which remains in the well, and the lower band is decatenated DNA. The results are representative of four individual experiments.

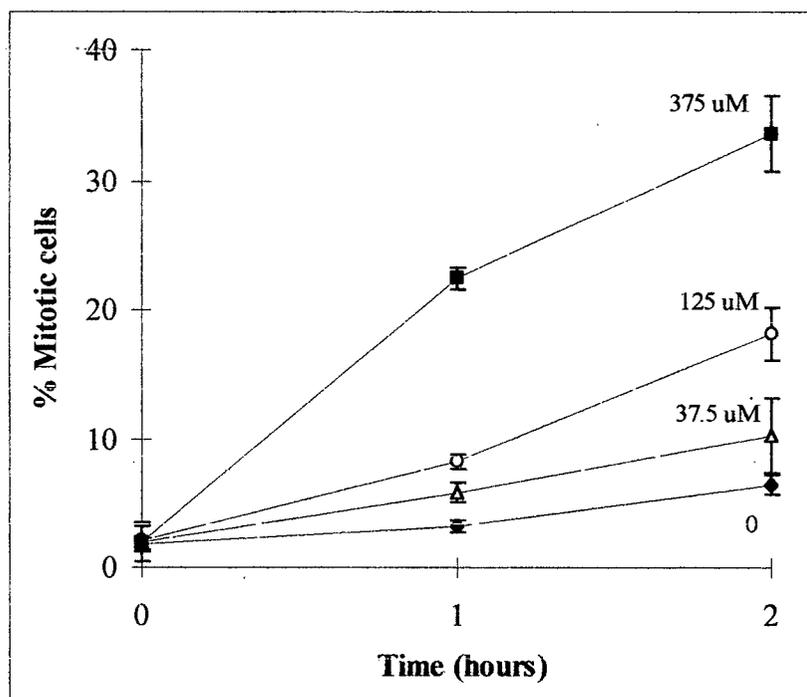
treatment, or higher fostriecin concentrations, the chromosomes exhibited a less-condensed morphology, similar to that observed in Figure 2B, and the degree of chromosome decondensation progressed as the period of incubation increased (Figure 2C - D). Cells incubated with or without colcemid for identical periods contained the same chromosome morphology as cells co-treated with colcemid and fostriecin. Cells treated with colcemid alone displayed similar chromosome morphology as untreated cells for at least 12 hours (discussed below, and illustrated in Figure 6). The degree of chromosome decondensation was not significant in cells treated in colcemid alone, but such chromosome morphologies were not scored as normal mitotic cells in the determination of the mitotic indices under any of the conditions analyzed.

It has been observed that okadaic acid (Sassa *et al.*, 1989; Dyban *et al.*, 1993), caffeine (Nishimoto *et al.*, 1978; Downes *et al.*, 1990) and 2-aminopurine (Andreassar and Margolis, 1992) also induce a premature mitosis. This work has predominantly been done with rodent cells, caffeine having been shown to induce mitotic events solely in rodent cells (Schlegel and Craig, 1991), without acceleration (Downes *et al.*, 1990). We therefore tested to see if similar effects also occur with simian COS, human leukaemia Jurkat and myeloid U937 cells treated with fostriecin. The mitotic indices of asynchronously growing cells treated with 375  $\mu$ M fostriecin and colcemid for 2 hours were compared to those treated with colcemid alone (Figure 4). In both cases, at least a 6-fold increase in cells having a normal to slightly decondensed mitotic chromosome morphology occurred upon treatment (Figure 2B). The acceleration of entry into mitosis of cells treated with fostriecin was therefore not limited to rodent cell lines.

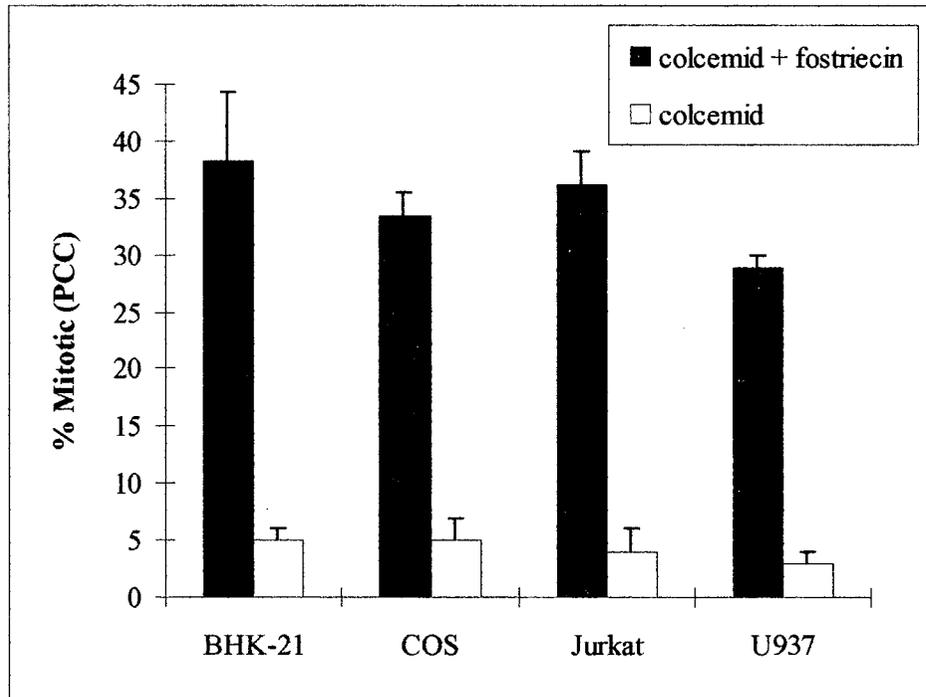
I next examined the long term effects of fostriecin treatment on asynchronously growing BHK-21 cells (Figures 5 and 6). Cells were treated with fostriecin for up to 12 hours and stained for fluorescent microscopy of the DNA. Slides were scrambled to ensure lack of bias in morphological determination. At least 250 cells were scored for each time point. Following approximately 3 hours of treatment, chromatin morphologies were marked by a mitotic catastrophe phenotype (Heald *et al.*, 1993) with condensed aggregated chromatin (Figure 5A). This fragmented and often pulverized appearance was identical to that seen when S phase cells are forced to undergo premature chromosome condensation



**Figure 2.** Morphology of fostriecin-induced premature chromosome condensation (PCC) in asynchronously growing BHK-21 cells. Colcemid was also added to all cultures at time 0. Following treatment, cells were collected, fixed and stained with bisbenzamide for fluorescent microscopy. Distribution of chromosome morphology varied depending upon the period of fostriecin incubation and was characterized as belonging to one of four different categories. (A) Chromosome condensation indistinguishable from that of cells treated with colcemid alone, with nuclear lamina depolymerization, 0 - 1.5 hr treatment. The chromosome morphology in this panel is representative of the chromatin morphology of BHK-21 cells incubated in the presence of colcemid for 12 hr. (B) Slightly decondensed, but readily recognizable chromosomes, 2 - 2.5 hr treatment. (C) Structures resembling very decondensed chromosomes, usually occurring in clumps, 2.5 - 3 hr treatment. (D) Structures not recognizable as chromosomes but displaying a higher degree of condensation than interphase chromatin, and with rough edges, > 4 hr treatment (6 hr). Panel A is indicative of normal chromosome condensation, whereas Panels C and D illustrates chromosome decondensation and is also similar to premature chromosome condensation. Also, observe the micronucleation, or apoptotic bodies, that is shown at the bottom of panel C.

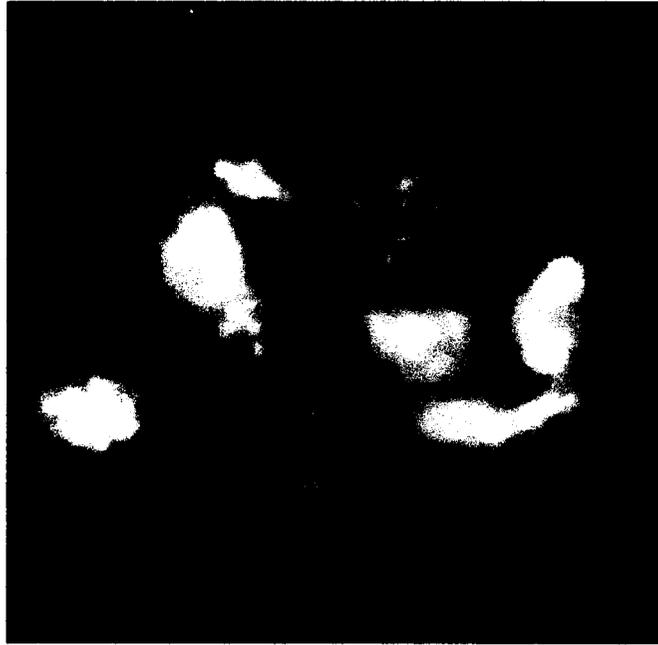


**Figure 3.** Acceleration of entry into mitosis by different concentrations of fostriecin. BHK21 cells were grown as monolayers in DMEM supplemented with 7.5% fetal bovine serum and antibiotics at 37°C in humidified 10% CO<sub>2</sub>. Different concentrations of fostriecin were added to cell cultures at time 0 together with 0.5 µg/ml colcemid. Floating and attached cells were collected, fixed, stained with the fluorescent DNA dye bisbenzimidazole and examined under the light microscope. The slides were coded and scrambled so that the number of mitotic cells and their morphological characteristics were determined without knowledge of the treatment. The percentage of mitotic cells was plotted against the time after addition of fostriecin. Error bars represent the S.D. of n=3 experiments.

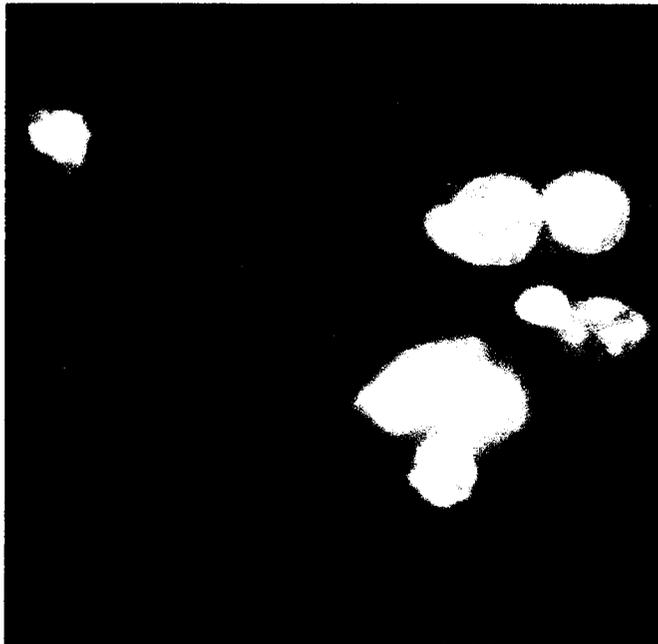


**Figure 4.** Fostriecin causes BHK-21, simian and human cancer asynchronously growing cells to accelerate into mitosis. The percentage of cells showing chromosome condensation after 2 hours treatment without (white bars) or with 375  $\mu$ M fostriecin (black bars) is shown. Error bars represent the S .D. of n=3 experiments.

**A.**

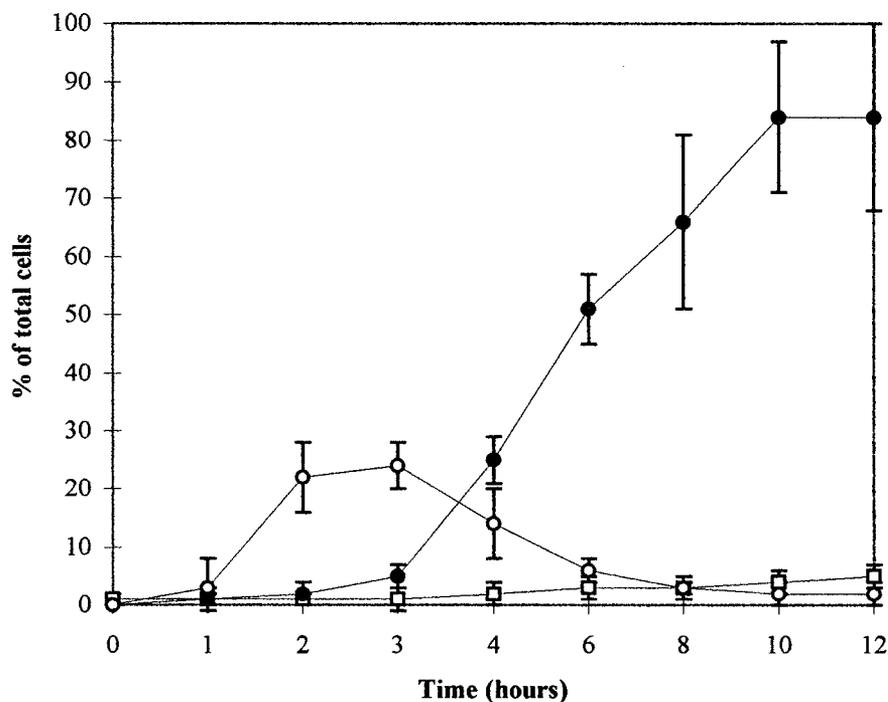


**B.**



**Figure 5.** Fostriecin treatment for extended periods of incubation in BHK-21 cells causes chromatin morphologies marked by a mitotic catastrophe phenotype. Cells treated for 3 hours (A) showed condensed aggregated chromatin that progressed to a micronucleated chromatin morphology following 6 hour treatment (B). Cells were treated similarly to those in Figure 2 for microscopy.

by fusion with mitotic cells (Johnson and Rao, 1970; Mullinger and Johnson, 1983). In many cases, fostriecin induced the formation of multinuclear cells following 8 hours, as has been observed with treatment of okadaic acid (Ghosh *et al.*, 1992). Further, cell death was imminent whether or not fostriecin was washed from the cells (not shown; based on trypan blue exclusion), and was probably due to mitotic catastrophe. Following 6 hours of fostriecin treatment, a substantial increase in micronucleated cells was observed (Figure 5B and 6). From these results alone, it could not be discerned whether cells were accelerated through the cell cycle, namely, that cells rapidly progressed from S to G2 followed by the G2 cells undergoing premature chromosome condensation and subsequent decondensation and fragmentation, or whether condensation was induced from other phases (addressed later) with a temporal lag due to accumulation of necessary mitotic factors (Nishimoto *et al.*, 1981; Yamashita *et al.*, 1985; Steinman *et al.*, 1991). I observed that cells synchronized to G1 via either prolonged incubation in isoleucine-free media, or serum deprivation, did not obtain abnormal chromosomal phenotypes (not shown).

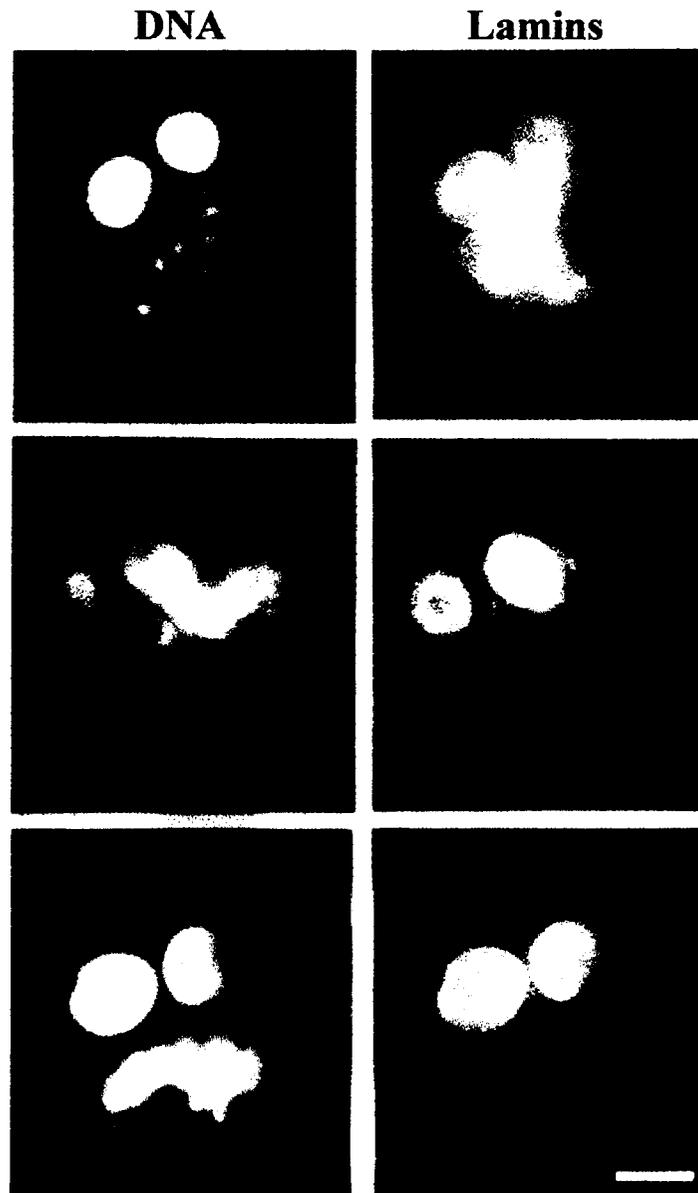


**Figure 6.** Fostriecin treatment induces a micronucleated morphology subsequent to PCC following prolonged drug treatment. Asynchronously growing BHK-21 cells were treated with 0.5  $\mu\text{g}/\text{ml}$  colcemid for 2 hours followed by 125  $\mu\text{M}$  fostriecin for 12 hours. The percentage of cells having micronucleated morphology subsequent to 2 hours colcemid pretreatment followed with fostriecin (●), without fostriecin (with colcemid; □) or having mitotic PCC following colcemid pretreatment with fostriecin (○) was determined. Colcemid was maintained during the entire incubation periods to block cells at mitosis. Cells were fixed and stained for DNA with bisbenzamide at the times shown, and at least 250 cells were counted at each time point (representing the 'total cell' population addressed on the ordinate axis. The values represent the mean  $\pm$  the S.D. of  $n=3$  experiments.

Fostriecin causes nuclear lamina depolymerization in BHK 21 cells and overcomes a G2 block in BHK-21 cells arrested with camptothecin or VM-26

At mitosis, the onset of chromosome condensation is accompanied by the depolymerization of the nuclear lamina, and nuclear envelope breakdown at prometaphase (Roberge *et al.*, 1990). To observe whether this occurred following fostriecin treatment, asynchronously growing BHK 21 cells were incubated for 1.5, 2 and 2.5 hours with 125  $\mu$ M fostriecin and subsequently collected and fixed. Lamins were detected by indirect immunofluorescence using FITC-conjugated anti rabbit IgG secondary antibody following preincubation with an antibody made against the lamin protein. Cells were also stained with *bisbenzimidazole*. Immunolocalization of nuclear lamins (Figure 7) showed that fostriecin also caused lamina depolymerization. Depolymerization and dissolution was observed in cases of both induced normally condensed (Figure 7, top, and Figure 2, panel A), and partially decondensed chromosomes (Figure 7, centre panel, and Figure 2, panel B), and fragmented chromatin (Figure 7, lower panel, and Figure 2, panels C and D). Given that the chromosome condensation appeared normal during early periods of fostriecin incubation, (followed by abnormal decondensation and fragmentation later during the treatment), and that nuclear lamina depolymerized normally, entry into a morphological state resembling mitosis triggered by fostriecin, therefore appeared normal but transient.

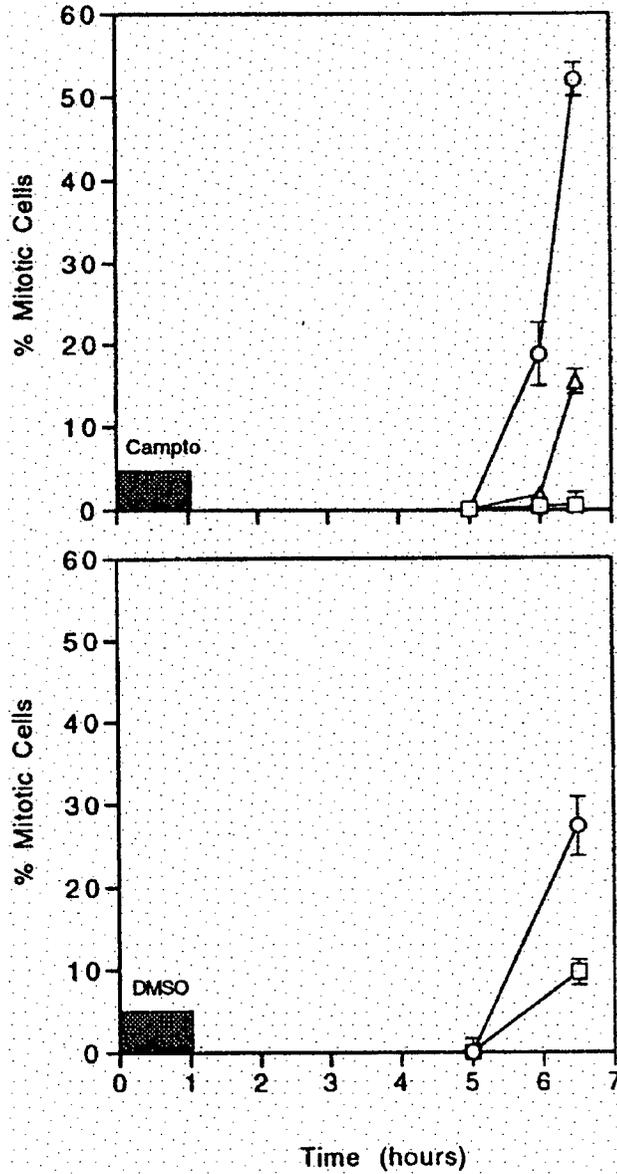
I have shown that the initial morphological effect of fostriecin was that of a normal mitotic one, indicating a rapid progression from the late G2/M phase checkpoint block. I therefore tested to see if fostriecin overcomes a G2 block imposed by the topoisomerase I and II inhibitors camptothecin and VM-26, respectively, which have been shown to impair the G2 to M transition in cycling cells (Liu, 1989; Wright and Schatten, 1990). VM-26 halts cell cycle progression prior to the step involving condensation of chromatin in prophase (Del Bino *et al.*, 1991) by inducing DNA lesions (Hartwell and Weinert, 1989). It has been shown that short (1 hour) incubation of S phase cells with camptothecin results in G2 block (Tsao *et al.*, 1992). This was confirmed by exposing BHK-21 cells synchronized in S phase using a double block method (described in 'Experimental Methods') to 0.5  $\mu$ g/ml camptothecin for 1 hour followed by incubation in camptothecin-free medium resulting in complete block of entry into mitosis between 5 and 6.5 hours after onset of



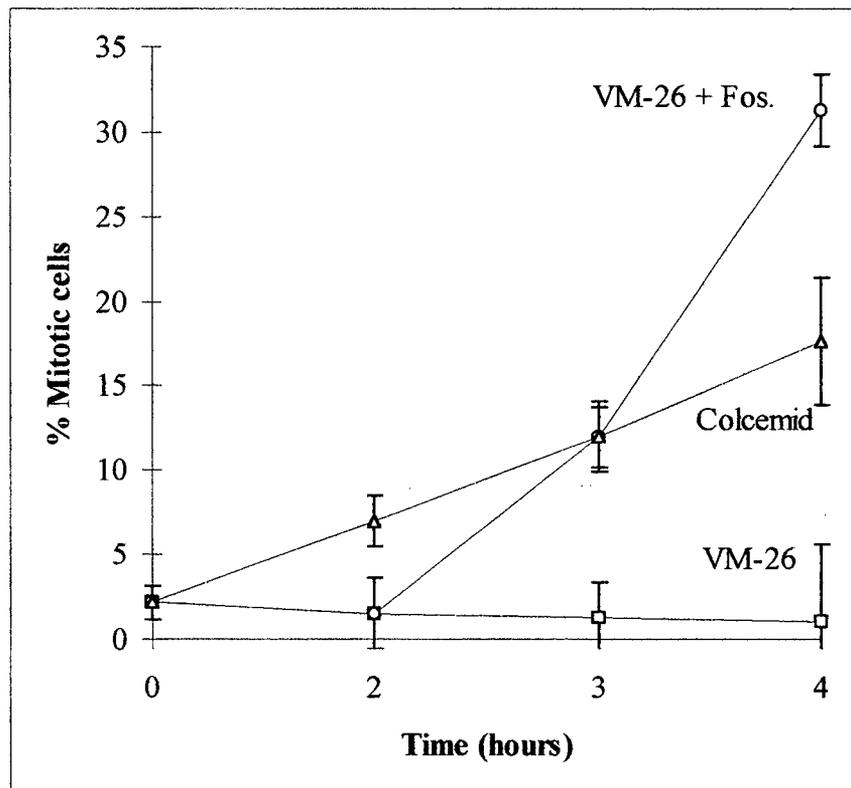
**Figure 7.** Chromosome condensation and nuclear lamina depolymerization in fostriecin treated asynchronously growing BHK-21 cells. Fixed cells were stained for DNA with bisbenzamide (left image) and indirect immunofluorescence of lamins (right). Cells were treated from top to bottom for 1.5, 2.0 or 2.5 hours with 375  $\mu$ M fostriecin. The morphology of chromosomes and the nuclear lamina in cells not treated with drug was similar to those illustrated above treated for 1.5 hours. Bar, 15  $\mu$ M.

treatment (Figure 8). Addition of fostriecin following 5 hours of colcemid treatment caused a large proportion of the cells to undergo mitotic-like chromosome condensation 1.5 hours later, compared to cells treated with colcemid alone, with a greater mitotic index at the higher concentration. The morphology of the mitotic cells was similar to that of cycling cells treated with fostriecin.

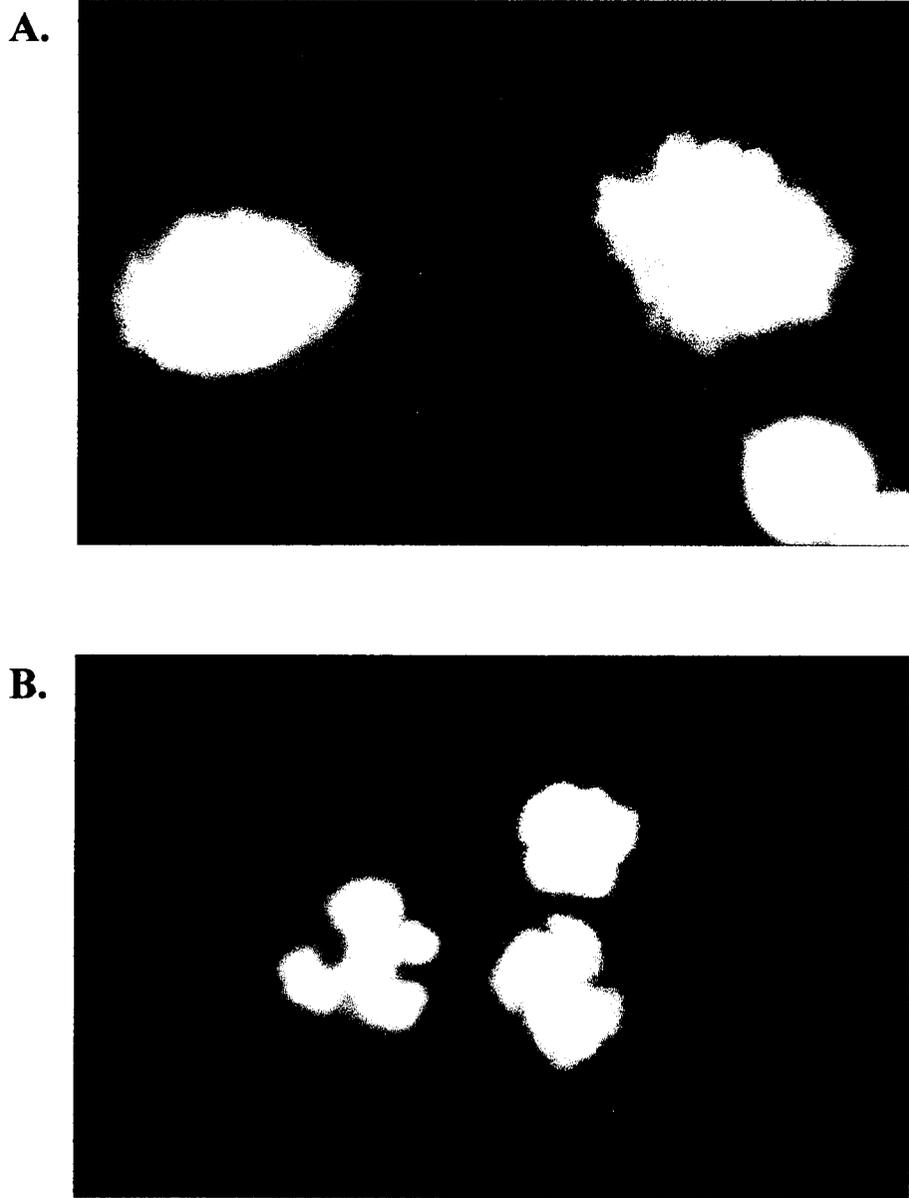
Previous studies in other laboratories have shown that a variety of anticancer drugs induced internucleosomal DNA fragmentation in cell culture. These compounds include lovastatin, cisplatin, camptothecin, mAMSA and Tenoposide (VM-26) (Roberge *et al.*, 1990; Hotz *et al.*, 1992; Borner *et al.*, 1995), and it has been demonstrated that they inhibit progression of cells through the cell cycle. Investigations undertaken in Darzynkiewicz's laboratory at approximately the same time as the work presented here, showed that MOLT-4 and HL-60 cells exposed to fostriecin at concentrations exceeding 5  $\mu$ M for 6 hours were blocked in the cell cycle at S phase with significant DNA degradation that was consistent with apoptosis as determined by flow cytometry and DNA laddering (Hotz *et al.*, 1992). Additionally, they observed that exposure of cycling cells to fostriecin (6 hours) made them insensitive to the DNA degradation effects otherwise induced by VM-26. My earlier results demonstrated that at periods earlier than 3 hours of fostriecin incubation, induction of cell cycle acceleration into a mitotic-like state with normally condensed chromosomes occurred. Cells treated with VM-26 and fostriecin were also observed to progress to the apparent M phase with reduced DNA decondensation and fragmentation triggered by VM-26. Figure 9 illustrates that the addition of 25  $\mu$ M VM-26 to asynchronously growing BHK-21 cells caused a reduction in cells containing condensed chromatin following 2 and 4 hour treatment as compared cells treated with colcemid alone. The addition of 375  $\mu$ M fostriecin to cells exposed to VM-26 also reversed the G2 block and induced the cells to escape the G2 phase block. Following 2 hours of fostriecin incubation, the percentage of cells having normally condensed chromosomes resembling mitosis was even greater than those cells treated with colcemid alone, showing again that fostriecin treated cells are accelerated into a mitotic state based on chromosome morphology. The combined treatment resulted in a chromosome morphology that was less



**Figure 8.** Inhibition of camptothecin-induced  $G_2$  arrest by fostriecin. The percentage of mitotic cells was plotted against time after 1 h treatment with DMSO (lower panel) or camptothecin (top panel). At 5 hours, 0.5  $\mu\text{g/ml}$  colcemid was added together with ascorbic acid (squares - Control), 125  $\mu\text{M}$  fostriecin (triangles), or 375  $\mu\text{M}$  fostriecin (circles). Error bars represent the S.D. of  $n=3$  experiments.



**Figure 9.** VM-26 treatment of asynchronously dividing BHK-21 cells induces G2 arrest which is overcome by fostriecin. At time 0, cells were treated with 0.5 ug/ml colcemid or 0.5 ug/ml colcemid and 25 uM VM-26. At 2 hours, fostriecin (125 uM) was added to cultures previously treated with VM-26 and colcemid. The percentage of mitotic cells was plotted against time, as determined in Figure 2. Cells treated with VM-26 showed no accumulation of mitotic cells. Those cells blocked at the G2/M boundary with VM-26, when treated with fostriecin for 2 hours showed a 31 % increase in mitotic cells. This should be compared to an 11% accumulation over the same period with cells treated with colcemid alone, illustrating both an acceleration of mitosis as well as the ability of cells to overcome the G2 block induced by VM-26 when treated with fostriecin. Error bars represent the S.D. of n=4 experiments.

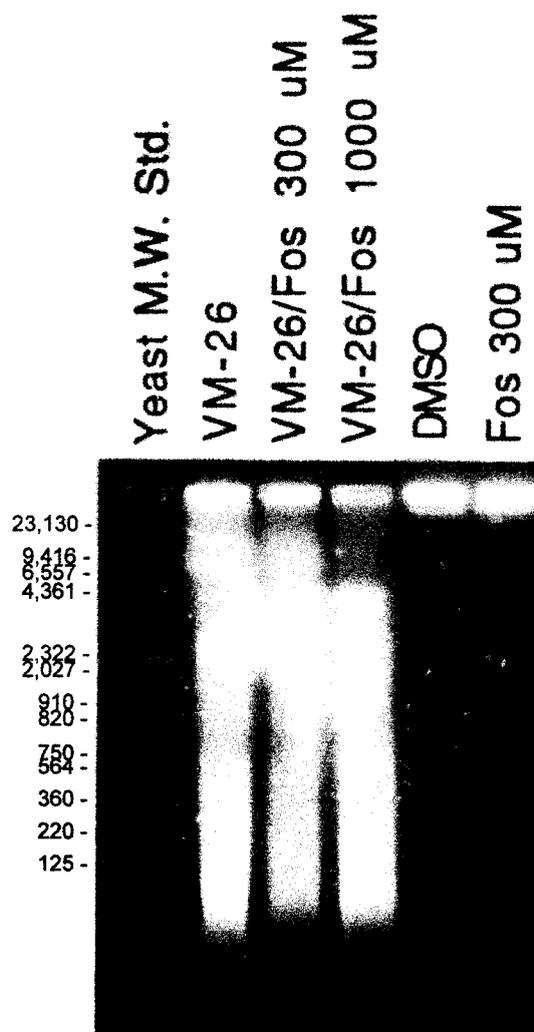


**Figure 10.** Combined treatment of BHK-21 cells with fostriecin (300  $\mu$ M) and VM-26 (5  $\mu$ M) for 2 hours (**A**) resulted in chromosome morphology that was less fragmented than cells treated with VM-26 alone (**B**). The cells were stained with bisbenzamide.

fragmented (Figure 10A) than cells treated with VM-26 alone (Figure 10B). The addition of fostriecin to cells exposed to VM-26 overcame the VM-26-associated G2 block and induced the cell to enter mitosis. Therefore, fostriecin can override the cell cycle arrest induced by camptothecin and VM-26. Additionally, I also observed nuclear apoptotic bodies, which I designated as micronucleated cells, in cells treated for extended periods of time, consistent with the findings of Hotz *et al.* (1992). I therefore sought to elucidate the effects of fostriecin on DNA degradation following shorter periods of drug treatment.

To determine whether DNA fragmentation occurred following treatment of BHK cells with various concentrations of fostriecin with and without VM-26, DNA isolated from these cells was subjected to pulsed-field gel electrophoresis (Figures 11 and 12). As described in other reports (Roberge *et al.*, 1990; Borner *et al.*, 1995), VM-26 induced DNA degradation of cycling cells (Figure 11, lane 2) whereas fostriecin-treated cells exhibited no DNA degradation (lane 6), which was observed in cells treated for 6 hours in Darzynkiewicz's laboratory (Hotz *et al.*, 1992). Further, cells treated with VM-26 and fostriecin simultaneously were observed to have triggered DNA degradation, but the staining of the DNA (lane 3) was less than that observed in cells treated with VM-26 alone. At earlier periods of incubation where DNA condensation and nuclear lamina breakdown occurred, DNA degradation was not triggered in cells following a 1.5 hour incubation with drug concentrations ranging from 50  $\mu$ M to 1 mM (Figure 12A). Figure 12B demonstrated that although 300  $\mu$ M fostriecin induced mitotic morphologies in cells treated with fostriecin for 2.0 hours without DNA degradation (lane 5), a higher concentration of drug induced a larger number of cells with to undergo chromosome condensation (lane 4) although DNA degradation was triggered. This concentration dependent effect was also observed in cells pretreated with VM-26, which usually results in an induction of a G2 block, where 1 mM fostriecin treated cells had a greater mitotic index (16.1%) compared to 300  $\mu$ M treated cells (6.2%). These results are consistent with the notion that fostriecin overcomes the G2/M phase checkpoint, inducing cells into a premature mitotic state that progresses to apoptosis following prolonged treatment. This effect was most likely not attributed to inhibition of topoisomerase II, but may have resulted from differential

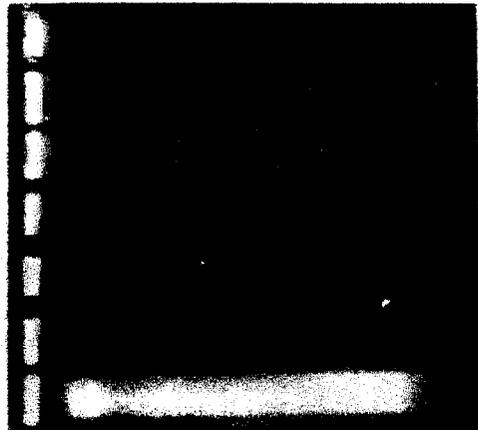
phosphorylation induced by fostriecin and induction of endonucleases later in the incubation period.



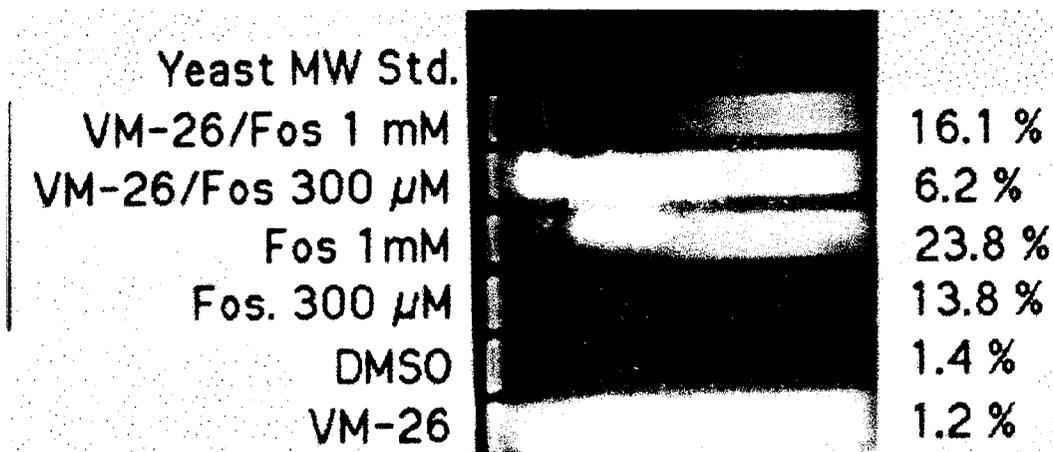
**Figure 11.** Pulsed-field agarose gel electrophoresis of DNA from BHK-21 cells treated with 25  $\mu$ M VM-26 without and with 100  $\mu$ M and 300  $\mu$ M fostriecin, demonstrating induction of DNA strand breaks. Cell treated with 0.5% DMSO or 300  $\mu$ M fostriecin alone showed no DNA degradation. Cells were treated with drug for 1 hour after which they were embedded in agarose, lysed and digested with proteinase K as described in 'Experimental Methods'. Agarose (1%) gel plugs inserted into loading wells are shown at the top of the gel. The molecular weight marker is *Saccharomyces cerevisiae* chromosomal DNA and Hind III digested lambda DNA with the range indicated in bp.

**A.**

Nocodazole 0.25  $\mu$ g/ml  
Fos. 1 mM  
Fos. 600  $\mu$ M  
Fos. 300  $\mu$ M  
Fos. 50  $\mu$ M  
DMSO  
VM-26 (25  $\mu$ M)



**B.**



**Figure 12.** (A) VM-26 (25  $\mu$ M) but not fostriecin induces DNA strand breaks in asynchronously growing BHK-21 cells. DNA from BHK-21 cells treated with fostriecin (50-1000  $\mu$ M) or VM-26 (25  $\mu$ M) for 1.5 hours or blocked at mitosis with nocodazole were subjected to Pulsed-field gel electrophoresis as described in Figure 8 and 'Experimental Methods'. (B) DNA from cells treated as illustrated in the Figure for 2 hours, were collected for pulsed-field agarose gel electrophoresis, or stained with bisbenzamide to determine chromosome morphology based on chromosome condensation, and shown as percentage of cells with condensed chromosomes to the right of the gel. The molecular weight standard is illustrated in Figure 11. Fostriecin treatment induced chromosome condensation in a concentration dependent fashion in this experiment, even in the presence of VM-26. DNA degradation was observed in all cells except those treated with fostriecin at 300  $\mu$ M.

### Fostriecin induces S phase premature chromosome condensation

The entry of cycling cells into mitosis requires them to bypass checkpoints at S and G2 phase, the G2/M phase interface being responsible for monitoring chromosome damage (Enoch and Nurse, 1991; Roberge, 1992). The S phase checkpoint acts to suppress the initiation of mitosis in the presence of unreplicated DNA. Both caffeine and the phosphatase inhibitor okadaic acid have been shown to be able to override BHK cells arrested at the start of S phase with hydroxyurea, a drug that inhibits DNA replication, and induce mitotic condensation even though the genome remains incompletely replicated (Downes, 1990). The resulting condensed nuclear structure is similar to that obtained with cell fusion. We have shown that fostriecin, like okadaic acid, inhibits phosphatases PP-2A and PP-1 (Roberge *et al.*, 1994). Further, we have already shown that following longer treatment with fostriecin (Figure 2), cells exhibited the fragmented chromosomes similar to the S phase premature chromosome condensation (S-PCC) induced with okadaic acid (Inomato *et al.*, 1995; Nuydens *et al.*, 1998). I therefore examined whether fostriecin can induce premature chromosome condensation in cells arrested in the cycle as a result of incomplete DNA replication.

BHK-21 cells were synchronized to the G1/S boundary with aphidicolin, an inhibitor of DNA polymerase. Measurement of DNA content showed that this method caused greater than 80% of the cells to accumulate in S phase, and 60% of the cells were released from S phase synchronously upon release from the aphidicolin block (Figure 13, bottom). When BHK 21 cells were released and treated immediately with fostriecin, up to 68% of the cells underwent S-PCC (Figure 14A). Similar results were attained with continuous treatment of aphidicolin except S-PCC was 20.9% with fostriecin versus 1.7% without fostriecin at 2 hours. The results are a mean of three experiments with a standard deviation not exceeding 2.8%. Similar experiments were performed with Jurkat, HL-60, U937 and CEM human cell lines, each displaying S-PCC (Figure 14B). Fostriecin caused nuclear lamina depolymerization under these conditions (not shown). These findings, and those previously observed with caffeine and okadaic acid, confirm that there is no strict dependence of mitosis on the completion of S phase, but rather, there exists a signal mechanism that couples these events which may be circumvented.

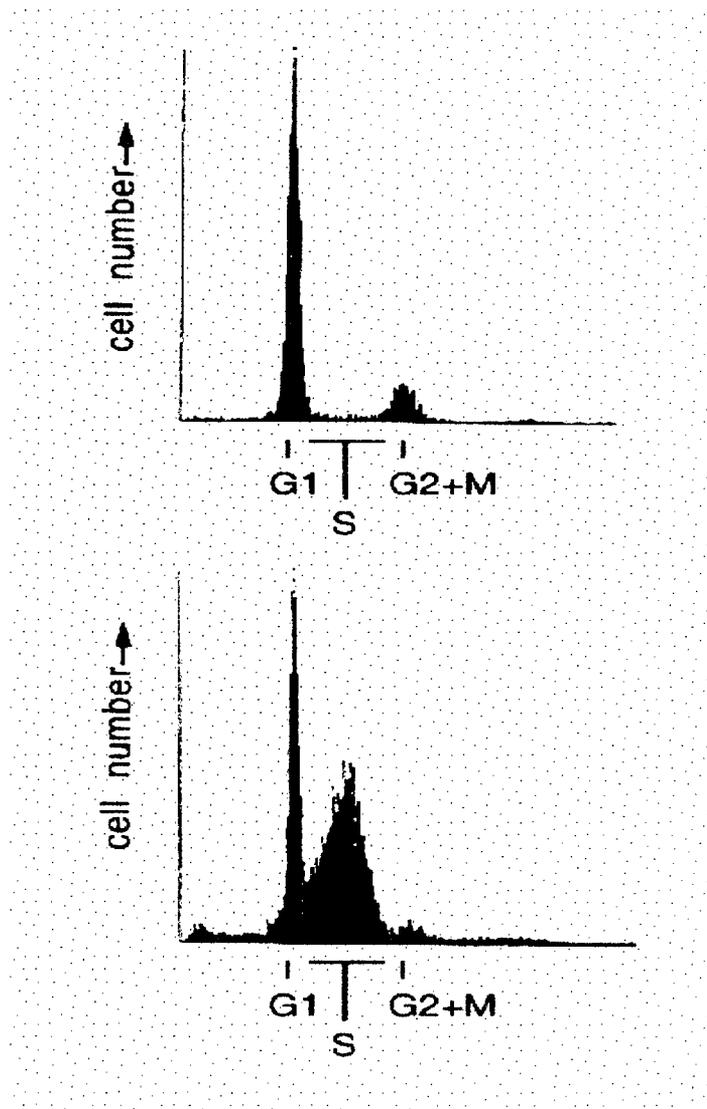
To determine if pulverized and micronucleated chromosomes result from prolonged exposure to fostriecin, which is common to other chemotherapeutic agents, BHK 21 and Jurkat cells synchronized and maintained in S phase with continuous incubation with aphidicolin, were treated with fostriecin. Cells were also released from the aphidicolin block and left untreated as a control. Figure 15 illustrates that the micronucleated and fragmented phenotypes occurred with prolonged treatment, similar to that observed with cells blocked in G2. The morphologies resulting from prolonged exposure support the notion that the mitotic catastrophes resulted from cells exiting S phase possessing incompletely replicated DNA. To ensure that the fostriecin-treated cells possessing S-PCC were S phase cells, and not cells having progressed first into G2 phase, the thymidine analog, 5-bromo-2-deoxyuridine (BrdU) was added to the synchronized BHK 21 cell cultures upon release from aphidicolin block to label cells undergoing DNA replication. After 20 min, the BrdU was washed out and the cells were put into fresh medium and colcemid with and without fostriecin for up to 2 hours. Individual cells were monitored for the incorporation of BrdU by immunofluorescence, and for S-PCC with the DNA dye *bisbenzimidide*. All the cells were stained with BrdU, demonstrating that cells did not undergo PCC from G1. Figure 16 illustrates that S phase cells undergo PCC when treated with fostriecin at a rate far exceeding those cells treated with colcemid alone. Similar results were obtained with cells treated further with aphidicolin at the onset of fostriecin treatment where as many as 68% of the BrdU positive cells had S-PCC (Table 1). Additionally, 100% of the cells that had undergone S-PCC incorporated BrdU. These results demonstrate without any ambiguity, that fostriecin also overcomes the cell cycle block imposed by the S phase checkpoint that monitors incomplete DNA replication.

I have determined that fostriecin overcomes cell cycle blocks imposed at S and G2 phases when the DNA is incompletely replicated or its integrity is not sufficient for progression into prophase. To determine if fostriecin affects cells in G1 phase and causes them to enter mitosis prematurely based on chromosome morphology, BHK cells were arrested by isoleucine deprivation and released into G1 by incubation in complete medium for 1 hour and then treated with 125  $\mu$ M fostriecin for 1.5 hours. This protocol has been used to consistently block cells in G1 phase prior to entry into S phase (Roberge et al.,

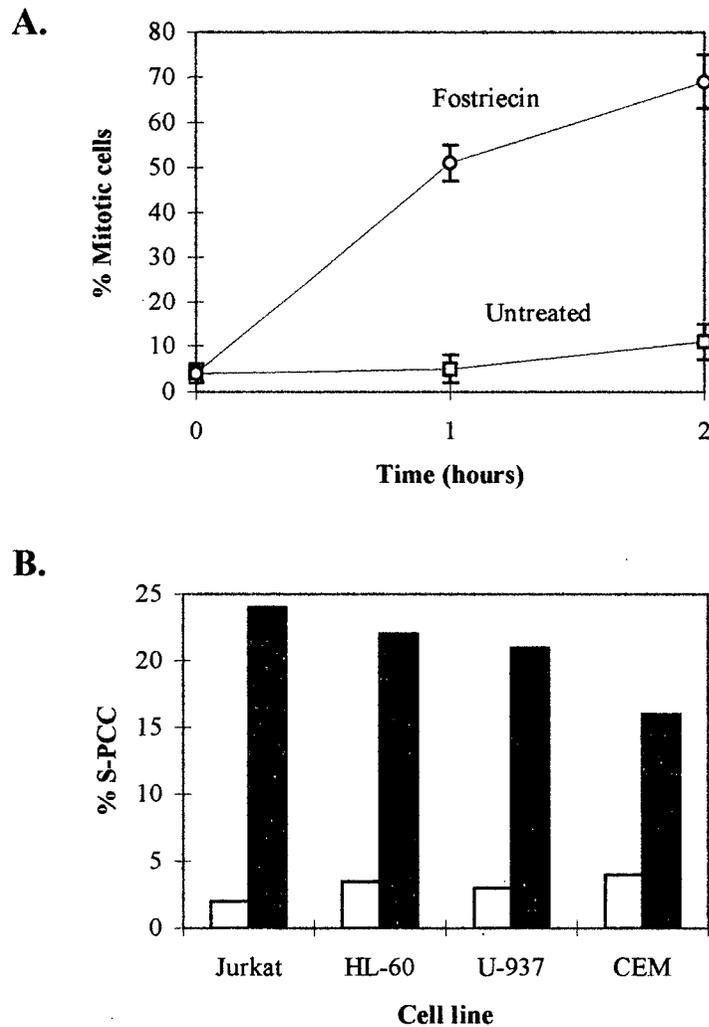
1992; Th'ng *et al.*, 1994). I observed that only 0.4% of the cells entered underwent chromosome condensation containing punctated chromatin during this period. These experiments were repeated in Dr. Bradbury's laboratory as part of a collaboration (communication with Dr. Bradbury; Th'ng *et al.*, 1995). Therefore, fostriecin affects the cell cycle only when cells have progressed beyond the initiation of S phase.

| Treatment                     | BrdU <sup>+</sup> cells (%) | BrdU <sup>+</sup> in S-PCC (%) | S-PCC cells that are BrdU <sup>+</sup> (%) |
|-------------------------------|-----------------------------|--------------------------------|--|
| ascorbic acid                 | 39                          | 0                              | -  |
| fostriecin                    | 40                          | 58                             | 100  |
| ascorbic acid and aphidicolin | 33                          | 0                              | -  |
| fostriecin and aphidicolin    | 41                          | 68                             | 100  |

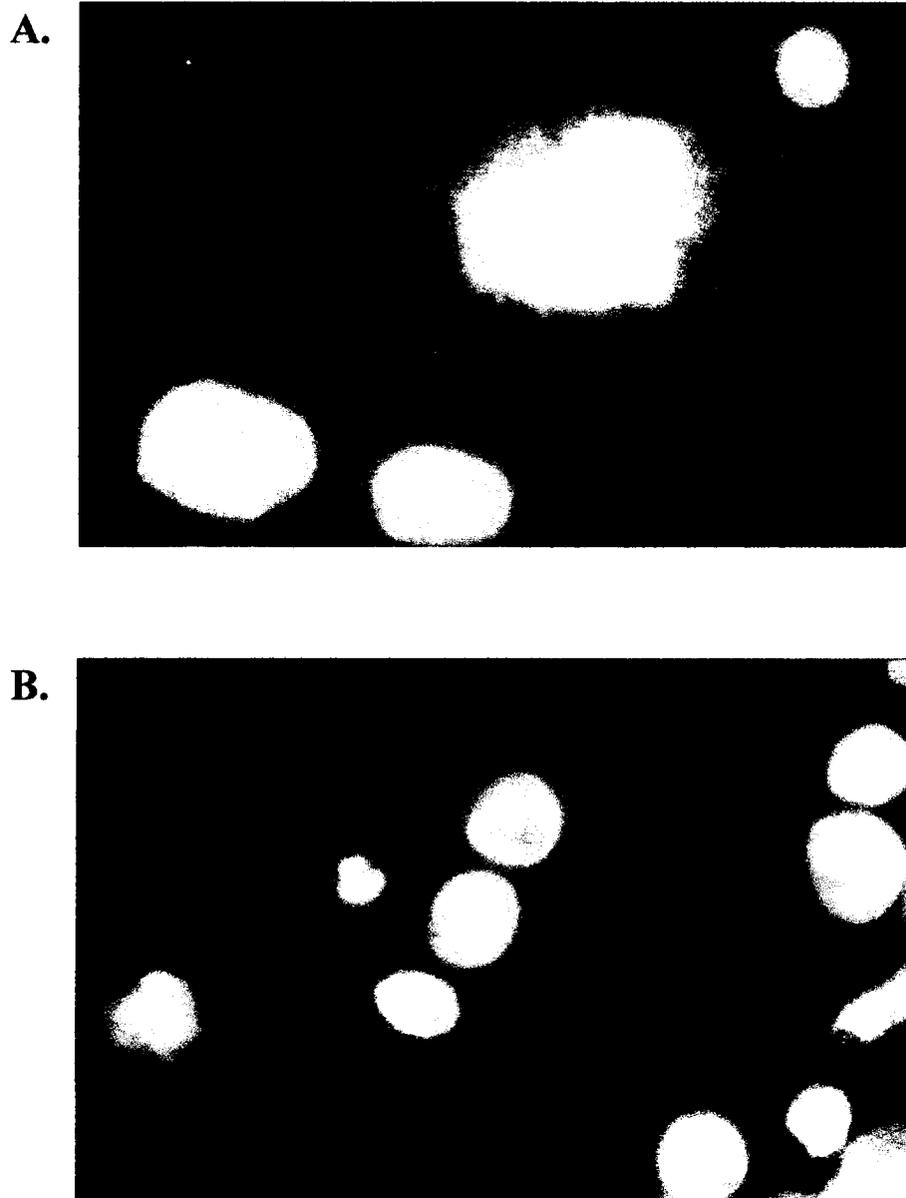
**Table 1.** Induction of S Phase PCC by fostriecin. To determine the percentage of cell containing PCC that were in S phase, 125  $\mu$ M Fostriecin was added for 2 hours to cells previously pulse-labeled for 20 min with the thymidine analog, 5-bromo-2-deoxyuridine (BrdU<sup>+</sup>). The percentage of BrdU<sup>+</sup>-labeled cells was determined by first localizing BrdU<sup>+</sup>-labeled cells by immunofluorescence with an FTIC-conjugated BrdU secondary antibody, and then determining the condensation state of their chromosomes with *bis*benzimidazole.



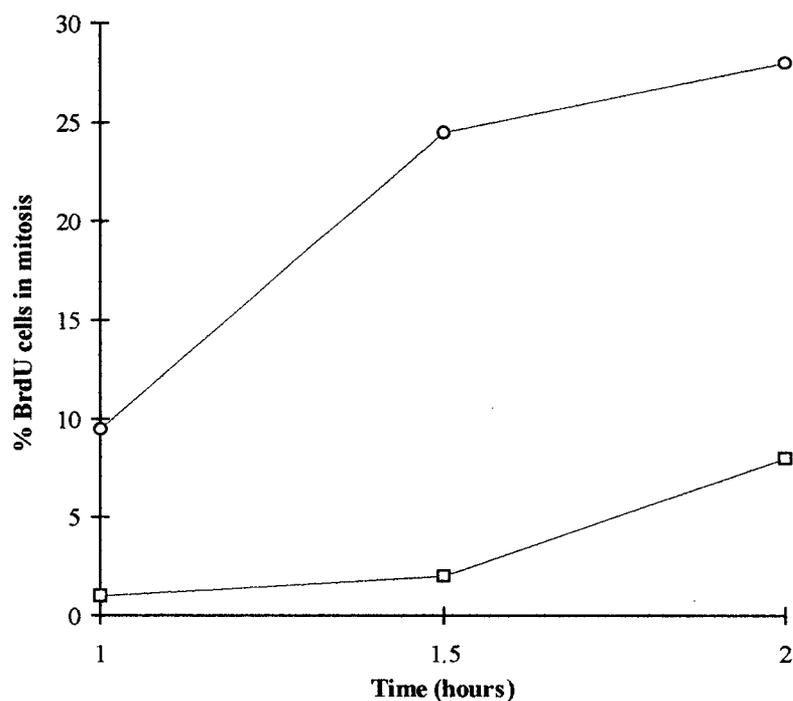
**Figure 13.** Cell cycle distribution determined by flow cytometric analysis of DNA content in asynchronously growing BHK-21 cells (upper plot) and cells synchronized in S phase with the DNA polymerase inhibitor aphidicolin (lower plot). Approximately 80% of the cells accumulated at the G1/S boundary in response to aphidicolin.



**Figure 14.** Inhibition of aphidicolin-induced cell cycle arrest by fostriecin. **(A)** BHK-21 cells were synchronized by treatment with DMEM containing 0.25% bovine serum for 24 hours, followed by incubation in complete culture medium containing 2.5  $\mu\text{g/ml}$  aphidicolin. At time 0, the medium was replaced with fresh medium with 375  $\mu\text{M}$  fostriecin (circles) or without (squares) and the percentage of mitotic cells was determined. **(B)** Fostriecin causes entry into mitosis of human cancer cells with incompletely replicated DNA (aphidicolin block). The percentages of cells showing S-PCC after the treatment without (white bars) or with 375  $\mu\text{M}$  fostriecin (black bars) is shown.



**Figure 15.** S phase premature chromosome condensation morphology of BHK-21 cells synchronized in S phase with aphidicolin and treated with 300  $\mu$ M fostriecin for 3 hours (A), and for 6 hours (B) where micronucleated and fragmented DNA phenotypes were exhibited. The cells were stained with bisbenzamide.



**Figure 16.** Acceleration of the rate of entry into mitosis of BrdU-labeled BHK cells by fostriecin. Fostriecin was added for the indicated time to cells previously pulse-labeled with BrdU. The percentage of BrdU-labeled cells in mitosis was determined by first localizing BrdU-labeled cells by immunofluorescence and then determining the condensation state of their chromosomes with bisbenzimidazole. (□) colcemid and ascorbic acid; (O) colcemid and fostriecin. The results are representative of two individual experiments.

Fostriecin -induced chromosome condensation does not require Cdk1 activity

Entry into mitosis requires the activation of Cdk1 (Norbury and Nurse, 1992). During the S and G2 phases of the cell cycle, Cdk1 is present in a potentially active form, but maintained inactive in part by checkpoint controls that monitor DNA replication and damage (Roberge, 1992; Murray, 1992). Cdk1 activation at the onset of mitosis requires there to be the complex between the p34<sup>cdc2</sup> protein and cyclin proteins (Pines and Hunter, 1991) and the dephosphorylation of critical amino acid residues in p34<sup>cdc2</sup> (Krek and Nigg, 1991; Norbury *et al.*, 1991). BHK-21 cells were synchronized in S phase by release from aphidicolin block and were treated with fostriecin. Cdk1 isolated from these cells following immunoprecipitation with the anti-Cdc2-CT antibody, showed a downward electrophoretic mobility shift characteristic of the dephosphorylated and activated form of Cdk1 (Figure 17A). Similarly, addition of fostriecin to cells blocked at G2 phase by VM-26 also caused dephosphorylation of p34<sup>cdc2</sup> (Figure 17A). Immunoblots of Cdk1 incubated with anti-phosphotyrosine antibody illustrated further that Cdk1 was completely dephosphorylated at the tyrosine in cells treated with drug compared to those not treated with drug (Figure 17B). Cdk1 dephosphorylation was accompanied by an increase in kinase activity as measured by the phosphorylation of histone H1 *in vitro* (Figure 17A), as well as an increase in the mitotic indices. Therefore, fostriecin caused a dephosphorylation of p34<sup>cdc2</sup> and moderate activation of Cdk1 in rodent cells. It was also observed in other experiments conducted under the same conditions as those illustrated in Figure 17A (Figure 17 is indicative of 4 experiments) that the level of Cdk1 in fostriecin treated cells was less than in cells not treated with the drug. Boritzki *et al.* (1988) had initially described fostriecin as a protein synthesis inhibitor at concentrations exceeding those used in these experiments. It is possible that fostriecin treatment resulted in partial repression of Cdk1 expression. Whether fostriecin was acting as a protein synthesis inhibitor was not addressed other than Western analysis. Cdk1 is normally constitutively expressed throughout the cell cycle (Clarke and Karsenti, 1991). Therefore, the partial repression of Cdk1 expression in response to fostriecin was not considered given the more significant observation in human cells that fostriecin treatment resulted in an abrogation of Cdk1

activity. It may be possible that fostriecin induced a degradation of Cdk1, but since this has not been reported in the literature, this hypothesis was not investigated.

It has so far been demonstrated that fostriecin causes a moderate increase in Cdk1 phosphotransferase activity in BHK-21 cells. To investigate further whether Cdk1 activity was necessary for the chromosome condensation induced following incubation with fostriecin and whether the Cdk1 pathway was affected by fostriecin, we examined the response to fostriecin in the mouse FT210 cell line that contained a temperature sensitive *cdc2* lesion. These cells, when grown at their restrictive temperature of 40°C versus the normal growth temperature of 32°C, lose their Cdk1 phosphotransferase activity and become arrested at G2 phase (Th'ng *et al.*, 1992). FT210 cells were released from an isoleucine free/aphidicolin G1/S double-block and incubated at 40°C for 18 hours. Under these conditions, cells arrest at G2 phase and have been shown to have greater than 90% of their Cdk1 inactivated (Th'ng *et al.*, 1992; Yamaguchi *et al.*, 1992). Western analysis of the Cdk1 and immunoprecipitated activity of cells arrested in G2 phase at their restrictive temperature, versus cells arrested at prometaphase grown at their normal temperature in the presence of colcemid, indicated that 0.2 % of the activity detected in colcemid M phase blocked cells was observed in the cells blocked at their non-permissive temperature (Figures 18 and 19). The upper panel in Figure 18 illustrates the Cdk1 immunoprecipitated histone H1 phosphotransferase activity determined in FT210 cells incubated at each temperature with 2 hour 375 µM fostriecin treatment (designated with a +) or without the drug. Controls without the histone H1 substrate and without the antibody in the immunoprecipitate were included. The lower panel represents the Western blot with anti-Cdc2-CT antibody of the same samples. Microscopic examination of cells stained with *bis*benzamide revealed that 40.9 % of the cells at the non-permissive temperature, treated with fostriecin, contained condensed chromosomes versus 3.9 % for the cells not treated with drug. Guo *et al.* (1995) repeated these experiments two years after the results presented here, and found that after 1 hour incubation with 100 µM fostriecin, as much as 60 % of the cells were observed to have condensed chromosomes. Additionally, I demonstrated that cells grown at their normal (permissive) temperature and treated with fostriecin showed 37 % having condensed chromosomes, versus 3.8 % in cells not treated

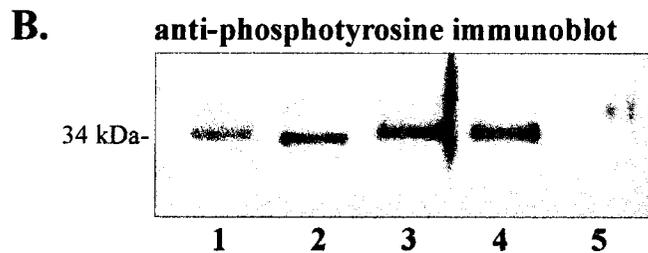
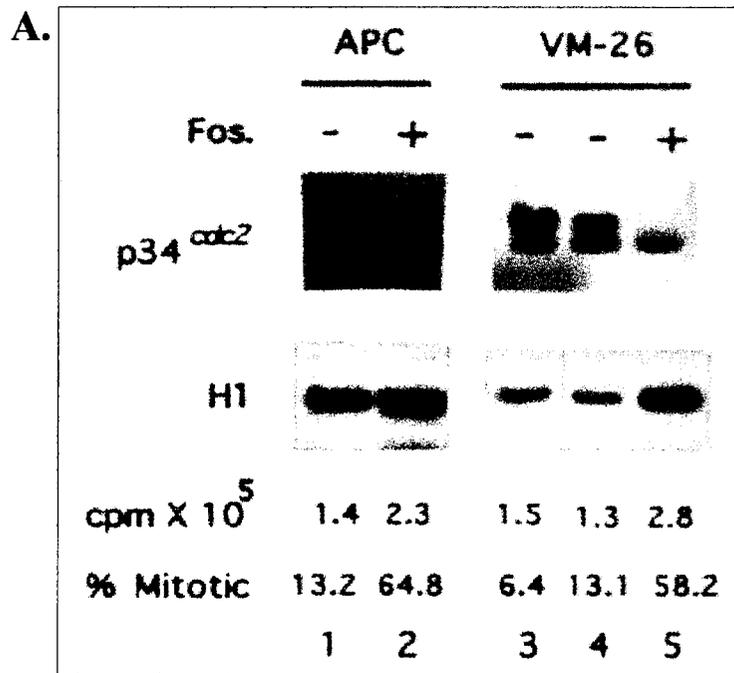
with drug (Figure 18A). The fostriecin-treated cells contained highly condensed chromosomes that were indistinguishable from those of cycling cells arrested with colcemid (Figure 7). Figure 18A illustrates that cells arrested at G2 at their non-permissive temperature exhibited almost undetectable amounts of Cdk1 protein as determined by Western analysis with antibody specific for the C-terminal domain of Cdk1, and immunoprecipitated Cdk1 phosphotransferase activity. When the immunoprecipitated protein was subjected to Cerenkov counting, a 500-fold decrease in Cdk1 phosphotransferase activity was detected in the cells arrested at their non-permissive temperature and treated with fostriecin versus cycling cells arrested with colcemid at the normal temperature, although at least a 10-fold increase in mitotic indices was observed with fostriecin treatment (Figure 18). No significant increase in the mitotic index was observed when the cells were maintained at 40°C in the absence of fostriecin. Also, the addition of fostriecin for up to 2 hours did not result in an increase in Cdk1 activity in either of the cell populations. Therefore, cells arrested in G2 at 40°C and treated with fostriecin had accumulated chromosome condensation similar to those treated with colcemid at the permissive temperature, although their Cdk1 phosphotransferase activity of the former was negligible.

The experiments presented thus far have illustrated a distinguishable increase in mitotic indices resulting from fostriecin treatment at approximately 1.5 hours post-treatment. Also, the experiment described in Figure 18 demonstrated a significant reduction of Cdk1 phosphotransferase activity following two hours fostriecin treatment. It is conceivable that a short, transient activation of Cdk1 occurred that was sufficient to induce premature chromosome condensation. To determine if PCC was induced because of the activation of transient Cdk1 activity not detected at two hours post-treatment, Cdk1 activity was assessed at 0.5 and 1 hour following addition of fostriecin. Incubation of FT210 cells at the non-permissive temperature resulted in loss of Cdk1 phosphotransferase activity (Figure 18B: 40°C), with only 0.2 % of the activity in cells measured at 32°C in the presence of colcemid. Therefore, even at earlier periods following fostriecin incubation, no transient increases in Cdk1 phosphotransferase activity were detectable, and

fostriecin was able to reduce the level of activity that was detectable at both the permissive and non-permissive temperatures (Figure 19).

|         | Mitotic | 30°C - | 30°C + | 40°C - | 40°C + |
|---------|---------|--------|--------|--------|--------|
|         | %       | %      | %      | %      | %      |
| 30 min  | 25      | 2.6    | 6.2    | 4.2    | 7.1    |
| 60 min  | 27      | 2.6    | 6.3    | 6.5    | 10.4   |
| 120 min | 35.7    | 3.8    | 37     | 3.9    | 40.9   |

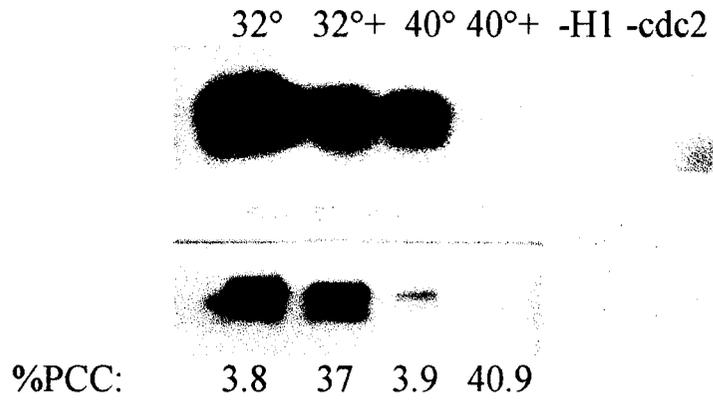
**Table 2.** The effect of fostriecin treatment on the mitotic indices in FT210 cells. FT210 cells were blocked at G2 phase subsequent to 12 hour isoleucine deprivation by incubating the cells at the non-permissive temperature for 18 hours. Cells were either released into the permissive temperature (30°C) in the presence (+) or absence (-) of 125 µM fostriecin, or maintained at the non-permissive temperature (40°C), in the presence or absence of fostriecin for the period indicated in the table. Cells were also co-treated with colcemid to ensure that they do not progress beyond metaphase. Cells were subsequently stained with *bisbenzimidide* and fixed for fluorescent microscopy to determine the percentage of cells containing normal mitotic chromosome condensation.



**Figure 17.** Cdk1 phosphorylation and histone H1 phosphotransferase activity in BHK-21 cells treated with fostriecin. (A) Immunoprecipitated Cdk1 (anti-Cdc2-CT antibody) was subjected to immunoblotting with Cdk1 antibodies (top), and its histone H1 kinase activity was determined (bottom). Extracts were from: Lane 1, cells blocked at S phase with aphidicolin (APC); lane 2, followed by treatment with 375  $\mu$ M fostriecin for 1.5 hours; Lane 3, cells released from aphidicolin block for 6 h followed by treatment with 25  $\mu$ M VM-26 for 2 hours to block in G<sub>2</sub>; Lane 4, same as for Lane 3, but with an additional 1.5 hour incubation without any other addition; Lane 5, same as for Lane 3, but with an additional 1.5 hour incubation with fostriecin. The radioactivity associated with the histone H1 band and the percentage of mitotic cells in each sample are also indicated. (B) Immunoprecipitated Cdk1 was probed for tyrosine phosphorylation with the 4G10 antibody.

**Figure 18. (1) (A)**, Induction of premature chromosome condensation and repression of histone H1 kinase activity following 2-hour fostriecin treatment of FT210 cells. FT210 cells were synchronized at permissive temperature (32°C) at G<sub>1</sub>/S with serum deprivation followed by aphidicolin treatment and released in regular medium containing colcemid at both permissive and non-permissive (40°C) temperatures for 6 and 18 hours respectively. Three hundred μM fostriecin (+) were then added to half of the cells under each condition for two hours. Following incubation, approximately 2 x 10<sup>5</sup> cells were removed and fixed with 3.7% formaldehyde and stained with Hoechst 33342 for observation of chromosome morphology. The percentage of cells undergoing premature chromosome condensation (%PCC) was determined by fluorescence microscopy where cells with condensed chromosomes were counted against total cells. The remaining cells were harvested and cellular proteins were extracted and half subjected to immunoprecipitation with a C-terminal-specific polyclonal anti-p34<sup>cdc2</sup> antibody and assayed for kinase activity using histone H1 as a substrate. Fractions were assayed without substrate (-H1) or without antibody during immunoprecipitation (-cdc2) as controls (see top insert). Fifty μg protein from the same cell lysates were size fractionated on a 10% SDS-PAGE gel and transferred to nitrocellulose. Immunoblotting was carried out with the same antibody to determine the total amount of Cdk1, shown in middle insert. The results are from identical conditions and indicate that fostriecin induced PCC and repressed Cdk1 activity at the permissive temperature, and induced PCC in the absence of Cdk1 at the non-permissive temperature. **(B)**, Histone H1 kinase activity of FT210 cells following 30 and 60 min incubations. Cells were synchronized as in Figure (A) and released in normal growth medium at both temperatures with colcemid as before. Fostriecin (300 μM) was added to half of the cells at each for 30 min (top) or 60 min (bottom).

A.



B.

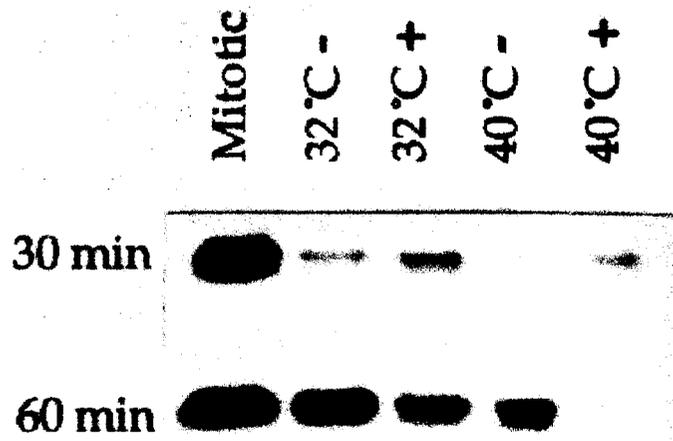
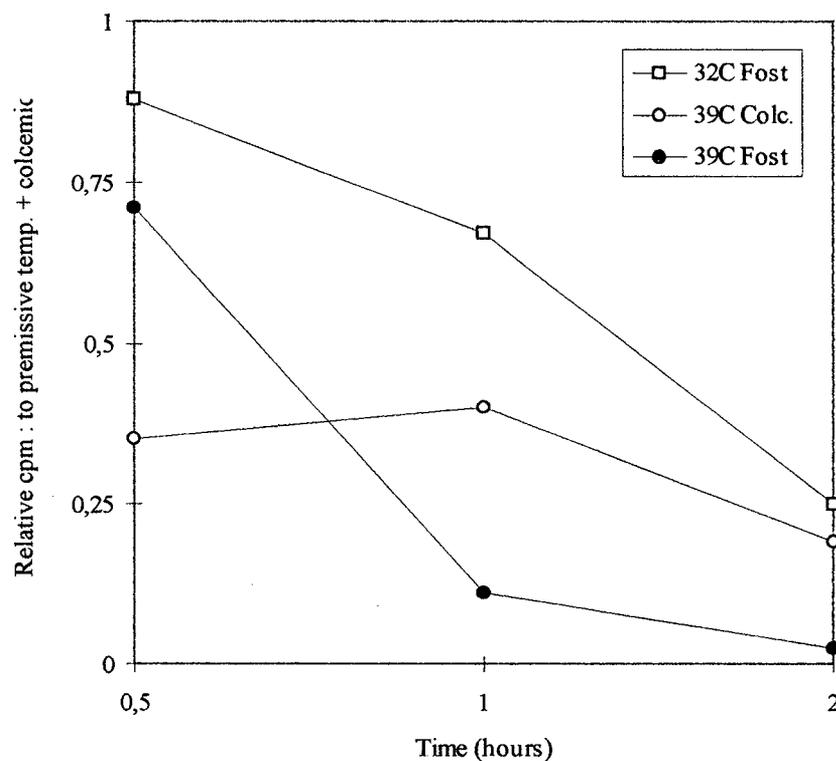


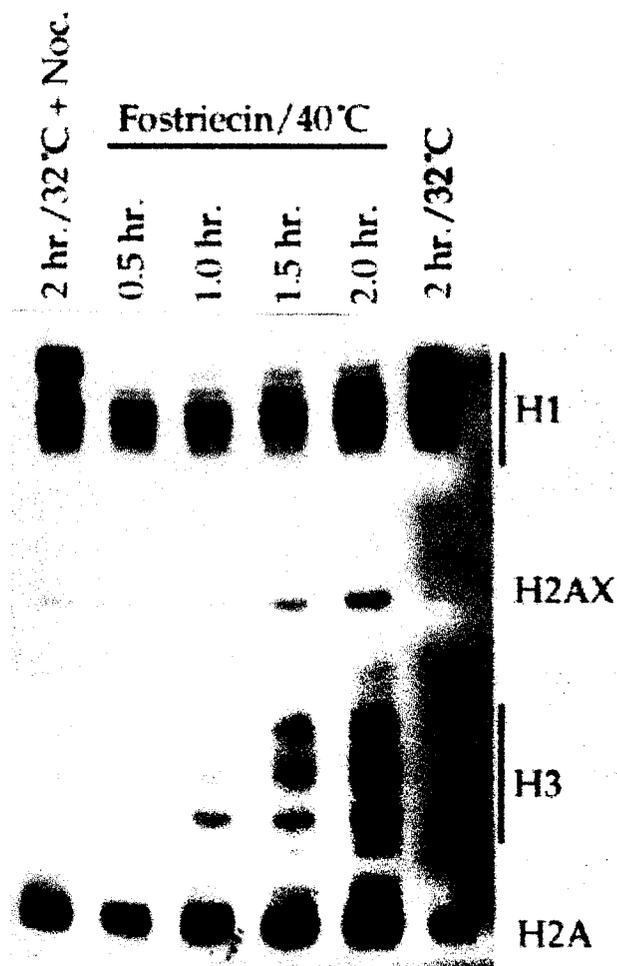
Figure 18.



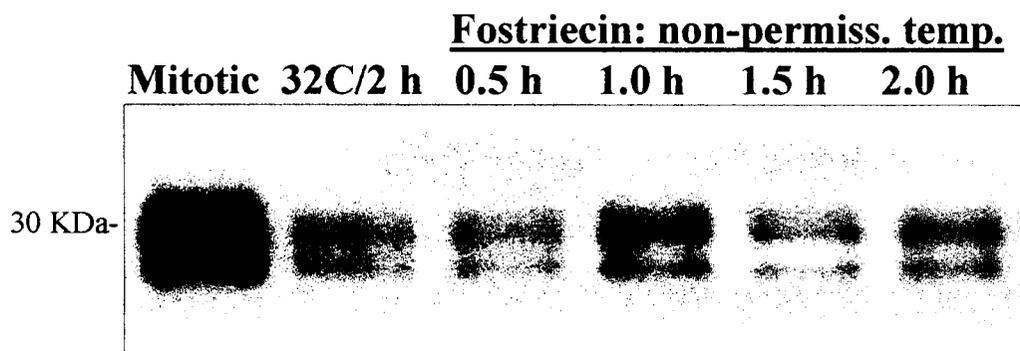
**Figure 19.** Cdk1 activity of FT210 cells described in Figure 18, relative to those cells grown at the permissive temperature (32°C) in the presence of colcemid. Densiometric analysis of the autoradiograms in Figure 18 was quantified at 30, 60 and 120 min. Cdk1 histone H1 phosphotransferase activity of cells treated with 300  $\mu$ M fostriecin at the permissive temperature (open square), the non-permissive temperature with colcemid (40°C, open circle), and at the non-permissive temperature in the presence of fostriecin (closed circle), were compared to cells treated with colcemid alone at the permissive temperature for the indicated drug exposure times.

Histones H2A and H3, but not H1, undergo hyperphosphorylation in fostriecin-treated cells that have undergone premature chromosome condensation

Mitotic phosphorylation of histone H1 correlates with chromosome condensation, and Cdk1 is the primary mitotic histone H1 kinase (Langan *et al.*, 1992). Since I have demonstrated that fostriecin can cause chromosome condensation in the absence of Cdk1 activity, we next determined its effect on histone H1 phosphorylation. FT210 cells were arrested in G2 at 40°C, or in mitosis with nocodazole, in the presence of [<sup>32</sup>P]orthophosphate and treated with fostriecin for 0.5, 1, 1.5 and 2 hours or without fostriecin. Cells were also released at 32°C with or without nocodazole and without fostriecin as a control. Histones were extracted via acid solubilization, separated on an acid urea-polyacrylamide gel and visualized by autoradiography. In this gel system, increasingly phosphorylated histones migrated as discrete bands of decreasing mobility (Th'ng *et al.*, 1992, 1994). Figure 20 illustrates that cells treated with fostriecin were observed to contain negligible increases in histone H1 phosphorylation compared to the controls (2 hour treatment at 32°C with or without nocodazole). It was demonstrated that the level of histone H1 phosphorylation remained in a lower phosphorylation state indicative of interphase cells (Guo *et al.*, 1995). Cdk1 was immunoprecipitated with the anti-Cdc2-CT antibody from the same cells and subjected to histone H1 phosphotransferase activity determination (Figure 21). Cells that were maintained at 40°C with fostriecin treatment displayed almost undetectable Cdk1 activity (Figure 21), although a 10-fold increase in the mitotic index was again observed (48 % for cells at 40°C with fostriecin versus 5.1 % for cells not treated with fostriecin). These results demonstrate that chromosome condensation was induced in G2 by fostriecin in the absence of dramatic increases of histone H1 hyperphosphorylation, and further, that this condensation of chromosomes did not require Cdk1 phosphotransferase activity, since it has already been shown that fostriecin inhibits Cdk1 activity. Figure 20 showed that the phosphorylation state of the fastest migrating histone H1 species remained unchanged in fostriecin treated cells while that of the slower migrating bands decreased relative to cells not treated with fostriecin. This would indicate that fostriecin affects interphase phosphorylation differently than that of mitotic cells. Similar effects were observed in FM3A cells treated



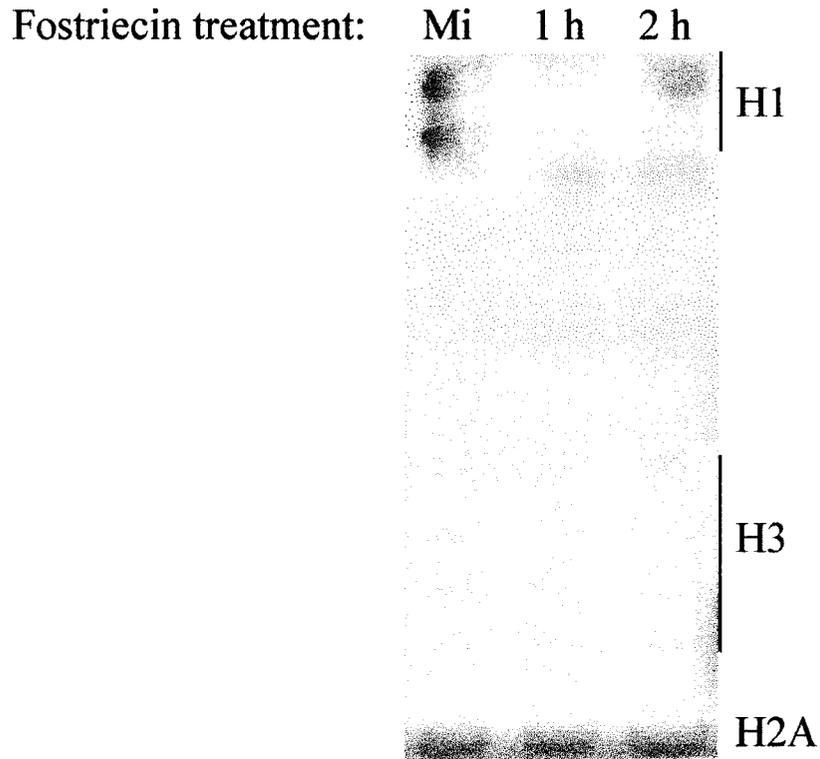
**Figure 20.** The effects of fostriecin on the phosphorylation of acid solubilized core histones. FT210 cells were arrested in G2 following incubation at 40°C for 18 hours and subsequently incubated with fostriecin for a period of 0.5 to 2.0 hours (hr). The figure illustrates the autoradiogram of the acid-extracted core histones separated in an acid-urea gel. Histones were also extracted from cells blocked at metaphase with colcemid and cells released from the non-permissive temperature to 32°C for two hours without fostriecin. The 2 hr/32°C condition represents the 'starting point,' whereas the 2 hr/32°C + Noc. represents cells released at 32°C for 2 hours with a block at metaphase. This figure illustrates the immediate and sustained histone H1 dephosphorylation and gradual hyperphosphorylation of histones H3 and H2A induced by fostriecin treatment.



**Figure 21.** Cdk1 activity of cells treated concurrently with those described in Figure 20, demonstrating an autoradiogram of histone H1 phosphorylation by Cdk1 immunoprecipitated with the anti-cdc2-CT antibody. The level of SDS-PAGE purified histone H1 phosphorylation was decreased in cells treated with fostriecin compared to cells blocked at mitosis.

with fostriecin (Figures 22) where endogenous histone H1 phosphorylation was also observed in the parental cell line, FM3A, to ensure that the histone phosphorylation inhibitory effect of fostriecin was not an anomaly characteristic of the temperature-sensitive cell line. FM3A cells that were blocked in mitosis in the presence of nocodazole and subsequently treated with fostriecin (with nocodazole) were observed to have a significantly reduced histone H1 phosphorylation profile compared to that observed in cells blocked at metaphase with nocodazole alone (Figures 22). These results show that chromosome condensation could be induced in G2 phase by fostriecin with the absence of histone H1 phosphorylation. Further, in addition to inhibiting Cdk1, fostriecin treatment also affects a phosphatase, or inhibits a kinase that targets histone H1, reducing the phosphorylation of this histone, even when cells are resting in mitosis. It is important to note that sea star Cdk1 purified in its active form was not directly inhibited by the presence of fostriecin (data not shown).

Histone H3 is normally phosphorylated only at metaphase, whereas histone H2A is phosphorylated throughout the cell cycle. Histones H2A, H2AX and H3 were also resolved on the acid-urea gel, where again, the more highly phosphorylated species are represented as the slower migrating band. Contrary to observations with histone H1, histones H2A, H2AX and H3 underwent significant increases in phosphorylation in FT210 cells treated with fostriecin (Figure 20). To determine if the degree of histone H3 phosphorylation in mitotic cells was affected by incubation with fostriecin, FM3A cells blocked at mitosis with nocodazole were subsequently treated with fostriecin for a period of one and two hours in the presence of radiolabeled orthophosphate prior to histone extraction (Figure 22). Subsequent to fostriecin incubation, the degree of histone H3 phosphorylation was significantly increased, with phosphorylation occurring at an additional residue as determined by observation of the additional mobility shift. Additionally, cells treated with fostriecin exhibited phosphorylated bands with lower mobility than phosphorylated histone H3 from mitotic cells. This would indicate that there exists an additional potential phosphorylation site that is not normally phosphorylated during mitosis, but is susceptible to a histone H3 kinase in the presence of fostriecin. Further, it was observed that histone H2A increases in phosphorylation following fostriecin treatment although no additional,

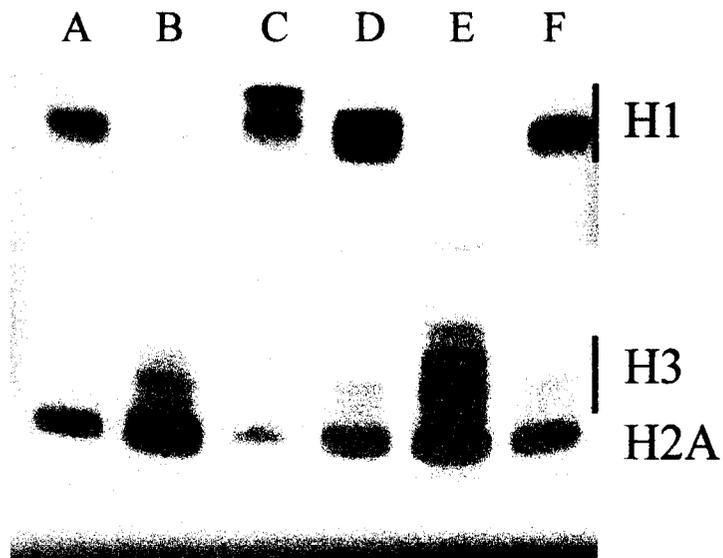


**Figure 22.** Effects of fostriecin on the phosphorylation of acid solubilized core histones extracted from FM3A cells incubated with [ $^{32}\text{P}$ ]orthophosphate and arrested in mitosis with colcemid. Cells arrested at mitosis (left Lane) were treated with fostriecin for 1 hour (h) (centre Lane) or 2 hours (right Lane) (incubation with colcemid was maintained during fostriecin treatment). Densitometric analysis of the autoradiogram showed a 50% decrease in histone H1 phosphorylation and a 4-fold increase in histone H3 hyperphosphorylation after 2 hours (2 h) fostriecin incubation compared to the normal mitotic (Mi) histone profile.

slower migrating bands were detected observed, indicating that additional histone H2A proteins were phosphorylated (Figure 20). These observations may imply that the enhanced phosphorylation of histones H2A and H3, induced by fostriecin incubation, may cause chromosomes to condense without histone H1 hyperphosphorylation. Also, given that fostriecin has been shown to be an effective PP2A inhibitor (Roberge *et al.*, 1994), fostriecin may induce the mitotic associated morphological event of chromosome condensation by inhibiting the phosphatase that is responsible for the dephosphorylation of histones H2A and H3, which when induced to higher than normal phosphorylation states, compensates for the reduced histone H1 kinase. It is possible that histone H1 is not necessary for chromosome condensation. Chromosome condensation may result in cells treated with fostriecin due to the activation of a histone H3 kinase, or the inhibition of an H3 phosphatase, perhaps in the PP2A family, resulting in metaphase-associated phosphorylation of histone H3.

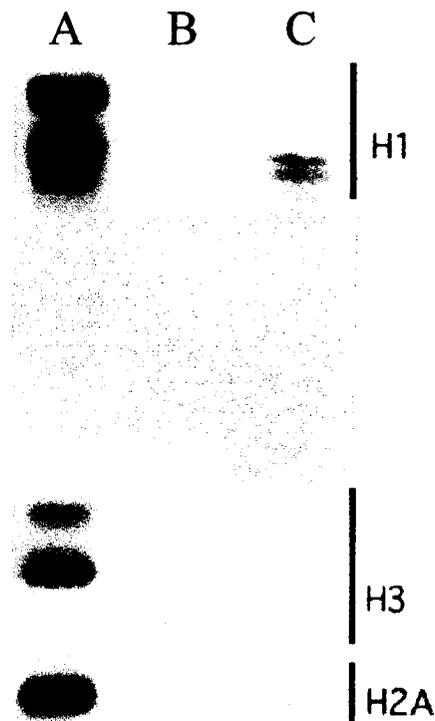
The correlation between enhanced histone H3 phosphorylation in the presence of fostriecin and premature chromosome condensation in cells blocked at G2 phase was also observed in cells synchronized to S phase with aphidicolin (Figure 23). Cells blocked at S phase and treated with fostriecin were observed to have PCC. Additionally, endogenous histone H3 was hyperphosphorylated in the presence of the drug (Figure 23, lanes B and E) compared to S phase cells not treated with fostriecin (Figure 23, lanes D and F). Again, the incorporation of phosphate into endogenous histone H3 protein was greater than that of cells blocked at M phase with colcemid (Figure 23, lane C). The level of histone H1 phosphorylation that normally occurs in S phase cells was observed to be reduced in the presence of fostriecin, which may be a result of indirect inhibition of Cdk1 by the drug.

To determine whether fostriecin-induced histone H3 hyperphosphorylation was regulated by a protein kinase, the effects of the kinase inhibitor staurosporine on these mitotic events were analyzed. Staurosporine has been demonstrated to inhibit histone H1 and H3 kinase activities and chromosome condensation, and cause G2 arrest (Th'ng *et al.*, 1994; Guo *et al.*, 1995). FT210 cells incubated at their permissive temperature were released from an aphidicolin induced S phase block for 18 hours with 100 ng/ml

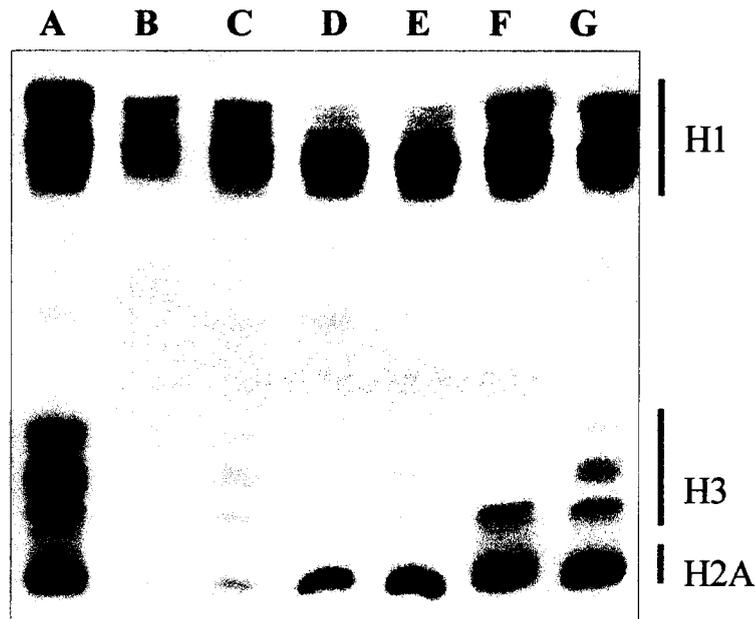


**Figure 23.** Fostriecin induces histone H3 hyperphosphorylation in FM3A cells released from an aphidicolin induced S phase block. Acid solubilized core histones were extracted from FM3A cells incubated with [<sup>32</sup>P]orthophosphate following synchronization to S phase in the presence of 2.5 μg/ml aphidicolin for 16 hours with 375 μM fostriecin for an additional 2 hours (B), without fostriecin (A), and from cells which were released from an S phase block for 12 hours in the presence of colcemid (0.5 mg/ml) (D), fostriecin (E), or without drugs (F). Endogenous core histone phosphorylation was also determined for cells blocked at mitosis in the presence of colcemid for 2 hours without prior synchronization (C).

staurosporine, together with nocodazole to capture cells that enter mitosis. Figure 24 illustrates the resulting phosphorylation states of the acid-solubilized core histones. The addition of 150  $\mu$ M fostriecin for two hours did not overcome the G2 arrest or cause cells to undergo PCC (not shown). The results demonstrate the requirement of protein phosphorylation and that both protein kinases and phosphatases are required for the regulation of chromosome condensation. Further, histone H3 appears to be important for chromosome condensation as illustrated in the previous figures. Noteworthy in Figure 24 was the reduction of histone H1 phosphorylation of mitotic cells, which is consistent with previous experiments. The experiment was repeated in FM3A cells resulting in similar results (data not shown). Histone H1 and H3 hyperphosphorylation associated with mitosis (lane 1) was inhibited by the presence of staurosporine (lane 2) and fostriecin and staurosporine (lane 3). Since no PCC was observed in cells treated with staurosporine and fostriecin together, this is consistent with the requirement for histone H3 hyperphosphorylation for the onset of premature chromosome condensation following fostriecin treatment. The necessity for protein phosphorylation in the induction of histone H2A and H3 kinase activity was demonstrated again in Figure 25, where mitotic cells were treated with combinations of staurosporine and fostriecin, and it was observed that staurosporine inhibited the ability of fostriecin to induce histone H3 hyperphosphorylation.



**Figure 24.** Histone H1 and H3 mitotic associated hyperphosphorylation was inhibited by staurosporine treatment, even in the presence of fostriecin. FT210 cells were synchronized to S phase in aphidicolin and released in the presence of colcemid (0.5 mg/ml) for 12 hours (mitotic, **A**), 100 ng/ml staurosporine for 12 hours (**B**), or staurosporine for 12 hours with fostriecin (150  $\mu$ M) for the last 2 hours (**C**), in the presence of [ $^{32}$ P]orthophosphate. Histones were extracted, separated on an acid urea-PAGE gel and visualized by autoradiography. The positions of histone H1, H2A and H3 are indicated.



**Figure 25.** Staurosporine inhibits fostriecin-induced histone H3 hyperphosphorylation in FT210 cells. Asynchronously growing FT210 cells, incubated at their permissive temperature, were blocked at mitosis in the presence of 50 ng/ml nocodazole for 18 hours without (A), or with 150 ng/ml staurosporine co-treatment (B), or with staurosporine co-treatment followed by 2 hours of fostriecin treatment (C). FT210 cells were also grown in the presence of staurosporine alone for 18 hours without (D), or with fostriecin for an additional 2 hours (E), or in the presence of fostriecin alone for 1 and 2 hours following 18 hour nocodazole incubation (F and G, respectively). Nocodazole was maintained during fostriecin treatment and incubations were co-incubated with orthophosphate for the same period as drug incubations under each condition. Histones were extracted and separated on acid urea-PAGE gels and visualized by autoradiography.

## DISCUSSION

Topoisomerase II is an ATP-dependent homodimeric protein (approximately 170 kDa) that alters the topological state of DNA. It is essential for survival of eukaryotic cells and is required for the segregation of mitotic (and meiotic) chromosomes and maintenance of chromosome structure. Its activity and expression has been linked with DNA replication, transcription, recombination as well as being required for chromosome condensation and cell cycle progression (Chow and Ross, 1987; Adachi *et al.*, 1991; Osheroff *et al.*, 1991). The catalytic cycle of topoisomerase II consists of its covalent binding to the nucleotide substrate to form a cleavable complex with the second strand of DNA that will be passed through the first. The 'pre-strand' of DNA is ligated in the presence of magnesium and ATP followed by post-strand passage, re-ligation of the DNA complex, and ATP hydrolysis to allow for enzyme turnover. Several classes of antitumour drugs act as topoisomerase II poisons, primarily by stabilizing the topoisomerase-DNA complex. This complex is susceptible to cleavage by strong protein denaturants revealing topoisomerase II-sequestered DNA single- or double-stranded breaks (Cummings and Smyth, 1993). But not all topoisomerase II poisons function by stabilizing the cleavable complex. For example, the disruption of ATP turnover by coumarins and DNA intercalating agents can inhibit the activity of the topoisomerase II enzymes (Liu, 1989). Fostriecin's mechanism of action as an antitumour compound has been described as a function of inhibition of topoisomerase II without forming a cleavable complex (Ishimi *et al.*, 1992; Francis *et al.*, 1994). This inhibition was suggested to be the mechanism of action in cells treated with fostriecin, including a decreased rate of DNA, RNA and protein synthesis, and cytostasis (Fry *et al.*, 1984; Hotz *et al.*, 1992).

Topoisomerase II experiments described in this thesis revealed that virtually no inhibition of topoisomerase II was observed in cells treated with up to 400  $\mu\text{M}$  fostriecin. Since all of the nuclear morphological and biochemical studies with fostriecin in this report were conducted with drug concentrations not exceeding 375  $\mu\text{M}$ , any effect observed was not likely a result of inhibition of topoisomerase II. It is possible that because the topoisomerase II enzyme utilized in the study was purified yeast

topoisomerase II, and not that of human, fostriecin may have an inhibitory effect on the activity of the human form of topoisomerase II at the concentration that chromosome condensation was observed, and therefore, may contribute to the observations described in the Results. This is unlikely, although, since to date, no topoisomerase inhibitors have been observed to induce any events associated with mitosis, but all have been demonstrated to induce a G2 phase block (Adachi *et al.*, 1991; Anderson and Roberge, 1996). Phosphatase 2A inhibitors such as okadaic acid and caffeine have been shown to induce mitotic events including chromosome condensation, nuclear lamina dissolution and aster formation (Downes *et al.*, 1990; Yamashita *et al.*, 1990; Ghosh *et al.*, 1992; Dyban *et al.*, 1993; Minshull *et al.*, 1996). Since we have demonstrated that fostriecin is a phosphatase 2A inhibitor (Roberge *et al.*, 1994), it is likely this mechanism, and not the inhibition of topoisomerase II that contributes to the observations discussed in this thesis.

Cells possess a network of regulatory mechanisms that ensure that the initiation of mitosis occurs only after the completion of DNA replication and subsequent DNA repair. These events are monitored by checkpoint controls at S and G2 phases that regulate the progression through the next phase of the cell cycle towards mitosis. It has been shown that these checkpoints can be overridden by fusion of interphase cells with mitotic ones (Mullinger and Johnson, 1983), loss of RCC1 function (Nishitami *et al.*, 1991; Matsumoto *et al.*, 1991), overexpression of *cdc25*, mutations of a number of yeast genes (Weinert and Hartwell, 1988; Rowley *et al.*, 1992), and exposure of cells to methylxanthines and caffeine which can suppress G2 delay induced by DNA damage (Shlegal and Craig, 1991) as well as to other compounds, including okadaic acid and 2-aminopurine, which can induce entry into mitosis from S phase in the presence of unreplicated DNA. This study has demonstrated that another compound, fostriecin, can induce PCC in cells blocked at S and G2 phases in the presence of DNA replication inhibitors and damaging agents, respectively. Fostriecin was found to induce the rounding up of cells, premature chromatin condensation and nuclear lamina dissolution. These mitotic phenomena occurred as early as 1 hour post-treatment in cells grown asynchronously, or in synchronized cells between the G1/S and G2/M transitions of the cell cycle. These effects did not appear to be reversible as similar mitotic indices were observed in cells when fostriecin was washed

from cells following 30 and 90 min incubations and scored for chromosome condensation at 2 hours (data not shown). Further, fostriecin treatment ultimately lead to mitotic catastrophe and cell death. This is unique to fostriecin, unlike other compounds including okadaic acid, caffeine and 2-aminopurine which can override the S phase checkpoint, since fostriecin is presently being investigated clinically as a possible therapeutic agent (Gorczyca *et al.*, 1993).

One of the most dramatic and immediate effects following fostriecin treatment is the induction of chromosome condensation. Striking in this morphological induction are its speed and apparent cell cycle independence subsequent to G1 phase. This was dramatically demonstrated in cells blocked at S phase with the DNA synthesis inhibitor, aphidicolin, where addition of fostriecin induced premature chromosome condensation (PCC) within 1.5 hours of drug treatment. Like asynchronously growing cells, S phase PCC (S-PCC) was concentration dependent. This was surprising, as among the anti-tumour agents studied to date, most produce similar effects on the cell cycle; namely, inducing G2 arrest (Konopa *et al.*, 1988). These agents also caused progressive enhancement of cellular accumulation in G2 phase with an increase in drug dosage (Dean *et al.*, 1983). Rao *et al.* (1970) have suggested that this arrest by anti-tumour drug action causes depletion of mitotic factors required for mitotic transition. These factors have been proposed to be one of, or a combination of cyclin B (O'Connor *et al.*, 1992), phosphatase inhibitor-2 (Nishitani *et al.*, 1991; Brautigan *et al.*, 1990), or p50<sup>Wee1</sup> (Heald *et al.*, 1993). The requirement of such factors was initially suspected, since asynchronously growing cells treated with fostriecin are induced to undergo a normal chromosome condensation prior to any observed S-PCC or chromatin fragmentation. The possibility of avoiding the lethality of fostriecin by preventing synthesis of mitosis-inducing proteins prior to repair of damaged DNA, as has been observed with caffeine (Downes *et al.*, 1990), was ruled out since fostriecin has been suggested to be a macromolecular synthesis inhibitor (Fry *et al.*, 1984; Leopold *et al.*, 1984; Hotz *et al.*, 1992). Additionally, S-PCC was observed in fostriecin treated cells following synchronization with aphidicolin and preincubation with cyclohexamide (not shown). Therefore, fostriecin, like okadaic acid, can induce premature chromosome condensation in the absence of DNA replication and protein synthesis. It cannot be

discerned, though, if in asynchronously growing cells, the cell cycle is accelerated and fragmentation occurs following initial normal chromosome condensation as a result of lesions in the condensed chromosomes, or if condensation of chromatin prior to G2 is delayed by some unknown mechanism. I have demonstrated that S phase cells, identified with BrdU, and cells blocked at G2, undergo PCC prior to DNA fragmentation, even in the presence of VM-26 (discussed later).

Unlike caffeine, which only restores the normal cycle timing by overcoming a restriction point in cell cycle progression that is otherwise imposed in the presence of unreplicated DNA, fostriecin accelerated mitotic progression, even in the presence of aphidicolin, based on chromosome condensation. It was demonstrated in collaboration with Dr. Morton Bradbury's laboratory (communication with Dr. Bradbury) that the rate of S-PCC induction far exceeded that of 2-aminopurine and okadaic acid. This effect was unique to fostriecin, since S-PCC in the presence of a DNA synthesis inhibitor was observed in human cells, contrary to okadaic acid, which has been shown to do so only for rodent cells (Ghosh *et al.*, 1992). This is significant in elucidating the mechanisms of mitotic induction as Steinmann *et al.* (1991) have suggested that the onset of mitosis is regulated differently among mammalian cell types, which can affect the susceptibility of cells to drug-induced mitotic aberrations, such as premature chromosome condensation. Our findings confirm that there is no obligatory dependence of mitosis on the completion of S phase, but rather, there exists a signalling mechanism that normally couples these events that may be circumvented.

Cells respond to DNA damage by delaying the onset of mitosis to allow for time to repair DNA prior to the initiation of cellular division. Prevention of such a delay would result in increased cytogenetic damage and cell death (Schlegel and Craig, 1991; Roberge, 1992), and loss of control may even contribute to genomic instability leading to cancer (Hartwell, 1992). Cdk1 has been implicated in playing a critical role in the G2 to M transition in all eukaryotic cells (Pines and Hunter, 1991; Enoch and Nurse, 1991; Nigg, 1993). At the onset of mitosis, the cell undergoes structural modifications in preparation of cellular division. Many of the substrates of Cdk1 play a structural role in mitosis, including histone H1, the lamins, and nucleolin (Lewin, 1971). To determine if fostriecin can

overcome the checkpoint associated with this transition, we blocked cells in G2 prior to fostriecin treatment with the topoisomerase I and II inhibitors camptothecin and VM-26, respectively. Both of the inhibitors have been observed to halt cells in late G2 prior to the initiation of chromatin condensation in prophase (Del Bino *et al.*, 1991; Tsao *et al.*, 1992). When applied in late S or early G2 phase, camptothecin has its inhibitory effect, probably by causing interference with transcription of genes necessary for the passage into mitosis, and like VM-26, creates lesions in the DNA. VM-26 blocks G2 progression by stabilizing topoisomerase II-DNA complexes that induce nicks in the DNA, thus disrupting chromosome condensation (Charron and Hancock, 1990; Wright and Schatten, 1990). Additionally, VM-26 was found to completely inhibit activation of Cdk1 and the increase in H1 phosphorylation that would normally occur at this point of the cell cycle (Roberge *et al.*, 1990; Lock, 1992). Addition of fostriecin to cells exposed to either inhibitor reversed the G2 block and induced cells to enter mitosis. Since the G2 block was caused by lesions in the DNA induced by the inhibitors, I have demonstrated that fostriecin inhibits the checkpoint control that imposes a G2 block when DNA is damaged. The chromosome morphology of co-treated cells was similar to that of cycling cells treated with fostriecin alone. This was surprising as it has been shown that chromosomes become partially decondensed after 1 hour addition of VM-26, and adopt a partially unfolded conformation after 2 hours (Roberge *et al.*, 1990). In our experiments, combined treatment produced chromosomes that did not unfold to the extent of those treated with VM-26 alone. These results imply that the premature timing of chromosome condensation precedes by more than 2 hours the fragmentation observed with fostriecin treatment. This was because the DNA lesions were not additive with combined treatment as compared to VM-26 treatment alone, but instead transgress the initial extent of decondensation resulting from DNA lesions due to VM-26 treatment alone.

Rao *et al.* (1979) has shown previously that G2 arrest caused by a number of anti-tumour drugs is associated with extensive chromosome damage including breaks and gaps (Rao *et al.*, 1979). Some of these compounds, such as the crosslinking agent mitomycin C, induce the cell to undergo chromosome condensation even though DNA synthesis is not fully completed yet still accumulate at G2 (Konopa, 1988). Thus, DNA repair mechanisms

may be limited to the generation of the G2 block, and fostriecin appears to circumvent this and other activities that play a role in inhibiting premature cell cycle progression. Therefore, fostriecin serves as a novel tool for studying how defects in a cell's ability to control cell cycle progression lead to premature mitosis, death and/or cancer. Additionally, fostriecin may be utilized to ascertain those pathways that regulate cell cycle progression. Fostriecin has been shown to be implicated in the acceleration of cells into a pseudo-mitotic state under conditions where there is a depletion of mitotic factors required for mitotic transition, incomplete DNA synthesis or excessive DNA lesions (dysfunction of DNA repair mechanisms).

The ability of fostriecin to overcome the checkpoint that monitors DNA damage at the G2/M transition indicates that fostriecin may indirectly affect the phosphotransferase activity of Cdk1, and therefore initiate chromosome condensation and lamina depolymerization. Fostriecin does not inhibit Cdk1 directly *in vivo* or *in vitro* (data not shown), but I have discovered that it inhibits protein phosphatase 2A strongly, with an  $IC_{50}$  of 40 nM, and protein phosphatase 1 to a much lesser extent. Fostriecin exhibits additional specificity, as it did not inhibit tyrosine phosphatases (Roberge *et al.*, 1994). This effect on protein phosphatase 2A (PP2A) may be responsible for the morphological events observed in this thesis. PP2A has been suggested to be important in regulating chromosome condensation by restricting histone phosphorylation during interphase, and thereby preventing PCC (Dasso and Newport, 1990; Mahadevan *et al.*, 1991; Ohsumi *et al.*, 1993; Ajiro *et al.*, 1996). Additionally, phosphatase activity causes the binding of DNA to the nuclear matrix, the release of which precedes nuclear dissolution, which is itself, mediated by a kinase (Moreno and Nurse, 1990; Pyrpasopoulou *et al.*, 1996)). Therefore, the inhibition of PP2A following fostriecin treatment may result in the repression of the phosphatase that restricts the level of interphase histone phosphorylation, such as the phosphorylation of metaphase associated histone H3 phosphorylation, resulting in PCC. Also, inhibition of a phosphatase with fostriecin may result in the dissolution of the nuclear membrane due to the subsequent phosphorylation of the lamin proteins. The observation that the chromosome condensation induced with fostriecin correlated with histone H3 hyperphosphorylation, and not histone H1, may be due to the possibility that a major factor

in chromosome condensation is simply the 'degree' to which positive charges of the histone tails are neutralized to offset electrostatic repulsion between histones or moderate the charge balance between histones and DNA. Therefore, the overall degree of histone phosphorylation may be more important for chromosome condensation than the particular identity of the phosphorylated histone.

I have shown that Cdk1 immunoprecipitated from extracts of cells blocked at either S phase with aphidicolin or G2 with VM-26, and treated with fostriecin becomes dephosphorylated at the tyrosine as we observed changes in the protein's mobility on Cdk1 Western blots, and the immunoreactivity with anti-phosphotyrosine antibodies. Tyrosine dephosphorylation of Cdk1 is associated with its activation (Dunphy and Kumagai, 1991). Although dephosphorylation of Tyr-15 of Cdk1 is necessary for phosphotransferase activity, so too is dephosphorylation of the Thr-14 and continued phosphorylation of Thr-161 (Solomon, 1994). Histone H1 is often utilized as a substrate to measure endogenous Cdk1 (histone H1 kinase) activity (Lohka, 1989). BHK-21 cells synchronized to S and G2 followed by treatment with fostriecin did not exceed a 2.2-fold histone H1 phosphotransferase activity compared to untreated cells, although a 5-fold increase in the mitotic indices did occur. This was surprising, as an excess of 5- to 10-fold increase in histone H1 phosphotransferase activity is usually associated with the onset of mitosis and chromosome condensation (Yamashita *et al.*, 1990; Solomon, 1994). It is interesting that although all of the immunoprecipitated protein underwent tyrosine dephosphorylation, the level of activity was comparatively moderate. This discrepancy, and that of the high degree of premature chromosome condensation (mitotic indices) relative to Cdk1 activity obtained by other methods of inducing mitosis, or of collected mitotic cells, may be explained by a number of possibilities. First, the level of activity observed here may be sufficient to initiate the characteristics of mitosis associated with fostriecin treatment. Second, Lamb *et al.* (1991) suggested that although contradictory to the dogma linking histone H1 phosphorylation by Cdk1 to chromatin condensation, different lines of evidence argue against periodic Cdk1 activation being solely responsible for chromatin condensation. In mammals, both histone H1 phosphorylation and chromatin condensation increase continuously from late G1 to late G2 when Cdk1 is still inactive (Gurley *et al.*, 1975).

Furthermore, the unscheduled activation of Cdk1 may not be sufficient to induce all of the events characteristic of mitosis, but may require a cooperation of Cdk1 with other factors (Krek and Nigg, 1991). Candidates for such factors include members of the MAP kinase family (Gotoh *et al.*, 1991), or NimA kinase (Osmani *et al.*, 1991). Lock and Ross (1990) have shown that Cdk1 is significantly inhibited following brief exposure to etoposide, an analog of VM-26, which supports the possibility that G2 arrest resulting from drug treatment may be caused by inhibition of an enzyme required for mitosis. CHO cells have been synchronized at the G1/S boundary and exposed to etoposide (25  $\mu$ M) following release of the block. On the eighth hour post release, Lock (1992) observed the change in histone H1 phosphorylation was approximately 3.2-fold greater without drug although the increase in the percent of mitotic cells was only 5-fold (Lock, 1992). I observed only a 2.3-fold increase in histone H1 phosphorylation with complete tyrosine dephosphorylation while inducing greater than a 4.4-fold increase in mitotic cells following 2 hours combined treatment. This implies that in addition to affecting a protein ultimately responsible for inactivating or inhibiting the activation of Cdk1, another mitotic protein may have been activated with fostriecin treatment. For instance, cells expressing a Cdk1 mutant that cannot be phosphorylated on Thr-14 or Tyr-15 (a requirement for inactivation) (Cdc2AF), did not induce mitotic events when arrested at early S phase, but were observed to undergo premature chromosome condensation when progressing through S phase and G2 (Jin *et al.*, 1996, 1998). Further, cells containing DNA damage induced by X-irradiation during S phase and transfected with Cdc2AF were observed to have a reduced G2 phase delay. Therefore, it was suggested that the inhibitory phosphorylation of Cdk1 delayed mitosis after DNA damage (Jin *et al.*, 1996). This mechanism was not functional in cells treated with fostriecin as cells were observed to enter mitosis based on chromosome condensation and nuclear lamina breakdown without Cdk1. This implicates another mediator, and/or event, such as apoptosis, that is induced following fostriecin treatment that is mediated by some of the same morphological events.

Inhibition of Cdk1 may be mediated through upstream regulators of Cdk1, such as Myt1 (Mueller *et al.*, 1995), but evidence exists that would indicate otherwise. For instance, Wee1, the Cdk1 inhibitor that functions by phosphorylating and inactivating

Cdk1, is itself negatively regulated through phosphorylation. Wee1 activity was not found to be elevated in HeLa cells arrested in S phase as a result of unreplicated DNA (McGowan and Russell, 1995). Therefore, unreplicated DNA does not delay mitosis by hyperactivating Wee1, and in the systems investigated with fostriecin, the inhibition of Cdk1 and acceleration into an M-phase state with subsequent mitotic catastrophe, probably resulted through a mechanism that is not mediated through the Wee1 pathway. Further, mitotic catastrophe (described as premature entry of mitosis) has been observed to occur in loss-of-function Wee1 and Mik1 *S. pombe* mutants, which is consistent with the results demonstrated in this report (Berry and Gould, 1996). In a study described by Izumi and Maller (1995), it was demonstrated that a kinase distinct from Cdk1 and Cdk2 may initially activate Cdc25 (instead of Cdk1) *in vivo* and they suggest that this kinase may also phosphorylate mitotic substrates even in the absence of Cdk1. Such a kinase may lie on a pathway that is distinct from the Cdk1 one, and may be affected by fostriecin treatment leading to inhibition of Cdk1 and phosphorylation of mitotic substrates. Assembly and disassembly of the nucleus at mitosis involves reversible interaction of chromatin and the nuclear membrane. Membrane release of nuclear chromatin and microtubules during mitosis is promoted by an increase in kinase activity, whereas phosphatase activity stimulated membrane binding. Therefore an inhibitor of phosphatases, such as okadaic acid or fostriecin would result in mitosis-associated membrane release. This inhibition of a phosphatase is paralleled by the activity of a kinase that mediates membrane release, and it has been demonstrated that a release kinase exists that is distinct from Cdk1 (Pfaller and Newport, 1995). Another example of a protein kinase pathway that may be affected by fostriecin is that of the early response secondary messengers. For instance, PKC $\beta_{II}$  has been shown to be required at G2 phase for mitotic nuclear lamina disassembly and entry into mitosis, and PKC $\beta_{II}$ -mediated phosphorylation of nuclear lamin B is important in these events (Goss *et al.*, 1994).

Investigations done in Darzynkiewicz's laboratory at approximately the same time as the work presented here was conducted showed that MOLT-4 and HL-60 cells exposed to fostriecin at concentrations exceeding 5  $\mu$ M for 6 hours are blocked in the cell cycle at S phase with significant DNA degradation that is consistent with apoptosis as determined by

flow cytometry and DNA laddering (Hotz *et al.*, 1992). Additionally, they observed that exposure of cycling cells to fostriecin (6 hour) made them insensitive to the DNA degradation effects otherwise induced by VM-26. Our earlier results demonstrated that at periods earlier than 3 hours of fostriecin incubation, induction of cell cycle acceleration into mitosis with normally condensed chromosomes occurred. Cells treated with VM-26 and fostriecin were also observed to progress to M phase with reduced DNA decondensation and fragmentation triggered by VM-26. Additionally, we also observed nuclear apoptotic bodies, which we designated as micronucleated cells, in cells treated for extended periods of time, consistent with the findings of Hotz *et al.* (1992). This concentration dependent effect was also observed in cells pretreated with VM-26, which usually results in an induction of a G2 block, where 1 mM fostriecin treated cells had a greater mitotic indices (16.1 %) compared to 300  $\mu$ M treated cells (6.2 %), and each was observed to have undergone DNA degradation, probably triggered by VM-26. These results are consistent with the notion that fostriecin overcomes the G2/M phase checkpoint, inducing cells into a premature mitotic state that progresses to apoptosis following prolonged treatment. This effect is most likely not attributed to inhibition of topoisomerase II, but may result from differential phosphorylation induced by fostriecin and induction of endonucleases later in the incubation period. The initial inhibition of DNA degradation at the onset of mitotic catastrophe (determined with observations of nuclear polyploidy) followed by an onset of degradation without the presence of Cdk1 following fostriecin treatment is not the first observation of this phenomenon. Brefeldin induced apoptosis-associated DNA fragmentation independent of p53 and Cdk1, and brefeldin was actually observed to inhibit the phosphotransferase activity of Cdk1, similar to fostriecin (Shao *et al.*, 1996). Such an event may be regulated by a protein that regulates the integrity of the DNA, such as Cdc21. Cdc21, which is phosphorylated by Cdk1 *in vitro* and may be a component of the DNA licensing factor (MCM), is bound to DNA during S phase, and remains underphosphorylated until mitosis where it is hyperphosphorylated (probably by Cdk1) (Coue *et al.*, 1996). Fostriecin may affect this mechanism through its action as a phosphatase inhibitor, resulting in decondensation of DNA, due to the release of Cdc21 from DNA which may signal the end of DNA replication.

Polyploid cells are not quiescent. Their cell cycle is longer than a typical modal-ploidy population, and thus maintain cyclin B throughout the cell cycle, which may contribute to polyploidy development by impeding the exit from mitosis (Hall *et al.*, 1996). In FT210 cells, it has been shown that cyclin B is not present (Hall *et al.*, 1996), and therefore, polyploidy is being induced by a mechanism in response to fostriecin that is not preceded by a block at mitosis. Further, the observation that cells are accelerated into morphologically mitotic-like state in response to fostriecin may be followed by signalling that is not exclusive to the G2 checkpoint and mitosis, but indicative of cells that have progressed through mitosis, which would result in polyploidy (Zucker *et al.*, 1991; Ferguson *et al.*, 1996). For example, in a study with the topoisomerase II inhibitor, amsacrine, DNA polyploidy was explained by two mechanisms: at low concentrations, aneuploidy may occur through a failure to resolve catenated chromatids prior to anaphase, whereas at higher concentrations, changes in Cdk1 activity by the drug results in loss of Cdk1-related coordination of catalysis of chromosome condensation and reorganization of microtubules necessary for chromosome separation. Chromosome damage induced by agents such as amsacrine or VM-26 may lead to G2 arrest and reduction of cyclin A and Cdc2 levels, resetting the cell cycle clock to G1 phase and the ploidy to tetraploid (Ferguson *et al.*, 1996). Endomitosis is the phenomenon whereby cells become polyploid as a result of DNA replication in the absence of mitosis. Cdk1-cyclinB kinase activity is largely abolished in polyploidy megakaryocytes that have become polyploid during endoreduplication, and that this endomitosis was associated with reduced levels of cyclin B (Zhang *et al.*, 1996). Therefore, the reduction in Cdk1 activity appeared to result in polyploidy in this system, which is consistent with our observations.

The morphological events observed with fostriecin treatment including nuclear lamina depolymerization, chromosome condensation, DNA polyploidy and ultimately cell death may be a result of apoptotic versus mitotic signalling. Deregulation of the cell cycle components has been shown to induce mitotic catastrophe. For example, it has been argued that the morphology of cells undergoing apoptosis is similar to cells undergoing both normal mitosis and aberrant forms of mitosis called mitotic catastrophe. During each of the processes in mitotic catastrophe, cells release substrate attachments, lose cell

volume, condense their chromatin and disassemble the nuclear lamina (King and Cidlowski, 1995). Apoptosis, when it is a defective form of mitosis, is characterized by chromatin condensation and nuclear lamina breakdown, and can occur following G2 arrest. It has been demonstrated in thymocytes that Cdk1 is not involved in this form of apoptosis, and that chromatin condensation and lamina disassembly in apoptosis in this system occurs by signalling processes different from those that operate in mitosis (Norbury *et al.*, 1994). Lamin degradation is an early feature of apoptosis in thymocytes and this apoptosis-associated chromosome condensation and breakdown of the nuclear envelope may result from disruption of nuclear lamina architecture (Norbury *et al.*, 1994; Neamati *et al.*, 1995). For example, Neamati *et al.* (1995) demonstrated that glucocorticoid-induced degradation of lamin B in thymocytes was not accompanied by dephosphorylation-mediated activation of Cdk1. Although Yao *et al.* (1996) suggested that Cdk1 is critical for cell death mediated by Fas and ICE family proteases, this mechanism is probably not functional in this study as Cdk1/cyclin B kinase was either not present or active in the experiments described with fostriecin in this thesis. Cdk1-independent apoptotic signalling has been reported elsewhere, including the observation that overexpression of Cdk1, Cdk2, Cdk3 and cyclin A, but not cyclin B, elevates the incidence of apoptosis (Meikrantz and Schlegel, 1996). A criterion for apoptotic cells has included chromatin condensation paralleled by DNA fragmentation. Research conducted by Oberhammer *et al.* (1994) has indicated that chromatin condensation and lamina dissolution during apoptosis appears to be due to a rapid proteolysis of nuclear matrix proteins, which does not involve Cdk1.

Cells overexpressing Wee1 became resistant to apoptosis induced by granzyme B and perforin, therefore demonstrating that Wee1 rescues apoptosis by preventing Cdk1 dephosphorylation and implicating Cdk1 phosphotransferase activity in granzyme B induced apoptosis (Chen *et al.*, 1995). I propose that apoptosis induced by fostriecin treatment does not involve this mechanism, but is mediated by the state of the DNA and a Cdk1-like kinase. Chromatin condensation during apoptosis is a result of rapid proteolysis of nuclear matrix proteins, which does not involve Cdk1. During this apoptotic event, it has been observed that a 50-kDa PSTAIR-immunoprecipitable protein is highly expressed (Oberhammer *et al.*, 1994). Additionally, the 58-kDa kinase PITSLRE $\beta$ 1, a member of the

Cdc2 family, was shown to induce telophase delay, abnormal chromosome condensation (Lahti *et al.*, 1995). This protein may lie on the apoptotic-signalling pathway as it was observed that following induction of apoptosis, proteolysis of larger PITSLRE isoform resulted in an active 50-kDa kinase that was linked to these morphological phenomena. The observation that DNA degradation was delayed following fostriecin treatment may therefore be a result of an induction of apoptosis prior to this morphological change, since DNA condensation occurs initially with a delayed decondensation when the nuclear lamina is dissociated and endonuclease activity is initiated in progressed apoptosis. These events may be induced following fostriecin treatment by a Cdc2-like kinase, and does not require the phosphorylation of histone H1.

### Conclusion

Fostriecin treatment resulted in the following:

- Inhibition of protein phosphatase 2A (PP2A)
- Repression of expression of cyclins A and B
- Repression of Cdk1 activity
- Repression of an histone H2A and H3 phosphatase
- Repression of the mitotic, but not the interphase histone H1 phosphatase
- Repression of histone H1 kinase(s)
- Premature chromosome condensation
- Nuclear depolymerization and lamina dissolution
- Abnormal spindle formation
- Delayed DNA fragmentation
- DNA polyploidy
- Formation of apoptotic bodies, and later cell death.

Given these observations, a hypothesis of the events leading to cell death resulting from fostriecin treatment may be developed. I propose that the most significant effects of the treatment are the inhibition of PP2A, and repression of cyclin A and B expression. The

other observations probably resulted from these two fostriecin-induced phenomena that may be ordered such that the DNA replication and DNA damage checkpoints be abrogated. The repression of PP2A would increase the phosphorylation levels of Cdk substrates, but in fostriecin treated cells, these substrates would not be phosphorylated by Cdk1 or Cdk2. Other cell cycle progression mediating proteins would also become hyperphosphorylated, including the DNA licensing factor (such as Cdc21), nuclear lamins, and histone H3. The premature phosphorylation of histone H3 probably contributed to the premature chromosome condensation observed early following treatment, and the repression of PP2A would also trigger the release of the DNA from the nuclear matrix, as mentioned in the discussion. The later could trigger nuclear dissolution in conjunction with the phosphorylation of the lamins. Should apoptosis signalling also be triggered, perhaps as a result of the repression of cyclin A, the nuclear lamina may also be digested by apoptotic proteases such as CPP32 (Wuarin and Nurse, 1996). Although the cells were observed to enter mitosis based on morphological events including cell rounding, chromosome condensation, nuclear lamina dissolution, histone H3 hyperphosphorylation and aster formation, it cannot be concluded that the cell cycle actually progressed through G2 phase. I suggest that cell cycle progression occurs in response to fostriecin treatment, although further studies are required to validate this conjecture. Should this occur, although, it is probably the absence of a normal spindle formation that would cause the cells to fail to complete mitosis. In addition to the S phase become uncoupled with mitosis, at least in terms of chromosome and nuclear morphology, the repression of cyclin A could signal the cell to undergo transition into G1 phase and undergo continued round of DNA synthesis which would result in DNA polyploidy (Pagano *et al.*, 1992; Cotter *et al.*, 1992; King and Cidlowski, 1995). This polyploidy, coupled with the delayed DNA fragmentation observed with fostriecin would result in later apoptosis (Allera *et al.*, 1997).

Fostriecin has yet to be demonstrated that it would be effective as a chemotherapeutic agent administered alone. It can be envisioned, given its properties outlined above and its checkpoint inhibition effect on proliferating cells, that fostriecin treatment would be ideal to be administered synergistically with an agent that induces a cell cycle block due to DNA lesions, or as a result of inhibiting enzymes that are critical for

progression through S phase including topoisomerases. By inhibiting the checkpoints induced by these other agents, fostriecin would cause the cell to undergo a mitotic catastrophe. Thus, it may also be possible that lower doses of the other, often extremely toxic agent, would be required since it would only need to induce the cell cycle block (Hickman, 1992; Darzynkiewicz, 1995).

CHAPTER 4

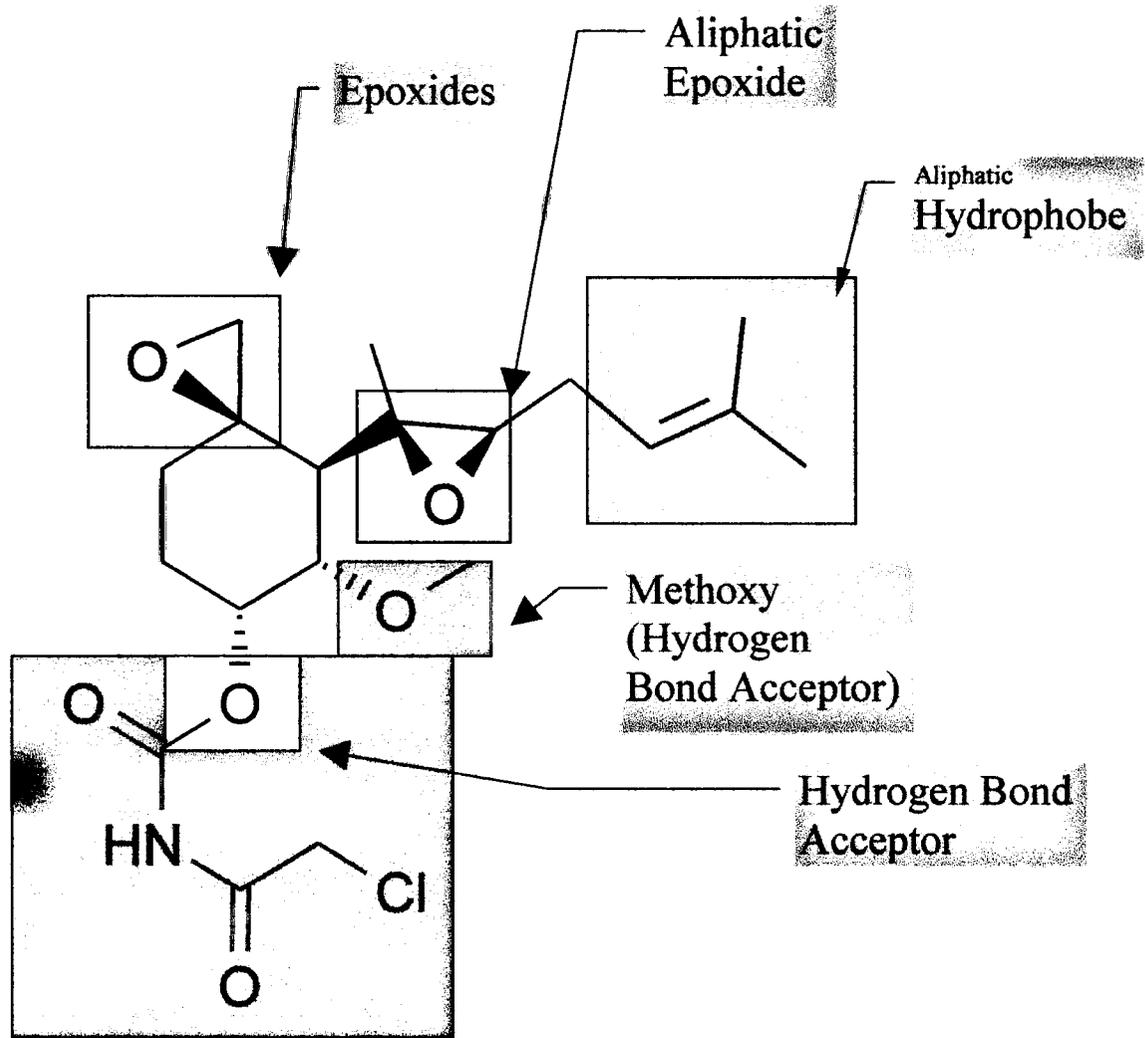
CHARACTERIZING THE ANTI-PROLIFERATION EFFECT  
OF THE ANGIOGENESIS INHIBITOR AGM-1470

## RESULTS

### AGM-1470 irreversibly inhibits the proliferation of microvascular endothelial cells

Various types of anti-angiogenesis agents have been identified based on chorioallantoic membrane (CAM) assays, which measure the inhibitory effect on capillary outgrowth, and proliferation assays specific to endothelial cells, such as the HUVEC assay. Both neovascularization and proliferation of endothelial cells are key morphological features of angiogenesis. Fumagillin analogs have a demonstrated anti-proliferative effect both *in vivo*, and *in vitro*, and this is the primary anti-angiogenic action of this class of compounds (Ito *et al.*, 1996). Their mechanism of action has yet to be determined, but their specificity for normal cells, as opposed to tumorigenic or immortalized cells (Antoine *et al.*, 1996), may reflect interference with p53 or pRb-mediated signalling. Figure 26 illustrates the molecular structure of AGM-1470, and its functional groups are highlighted. These moieties were determined to be functional based on a Structure Activity Relationship (SAR) developed from HUVEC [<sup>3</sup>H]thymidine incorporation assays (see 'Experimental Methods') and pharmacophore development based on approximately 50 analogs developed and/or synthesized at BioChem Pharma Inc. The chloroacetyl-carbamic acid moiety at the C6 does not affect HUVEC proliferation results unless alkyl substituents are used, but a strong hydrogen bond acceptor is important. Hypothetically, a hydrogen bond acceptor could be placed on the amide nitrogen or a different hydrogen bond accepting group on the adjacent carbonyl oxygen would be functional. For instance, AGM-1470 that was modified at the chloroacetyl-carbamic acid moiety to give a cyclic ketone still yielded similar HUVEC proliferation results. Other moieties of the fumagillin analogs that appear to be important for the anti-proliferative response as determined from the SAR, are the 5-methoxy, and the two epoxide functional groups. The conformations of the epoxides seems to be consistent throughout for low HUVEC IC<sub>50</sub> values. The C-4 chain (blue) with the 2'-methyl-oxyranlyl function cannot be shortened from the AGM-1470 structure, and the two methyl groups appear to be important, where their hydrophobic feature appears to be functional as determined by the SAR. A 6'-benzyl group in exchange for the dimethyl is functional, and an analog has recently been patented, (1, 2, 4, 5-di-O-isopropylidene-

# AGM-1470 Functional Groups

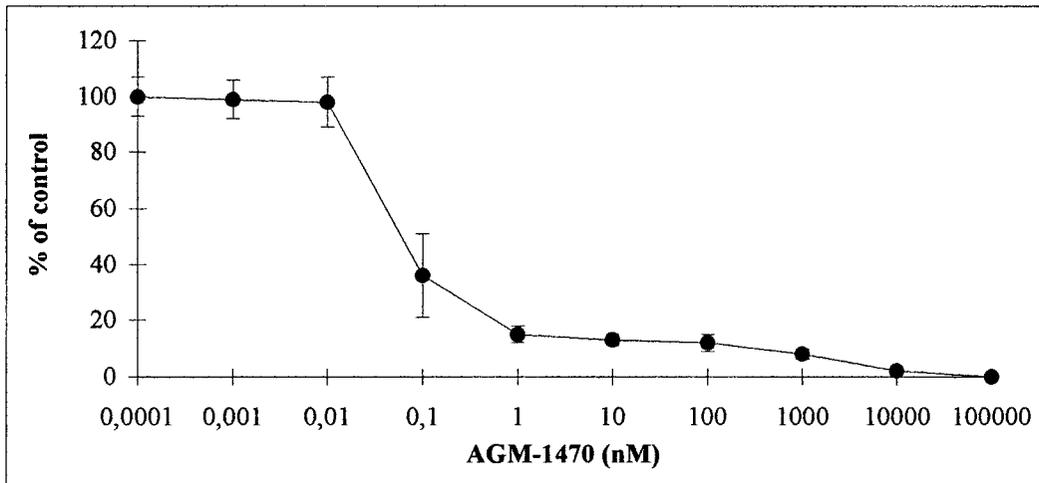


**Figure 26.** Molecular structure of AGM-1470. The structure of AGM-1470 is illustrated with the structural “hypothesis” high-lighted in different colours.

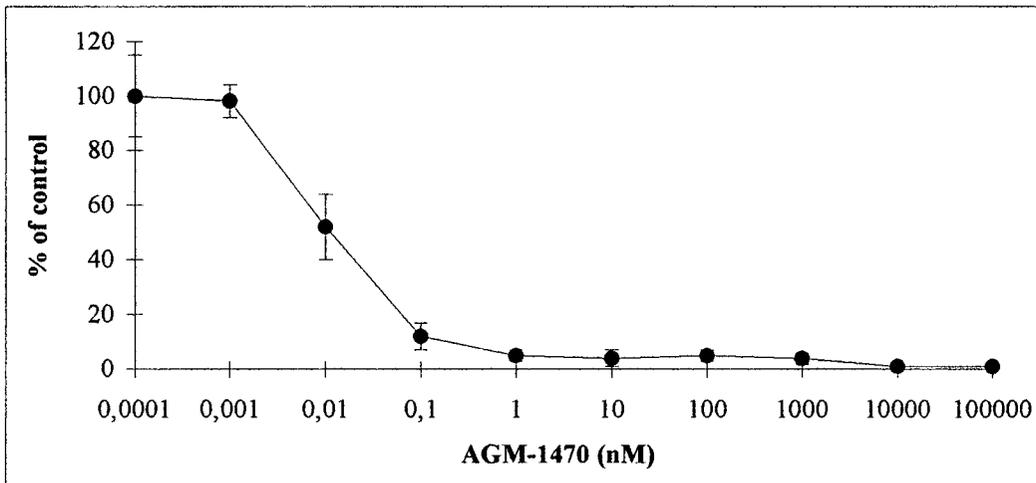
3-methyloxalyl-3-(5-phenylpent-1-yn-1-yl)- $\beta$ -D-fructopyranose) that was efficacious in CAM and HUVEC tests. The epoxide on the C4 aliphatic chain is most likely functional in terms of its acid lability, making it amenable for covalent binding to its effector. A 'hypothesis' for pharmacophore development was programmed in the *Catalyst* software (Molecular Simulations, San Diego, CA) and analogs with HUVEC proliferation  $IC_{50}$  values ranging from 10 to 80,000 pM fit perfectly into the pharmacophore developed by the software derived from all conformers with conformation energies not exceeding 10 kcal/mol. The chemical functions utilized in these hypotheses consisted of the C-6 hydrogen bond acceptor (green), the methoxy as a hydrogen bond acceptor, the epoxide groups (yellow), 1 or 2, and an aliphatic hydrophobe, therefore confirming the functionality of the chemical moieties described in Figure 26.

The structural interplay between the extracellular matrix (ECM), microtubules and microfilaments is required for the changes in cell and nuclear structure that are necessary for cellular progression (Ingber *et al.*, 1995). Indeed, ECM interaction with integrin receptors has been demonstrated to have a direct effect on intracellular signal transduction processes associated with proliferation including activation of G protein-coupled PLC $\gamma$ , pp60<sup>c-src</sup>, pp125<sup>FAK</sup>, PI 3-kinase, and urokinase (Plopper *et al.*, 1995; Chang *et al.*, 1995; Koolwijk *et al.*, 1996). To demonstrate that AGM-1470 mediates an endothelial cell proliferation inhibitory effect, HUVEC thymidine incorporation assays were conducted on a planar versus a three-dimensional model of matrix to allow for distinction between planar and a more endothelial tissue-like environment where the entire cell can be in contact with the matrix. Cells were grown on either collagen (Figure 27A) or 1 mm Matrigel<sup>®</sup> (distributed by Gibco BRL) (ECM, Figure 27B), to simulate a three-dimensional ECM environment. Presumably, should the anti-proliferative effect of AGM-1470 be mediated through the signal transduction pathway that integrates bFGF and/or VEGF, and integrin binding to ECM molecules, then the incorporation of [<sup>3</sup>H]thymidine should be reduced in the presence of Matrigel<sup>®</sup>, versus collagen, giving a lower  $IC_{50}$  value, since the three-dimensional Matrigel matrix would provide enhanced integrin binding. Matrigel<sup>®</sup> was digested with collagenase prior to harvesting the cells for scintillation counting to ensure all thymidine incorporation was detected. This method was validated prior to this

**A.**



**B.**



**Figure 27.** AGM-1470 inhibits thymidine incorporation into HUVEC growing on Matrigel® more effectively than HUVEC growing on collagen. The incorporation of [<sup>3</sup>H]thymidine (18 hours) into HUVEC following 24 hours of incubation with various concentrations of AGM-1470 after harvesting DNA is shown. Control values represent the incorporation of [<sup>3</sup>H]thymidine without drug (with vehicle, 0.1% DMSO). Asynchronously growing HUVEC were grown on collagen coated 96 well plates (**A**), or plates coated with 1 mm Matrigel® (**B**) extracellular matrix. Error bars represent the S.D. of n=3 experiments.

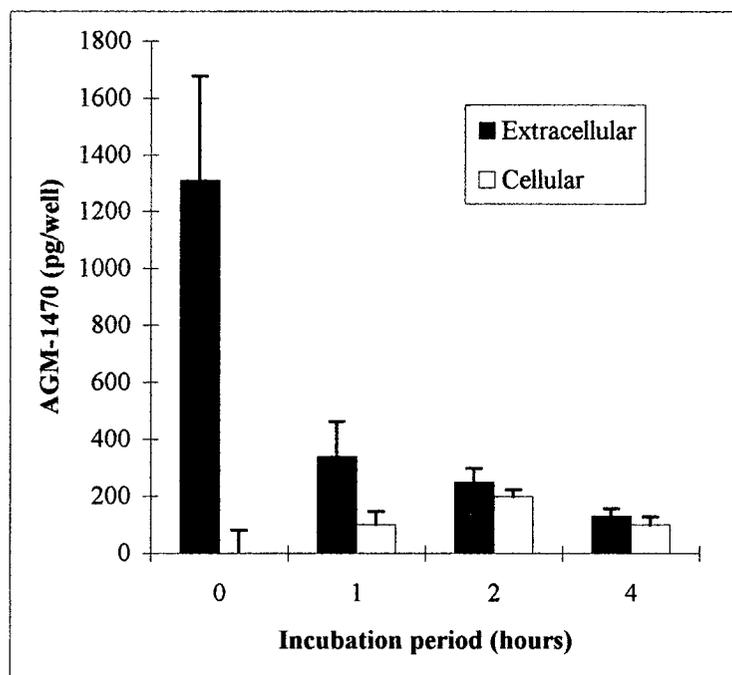
experiment in a side-by-side analysis with uncoated plates containing the same number of cells under identical conditions. HUVEC were incubated in the presence of CS-5.0 HUVEC SFM growth media containing mitogen (10 ng/ml VEGF and 5 ng/ml bFGF) (from Cell Systems Co.) and a final concentration of AGM-1470 from  $10^{-13}$  M to  $10^{-4}$  M. After 24 hours, [ $^3$ H]thymidine was added, and cells were incubated for a further 18 hours prior to washing and harvesting. All experiments were conducted 4 times except for Matrigel<sup>®</sup> covered plates, which was done in duplicate because of the cost of the ECM. Figure 27 illustrates that the presence of additional ECM molecules on the surface of the wells versus collagen alone reduces the IC<sub>50</sub> of AGM-1470 approximately 8-fold. [ $^3$ H]Thymidine incorporation was approximately 25% at  $10^{-11}$  M AGM-1470 on Matrigel<sup>®</sup> coated plates, indicating AGM-1470 has a greater effect when cells are incubated in a three-dimensional ECM environment. The necessity for the presence of growth factor was further verified via measurement of the change of extracellular acidification rates in the presence of AGM-1470 +/- growth factors (10 ng/ml VEGF and 5 ng/ml bFGF). HUVEC were seeded in gelatin-coated Capsule Cups (Molecular Devices product) at a density between 80-120,000 cells per cup, and were allowed to incubate for 44 hours prior to loading onto the Cytosensor<sup>®</sup> Microphysiometer (from Molecular Devices). Cells were bathed by a modified RPMI medium (Molecular Devices product) that was supplemented with 1 mg/ml human serum albumin (not shown here). The acidification rate profile did not resemble that of one produced by AGM-1470 pretreated-proliferating HUVEC cells, unless the growth factors were present. These results indicate that AGM-1470 mediates its inhibitory effect on the proliferation of HUVEC cells through the mechanism that is dependent on matrix binding and the presence of soluble growth factors bFGF and VEGF.

HPLC analysis of extracellular and intracellular AGM-1470 in human hepatocytes following incubation at 1 minute, 1 hour and 4 hours was conducted. Initial AGM-1470 metabolic studies in hepatocytes were conducted with radiolabeled (tritiated) AGM-1470 and an HPLC mobile phase consisting of potassium phosphate (Placidi *et al.*, 1995). Additionally, only the soluble fraction of the cell lysate was analyzed following centrifugation. Since radiolabeled AGM-1470 was not available, and given the absence of

an effective U.V. chromophore on the molecule, various protocols were assessed for optimal detection. Reproduction of the method described by Placidi *et al.* (1995) that described the half-life of AGM-1470 as 10.4 min did not provide sufficient sensitivity for cold compound. Ammonium acetate (2 mM, pH 5.0) was used in the mobile phase with acetonitrile as described in 'Experimental Methods.' This method allowed for reproducible detection of 25 ng of drug. NP-40 (1%) and SDS (0.2%) were added to the pellet to solubilize compound that may be present in the particulate fraction that would not have been detected in the soluble fraction alone. A peak at 30.6 min was resolved that was not detected when mobile phase alone was injected (data not shown). This peak was determined to be AGM-1470 (1.44  $\mu$ g) based on analysis with AGM-1470 in acetonitrile alone. Following 4 hours of incubation, significant metabolism and uptake of the drug was observed. The presence of AGM-1470 at 1 and 4 hours of incubation was detected showing that contrary to earlier findings (Placidi *et al.*, 1995), AGM-1470 was detectable in the intracellular fraction. Analysis of extracellular and intracellular AGM-1470 over 1 min to 4 hours after exposure to human cultured hepatocytes is illustrated in Figure 28, where it is shown that although metabolism of AGM-1470 did occur, it was not completely metabolized, and was detectable in the intracellular fraction for at least 4 hours. I propose that our observations differ from those of Placidi *et al.* (1995) due to the inclusion of the detergent solubilized fraction, and because AGM-1470 probably bound to a protein that was present in the particulate fraction.

The observation that AGM-1470 binds to a protein that has been demonstrated to be present in the membrane (Yoshida *et al.*, 1998), and that AGM-1470 is found in the particulate fraction indicates that it is possible that the antiproliferative effect of AGM-1470 may be irreversible (Figure 28), or affect the proliferating cell when it is in a particular phase of the cell cycle. To test this conjecture, I carried out a kinetic study in which [<sup>3</sup>H]thymidine labeled cells were pretreated with 5 nM AGM-1470 for various periods of time. A concentration of 5 nM was chosen, since it was sufficient to inhibit greater than 80% of the thymidine incorporation as shown in Figure 29A. To date, no pulse-chase experiments have been reported to determine the effects of shorter periods of drug incubation followed by a wash prior to the addition of [<sup>3</sup>H]thymidine. Such

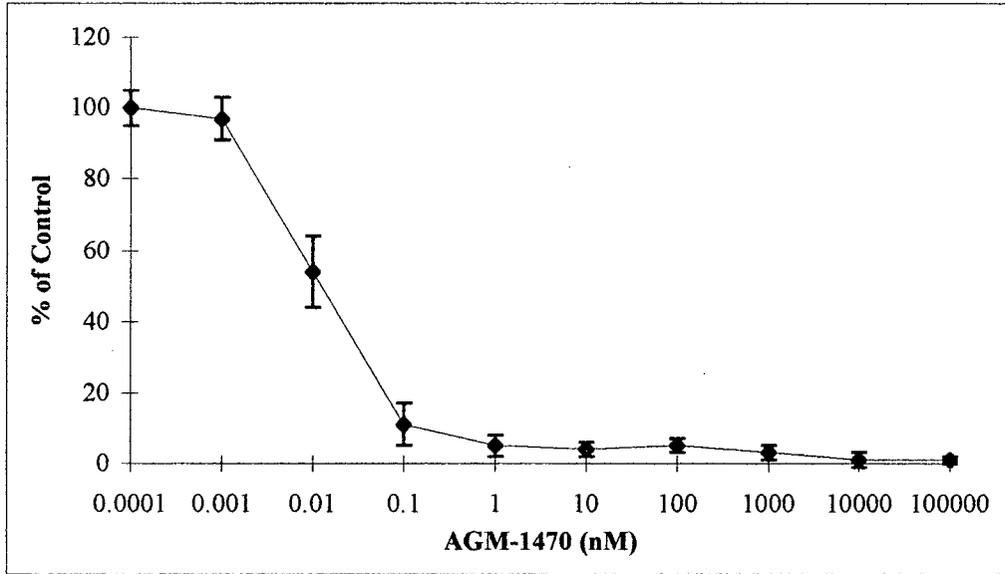
experiments are fruitful in elucidating whether the drug's effect is reversible and gives further insight into the kinetics of the compound of interest. To determine the incubation period kinetics, a [<sup>3</sup>H]thymidine incorporation assay on 96 well plates was done where all cells were treated with AGM-1470 at the same time, and at various periods following the drug charging, cells were washed 3 times with fresh media and incubated with media without drug. Following 15 min to 32 hours to of drug incubation, [<sup>3</sup>H]thymidine was also added to the wells (except control without) for a period of 8 or 16 hours. The plates were centrifuged at 500 rpm for 5 min to allow for any mitotic cells, which may have begun proliferating should the drug not be irreversible, to bind to the plate prior to harvesting. Mitotic cells are known to obtain a rounded morphology and hence are prone to becoming suspended. Figure 29B illustrates the degree of thymidine incorporation compared to cells not treated with AGM-1470 but incubated with [<sup>3</sup>H]thymidine at the 24 hour period (illustrated in Figures 27 and 29A). Thymidine incorporation was for 16 hours in these experiments. Similar results were obtained when the thymidine incubation prior to cell harvest was decreased to 8 hours (data not shown). These results demonstrate that a period of only 15 min was necessary for the antiproliferative effect of AGM-1470 when added to asynchronously growing cells. Additionally, the antiproliferative effect of a 15 min incubation with 50 nM AGM-1470 was sustained for at least 2 cell cycles (21.3 hours per cell cycle). This was verified in another experiment where asynchronously growing HUVEC were incubated with AGM-1470 for periods of 15 min to 32 hours and then washed and incubated in fresh drug-free medium for the period of 32 hours less the incubation period, prior to the subsequent 16 hour incubation with [<sup>3</sup>H]thymidine. Similar results to those discussed above and illustrated in Figure 29B were observed in these experiments, i.e., the cells were unable to replicate their DNA (not shown).



**Figure 28.** AGM-1470 is stable in primary human hepatocytes following at least 4 hours of incubation following initial degradation and metabolism. AGM-1470 separated by HPLC from the extracellular medium (dark bars) or detergent-solubilized extracts (white bars) of cells treated as described in Figure 29 (for 0, 1, 2 and 4 hours in the presence of 50  $\mu$ M AGM-1470), was quantified against a standard curve, and the measure of drug in each fraction for the indicated periods is shown. The results represent the mean of two experiments, and error bars represent the difference of the means.

**Figure 29.** AGM-1470 inhibits HUVEC proliferation with only a 15 min pulse incubation. **(A)** [<sup>3</sup>H]Thymidine incorporation into HUVEC cells following 16 hours of incubation with various concentrations of AGM-1470, followed by 16 hours of [<sup>3</sup>H]thymidine prior to harvesting the DNA and assessment. **(B)** The results of a kinetics study where asynchronously growing HUVEC cells were treated with 5 nM AGM-1470 for periods between 15 min and 32 hours is presented. Cells were subsequently washed and re-incubated with drug-free complete media in the presence of [<sup>3</sup>H]thymidine for 16 hours. The results are presented as the percentage of [<sup>3</sup>H]thymidine incorporation compared to HUVEC cells incubated with [<sup>3</sup>H]thymidine for 24 hours (100%). Error bars represents the S.D. of n=3 experiments.

**A.**



**B.**

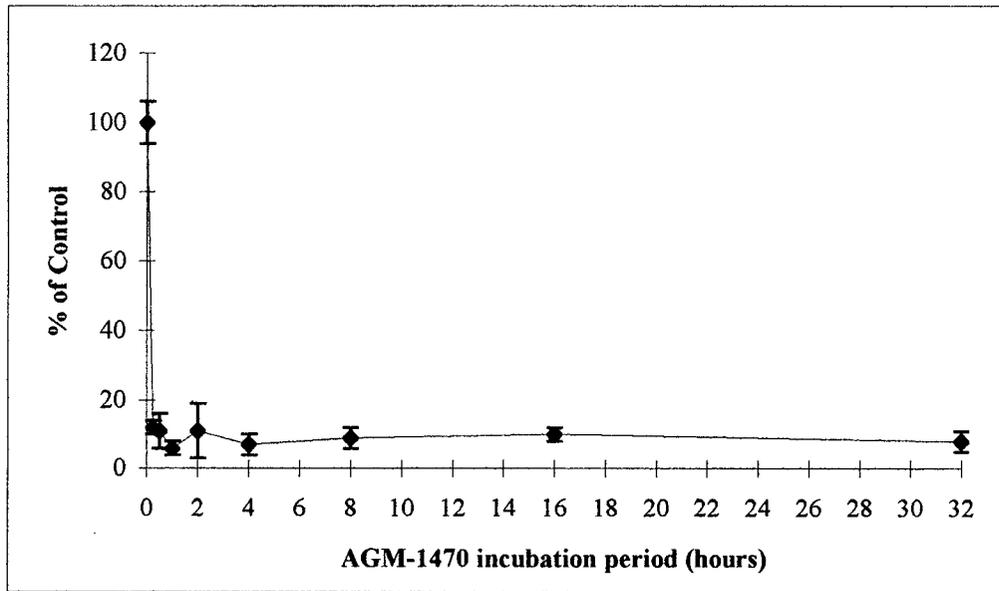
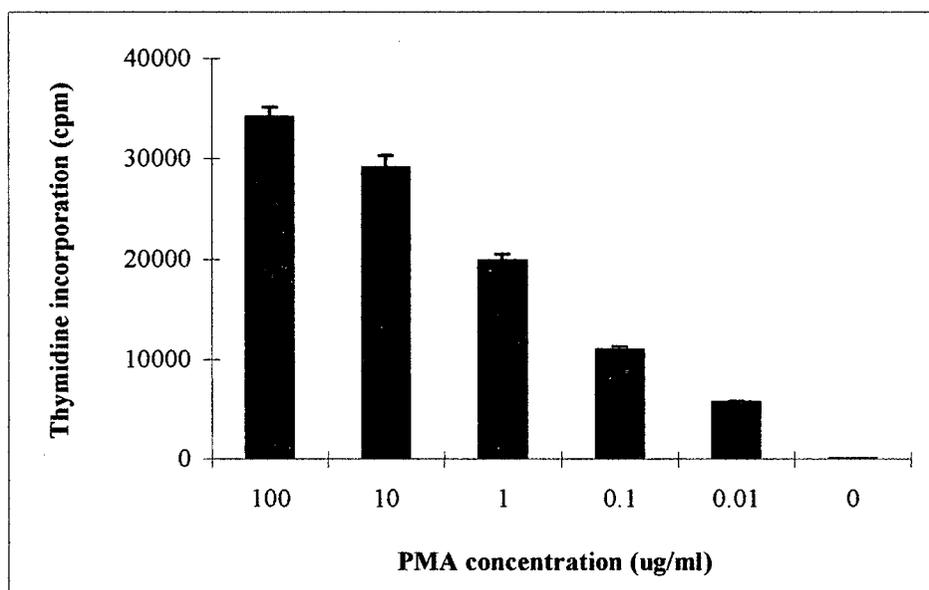


Figure 29.

*PMA rescues the antiproliferative effect of AGM-1470 pretreatment in HUVEC cells*

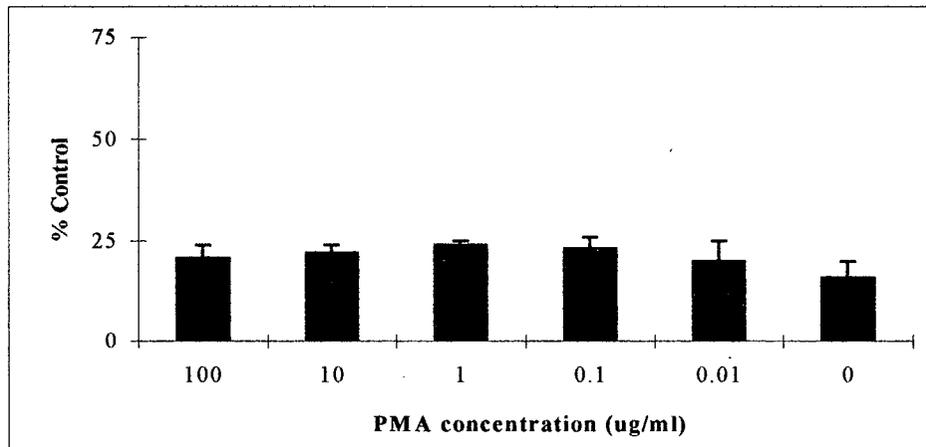
It was demonstrated that a 15 minute incubation with 5 nM AGM-1470 with subsequent washing and re-incubation with drug-free media and [<sup>3</sup>H]thymidine for 16 hours resulted in an inhibition of thymidine incorporation (proliferation). We hypothesized that AGM-1470 may be specifically acting at the cell membrane or a site proximal to the cell membrane given our results in the HPLC analysis of AGM-1470 (Figure 28). This was investigated by measuring the effects of AGM-1470 on the protein kinase PKC. PKC has been demonstrated to mediate mitogen-stimulated signal transduction that leads to DNA synthesis in HUVEC cells (Zhou *et al.*, 1993). PKC has been further implicated in the activation of angiogenesis (Wright *et al.*, 1992; Chang *et al.*, 1995; Stoltz *et al.*, 1996). PMA, which activates PKC directly by substituting for diacylglycerol with greater affinity and having greater stability (Newton, 1995), has been shown to promote capillary tube formation which is subsequently inhibited with PKC inhibitors (Wright *et al.*, 1992). To determine if AGM-1470 acts within the PKC pathway, HUVEC were treated with 5 nM AGM-1470 for 30 min followed by a 1 hour incubation period with various concentrations of PMA (Figure 30). Cells were analyzed for [<sup>3</sup>H]thymidine incorporation as before. It was observed that PMA was able to rescue approximately 68% (at 100 ng/ml) of the AGM-1470 induced reduction in thymidine incorporation in a concentration dependent manner. This data implies that the activation of PKC by PMA overrides the proliferation inhibitory mechanism of AGM-1470. The observation that PMA 're-activated' PKC that was down-regulated by AGM-1470 pretreatment does not solely imply that PMA was inducing re-synthesis of PKC's since PKC can be regulated by other mechanisms including membrane translocation and phosphorylation (Mellor and Parker, 1998; Ohmori *et al.*, 1998). The next section in this chapter illustrates this further, but PMA induction of PKC subsequent AGM-1470 treatment was not regulated via protein expression as Western analysis with antibodies to PKC of extracts from cells treated with and without subsequent phorbol ester treatment revealed no change in PKC expression (data not shown).

When HUVEC were treated with PMA for 30 min prior to the addition of AGM-1470 (Figure 31A), the inhibition of thymidine incorporation was not titrated out by

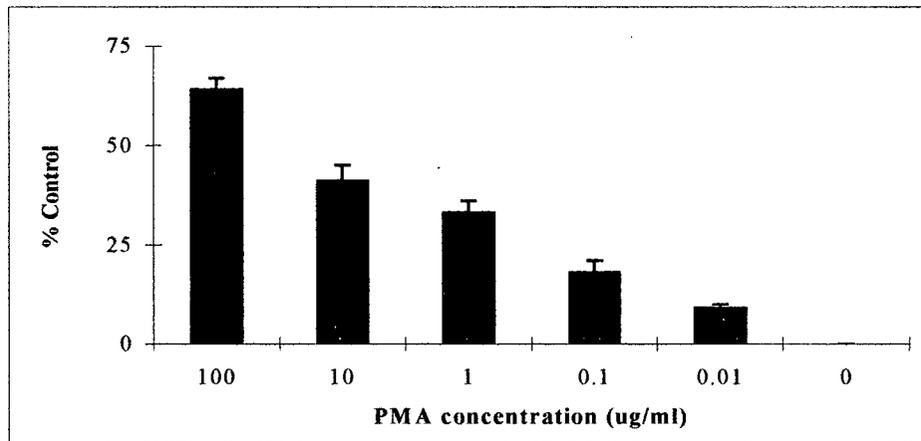


**Figure 30.** PMA rescues the antiproliferative effect of AGM-1470 preincubation. HUVEC pre-incubated with 5 nM AGM-1470 for 30 min were titrated with PMA from 0.01 to 100  $\mu\text{g/ml}$  for 1 hour followed by [ $^3\text{H}$ ]thymidine for 16 hours. Cells were harvested and monitored for thymidine incorporation. The 0 time point represents HUVEC treated with AGM-1470 for 1.5 hours prior to charging with [ $^3\text{H}$ ]thymidine without PMA treatment. Error bars represent the S.D. of n=3 experiments.

A.



B.



**Figure 31.** PMA pretreatment does not result in a complete rescue of HUVEC anti-proliferation induced by AGM-1470. (A) HUVEC were treated with 0-100  $\mu\text{g/ml}$  PMA for 30 min followed by a 1 hour incubation with 5 nM AGM-1470 and 16 hours of [ $^3\text{H}$ ]thymidine prior to harvesting and assessment of thymidine incorporation. The 0 PMA represents [ $^3\text{H}$ ]thymidine incorporation into cells treated with 1  $\mu\text{g/ml}$  PMA without subsequent AGM-1470 treatment. (B) The effect of incubation for 1 hour with 0-100  $\mu\text{g/ml}$  PMA incubation in cells pretreated with AGM-1470 for 30 min was determined, similar to Figure 30, but [ $^3\text{H}$ ]thymidine incorporation was allowed to proceed for only 8 hours. Results are presented as the percentage of [ $^3\text{H}$ ]thymidine incorporation compared to cells incubated with [ $^3\text{H}$ ]thymidine without drugs (Control). Error bars represent the S.D. of  $n=3$  experiments.

PMA as observed with AGM-1470 pretreatment (Figure 30 and 31B). An explanation for this observation might be that AGM-1470 acts either upstream of PKC or induces the transcription or translation of a protein that lies upstream of PKC, such as through the activation of the MAP kinase pathway. This is consistent with the interpretation of the previous figure. Phorbol ester is known to be resistant to washing and to bind tightly to PKC, and additionally, induction via phorbol ester is irreversible (Ohmori *et al.*, 1998). But the observation in Figure 31A that degree of thymidine incorporation was similar, approximately 23%, under all experimental conditions, and greater than that of HUVEC cells treated with AGM-1470 alone (0  $\mu\text{g/ml}$  PMA, Figures 30 and 31B), indicates that there may be a function of PMA induction of PKC activity specific to endothelial cell proliferation. In an experiment where HUVEC were pretreated with PMA for 4 hours prior to AGM-1470 incubation (or not) demonstrated results similar to those illustrated in Figure 33A (data not shown). Therefore, the ability of PMA to rescue the HUVEC pretreated with AGM-1470 was a result of the activation of PKC. Since PMA pretreatment prior to AGM-1470 incubation yielded results that were similar with or without subsequent AGM-1470 treatment suggests that the initial transient activation of PKC in endothelial cells may be a cycle specific event with regards to mediating DNA synthesis.

#### AGM-1470 inhibits PKC in proliferating HUVEC cells

PKC was immunoprecipitated with an affinity purified polyclonal antibody directed toward the catalytic domain of PKC isoforms, from lysates prepared from asynchronously growing HUVEC treated with 5 nM AGM-1470, or with 0.5  $\mu\text{g/ml}$  PMA for 4 hours as a control. Prolonged treatment of cells with PMA results in downregulation of PKC (Malarkey *et al.*, 1996) except for the PKC $\delta$  isoform (Bharti *et al.*, 1998; Ohmori *et al.*, 1998). Histone H1 phosphotransferase activity of the immunoprecipitated PKC was determined in the presence of [ $\gamma$ - $^{32}\text{P}$ ]ATP and magnesium with the PKC cofactors phosphatidylserine (PS), the diacylglycerol analog diolein (DO), and calcium to measure  $\text{Ca}^{2+}$ -dependent PKC isoenzyme activity; PS and DO to measure  $\text{Ca}^{2+}$ -independent PKC isoenzyme activity, or PS and calcium as a negative control (Figure 32A). Asynchronous proliferating HUVEC cells (control) not treated with drug were observed to have active

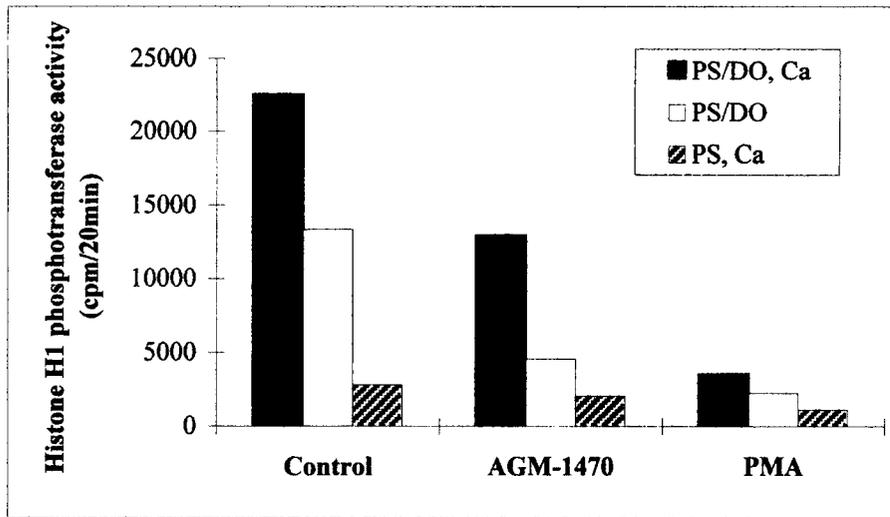
calcium-dependent and independent PKC histone H1 phosphotransferase activity. Treatment with PMA resulted in almost complete inhibition of both isoforms of PKC, as expected. Pretreatment with AGM-1470 for 1 hour resulted in an equivalent reduction of histone H1 phosphotransferase activity of both isoforms, with the calcium-independent isoform being almost completely lost, indicating that AGM-1470 pretreatment induced an abrogation of a calcium-independent isoform of PKC.

Following MonoQ fractionation of HUVEC lysates from cells treated without or with 5 nM AGM-1470 for 2 hours, or PMA for 4 hours, a peak of histone H1 phosphotransferase activity was detected in lysates from cells not treated with AGM-1470 or PMA (Figure 32B). Both of which were shown to mediate inhibition of PKC activation as shown in the immunoprecipitation assay (Figure 32A). To distinguish whether this histone H1 phosphotransferase peak represented PKC activity, or the activity of another kinase that co-eluted and phosphorylated histone H1, each fraction was assayed in the presence of the PKC cofactors phosphatidylserine, the diacylglycerol analog diolein, and calcium, or magnesium alone. The conventional PKC isoenzymes  $\alpha$ ,  $\beta$ ,  $\beta$ II and  $\gamma$ , have been shown to require phosphatidylserine, diacylglycerol, and calcium, whereas the non-conventional PKC isoforms  $\delta$ ,  $\epsilon$ ,  $\eta$ , and  $\theta$  isoforms are calcium-independent and require only phosphatidylserine and diacylglycerol. The other, atypical PKC isoforms are not activated by DAG and do not require calcium as a cofactor (Malviya and Black, 1993; Nishizuka, 1995). Therefore, the histone H1 kinase activity was fractionated with this methodology, and both prolonged incubation with PMA or AGM-1470 pretreatment, inhibited the PKC histone H1 phosphotransferase activity. The presence of PKC in fractions 24-27 was further confirmed following SDS-PAGE and Western analysis using a monoclonal antibody to the catalytic domain of PKC (Kinetek Pharmaceuticals Inc., Vancouver, BC). Western blotting analysis revealed a protein band that migrated with an apparent molecular mass of approximately 74 kDa (not shown). PKC $\delta$  has been described to have the same apparent molecular mass (Hug and Sarre, 1993). Therefore we reprobated the membrane with an anti-PKC $\delta$  antibody (Gibco BRL) and detected the presence of the calcium-independent PKC $\delta$  isoform (Figure 32C). These results indicate that AGM-1470 inhibited PKC in proliferating HUVEC, and more specifically, pretreatment with

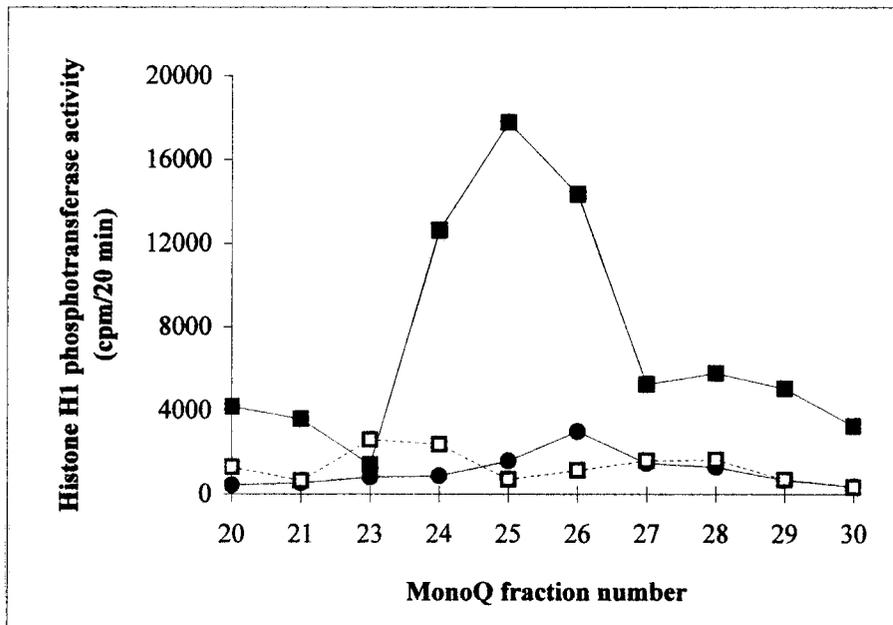
AGM-1470 inhibited a calcium-independent isoenzyme, which we identified as PKC $\delta$ , as determined by immunoprecipitation, and phosphotransferase assays following anion exchange chromatography and Western analysis. Immunodepletion experiments would be necessary to prove that this PKC isoform is exclusively responsible for mediating the proliferation effect in HUVEC that is abrogated in response to AGM-1470 treatment.

**Figure 32.** AGM-1470 inhibits PKC $\delta$  in proliferating HUVEC cells. **(A)** The effects of 5nM AGM-1470 treatment (1 hour) on PKC activity in HUVEC was assessed. PKC was immunoprecipitated from lysates prepared from asynchronously growing cells (control), cells treated with AGM-1470, or with 0.5  $\mu$ g/ml PMA for 4 hours (results in down-regulation of PKC), with a monoclonal antibody having affinity for the catalytic domain of PKC (Kinetek Pharmaceuticals, Inc.). Histone H1 phosphotransferase activity was determined in the presence of [ $\gamma$ - $^{32}$ P]ATP and magnesium with the PKC cofactors phosphatidylserine (PS), diolein (DO), and calcium (Ca $^{2+}$ -dependent PKC isoforms), PS and DO (Ca $^{2+}$ -independent isoforms), or PS and calcium (negative control). **(B)**, MonoQ fractions from extracts derived from cycling HUVEC cells (closed squares), cells treated with AGM-1470 for 2 hours (open squares) or with PMA for 4 hours (closed circles) were incubated with [ $\gamma$ - $^{32}$ P]ATP with histone H1 in the presence of PS, DO, calcium and magnesium. **(C)**, Fractions from **(B)** (cycling cells; closed squares) were subjected to SDS-PAGE and Western analysis with the monoclonal anti-PKC $\delta$  antibody (Gibco BRL). The values represent the mean of two experiments.

**A.**



**B.**



**C.**

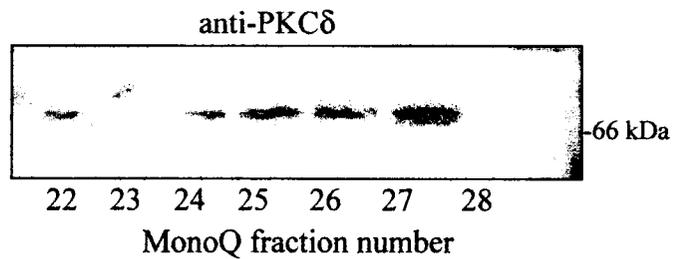


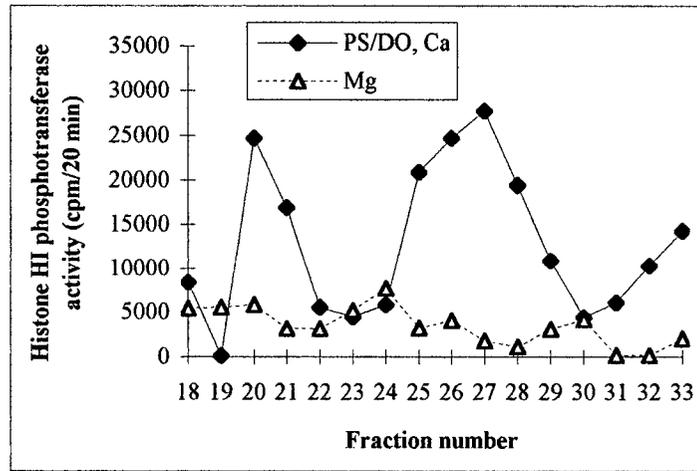
Figure 32.

The observation that PMA rescued the antiproliferative effect of AGM-1470 pretreatment, presumably through activation of PKC, was revisited. MonoQ fractionation of HUVEC-treated without, or with 5 nM AGM-1470 for 1 hour followed by a 30 min incubation with PMA (0.5  $\mu\text{g/ml}$ ) was conducted (Figure 33). PKC activity in the resulting fractions was determined by analyzing the histone H1 phosphotransferase activity in the presence of the PKC cofactors PS, DO and calcium, or magnesium alone (negative control), identical to the conditions described in Figure 32. Figure 33B illustrates that the PKC histone H1 phosphotransferase peak observed between fractions 24 and 27 in Figure 32B and 33A was inhibited by AGM-1470. Figure 33C shows that incubation of AGM-1470 pretreated HUVEC with PMA resulted in the activation of PKC. This confirmed that PMA induced PKC activity in AGM-1470 pretreated cells, permitting their proliferation that was inhibited by AGM-1470.

PKC $\delta$  has been implicated in the proliferation of cells, particularly because of its ability to induce the expression of cyclin D (Fukumoto *et al.*, 1997). The regulation of this isoform has therefore been the subject of numerous studies, and its activation has been shown to be regulated by several methods. These methods include G protein-coupled receptor-induced hydrolysis of phospholipids (Iredale *et al.*, 1993), phosphorylation (Li *et al.*, 1994a), translocation (Ohmori *et al.*, 1998) and by direct activation by phorbol ester (Castagna *et al.*, 1982; Li *et al.*, 1994b). All the methods of PKC $\delta$  induction are transient except for phorbol ester activation, which is irreversible (Ohmori *et al.*, 1998), and has been shown to mediate PKC $\delta$  activation via slow-translocation (Ohmori *et al.*, 1998) or tyrosine phosphorylation (Li *et al.*, 1994b). Ubiquitously expressed PKC $\delta$  is unique among PKC isoforms as a substrate for tyrosine phosphorylation (Li *et al.*, 1997), and this phosphorylation seems to be related to cell proliferation and differentiation (Watanabe *et al.*, 1992). Therefore, PKC $\delta$  induction by PMA is probably mediated via phosphorylation and/or phospholipid breakdown since changes in PKC $\delta$  expression in response to PMA treatment was not observed. Nishizuka and associates (1995) have already demonstrated that PKC $\delta$  activation is mediated via a synergistic mechanism which involves in addition to phospholipid breakdown, tyrosine kinase signalling (Nishizuka *et al.*, 1995).

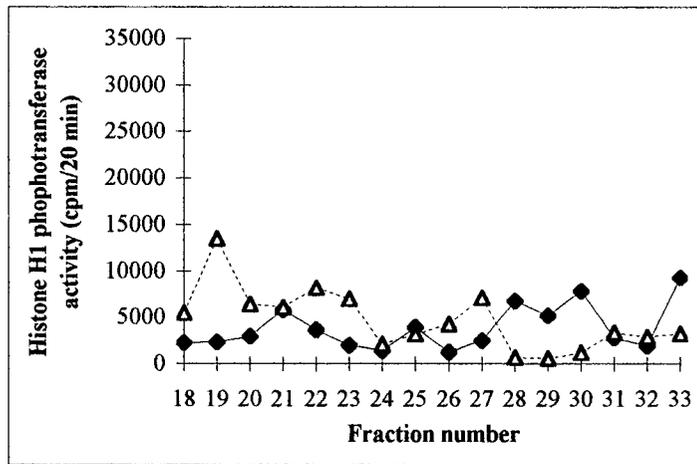
**Figure 33.** AGM-1470 inhibition of PKC activity in proliferating HUVEC cells is rescued with PMA. MonoQ fractionation was conducted on detergent solubilized extracts from asynchronously growing HUVEC cells (A) treated with 5 nM AGM-1470 alone (B), or from cells pretreated with AGM-1470 (1 hour) followed by 30 min incubation with PMA (0.5  $\mu$ g/ml) (C). PKC activity was determined by analyzing the histone H1 phosphotransferase activity in the presence of the PKC cofactors PS, DO, calcium and magnesium, or magnesium alone (negative control; triangle). The PKC peak was represented between fractions 25-28 as determined in Figure 35.

**A.**



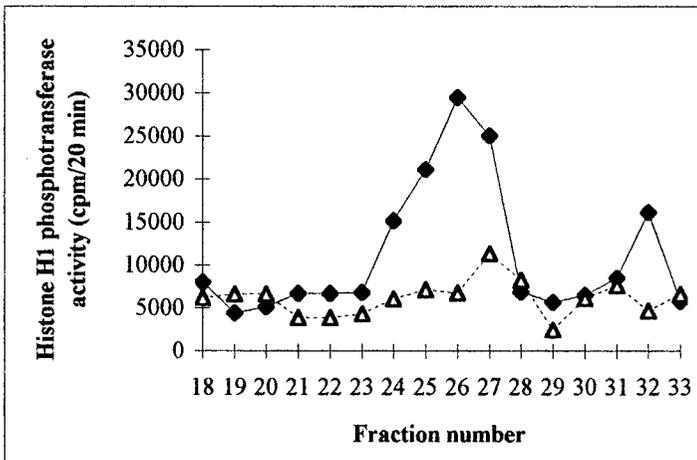
**Asynch.  
HUVEC**

**B.**



**+ AGM-1470  
1 hr**

**C.**

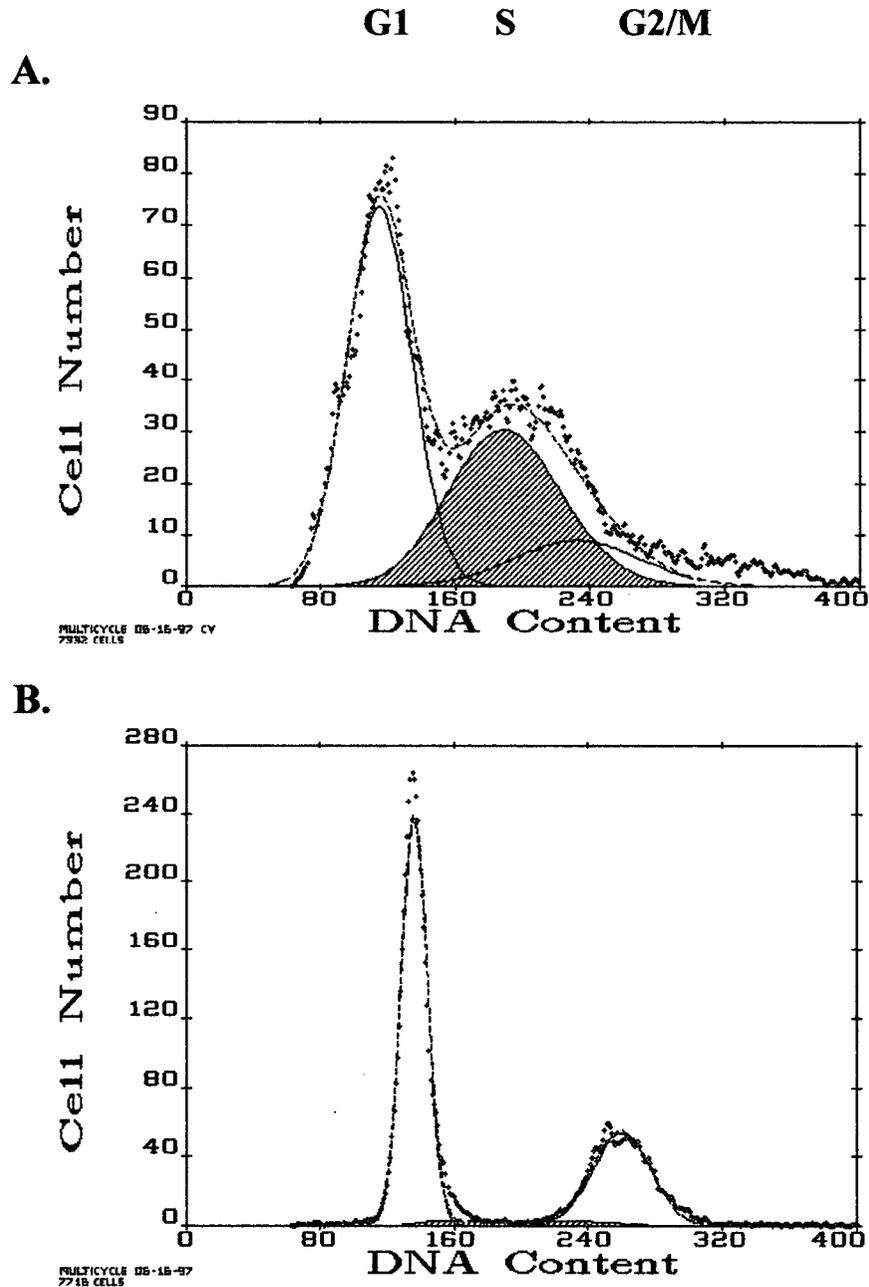


**1. AGM 1 hr  
2. PMA + 30 min**

Figure 33.

AGM-1470 regulates proliferating HUVE cells in G1 phase of the cell cycle

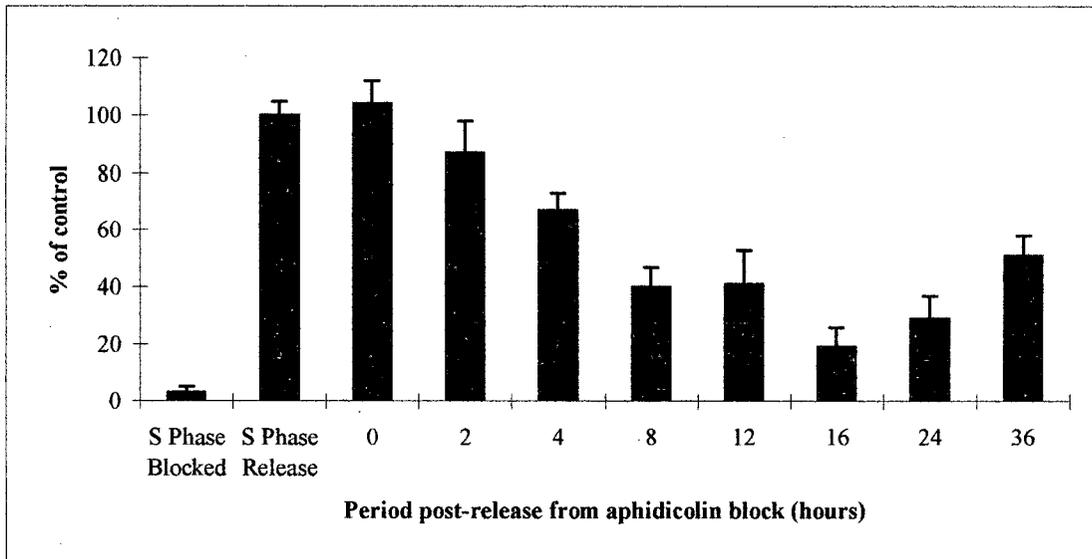
To determine whether AGM-1470 selectively affected cells when they are in a particular phase of the cells cycle, HUVEC were synchronized in S phase with aphidicolin and released into the cell cycle by washing and re-incubation in complete media without drug. At various periods following release, cells were treated with 5 nM AGM-1470 for 30 min followed by washing and re-incubation in drug-free media containing [<sup>3</sup>H]thymidine for 16 hours prior to harvesting the DNA and assessing incorporation of thymidine as a measure of proliferation. Figure 34A illustrates that aphidicolin treatment effectively caused a significant number of cells (approximately 70%) to be blocked at G1/S phase. Cell cycle distribution of HUVEC released from this block for 16 hours (also determined by flow cytometry) is illustrated in Figure 34B and shows that a predominant proportion of cells are in G1 and G2 phase, with few cells in S phase. Figure 35A illustrates the resulting incorporation of [<sup>3</sup>H]thymidine into HUVEC following AGM-1470 treatment initiated at various phase of the cell cycle subsequent to release from the aphidicolin-induced S phase block. Cells that were treated with AGM-1470 while at S phase were observed to incorporate thymidine normally, but the degree of incorporation diminished significantly as cells entered G1 phase (16 hours post-treatment, Figure 34B). Cells that were blocked at S phase did not incorporate thymidine. These results indicate that inhibition of proliferation of HUVEC following a 30 min pulse AGM-1470 incubation was cell cycle dependent. Figure 35B illustrates the results of 16 hour thymidine incorporation into cells that were treated for 1 hour with AGM-1470 at a specific phase of the cell cycle. HUVEC in G1 phase appeared to be sensitive to AGM-1470.



**Figure 34.** S phase synchrony of HUVEC following aphidicolin treatment. (A) Asynchronously growing HUVEC were treated with 2.5  $\mu\text{g/ml}$  aphidicolin in complete medium for 16 hours. Cells were fixed and stained with propidium bromide and the cell cycle distribution was analyzed via flow cytometry. Cells released from the S phase block (shaded region) in the presence of complete medium without drug for 16 hours (B) were fixed, stained and analyzed similar to (A).

**Figure 35.** AGM-1470 mediates its anti-proliferative effect when cells are in G1 phase of the cell cycle. **(A)** HUVE cells synchronized to S phase with aphidicolin as described in Figure 35 were released following washing and re-incubation in complete media without drug. At various periods following release (shown on *x-axis*), cells were treated with AGM-1470 for 30 min, followed by washing and re-incubation in drug-free media charged with [<sup>3</sup>H]thymidine for 16 hours prior to harvesting the DNA and assessing incorporation of thymidine. Values are presented as the percentage of [<sup>3</sup>H]thymidine incorporation compared to cells released from the aphidicolin block for 16 hours without drug (S Phase Release) (100 %, Control). Cells incubated with [<sup>3</sup>H]thymidine for 16 hours without release is presented (S Phase Blocked). **(B)** HUVE cells synchronized in S phase and released until they were detected via flow cytometry (not shown) to be at the cell cycle phase depicted on the *x-axis* were treated with AGM-1470 (5 nM) for 1 hour with subsequent incubation with [<sup>3</sup>H]thymidine for 16 hours. Error bars represent the S.D. of the mean of n=3 experiments.

**A.**



**B.**

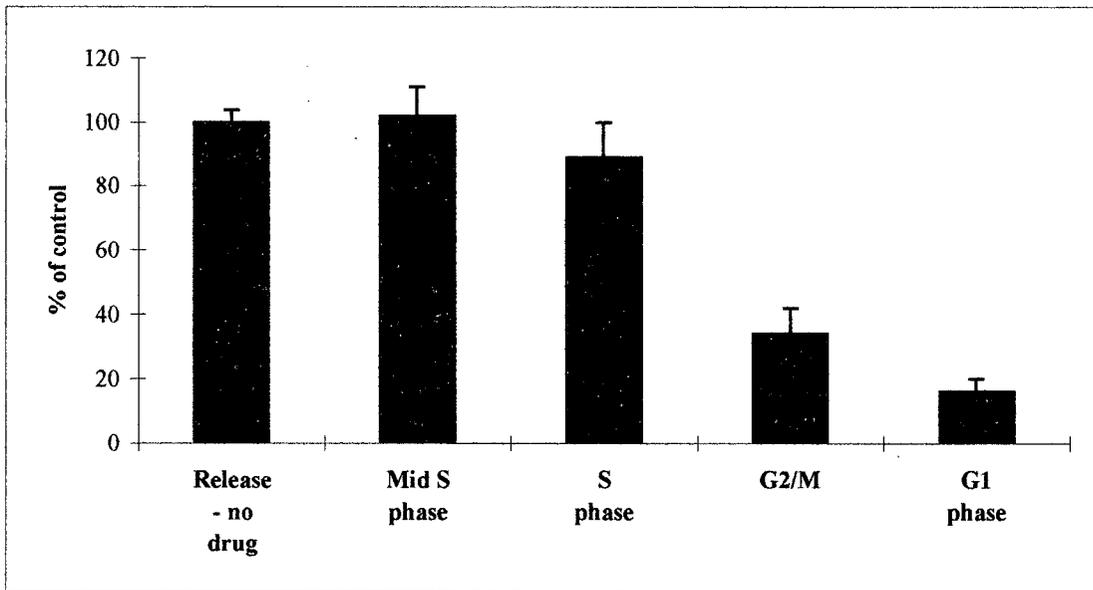
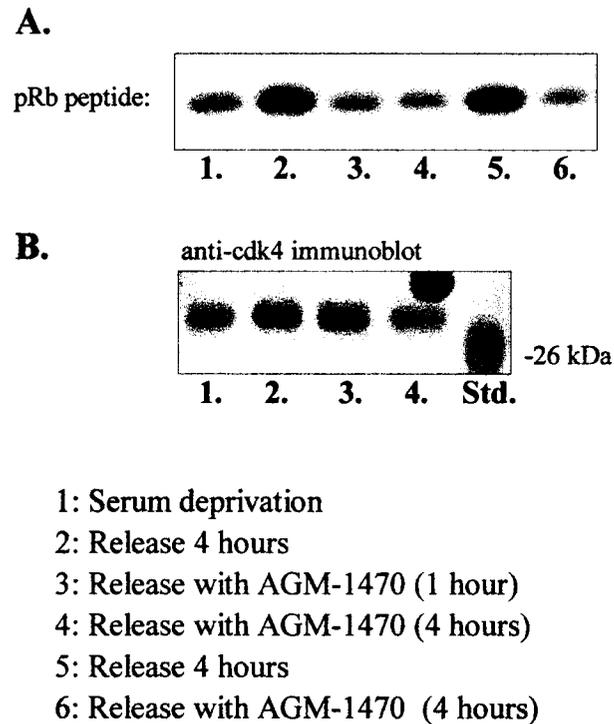


Figure 35.

AGM-1470 inhibits the cdk4-cyclin D complex

The mechanism(s) of AGM-1470 action is not yet clear other than its ability to inhibit proliferation by inducing a G1 phase block due to suppression of cyclin D expression, as was observed in HUVEC cells and AGM-1470 sensitive WiDr human tumour cells following incubation with the drug (Hori *et al.*, 1994). Cdk4-cyclin D complexes phosphorylate the retinoblastoma protein (pRb) in early G1 phase, prior to activation of the Cdk2-cyclin E complex. This mechanism regulates G1/S phase progression. Should AGM-1470 cell incubation result in abrogation of activity of this complex, this would demonstrate that inhibition of proliferation following AGM-1470 treatment occurs in early G1 phase of the cell cycle, and further confirm the cell cycle specificity of this compound in proliferating endothelial cells. Additionally, if Cdk4 activity is repressed following AGM-1470 treatment, then it can be inferred that because cyclin D expression has been shown to be repressed in response to AGM-1470 incubation, the target of AGM-1470 functions upstream of cyclin D expression. This was investigated in HUVEC released from serum deprivation that resulted in a G<sub>0</sub>/G1 phase block. Along with serum, the released cells were incubated without or with AGM-1470 for 1 to 4 hours and Cdk4 phosphotransferase activity was assessed by measuring the pRb-peptide (Santa Cruz) phosphorylation by immunoprecipitated Cdk4 from lysates of these cells Figure 36. The results show that early G1 phase associated activation of Cdk4 was inhibited by AGM-1470 treatment.



**Figure 36.** AGM-1470 treatment in HUVEC results in the inhibition of the Cdk4-cyclinD complex. HUVEC blocked at the  $G_0/G_1$  interphase following serum deprivation for 36 hours were released by incubation in complete media for 4 hours without or with 5 nM AGM-1470 for 1 or 4 hours. Cdk4 was immunoprecipitated with the anti-Cdk4 antibody (Santa Cruz) and activity was determined by measuring pRb-peptide (Santa Cruz) phosphotransferase activity in the presence of cofactors and  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ . The reaction mixture was separated on SDS-PAGE gels and the labeled peptide was detected via radiography (A). Western analysis of the Cdk4 protein transferred onto nitrocellulose from the gels in (A) was conducted with the same antibody to ensure an equivalent amount of protein was immunoprecipitated under each condition (B).

## DISCUSSION

This chapter indicates that the anti-proliferative effect of AGM-1470 may be mediated through a calcium-independent PKC pathway in endothelial cells. This was supported by experiments that demonstrated that when PKC activity was induced by PMA in AGM-1470 pretreated HUVEC, they were released from being blocked in early G1 phase (Figure 33). Since AGM-1470 inhibited PKC upstream of the kinase, it is still possible that the inhibition of proliferation by AGM-1470 was not strictly dependent on PKC inhibition. These cells were also observed to overcome the G1 block and progress through the cell cycle as determined by DNA synthesis (Figures 30 and 31). The mechanism of cell growth inhibition associated with suppression of DNA synthesis is not understood. It has previously been shown that AGM-1470 incubation with endothelial cells results in a cell cycle arrest at early G1 (Antoine *et al.*, 1994) and support for this was demonstrated with the resulting AGM-1470-induced inhibition of G1 phase associated expression of cyclins A, D, and E, Cdk4 and Cdk2, and the activity of Cdc2 and Cdk2 (Abe *et al.*, 1994). Because AGM-1470 has not been observed to show an inhibitory effect on the expression of c-Fos or c-Myc in endothelial cells, Abe and associates (1994) have suggested that AGM-1470 probably inhibits the activation of the Cdk4/cyclin complex at a site distal to the early G1 events. Although pRb phosphorylation has been shown to be inhibited in response to AGM-1470, this is the first report demonstrating directly that the Cdk4 phosphotransferase activity is abrogated in response to drug treatment. AGM-1470 treatment of HUVEC was also shown to inhibit the increase of endothelial cell proliferation without inhibiting mitosis, but instead preventing the entry of cells into G1 phase of the cell cycle (Antoine *et al.*, 1994).

Evidence of a growth regulatory consequence of PKC inhibition indicating a link between PKC signalling and cell cycle progression is extensive. PKC has been associated with endothelial cell proliferation and tumour angiogenesis in several independent studies (Davis *et al.*, 1993; Zhou *et al.*, 1993; Kieser *et al.*, 1994; Tang *et al.*, 1995; Stanimirovic *et al.*, 1995; Lewis *et al.*, 1996). The evidence presented here indicates that AGM-1470 regulates endothelial cell proliferation via PKC, which is consistent with the

aforementioned studies. AGM-1470 has been determined to inhibit angiogenesis by specifically down-regulating endothelial cell proliferation versus chemotaxis or formation of capillary-like tube formation (Kusaka *et al.*, 1994; Antoine *et al.*, 1994, 1996; Ito *et al.*, 1996). Therefore, I suggest that the regulation of PKC activity is functional in endothelial cells as the mechanism for the anti-angiogenic effect of AGM-1470 treatment. Another point of interest with the growth-inhibitory mechanism of AGM-1470 is its relative specificity for endothelial cells. In addition to tumour or immortalized cells being insensitive to AGM-1470, sometimes requiring 100,000 - 1,000,000 fold drug concentration to inhibit cell proliferation versus normal endothelial cells (Yamoaka *et al.*, 1993; Kusaka *et al.*, 1994; Abe *et al.*, 1994; Antoine *et al.*, 1994, 1996), transformed endothelial cells are also insensitive to AGM-1470 (Antoine *et al.*, 1994). In animal models, AGM-1470 was shown to have no suppressive effects upon the proliferation of leukemic or malignant cells disseminated in the peritoneal cavity, which do not depend on neovascularization (Ingber *et al.*, 1990). Therefore the anti-tumour and anti-metastatic effects of AGM-1470 is thought to be exerted by its anti-angiogenic action by affecting the cellular pathway that controls the passage of endothelial cells, that are bypassed in the genesis of cancer.

The cell cycle-dependent PKC-mediated bi-directional growth regulation associated with endothelial cells explains the specificity of AGM-1470 with endothelial cell. Work done by Zhou *et al.* (1993) with human umbilical vein endothelial cells demonstrated that activation of PKC during the early G1 phase potentiates growth-factor induced DNA synthesis, whereas activators of PKC completely inhibited the initiation of DNA synthesis in HUVEC when applied in late G1 or after entry into S phase. This cell cycle-dependent regulation mediated by PKC was found to be associated with either the induction or inhibition of pRb phosphorylation, and Cdk2-dependent histone H1 phosphotransferase activity which usually results following pRb phosphorylation by Cdk4 in early G1, and mediates the G1 to S phase transition (Sherr, 1993; Hunter, 1997). Additionally, Zhou *et al.* (1993) were able to show that the effects of PKC activators in both stimulatory and inhibitory directions were abolished in PKC down-regulated cells. I found that AGM-1470 inhibited PKC in HUVEC, and inhibited DNA synthesis, probably by indirectly down-

regulating the Cdk4/cyclinD complex, thus blocking cells from progressing into G1 phase. In addition to supporting the functional role of abrogating calcium-independent PKC by AGM-1470 in inhibiting endothelial cell proliferation, the observations described by Zhou *et al.* (1993) may explain why subsequent treatment of AGM-1470 pretreated cells with the PKC activator PMA induces the cells to progress normally through the cell cycle. AGM-1470 is able to block cells at early G1, without toxicity (Kusaka *et al.*, 1994), by indirectly inhibiting PKC activity, probably upstream of the calcium-independent isoform (Figure 32), and since activation of PKC in endothelial cells at early G1 phase results in DNA synthesis, AGM-1470 pretreated HUVEC are rescued from the cell cycle block with subsequent treatment with PMA. The observation that activation of PKC in AGM-1470 induced PKC down-regulated cells by PMA resulted in DNA synthesis may at first appear to contradict the aforementioned finding that the effects of PKC activators are abolished in PKC down-regulated cells. PKC down-regulation in that study was induced by long term (6 hours) incubation with 12, 13-dibutyrate, which interacts with PKC directly. Since I was able to demonstrate that AGM-1470 did not reduce the level of DNA synthesis in PMA-treated cells (Figure 31), and that PKC was activated in AGM-1470 pretreated cells following subsequent PMA incubation (Figure 33), AGM-1470 appears to act upstream of PKC. Further, I was not able to inhibit MonoQ fractionated or immunoprecipitated PKC histone H1 phosphotransferase activity with AGM-1470 directly (data not shown). Therefore, PMA was able to induce DNA synthesis in AGM-1470 pretreated cells since PKC was not down regulated directly. Treatment of HUVEC with PMA alone resulted in only 20-25% of the cells undergoing DNA synthesis. This fraction of asynchronously growing cells was most likely in G1 phase when incubated with PMA. Those cells that were in late G1 or S phase would not be affected by PKC stimulation, since PKC activation during these periods of the cell cycle resulted in the down-regulation of DNA synthesis (Zhou *et al.*, 1993). The observation in Figure 31A that PMA induced that same degree of HUVEC proliferation with or without subsequent treatment with AGM-1470 is not surprising given that AGM-1470 probably acts upstream of PKC. Therefore, the presence, or not of a compound that inhibits PKC upstream of the agonist, PMA, is inconsequential. Again, the same percentage of cells were observed to undergo DNA synthesis without the

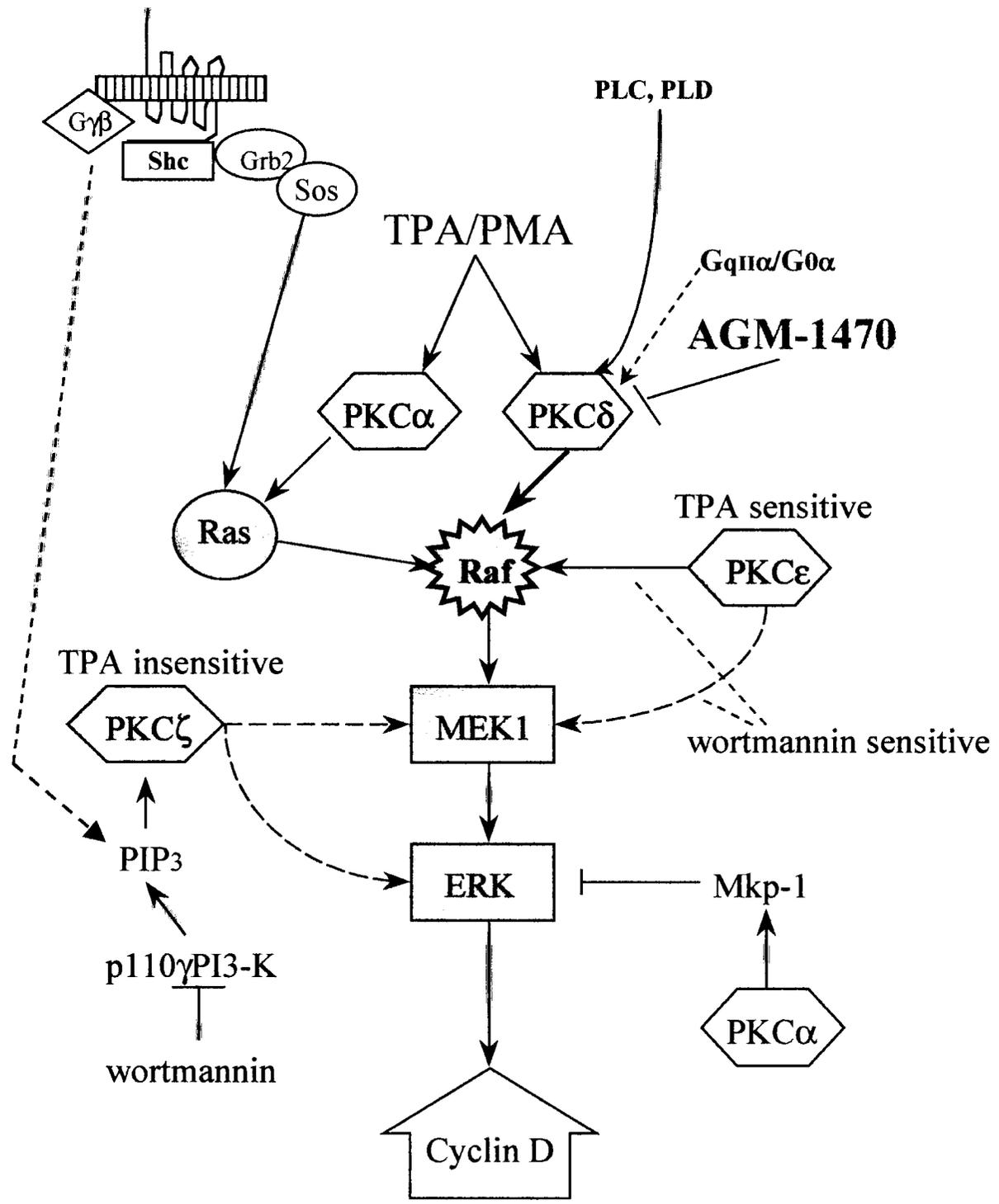
presence of AGM-1470, since induction of PKC in the window of cells at early G1 phase was stimulated by PMA treatment, and those cells were committed to progress through the cell cycle to S phase. Those cells that were already beyond the 'Restriction Point' would not progress through G1 phase into S phase, but remain blocked at G1 phase.

The cell cycle-specific effects of AGM-1470 treatment in HUVEC was further characterized by observing the effects of 30 min incubations with AGM-1470 on DNA synthesis in synchronized cells at various stages of the cell cycle. The observation that cells in S phase were not affected by AGM-1470 treatment, whereas mitotic and early G1 phase cells were inhibited from undergoing DNA synthesis is consistent with the suggestion by Abe *et al.* (1994) that AGM-1470 prevents entry of endothelial cells into G1 phase (versus induction of a mitotic block). Whether this specificity is a result of the inhibition of an effector of PKC that is active or expressed only during the AGM-1470 sensitive period of the cell cycle has yet to be determined, but this observation is also consistent with the PKC-mediated bidirectional regulation of DNA synthesis in endothelial cells. Although I demonstrated 70% of the cells were blocked at S phase immediately following the aphidicolin treatment, a more rigorous reversible cell cycle block, such as a the double-block utilized in the fostriecin study, could have been used in this study to obtain better synchrony. This alternative form of cell synchrony that requires serum deprivation might result in changes in the expression of various G1 phase associated cell cycle mediators that would not exist in normal cycling cells. As mediators of cyclin D expression are a putative target for AGM-1470, only the aphidicolin treatment-associated S phase block was used here. In this study, the degree of proliferation was based on the incorporation of thymidine into the DNA. An alternative method of analyzing the cell cycle dependence of AGM-1470 on endothelial cell proliferation would be to release cells from a cell cycle block, in the presence of BrdU, and conduct BrdU-propidium iodide 2-dimensional FACS analysis on cells at the time of treatment following the release, and 24 hours following that time for comparison of BrdU incorporation relative to the phase of the cell cycle. This is the first study to indicate that in addition to inducing a cell cycle block in endothelial cells, cellular response to AGM-1470 is also cell cycle specific.

The data presented provide strong support for the involvement of one or more specific PKC isoenzymes in the positive regulation of HUVEC in G1 phase, and demonstrates that by regulating this pathway, AGM-1470 effectively blocks endothelial cell proliferation resulting in an anti-angiogenic therapeutic result. Despite extensive evidence for the role of the PKC family of secondary signal transduction molecules in the positive regulation of growth control (Zhou *et al.*, 1993; Kieser *et al.*, 1994; Stanimirovic *et al.*, 1995; Perletti *et al.*, 1996; Cacace *et al.*, 1996), as well as negative control (Hughes *et al.*, 1997; Frey *et al.*, 1997), understanding of the mechanisms involved as well as the specific growth-regulatory functions of individual isoenzymes remains limited. Knowledge of the function of inhibiting specific PKC isoforms in endothelial cells in mediating a G1 arrest with AGM-1470 would provide insight into determining the target of AGM-1470, given its specificity (37 pM IC<sub>50</sub>, Kusaka *et al.*, 1994) for a unique molecule(s), as well as lead to a better understanding of cell cycle progression and the signal transduction alterations involved in the genesis of cancer. The latter insight would demonstrate how cells might develop resistance to chemotherapeutic compounds, and AGM-1470 provides an ideal model since cancer and transformed endothelial cells are not affected by AGM-1470. Given that AGM-1470 treatment results in the inhibition of Cdk4 pRb kinase activity, a hypothesis pertaining to the function of inhibition of PKC in the induction of a G1 block can be developed. Although p16<sup>INK4</sup> is responsible for down-regulating Cdk4 activity specifically (Serrano *et al.*, 1995), deregulation of cdk inhibitory proteins (including p21<sup>CIP1</sup> and p27<sup>KIP1</sup>) is most likely not the function of AGM-1470, since treatment results in a loss in expression of cyclin D1 (Abe *et al.*, 1994; Hori *et al.*, 1994). The MAP kinase pathway consisting of ERK1 and ERK2 isoforms, is involved in many of the intracellular signalling pathways that lead to endothelial cell growth, migration, and adhesion (Clark and Brugge, 1995; Lamarche *et al.*, 1996; Wary *et al.*, 1996; Hughes *et al.*, 1997; Renshaw *et al.*, 1997), and is the major cellular signalling pathway that mediates the effects of growth factors including bFGF, VEGF, as well as integrins, on cell cycle progression (Cobb *et al.*, 1995; Clark and Brugge, 1995; Rosales *et al.*, 1995; Yang *et al.*, 1996; Wei *et al.*, 1997). Another link between cell cycle progression and growth factor signalling is provided by cyclin D1, whose gene is induced as a secondary response gene following

mitogenic stimulation. Progression through G1 phase in mammalian cells is regulated by Cdk4 and Cdk6, which forms complexes with cyclin D1 (Sherr, 1993). It is now well established that cyclin D expression is dependent upon MAP kinase activity, and regulation of cyclin D1 is a critical target of the Ras-Raf-MEK1-ERK signalling cascade (Lavoie *et al.*, 1996; Aktas *et al.*, 1997; Sewing *et al.*, 1997; Takuwa *et al.*, 1997). I have shown that AGM-1470 treatment with neutrophils result in the inhibition of both PKC and MAP kinases activated in response to fMLP and PMA. Given that the literature is replete with observations that PKC isoforms regulate the Ras-Raf-MEK1-ERK pathway (Nishioka *et al.*, 1995; Clark and Brugge, 1995; Cacace *et al.*, 1996; Ueda *et al.*, 1996; Marquardt *et al.*, 1994; Tang *et al.*, 1994; Zou *et al.*, 1996; Xing and Insel, 1996; Liao *et al.*, 1997), it is possible that AGM-1470 inhibits endothelial cell progression by inhibiting MAP kinase through PKC (see Figure 37 for an illustration of where PKC may interact in this pathway). Various isoforms have been shown to mediate signalling via MAP kinase through interactions with Ras, Raf as well as MEK1 (Figure 37). Thus, the regulation of cyclin D through MAP kinase regulation by PKC may explain the PKC biphasic regulation of endothelial cell proliferation following AGM-1470 treatment, and therefore, cellular specificity. Antibodies against cyclin D were shown to inhibit cell cycle progression when microinjected during mid G1, but was ineffective near the G1-S boundary (Musgrove *et al.*, 1994). Haas and associates (1997) found that Ras was required for both induction of cyclin D and down-regulation of p27<sup>KIP1</sup> until cells pass the restriction point. Induction of a dominant negative Ras mutant either at G<sub>0</sub> or throughout G1 up to the restriction point has been observed to abrogate the expression of cyclin D1 at the level of the mRNA accumulation, consistent with studies indicating that the cyclin D1 promoter contains regulatory elements that are induced in response to oncogenic Ras proteins or activation of the ERK protein kinases (Albanese *et al.*, 1995; Lavoie *et al.*, 1996). Therefore, the PKC biphasic regulation of endothelial cell growth correlates closely to the expression and function of cyclin D.

**Figure 37.** Putative AGM-1470 sensitive signalling pathway in endothelial cells. Endothelial cell growth is mediated through the Cdk4/cyclin D complex in early G phase, where cyclin D transcription is dependent upon ERK activity coordinated through crosstalk between integrins and growth factors (i.e. bFGF and VEGF). This scheme illustrates possible mechanisms of activating the ERK pathway. Raf appears to be central, and its activation can be mediated through Ras following Shc phosphorylation at the integrin, or via PMA sensitive PKC stimulation. In addition to the  $\alpha$  and  $\beta$  isoforms, PKC $\epsilon$  is PMA sensitive and has been shown to activate Raf directly, whereas PKC $\zeta$  is PMA resistant but wortmannin sensitive, and is probably not functional in endothelial cell proliferation. PKC $\delta$  is also activated through G<sub>0</sub>/G<sub>q</sub> coupled receptors. The illustration shows experiments that may be conducted to delineate which effectors of ERK are responsible for cyclin D expression through ERK. The activities and phosphorylation profiles of the proteins highlighted in yellow should be determined in response to bFGF and VEGF +/- AGM-1470, as well as their function in this pathway (i.e. does Shc complex to caveolin, and does it mediate Ras activation through Grb2 directly, or via another mediator such as Gab1). The types of experiments that are suggested are indicated in red text.



**Figure 37.** Putative AGM-1470 sensitive signalling pathway in endothelial cells. The role of PKC isoforms (yellow) in the ERK (green) pathway.

Aberrant expression of cyclin D1 through chromosomal translocation, gene amplification or over expression is common in a number of human cancers including breast and bladder carcinoma and B-cell lymphomas where cyclin D1 is the favoured oncogene activated by the t(11; 14)(q13; q32) translocation. The chromosomal locus 11q13 is one of the most frequently amplified regions in human carcinoma (Lammie and Peters, 1991; Musgrove *et al.*, 1994, and discussion within). It is well established that oncogenic forms of Ras are also predominant in human tumours and cancer cell lines. Therefore, since AGM-1470 treatment results in the abrogation of cyclin D expression and also inhibits the activation of MAP kinase (unpublished observations), probably through the inhibition of PKC, cells containing aberrant expression of cyclin D1 or Ras, or pRb<sup>-/-</sup> cells, would be resistant to AGM-1470 treatment.

Uncontrolled cell proliferation or the inability of a cell to undergo apoptosis is clearly the hallmarks of tumorigenesis. At present, the chemotherapeutic agents utilized for the treatment of cancer are designed to kill these proliferating cells and are therefore limited in their efficacy, as treatment also results in toxicity to normal cells which also undergo constant division. Our understanding of the molecular mechanisms involved in cell proliferation and apoptosis is expanding rapidly, and so is the complexity of identifying targets for potential therapeutics that are not systemic, but unique to neoplastic signalling versus that of normal cells. Of course, identifying a unique target should be part of the paradigm in developing a therapy. Ideally, the drug should also have specificity towards the target to reduce toxicity often resulting from the redundancy of a target with another protein(s) in a specific pathway, or because the target is ubiquitous. But the recent studies in angiogenesis have identified an alternative approach to finding novel targets for combating cancer. During malignant conversion from normal to transformed tissue, various tissue and cellular morphologic events occur. In addition to uncontrolled proliferation of the tumour cells, extracellular matrix boundaries lose their integrity and continuity, and new blood vessels are recruited towards the interstices of the growing tumour mass, which is usually no more than 1 mm in diameter at the onset of this angiogenic response. Therefore, the modulation of any of these properties could be utilized as a therapeutic basis for cancer treatment. Judah Folkman first proposed that an

angiogenic based chemotherapy could specifically inhibit tumour expansion (Folkman, 1972). A compound that targets normal growing endothelial cells would most likely exhibit specificity for the tumour vasculature, since capillary endothelial cells grow significantly slower than cells in tumour vessels. AGM-1470 is now in phase III clinical studies as an anti-angiogenesis drug and its normal versus cancer cell specificity has already been described (Yamaoka, *et al.*, 1993; Antoine *et al.*, 1994, 1996). Its unique molecular structure, which contains a labile epoxide moiety conducive to covalent ligation that is found in other compounds that elicit an anti-angiogenic response, indicates the molecular target of AGM-1470 may be a specific one. The data presented in this thesis imply that AGM-1470 probably binds to a target that may reside in the vicinity of the cell membrane, co-localizes at the membrane, or is found in the particulate fraction of detergent solubilized hepatocytes (Figure 28). Further, in addition to the compound remaining stable in human hepatocytes (within the membrane) for at least 4 hours, although it has been demonstrated to be rapidly metabolized intracellularly in human hepatocyte cell culture by endogenous esterases and microsomal epoxide hydrolases (Figg *et al.*, 1994; Placidi *et al.*, 1995; Cretton-Scott *et al.*, 1996), I have demonstrated that its effect is probably irreversible (Figure 29) given that its DNA synthesis inhibitory effect is sustained for at least 2 cell cycles following a 30 min incubation with subsequent washing in drug-free medium. It is important to note here that AGM-1470 is effective in inhibiting hepatic metastasis of human colon cancer (Tanaka *et al.*, 1995). This apparent irreversible effect, demonstrated here for the first time *in vitro*, as well as the aforementioned results of recent animal studies, is consistent with AGM-1470 binding covalently or by strong hydrogen binding to its target. Ingber *et al.* (1990) demonstrated significant growth inhibition of Lewis Lung Carcinoma and B16 melanoma in mice when administered with AGM-1470 subcutaneously every other day. I have observed in our laboratory similar results when the drug is administered only every seven days (unpublished results).

My findings support the notion that AGM-1470 is highly specific for a target that may be uniquely expressed in endothelial cells. Additionally, I have identified an important role for PKC in normal endothelial cells versus genetically unstable tumour cells, and I suggest that this may be beneficial as the likelihood of normal cells developing

resistance is negligible, as well as having a significantly reduced toxicity relative to the present therapies (Kusaka *et al.*, 1994). I also propose that because AGM-1470 was observed to inhibit a  $\text{Ca}^{2+}$ -independent isoform of PKC in endothelial cells, the effect of inhibiting specific isoform(s) of PKC is functional to its cellular specificity. It was recently reported that fumagillin covalently binds the methionine aminopeptidase, MetAP-2, and the authors have proposed that this is the physiological target of the drug. Their argument was based on the knowledge that MetAP-2 levels are greatly induced upon mitogen stimulation, and expression correlates with cell growth (Sin *et al.*, 1997; Borman, 1997; Griffith *et al.*, 1997). Further, because methionine aminopeptidases affect posttranslational processing required for protein myristoylation, inhibition of MetAP-2 may prevent the myristoylation of a signalling component that probably acts in early signalling in response to mitogens. The authors conceded that their model remains speculative, and no link between this model and a G1 block is provided. The significance of AGM-1470 binding to MetAP-2, in conjunction with the results presented in this thesis can be deduced based on other recent studies in the literature. It has been demonstrated that aminopeptidase activity is present in different cellular compartments and that several bacterial aminopeptidases are membrane-bound or have some interaction with the cytoplasmic membrane, particularly the intercellular side, without being considered as a membrane protein (Gonzales and Robert-Baudouy, 1996). Should MetAP-2 prove to be the physiological target of AGM-1470, how MetAP-2 functions in endothelial cell proliferation will require consideration. Given that MetAP-2 also acts as an inhibitor of eukaryotic initiation factor  $2\alpha$  (eIF-2 $\alpha$ ) phosphorylation (Ray *et al.*, 1993) and the recent finding that the human homologue of a rat initiation factor-2 associated protein is a methionine aminopeptidase (Li and Chang, 1996), indicates that AGM-1470 may inhibit endothelial cell proliferation by promoting eIF-2 kinase-catalyzed phosphorylation of the initiation factor by inhibiting MetAP-2 from binding to the eIF-2 phosphorylation site and preventing the regulatory event. Therefore, AGM-1470 may prevent the translation of a protein that regulates the activity of the PKC isoform whose activity is abrogated in response to AGM-1470 therapy.

In summary, AGM-1470 may specifically inhibit normal endothelial cells versus cancer cells or other normal cells, because it may deregulate the expression of a protein

that is functional at G<sub>0</sub> and/or G<sub>1</sub> that lies upstream of PKC, which in endothelial cells is bimodal in cellular growth regulation. Also, since this is likely upstream of cyclin D expression, which is mediated through the Ras-Raf-MEK1-ERK signalling pathway, cancer cells that have aberrant expression of cyclin D, Ras, or contain pRb<sup>-/-</sup> mutants, would be resistant to AGM-1470 treatment.

CHAPTER 5

SIGNAL TRANSDUCTION EVENTS IN NEUTROPHILS  
FOLLOWING INCUBATION WITH OPSONIZED CPPD  
CRYSTALS, FMLP, AND PMA, AND THE EFFECTS OF  
TAXOL AND AGM-1470 PRETREATMENT

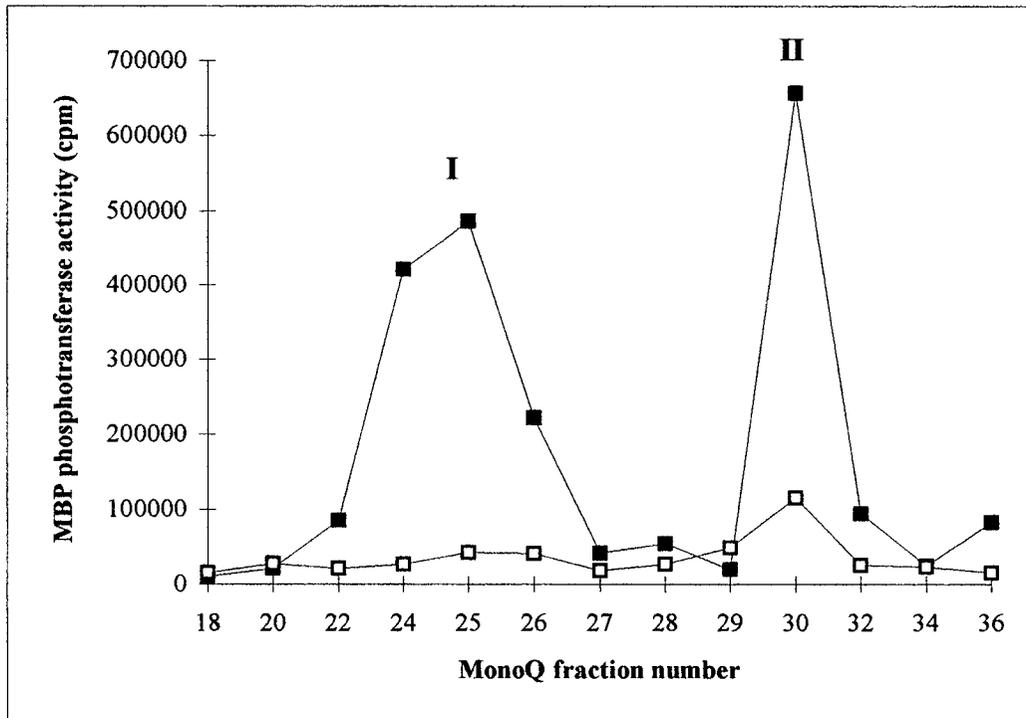
## RESULTS

### MAP kinase is activated in neutrophils in response to CPPD crystals

The primary function of neutrophils is the detection, migration to, and destruction of microbial pathogens, hence protecting the host from infections. The activation of neutrophils by chemoattractants proceeds via intracellular signalling pathways that include many of the components identified in particulate-activated, and calcium pyrophosphate dihydrate crystals (CPPD) and PMA-induced neutrophils. The inflammatory disease known as acute pseudogout arises from the deposition of CPPD in the synovial joints of humans (McCarthy, 1985). Neutrophil activation by both fMLP and CPPD crystals have been demonstrated to activate MAP kinase and PI 3-kinase (Worthen *et al.*, 1994; Krump *et al.*, 1997; Jackson *et al.*, 1997), together with neutrophil chemiluminescence, superoxide anion generation and degranulation (Jackson *et al.*, 1997a, b). To gain insight into the actions of AGM-1470 and Taxol, and a greater understanding of the signalling events leading to neutrophil responses and arthritis, I utilized the neutrophil system activated by CPPD(T) (triclinic) crystals, fMLP, and PMA. Here I investigated the effects of AGM-1470 and Taxol on agonist-induced PKC and MAP kinase activation and neutrophil activation based on chemiluminescence and superoxide anion generation.

MonoQ fractionation of neutrophil homogenates from cells incubated with CPPD crystals permits the resolution of two peaks of MBP phosphotransferase activity in fractions 20-30 and fractions 30-34 (Figure 38). The identity of the second peak between fractions 30 and 34 is unknown. However, immunoblotting of fractions 20-30 with anti-ERK1-CT antibodies specific to both ERK 1 and ERK 2 forms of MAP kinase revealed that both of these kinases were present in these fractions (Figure 39B; ERK1 at 44 kDa and ERK2 at 42 kDa). ERK activity in peak I was further validated following immunodepletion of the kinase from fractions 23-26, and MBP phosphotransferase activity re-evaluated. MBP phosphotransferase activity revealed that ERK was immunodepleted from peak I (data not shown). Both of the proteins featured a second slower migrating band representing an activated form of these kinases (illustrated in Figure 39B as 44 kDa and 42 kDa, respectively). Western analysis of the fractions containing MAP kinase with

the anti-phosphotyrosine antibody, 4G10, showed the ERK 1 and 2 bands were heavily tyrosine phosphorylated in fractions 24 and 25 (Figure 39C). The major immunoreactive band co-migrated with the hyperphosphorylated forms of the ERK proteins, indicating that the



**Figure 38.** MonoQ fractionation of MBP phosphotransferase activity. Neutrophils ( $5 \times 10^6$  cells/ml) were incubated with (closed square) or without (open square) plasma-opsinized CPPD(T) crystals (50 mg/ml) for 2 min. Cytosolic extracts (detergent solubilized) were subjected to MonoQ chromatography and MBP phosphotransferase activity was determined in the presence of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  and 1.5 mg/ml MBP substrate for 10 min at  $30^\circ\text{C}$ . MBP phosphorylation (peak I) represents the activity of MAP kinase, as determined by Western blotting and immunodepletion analysis, shown in Figures 39B.

immunoreactive peak contained both ERK 1 and ERK 2 in the active form. The presence of ERK 1 and 2 outside of fractions 24 and 25 corresponded to inactive forms of MAP kinase. The reason for this is unknown, but probably indicates that the most active forms of MAP kinase lie in fractions 24 and 25. Using rat muscle extracts, our laboratory has observed that MAP kinase eluted over a wide range of MonoQ fractions, whereas tyrosine phosphorylation was confined to just two fractions (Hei *et al.*, 1993).

The degree of activation of ERK1 and ERK2 in MonoQ MBP kinase peak I in control, plasma-coated and uncoated CPPD crystals following 1, 2 and 5 min incubation was determined. Figure 39A illustrated that MAP kinases were activated in response to CPPD crystal stimulation over the control, with strong activation at 1 min, followed by sustained activation until 5 min. Uncoated crystals gave slightly higher levels of MAP kinase activation than opsonized crystals. The reason for this is unknown. However, the effect was only significant at the 2 min value ( $P < 0.05$ ).

**Figure 39.** MAP kinase activation in neutrophils by CPPD(T) crystals. (A) Cytosolic extracts from neutrophils ( $5 \times 10^6$  cells/ml) treated for 1, 2 and 5 min with plasma-opsonized CPPD(T) (50 mg/ml) crystals (open circle), uncoated CPPD(T) crystals (closed square), or control cells (open square). Extracts were subjected to MonoQ fractionation and assayed for phosphotransferase activity using MBP as a substrate. MonoQ fractions that showed phosphotransferase activity were subjected to SDS-PAGE for Western blotting with (B) the affinity purified polyclonal antibodies that recognize the C-terminal region of MAP kinase, and (C) the 4G10 antiphosphotyrosine antibody. Error bars in (A) represent the S.D. of  $n=3$  experiments.

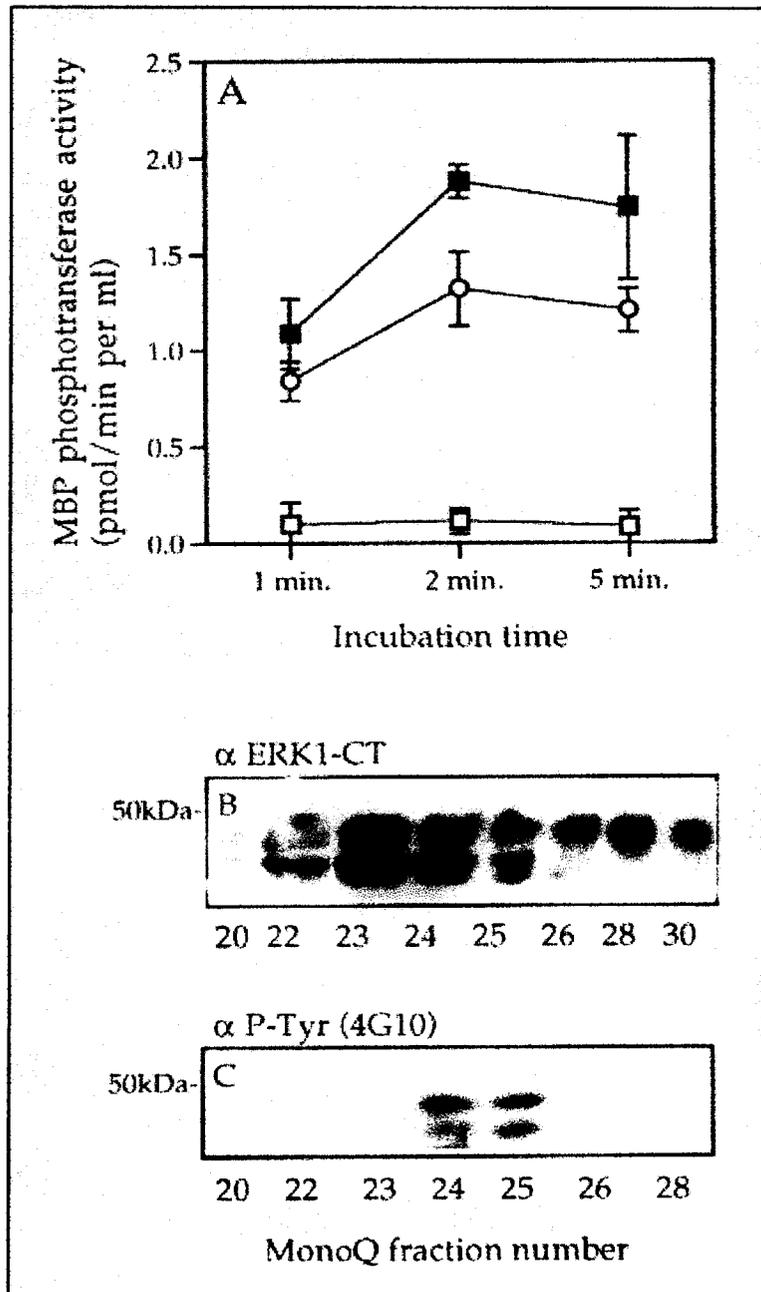


Figure 39.

### Activation of p70<sup>S6K</sup> via a PKC-dependent, PI 3-kinase-independent mechanism

To investigate crystal-induced activation of p70<sup>S6K</sup> I fractionated cytosolic extracts from neutrophils incubated with CPPD crystals by anion exchange chromatography on MonoQ. Phosphotransferase activity of the fractions was resolved using three different substrates of the p70<sup>S6K</sup>: MBP-NT peptide, S6-10 peptide and MBP. Assays with the MBP-NT peptide included the PKC inhibitor, Compound 3, since MBP-NT is also a substrate for PKC. Extracts from neutrophils treated for two min with plasma-coated CPPD crystals resolved a major phosphotransferase peak at approximately 0.4 M NaCl for each substrate (Figure 40A-C). To identify this activity, the MonoQ fractions were immunoblotted with the anti-p70<sup>S6K</sup>-NT antibody. The major immunoreactive protein coeluted with the phosphotransferase activity and migrated as a protein of approximately 70 kDa on SDS-polyacrylamide gels (Figure 40D).

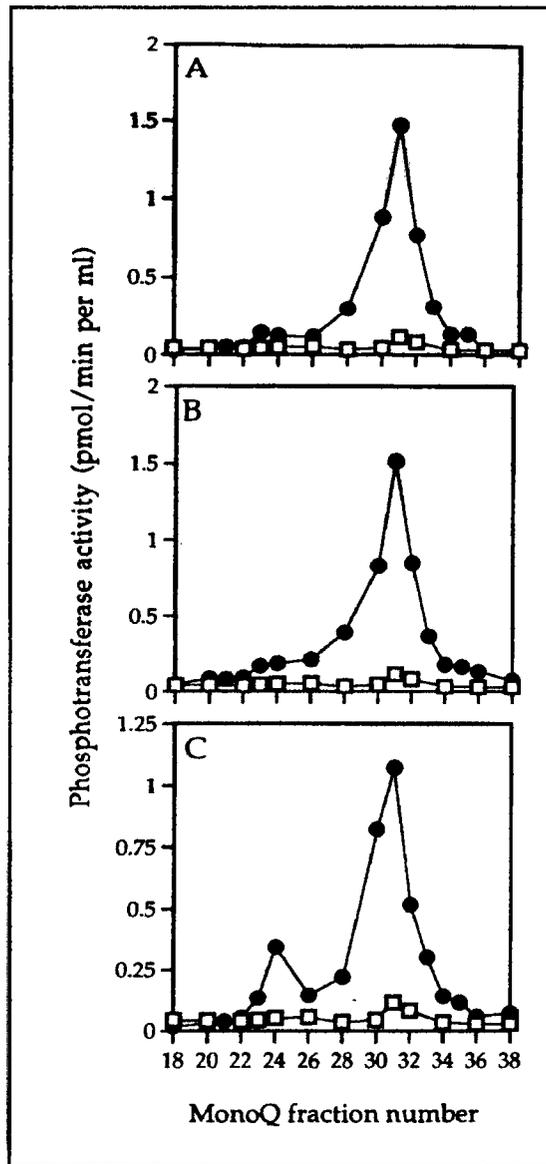
Previous work has established the p70<sup>S6K</sup> is activated through phosphorylation on multiple serine and threonine residues, likely by more than one upstream kinase (Mukhopadhyay *et al.*, 1992; Ferrari and Thomas, 1994; Petritsch *et al.*, 1995). The most highly phosphorylated and active form of p70<sup>S6K</sup> migrated more slowly than the basally (partially) phosphorylated or dephosphorylated inactive enzyme (Petritsch *et al.*, 1995; Han *et al.*, 1995). The corresponding band shift that can be visualized with immunoblotting of the enzyme correlated with p70<sup>S6K</sup> activity in many systems. In neutrophils not treated with crystals (control), no phosphotransferase activity was observed and p70<sup>S6K</sup> was present as a hypophosphorylated species (Figure 40D). Following 2 min neutrophil incubation with plasma coated CPPD, p70<sup>S6K</sup> activity was robust, and the hyperphosphorylated form of the kinase was observed by immunoblotting (Figure 40E). Hei *et al.* (1993) performed similar immunological studies on MonoQ fractions from rat skeletal muscle extracts with antibodies generated against the C-terminus and subdomain III of p70<sup>S6K</sup>, and the major immunoreactive protein coeluted with the p70<sup>S6K</sup> activity in an identical manner to that illustrated in Figure 40. These results indicate that stimulated p70<sup>S6K</sup> was present in the MonoQ fractions, and therefore this protocol was subsequently used for the rest of this study to investigate the effects of crystal incubation of neutrophils on this kinase.

To determine the kinetics of p70<sup>S6K</sup> activation following crystal incubation, cytosolic extracts from neutrophils incubated with plasma coated CPPD crystals for 1, 2, 5, 10 and 15 min were separated by anion exchange chromatography and the MBP-NT phosphotransferase activity was determined. The total phosphotransferase activity recovered in the column fractions (A.U.C.: Area Under the Curve) that contained p70<sup>S6K</sup> immunoreactivity was standardized to the amount of protein loaded onto the column and plotted against incubation time. The time course study showed that there was a marked and transient activation of cytosolic p70<sup>S6K</sup> activity following two minutes of crystal incubation as shown in Figure 41A. I have previously shown that both uncoated and plasma-coated CPPD crystals activated neutrophils and that plasma pre-coating amplifies neutrophil responses to CPPD (Burt and Jackson, 1993). Figure 41B shows that plasma pre-coating of CPPD crystals enhanced activity at least two-fold over uncoated CPPD crystals. Although PI 3- kinase and PKC are known to be activated in neutrophils, this is the first documented observation of neutrophil activation of p70<sup>S6K</sup>. p70<sup>S6K</sup> activation and regulation has generally been studied in mammalian cell systems treated with mitogens (Ballou *et al.*, 1991; Petritsch *et al.*, 1995), although Tsai *et al.* (1993) suggested a vital role of p70<sup>S6K</sup> in mast cells stimulated via the Fc receptor, where IgE was shown to stimulate p70<sup>S6K</sup> at 30 min, presumably through the Fc<sub>ε</sub>RI. This p70<sup>S6K</sup> activation was blocked by rapamycin, but rapamycin did not inhibit 5HT release.

The immunosuppressive compound rapamycin has become one of the most important and useful tools for dissecting the pathway leading to p70<sup>S6K</sup> activation. Rapamycin is a specific and potent inhibitor of p70<sup>S6K</sup> activation, blocking phosphorylation and stimulation of the enzyme by all known mitogens including EGF, v-Src, phorbol esters, calcium ionophore, heat shock, cyclohexamide, interleukin-2 and insulin (Jurivich *et al.*, 1991; Hei *et al.*, 1993; Ferrari *et al.*, 1994; Chung *et al.*, 1994). Although rapamycin inhibits p70<sup>S6K</sup>, it does not block the activation of other mitogen-activated kinases such as ERK1 and ERK2, p90<sup>ras</sup> or p74<sup>raf-1</sup> (Kuo *et al.*, 1992; Chung *et al.*, 1994). The drug does not directly interact with p70<sup>S6K</sup>, but binds to a protein termed FKBP12 (FK506-binding protein; Arcaro and Wymann, 1993) and the complex interacts with the target of rapamycin (mTOR, also known as FRAP). mTOR contains significant homology with the catalytic

**Figure 40.** MonoQ fractionation of p70<sup>S6K</sup> activation in neutrophils stimulated with CPPD(T) crystals. Neutrophils ( $5 \times 10^6$  cell/ml) were incubated with (closed circle) or without (open square) plasma-opsonized CPPD(T) crystals (50 mg/ml) for 2 min. Cytosolic extracts (non-detergent solubilized) were subjected to MonoQ chromatography and phosphotransferase activity was measured using the substrates (A) MBP-NT, (B) S6-10 peptide and (C) MBP. The same fractions were subjected to SDS-PAGE for Western blotting with affinity-purified rabbit polyclonal antibodies that recognize the N-terminal region of p70<sup>S6K</sup>. Immunodetection was conducted for both control cells (D) and CPPD(T) treated extracts (E).

**Figure 41.** Time course of p70<sup>S6K</sup> activation by CPPD(T) crystals, and the effect of rapamycin on CPPD(T) crystal-induced p70<sup>S6K</sup> activation in neutrophils. (A) Cytosolic extracts from neutrophils ( $5 \times 10^6$  cells/ml) treated for 1 to 15 min with plasma-opsonized crystals (50 mg/ml) and without crystals (control) were subjected to MonoQ fractionation and assayed for MBP-NT phosphotransferase activity detection (Compound 3 at 10 nM was included to inhibit PKC activity). p70<sup>S6K</sup> phosphotransferase activity within the S6 kinase immunodetected peak was determined. (B) Separate experiments were conducted to determine the p70<sup>S6K</sup> activity in neutrophils incubated for 2 min without crystals (white bar), with plasma-opsonized crystals (black bar), or with uncoated crystals (black and white hashed bar). Neutrophils +/- rapamycin pretreatment (10 nM, 10 min) were incubated with plasma-opsonized crystals for 2 min, and cytosolic extracts were prepared followed by MonoQ fractionation. (C) p70<sup>S6K</sup> activity was determined by phosphotransferase determination using the MBP-NT substrate. Control cells (open box), CPPD(T)-stimulated without rapamycin (closed box), CPPD(T) stimulated with rapamycin pretreatment (closed circle). (D) p70<sup>S6K</sup> phosphotransferase activity within the S6 kinase immunodetected peak was determined. Control cells (white bar), CPPD(T)-stimulated without rapamycin (black and white hashed bar), CPPD(T)-stimulated with rapamycin pretreatment (black bar).



**Western analysis**

**D. Control (anti-S6-NT)**



Fraction #      22    24    26    28    30    32    34    36



**E. 2 min treatment (anti-S6-NT)**

Figure 40.

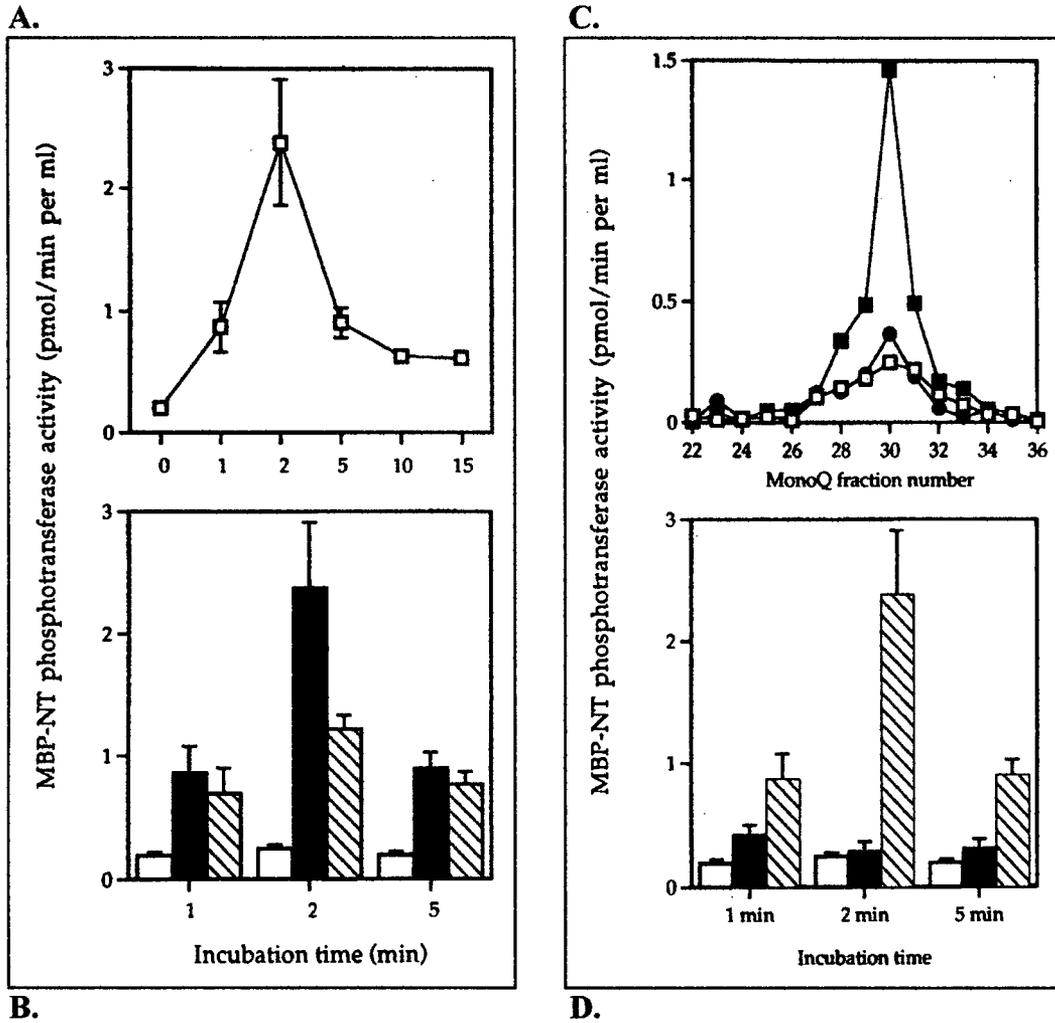


Figure 41.

subunit of PI 3-kinase, and has protein kinase activity. p70<sup>S6K</sup> has previously been shown to be activated by both PKC-dependent and -independent routes (Chung *et al.*, 1994 ; Han *et al.*, 1995), both of which are completely blocked by rapamycin. Rapamycin treatment has no effect on PI-3 kinase activity (Chung *et al.*, 1994; Ferrari and Thomas, 1994), but a recent report indicated that PI 3-kinase activity is required for TPA-induced AP-1 activation and transformation in mouse epidermal cells, linking PI 3-kinase to activation of PKC (Huang *et al.*, 1997).

Neutrophils were treated with rapamycin for 10 min prior to incubation with or without plasma-coated CPPD(T) (triclinic) crystals for 1, 2 and 5 min. Cytosolic extracts from treated neutrophils and non-treated controls were fractionated by MonoQ and phosphotransferase activity was determined as described previously. The activation of p70<sup>S6K</sup> at two minute crystal treatment was completely blocked by pretreatment with rapamycin (Figure 41C). The activity in the peak MonoQ fraction (fraction 30) was assayed and the mean of three separate experiments is shown in Figure 41D. Han *et al.* (1995) suggested that hyperphosphorylation of p70<sup>S6K</sup> was responsible for the mobility shift observed in Western blots following activation of this enzyme and that rapamycin may inhibit this phosphorylation. It is therefore likely that the activation of p70<sup>S6K</sup> by plasma-coated CPPD crystals is due to the phosphorylation of p70<sup>S6K</sup>, since a mobility shift was observed (Figure 40E) and the activation was rapamycin sensitive (Figure 41).

Increased tyrosine phosphorylation of several proteins has been shown to correlate with neutrophil activation, and it has been proposed that tyrosine kinases and phosphatases play a pivotal role in stimulus-response coupling in neutrophils (Burt *et al.*, 1992). MAP kinase is a protein serine/threonine kinase activated via tyrosine phosphorylation, and it has been reported that ERK1 (p42<sup>mapk</sup>) is stimulated in neutrophils by chemoattractant peptides (Worthen *et al.*, 1994; Grinstein and Furuya, 1992). Immunoblotting with the anti-ERK1-CT antibody specific to both ERK1 and ERK2 confirmed our previous findings that both of the MAP kinase isoforms coeluted with the first MBP phosphotransferase peak in fractions 21-26 (Figure 40C). Both isoforms had a more slowly migrating band that was characteristic of the activated form of these kinases. These fractions were subjected to Western blot analysis with the anti-phosphotyrosine antibody 4G10. The major

immunoreactive tyrosine phosphorylated band co-migrated with the hyperphosphorylated forms of the ERK proteins, indicating the first MBP peak contained active forms of both MAP kinases.

It has previously been suggested that MAP kinase and p70<sup>S6K</sup> lie on distinct signalling pathways (Ballou *et al.*, 1991). To test to see if this is similar in neutrophils following plasma-coated CPPD crystal incubation, I fractionated MAP kinase from extracts of neutrophils treated with or without 2 min crystal treatment +/- Compound 3 or wortmannin pretreatment. I have previously demonstrated that both ERK1 and ERK2 MAP kinases are activated in response to crystal incubation. The MonoQ profile is illustrated in Figure 42A, and the results of three separate MonoQ fractionated MAP kinase MBP phosphotransferase activation experiments in response to CPPD crystals with or without wortmannin is depicted in Figure 42B. The second peak represents p70<sup>S6K</sup>, which eluted 2 fractions later than normal during this experiment. Similar to p70<sup>S6K</sup>, wortmannin did not inhibit the activation of MAP kinase (Figure 42B). Unlike p70<sup>S6K</sup>, Roche 318220 (Compound 3) (a selective PKC inhibitor with an IC<sub>50</sub>  $\cong$  15 nM) did not prevent the activation of MAP kinase (Figure 42A). Therefore, MAP kinase can be activated by CPPD(T) crystals through a signalling pathway independent of PI 3-kinase, PKC, and p70<sup>S6K</sup>.

Neither CPPD(T)-induced MAP kinase activation (Figure 42A) nor p70<sup>S6K</sup> (Figure 43B) activation in neutrophils was substantially inhibited by wortmannin (only approximately 9 %), which indicated that PI-3 kinase activity was not required for their activation in this system. The PI 3-kinase independent activation of MAP kinase has previously been reported (Wang and Sul, 1998), but these studies of p70<sup>S6K</sup> indicated that PI 3-kinase is required for its activation (Chung *et al.*, 1994; Okada *et al.*, 1994; Welsh *et al.*, 1994; Petritsch *et al.*, 1995). Wortmannin at 0.1 to 1 mM (concentrations that are 10- to 100-fold greater than are necessary to suppress crystal induced PI 3-kinase activation in neutrophils; Bos, 1995) failed to inhibit crystal-induced activation of p70<sup>S6K</sup> (Figure 44B). LY294002 at 50  $\mu$ M (a concentration determined to inhibit PI-3 kinase and p70<sup>S6K</sup> in HepG2 cells following PDGF treatment; Chung *et al.*, 1994) was also ineffective at inhibiting CPPD-induced p70<sup>S6K</sup> activation. CPPD crystals also induced the strong

activation of both protein kinase B (PKB) and protein kinase C (PKC) after a 2 min crystal-neutrophil incubation (Figures 43A and 44A, respectively). Wortmannin was able to fully suppress the activation of PKB, indicating that activation of PKB in neutrophils was downstream of PI 3-kinase, consistent with other reports (Figure 44). In other cells, PKB (also known as Akt or Rac) has been proposed to be an indirect target of PI 3-kinase and a possible link to p70<sup>S6K</sup> activation (Burgering and Coffey, 1995; Chou and Blenis, 1996). In fact, PKB has recently been shown to be activated by interaction of its pleckstrin homology domain with phosphatidylinositol-3,4-bisphosphate (PIP<sub>2</sub>) generated by the action of PI 3-kinase (Franke, 1995). Cohen and associates (Cohen *et al.*, 1997; Alessi *et al.*, 1997) have also recently identified a protein that is not a member of the PI 3-kinase family but is active in the presence of PtdIns(3,4,5)P<sub>3</sub> or PtdIns(3,4)P<sub>2</sub>, that activates PKB directly. The wortmannin-sensitive activation of PKB and wortmannin-insensitive activation of p70<sup>S6K</sup> described in this study shows that in neutrophils, crystal induced p70<sup>S6K</sup> activation was not dependent on PKB activation. However, both PKC and p70<sup>S6K</sup> activation were inhibited by Compound 3, clearly implicating PKC as a putative mediator p70<sup>S6K</sup> activation in neutrophils. Figure 44 represents the results of MonoQ fractionation and histone H1 (Panel A) and S6-10 peptide (Panel B and C) phosphotransferase activities for p70<sup>S6K</sup> activity determination. Figures 44A and 44C represent the mean of three separate experiments where the activity indicates the A.U.C. of the immunosensitive peak of the respective enzyme activities. It has been reported that there are at least two kinase signalling pathways that lead to activation of p70<sup>S6K</sup> and that only one pathway is sensitive to wortmannin (Kishi *et al.*, 1996). Furthermore, p70<sup>S6K</sup> is activated by PKC-dependent and -independent mechanisms and rapamycin is able to block both (Chung *et al.*, 1994). The physiological role of p70<sup>S6K</sup> activation in neutrophils is unknown, but it is unlikely to be related to rapid responses such as respiratory burst or degranulation activity since rapamycin had no inhibitory effects on these in neutrophils. The activation of p70<sup>S6K</sup> in proliferating cells is associated with protein synthesis and non-mitogenic activation of this enzyme in neutrophils may be related to more long term neutrophil responses dependent on protein synthesis such as increases in membrane receptor expression.

**Figure 42.** The effects of Compound 3 and wortmannin on MBP phosphotransferase activity in neutrophils. Cytosolic extracts from neutrophils treated for 2 min with plasma-opsinized crystals (50 mg/ml) with and without Compound 3 pretreatment (10 nM, 5 min) (**A**), or wortmannin pretreatment (100 nM, 5 min) (**B**) were subjected to MonoQ fractionation and assayed for MBP phosphotransferase activity. MAP kinase activity within the first MBP phosphotransferase, MAP kinase immunodetected peak (Figure 39 and 40) was determined in three separate experiments, and are expressed in (**B**). The error bars represent the S.D. of three experiments. The second peak in (**A**) encompasses the p70<sup>S6K</sup> peak identified in Figure 41C.

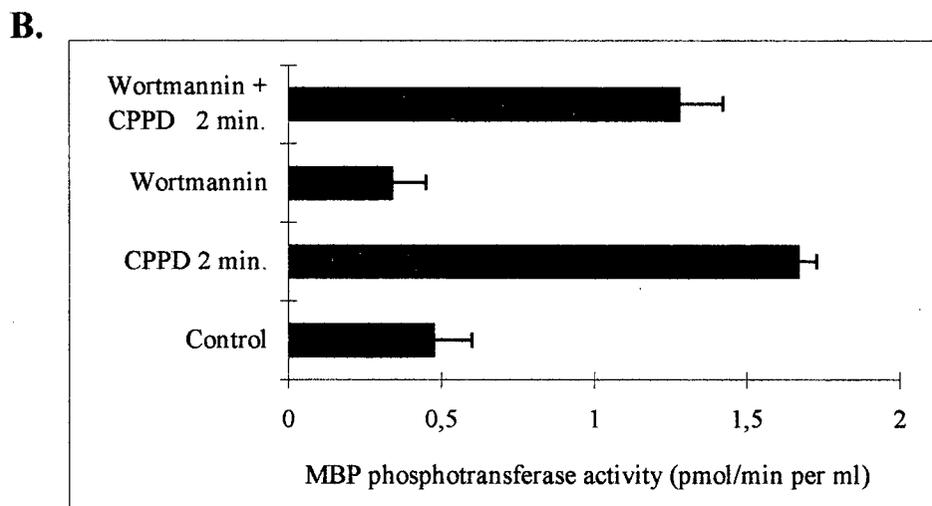
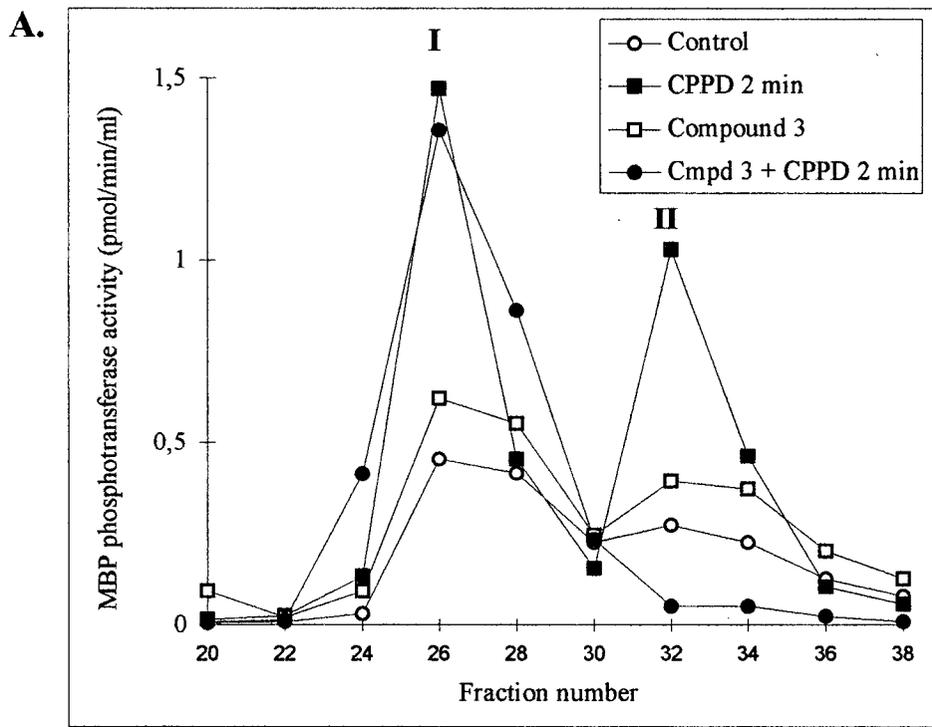
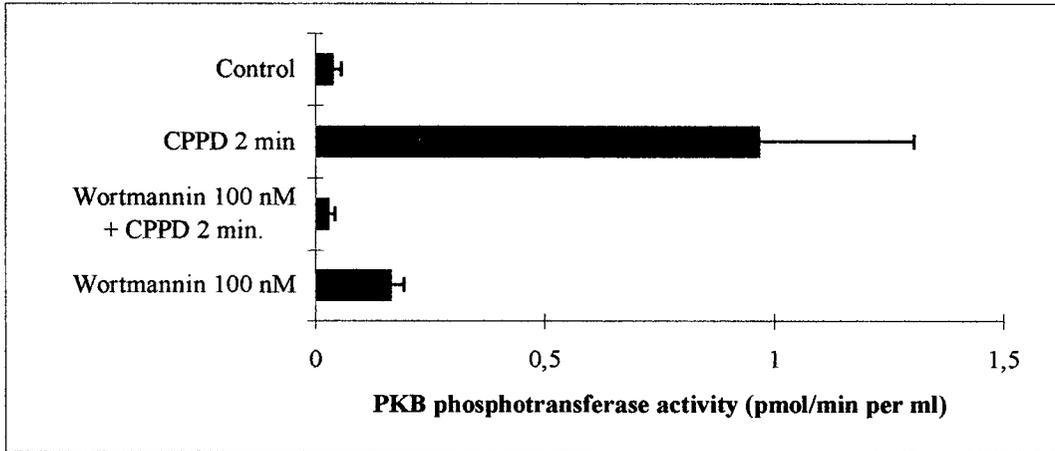


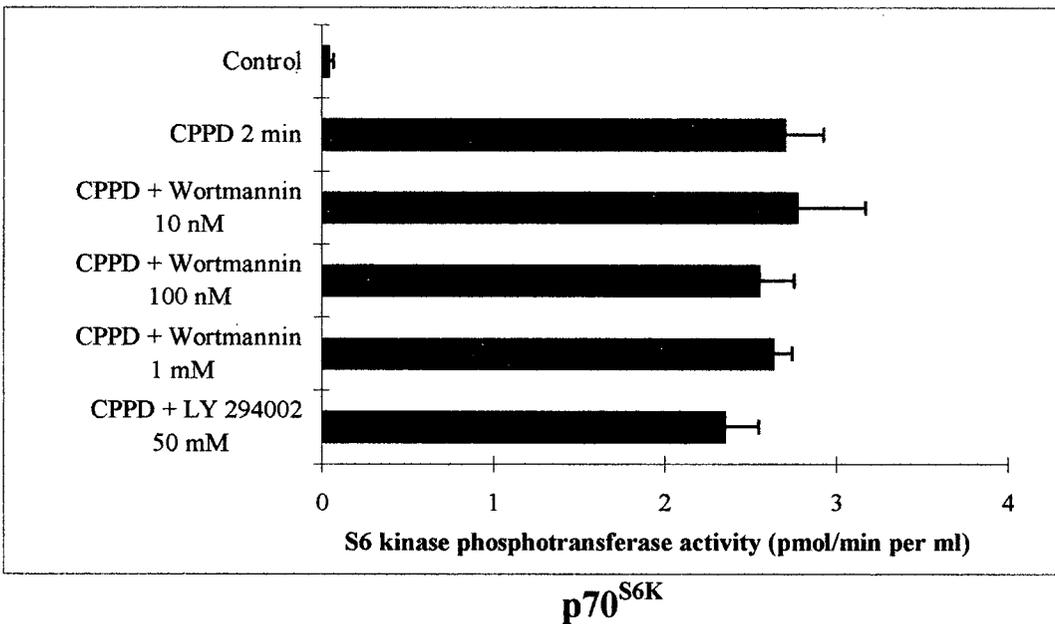
Figure 42.

## PKB

**A.**



**B.**



**Figure 43.** The effects of wortmannin on CPPD(T) crystal-induced PKB and S6 kinase activity. Neutrophils ( $5 \times 10^6$  cells/ml) +/- 5 min wortmannin (100 nM) pretreatment at the concentrations indicated were incubated with plasma-opsized CPPD(T) crystals (50 mg/ml) for 2 min, and cytosolic extracts were prepared. (A) PKB was immunoprecipitated and assayed with MBP in the presence of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  and (B)  $\text{p70}^{\text{S6K}}$  activity was determined by assaying S6-10 peptide phosphotransferase activity within the S6 kinase immunodetected peak from MonoQ fractionation. Values represent the mean +/- the S.D. of  $n=3$  experiments.

**Figure 44.** The effect of Compound 3 on CPPD(T) crystal-induced activation of PKC and p70<sup>S6K</sup> in neutrophils. Cytosolic extracts from neutrophils treated with plasma-opsonized CPPD(T) crystals (50 mg/ml) for 1, 2 and 5 min (**A**) or 2 min (**B, C**), where indicated, +/- Compound 3 (Cmpd3) pretreatment (10 nM, 5 min). PKC histone H1 phosphotransferase activity within the PKC immunodetected peak was determined. PKC phosphotransferase activity determined using histone H1 was determined from MonoQ fractions. (**B**) p70<sup>S6K</sup> Histone H1 phosphotransferase activity within the S6 kinase immunodetected peak was determined. p70<sup>S6K</sup> phosphotransferase activity determined using the S6-10 peptide was determined from MonoQ fractions (**B**) and the mean +/- the S.D. of three separate experiments similar to (**B**) is shown in (**C**). The values in (**A**) represent the mean +/- the S.D. of n=3 experiments.

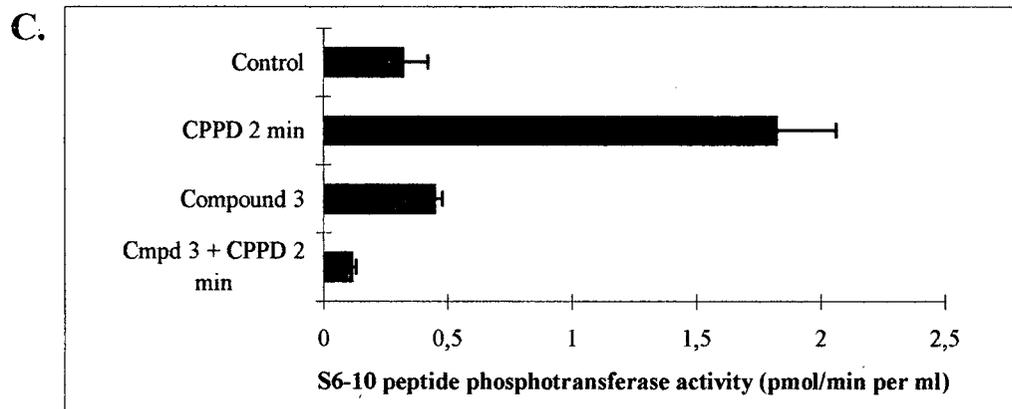
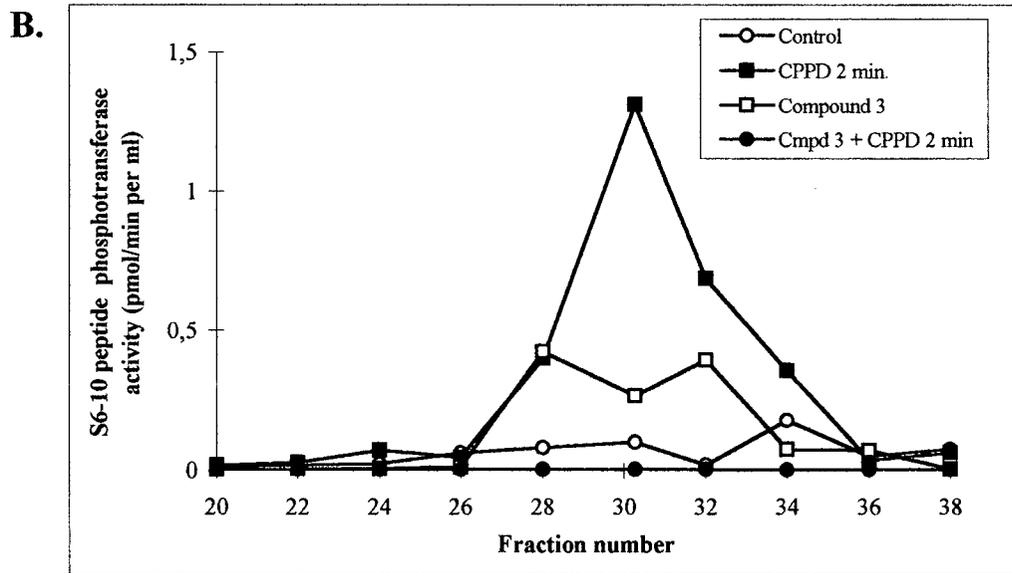
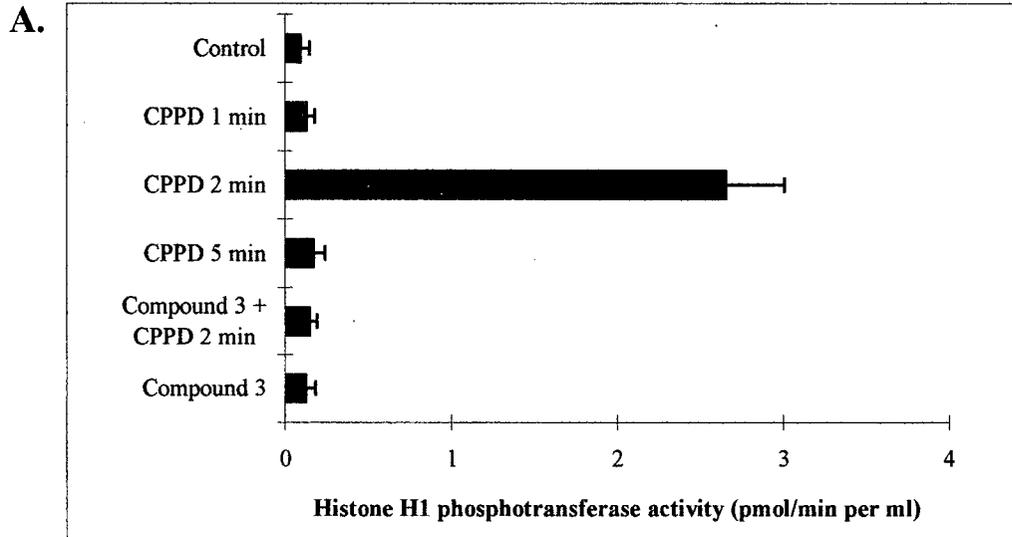
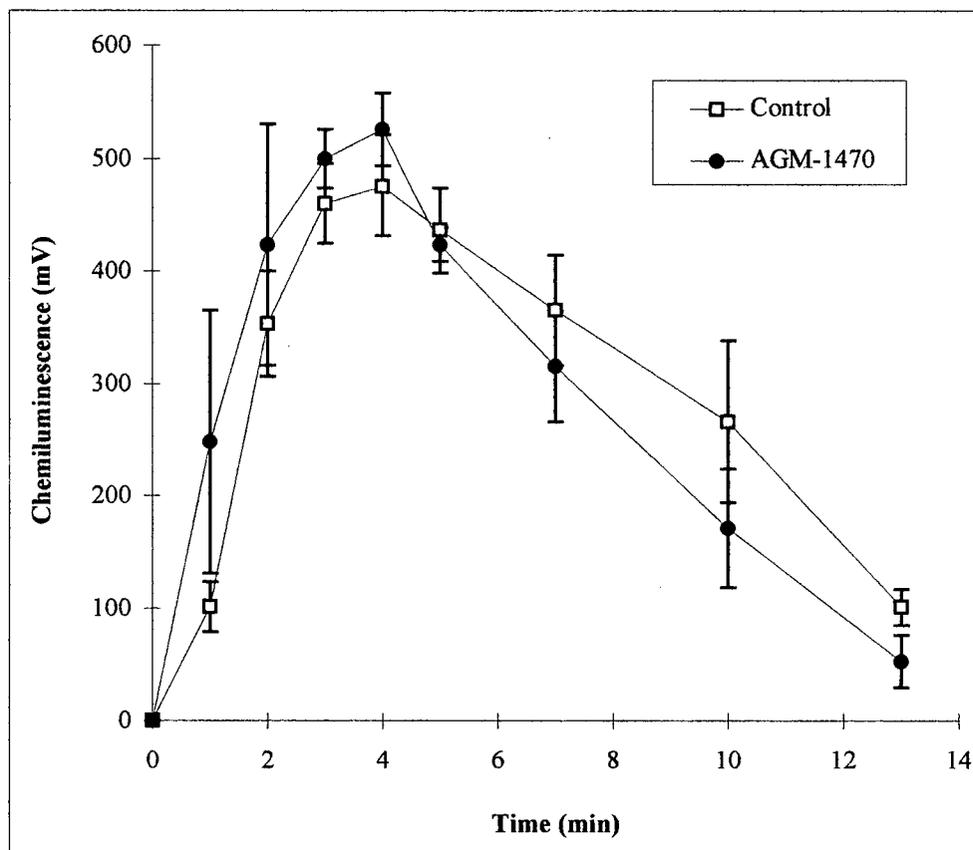


Figure. 44.

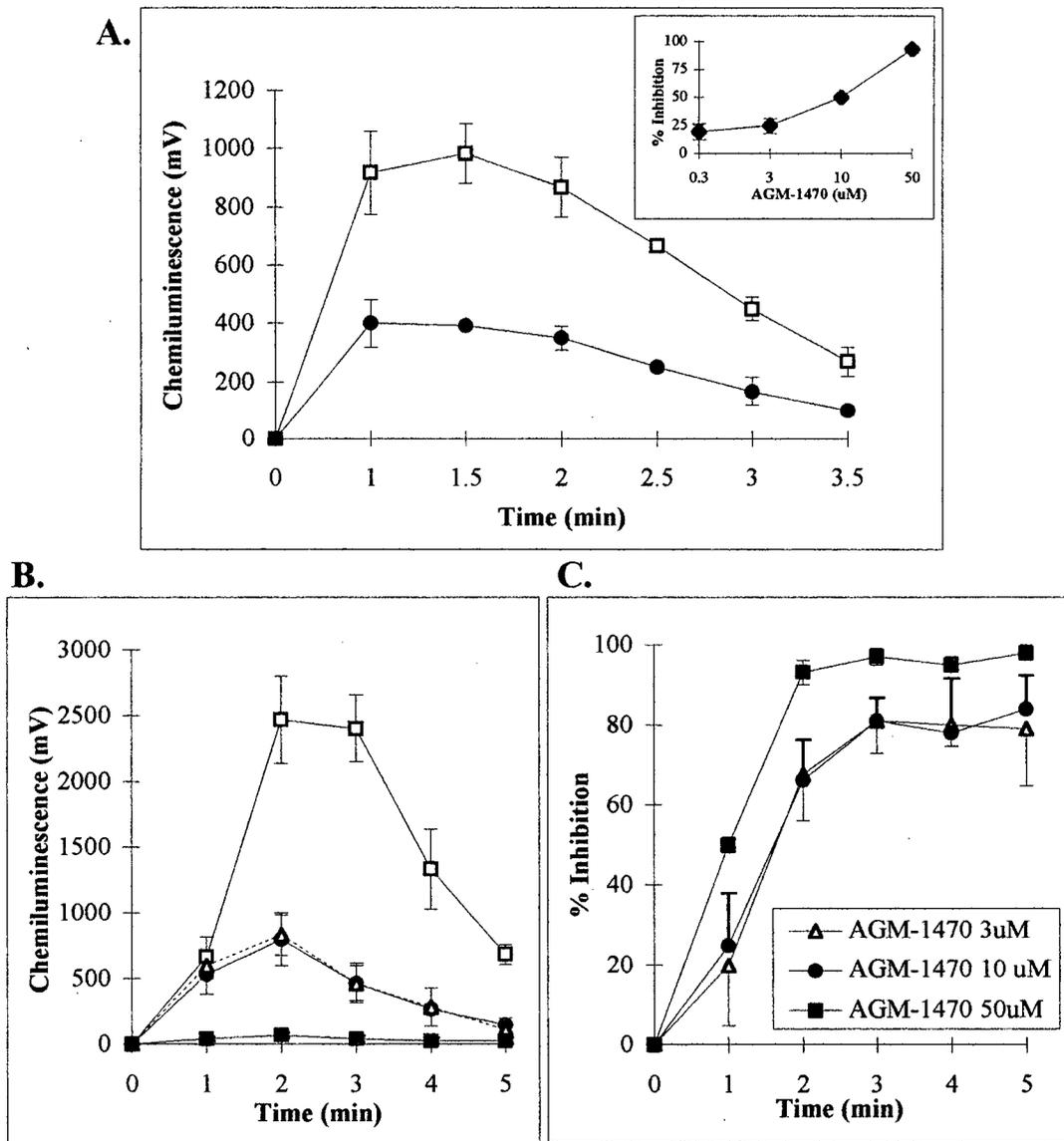
*The effects of AGM-1470 and Taxol on CPPD-, fMLP- and PMA-induced respiratory burst activity measured by chemiluminescence and superoxide anion generation*

Chemiluminescence arises primarily from the light emission associated with the myeloperoxidase initiated breakdown of hydrogen peroxide, a product of superoxide anion. As chemiluminescence only measures oxidase activation indirectly, I also assayed respiratory burst activity via discontinuous measurements of superoxide anion concentrations as determined by the superoxide dismutase inhabitable reduction of cytochrome c.

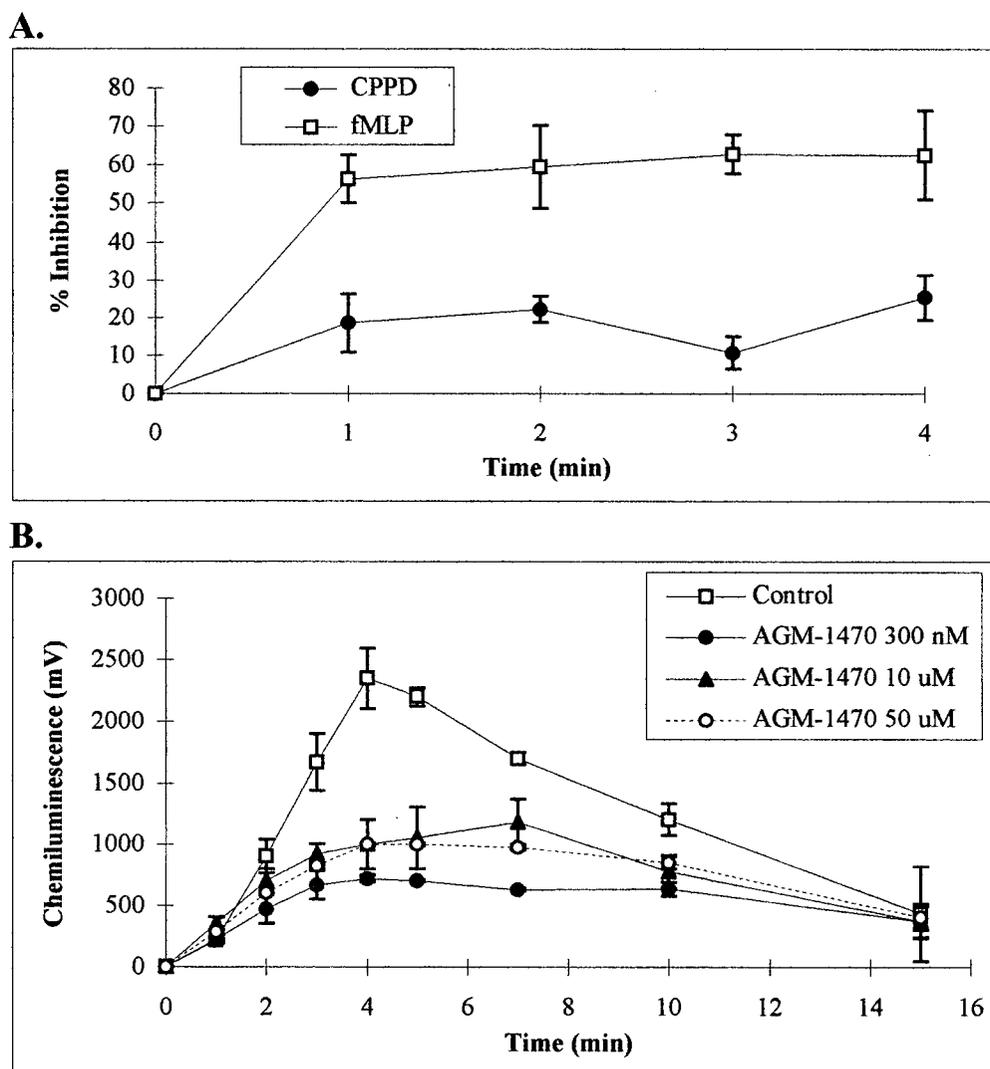
CPPD crystal-, fMLP- and PMA-induced stimulation of neutrophil chemiluminescence reached peak values in about 2-4 min (Figures 45 - 49). Time courses of chemiluminescence were generated from three separate experiments performed with each agonist, and with AGM-1470 at different concentrations. Time courses for CPPD crystal- and PMA-treated cells were completed within 15 min, and for 3.5 min with fMLP, as the rate of chemiluminescence diminished more rapidly after peaking with the latter agonist. All data for any given analysis were collected within 60 min. Figure 45 illustrates that AGM-1470 was not effective in inhibiting CPPD crystal-induced chemiluminescence, whereas activation in response to fMLP and PMA was significantly reduced in response to the drug (Figures 46 and 47). At AGM-1470 concentrations above 300 nM, concentration dependence of this inhibition was not apparent (Figures 46B and C and 47B), and I observed significant cell death based on trypan-blue exclusion microscopy at 50  $\mu$ M. Therefore, I did not further test the effect at higher concentrations. The degree of inhibition of chemiluminescence for fMLP treated cells did not exceed approximately 76%, and was lower (approximately 63%) in PMA treated cells (Figure 47B). An explanation for why inhibition was not greater as concentrations approached those that induced cell death is provided in the 'Discussion.' I also assessed a more direct measure of oxidase activity by assaying the superoxide anion concentration stimulated by fMLP or PMA, with or without AGM-1470 (Figure 48A). Consistent with the results observed in the chemiluminescence assays, AGM-1470 effectively inhibited the superoxide anion generation stimulated in response to fMLP and PMA. Again, the inhibition of superoxide anion generation by AGM-1470 (5  $\mu$ M) pretreatment was not complete. FMLP induction



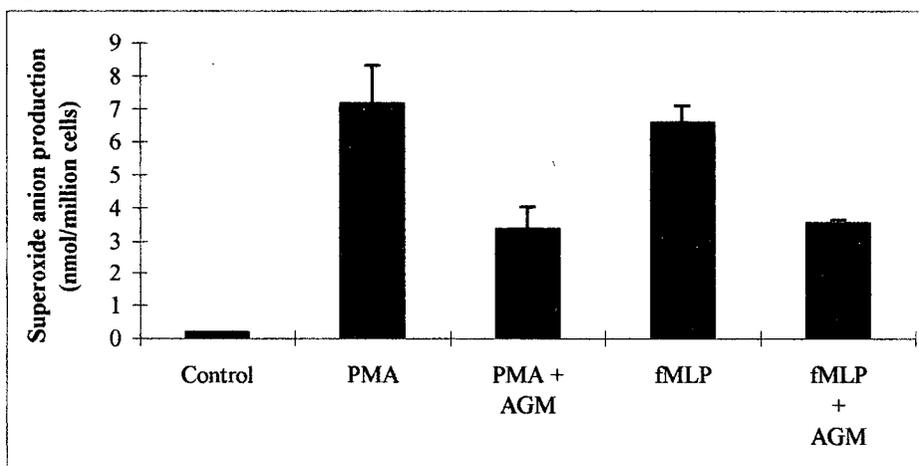
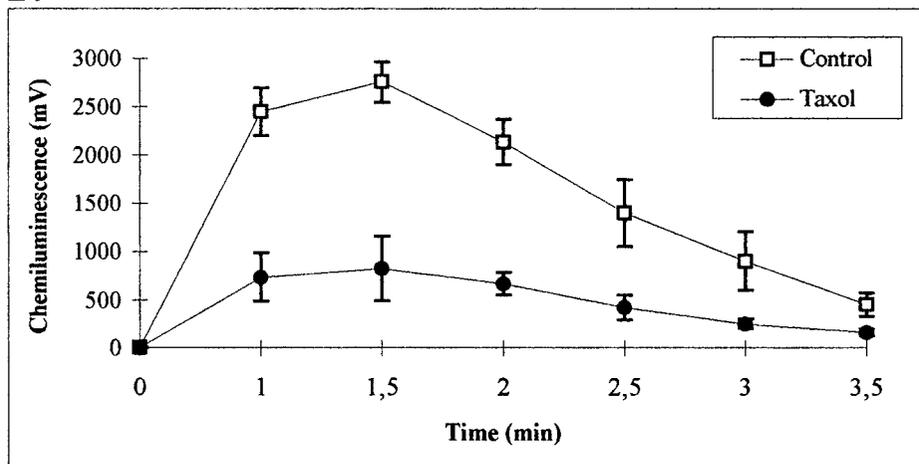
**Figure 45.** Effect of AGM-1470 on CPPD(T)-induced luminol-enhanced chemiluminescent responses in neutrophils. A time course of luminol-enhanced chemiluminescent responses in neutrophils treated without (open square) or with AGM-1470 (5  $\mu$ M, 1 hour) (closed circle) followed by stimulation with triclinic CPPD crystals (50 mg/ml). Values are represented as the mean  $\pm$  S.D. of n=3 experiments.



**Figure 46.** Effect of AGM-1470 on fMLP-induced luminol-enhanced chemiluminescent responses in neutrophils. (A) A time course of luminol-enhanced chemiluminescent responses in neutrophils treated without (open square) and with AGM-1470 (5  $\mu$ M, 1 hour) (closed circle) followed by stimulation fMLP (1  $\mu$ M). The inset represents a concentration dependent inhibition of chemiluminescence following 1 min of incubation with AGM-1470. (B) Represents the responses following preincubation in the absence (open square) and presence with different concentrations of AGM-1470, and (C - derived from B) also representing the inhibition of chemiluminescence from the mean of the maximal response following AGM-1470 treatment prior to fMLP incubation. Error bars represents the S.D. (n=3).



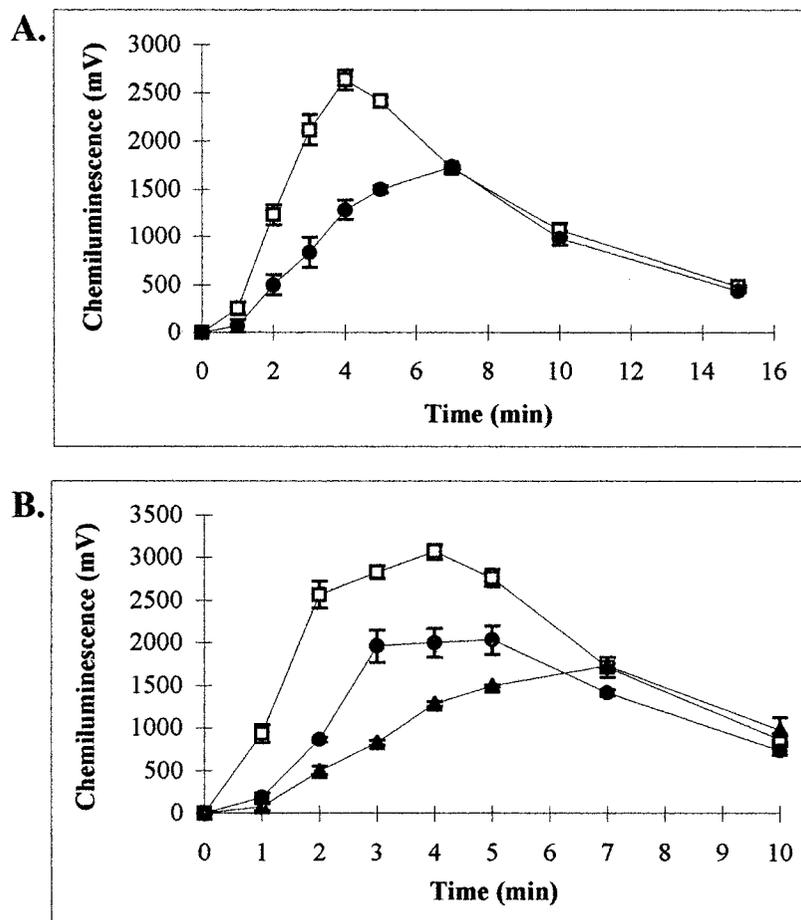
**Figure 47.** AGM-1470 inhibits fMLP and PMA but not CPPD(T) crystal-induced luminol-enhanced chemiluminescent responses in neutrophils. (A) Time course of the inhibition of luminol-enhanced chemiluminescent responses in neutrophils ( $5 \times 10^6$  cells/ml) treated with CPPD(T) crystals (25 mg/ml, 2 min) or 1  $\mu$ M fMLP followed by 5  $\mu$ M AGM-1470 pretreatment. (B) A time course of the luminol-enhanced chemiluminescent response in neutrophils pretreated without ( $\square$ ) or with AGM-1470 at 300 nM ( $\bullet$ ), 10  $\mu$ M ( $\blacktriangle$ ), or 50  $\mu$ M ( $\circ$ ), and each subsequently treated with 0.5  $\mu$ M PMA for 5 min. Values represent the mean  $\pm$  the S.D. of  $n=3$  experiments.

**A.****B.**

**Figure 48.** AGM-1470 inhibits PMA and fMLP-induced superoxide anion production, and Taxol inhibits fMLP-induced luminol-enhanced chemiluminescence in neutrophils ( $5 \times 10^6$  cell/ml). Myeloperoxidase activity expressed as the rate of oxidation of dianisadine, stimulated by PMA ( $0.5 \mu\text{M}$ , 5 min) or fMLP ( $1 \mu\text{M}$ , 1 min) with or without  $5 \mu\text{M}$  AGM-1470 pretreatment for 1 hour at  $37^\circ\text{C}$  (A). The values represent the peak activities for each condition, and are expressed as the mean  $\pm$  the S.D. of  $n=3$  experiments. (B) The time course of luminol-enhanced chemiluminescent response stimulated by fMLP without ( $\square$ ) or with ( $\bullet$ )  $25 \mu\text{M}$  Taxol pretreatment for 30 min.

was inhibited by only 46% ( $\pm 9.6\%$ ), and PMA was inhibited by 54% ( $\pm 3\%$ ). These results illustrate that AGM-1470 inhibited neutrophil respiratory burst activity based on chemiluminescence and superoxide anion production assays in cells treated with fMLP or PMA. AGM-1470 was ineffective in inhibiting respiratory burst activation induced with CPPD crystals. The observation that AGM-1470 selectively inhibited the fMLP- and PMA-induced respiratory bursts correlates with the observation that AGM-1470 pretreatment selectively inhibited fMLP- and PMA-stimulated MAP kinase activity, but not CPPD crystal-induced MAP kinase activation (shown later).

In collaboration with this study, it was demonstrated in Dr. Helen Burt's laboratory that 28  $\mu\text{M}$  Taxol produced strong inhibition of plasma-opsonized CPPD-induced neutrophil chemiluminescence (Jackson *et al.*, 1997a). The percentage inhibition of the peak chemiluminescence in response to plasma-opsonized CPPD with Taxol was 52% ( $\pm 12\%$ ) and the inhibition by Taxol was concentration dependent for all times from 3 to 16 min. The concentration dependence to Taxol inhibition of superoxide anion production and myeloperoxidase release (degranulation) by plasma-opsonized CPPD crystal-stimulated neutrophils was also observed (Jackson *et al.*, 1997a). The effect of Taxol treatment on chemiluminescence was assessed with neutrophils that were induced in the presence of fMLP or PMA (Figures 48B and 49A, respectively). The results demonstrated that neutrophil pretreatment with Taxol inhibited the fMLP- and PMA-induced respiratory burst response. Similar to the results observed with AGM-1470 and Taxol pretreatment in responses to CPPD crystals, Taxol did not induce a complete inhibition of chemiluminescence when cells were induced with either fMLP or PMA, although the degree of inhibition of PMA-induced chemiluminescence was greater as a result of Taxol pretreatment versus AGM-1470 (Figure 49B). These results indicate the presence of distinct pathways that independently contribute to neutrophil activation, which is dealt with in greater detail in the 'Discussion.'

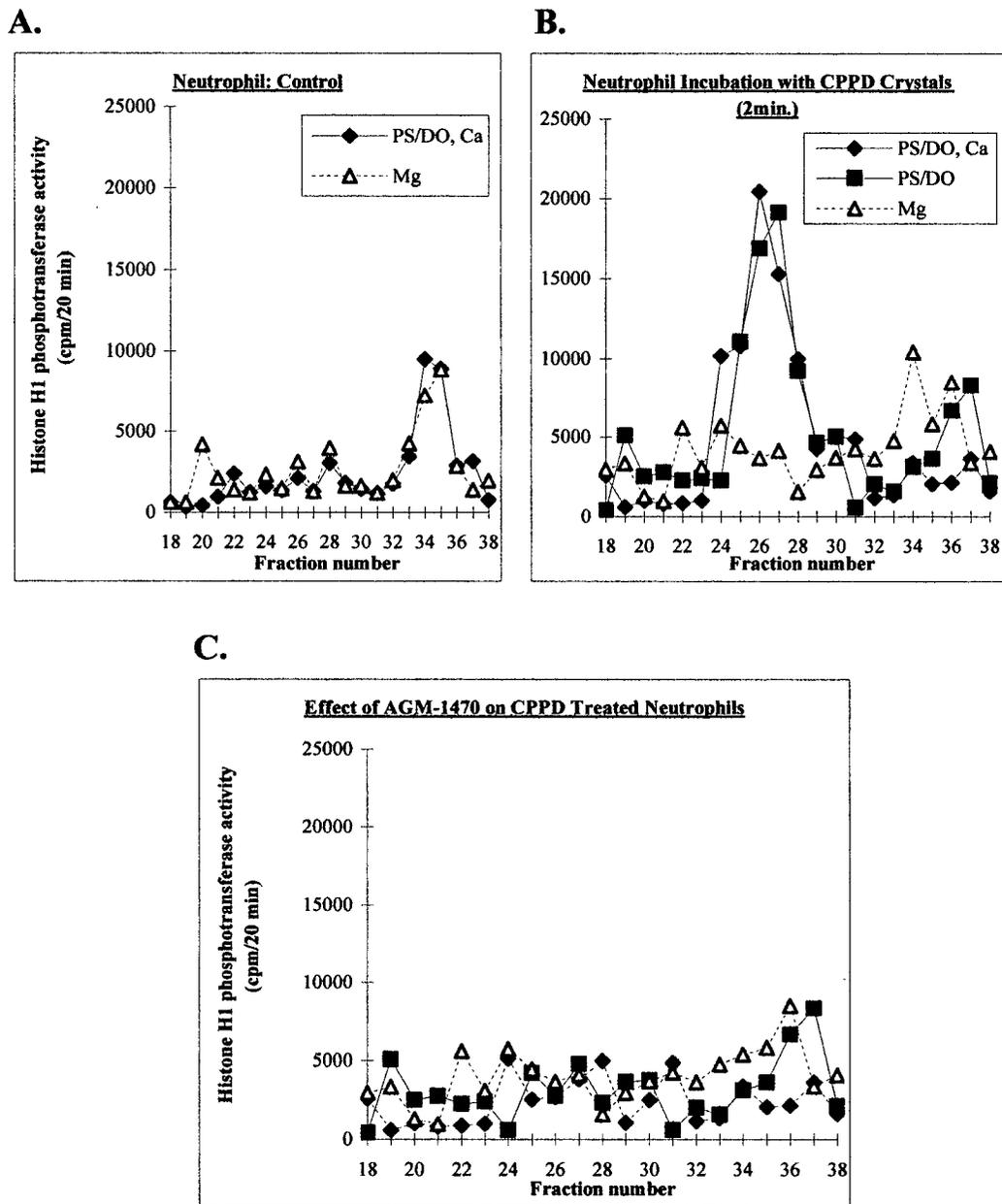


**Figure 49.** AGM-1470 and Taxol inhibit the luminol-enhanced chemiluminescent response in neutrophils stimulated with PMA. (A) A time course of luminol-enhanced chemiluminescence from neutrophils ( $5 \times 10^6$  cell/ml) pretreated with Taxol ( $25 \mu\text{M}$ , 30 min) (●) or without (□), followed by 5 min of PMA ( $0.5 \mu\text{M}$ ) stimulation at  $37^\circ\text{C}$ . (B) Comparison of the inhibitory effects of AGM-1470 (●) versus Taxol (▲) pretreatment on PMA-induced luminol-enhanced chemiluminescence. Cells treated with PMA alone (□). Values represent the mean  $\pm$  the S.D. of  $n=3$  experiments.

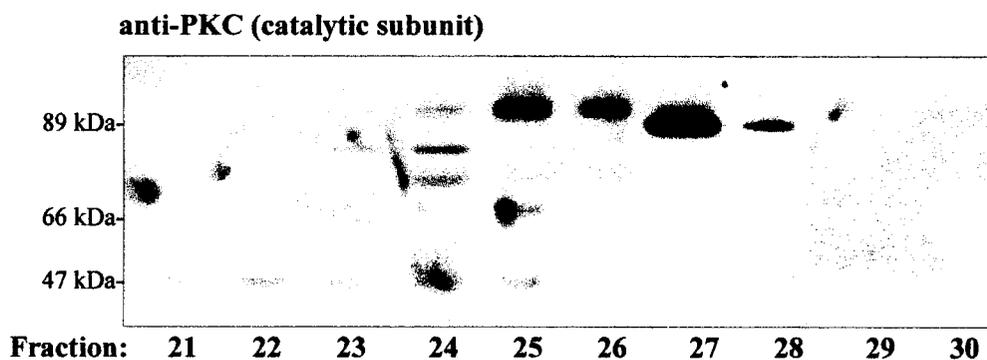
Effects of AGM-1470 and Taxol on CPPD crystal-, fMLP- or PMA-induced PKC activity

Following MonoQ fractionation of neutrophil homogenates from cells incubated with CPPD crystals, with and without AGM-1470 (5  $\mu$ M) pretreatment, and control cells, two peaks of histone H1 phosphotransferase activity were detected (Figure 50). To distinguish whether a peak represented PKC activity, or the activity of another kinase that co-elutes and phosphorylates histone H1, each fraction was assayed in the presence of the PKC cofactors PS, the diacylglycerol diolein (DO), and calcium. The control was represented with magnesium alone. The conventional isoenzymes of PKC  $\alpha$ ,  $\beta$ ,  $\beta$ II and  $\gamma$ , have been shown to require PS, DAG, and calcium, whereas the non-conventional PKC $\delta$ ,  $\epsilon$ , and  $\eta$  isoforms are calcium-independent and require only phosphatidylserine and diacylglycerol. The other, atypical isoforms, are not activated by DAG and do not require calcium as a cofactor (Malviya and Black, 1993; Nishizuka, 1995). Therefore, the peak observed in Figure 50A was unlikely to be from PKC since the peak representing the histone H1 phosphotransferase activity in the presence of the cofactors was approximately the same area as the peak assayed with magnesium alone. The peak (fractions 25-28) in Figure 50B illustrates the histone H1 phosphotransferase activity of a calcium-independent isoform of PKC, since the two peaks assayed with PS and DO with and without calcium represented approximately the same area, and significantly exceed the activity detected in the fractions assayed with magnesium alone. The presence of PKC in fraction 24-28 was further confirmed following SDS-PAGE and Western analysis using a monoclonal antibody to the catalytic subunit of PKC (Figure 51), revealing 90 and 93 kDa bands consistent with the PKC $\epsilon$  isoform (Figure 51, bottom) (Hug and Sarre, 1993). This PKC histone H1 phosphotransferase peak was completely abolished in cells treated with 2 min CPPD crystals that were pretreated with AGM-1470.

To further verify that AGM-1470 inhibited PKC activated in neutrophils in response to CPPD crystal treatment, I assessed the effect of AGM-1470 pretreatment on p70<sup>S6K</sup> activity. I previously observed a PKC-dependent, PI 3-kinase-independent p70<sup>S6K</sup> activity in a neutrophil signal transduction pathway induced by CPPD crystals. In an experiment identical to that described previously (Figure 40), and in 'Experimental Methods,' I fractionated cytosolic extracts from neutrophils incubated with CPPD crystals pre-



**Figure 50.** Partial MonoQ purification of PKC histone H1 phosphotransferase activity (cpm/20 min) from CPPD and AGM-1470 treated neutrophils. MonoQ fractions from neutrophil ( $5 \times 10^6$  cells/ml) detergent solubilized extracts derived from untreated control (A); CPPD(T) crystal (50 mg/ml, 2 min) incubated (B); or (C) cells incubated with 5  $\mu$ M AGM-1470 for 60 min prior to CPPD(T) incubation, were incubated with [ $\gamma$ - $^{32}$ P]ATP and histone H1 in the presence of the PKC cofactors phosphatidylserine (PS), and diolein (DO) with ( $\blacklozenge$ ) and without ( $\blacksquare$ ) calcium, or magnesium (without cofactors; open triangle). Results are representative of six independent experiments.



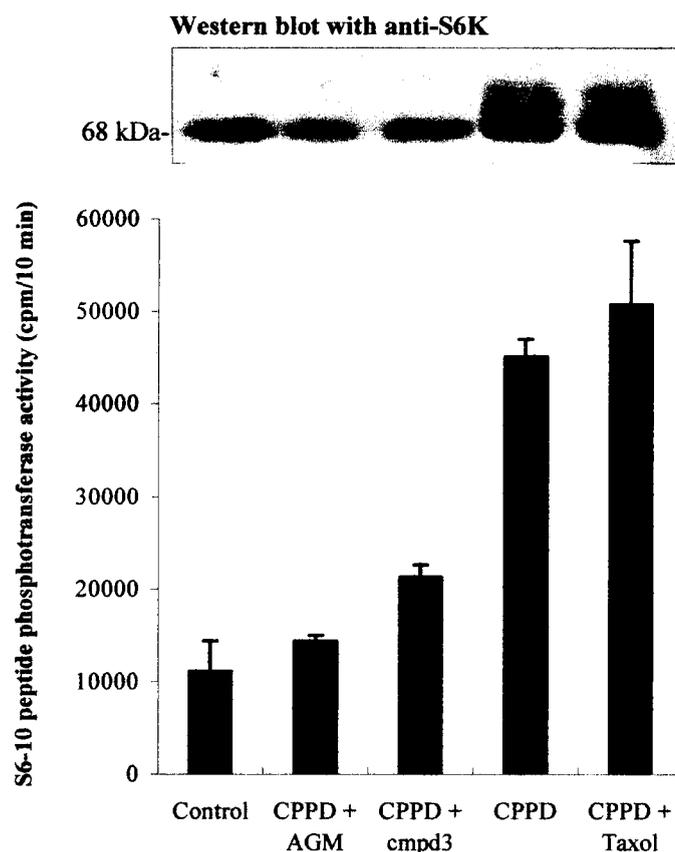
| <u>Isoenzyme</u> | <u>Cofactor requirements</u> | <u>Predicted</u> | <u>Apparent</u>          |
|------------------|------------------------------|------------------|--------------------------|
| $\alpha$         | Ca <sup>2+</sup> , PS, DAG   | 76.8             | 80-81                    |
| $\beta$          | Ca <sup>2+</sup> , PS, DAG   | 76.8             | 79-80                    |
| $\beta$ II       | Ca <sup>2+</sup> , PS, DAG   | 76.9             | 80                       |
| $\gamma$         | Ca <sup>2+</sup> , PS, DAG   | 78.4             | 82+84, 79-80, 77+80      |
| $\delta$         | PS, DAG                      | 77.5             | 78, 76+78, 74+76, 77-79  |
| $\delta$ II      | PS, DAG                      | 77.9             | 82, 84, 86               |
| $\epsilon$       | PS, DAG                      | 83.5             | 89, 90, 90-91, 93+96, 96 |
| $\zeta$          | PS, FA                       | 67.7             | 78, 76, 78-80            |
| $\theta$         | ?                            | 81.6,            | 79                       |
| $\eta$           | PS                           |                  |                          |
| $\lambda$        | ?                            |                  |                          |

**Figure 51.** Fractions from Figure 50B were subject to SDS-PAGE, and Western analysis using a monoclonal antibody (Kinetek Pharmaceuticals, Inc.) to the catalytic subunit of PKC. The data reveals an approximately 90 kDa calcium-independent isoform of PKC that is sensitive to AGM-1470 in the peak containing fractions 24 - 27.

incubated with AGM-1470 (AGM), the PKC inhibitor Compound 3 (cmpd3) or Taxol (Figure 52) by anion exchange chromatography on MonoQ. p70<sup>S6K</sup> phosphotransferase activity of the fractions was detected using the S6-10 peptide, and the fractions at the major phosphotransferase peak at approximately 0.4 M NaCl were pooled and quantitated as described in 'Experimental Methods.' The S6-10 peptide phosphotransferase activity is illustrated in Figure 52. The same fractions were subjected to SDS-PAGE and immunoblotted with the anti-p70<sup>S6K</sup>-NT antibody (Figure 52, inset) and the p70<sup>S6K</sup> phosphotransferase activity co-eluted with the major immunoreactive protein, which migrated as a protein of approximately 70 kDa on SDS-PAGE gels. Previous studies with p70<sup>S6K</sup> have established that it is activated through phosphorylation on multiple serine and threonine residues, and that the most highly phosphorylated and active form of p70<sup>S6K</sup> migrates more slowly than the basally phosphorylated or dephosphorylated inactive enzyme (Ferrari and Thomas, 1994; Petrisch *et al.*, 1995). The corresponding band shift can be visualized on a Western blot of the enzyme correlating with p70<sup>S6K</sup> activity in many systems, and the increased p70<sup>S6K</sup> activity illustrated in Figure 52 also correlates with the band shift observed in the Western blot produced from the same fractions. The results illustrated in Figure 52 demonstrate that the p70<sup>S6K</sup> activity induced by CPPD crystal incubation was inhibited with the PKC inhibitor Compound 3, as expected, and was also inhibited by AGM-1470 pretreatment. MonoQ fractionation of PKC from neutrophils pretreated with Taxol (28  $\mu$ M) and stimulated with fMLP (1  $\mu$ M) or PMA (0.5  $\mu$ M) was assessed for histone H1 phosphotransferase activity. It was observed that Taxol had no effect on CPPD-induced PKC activity (not shown) which correlated with the observation in Figure 52 that Taxol did not inhibit CPPD-induced p70<sup>S6K</sup> activity in neutrophils. MonoQ fractionation of histone H1 phosphotransferase activity from neutrophils stimulated with either fMLP (Figure 53A) or PMA (Figure 53B) was assessed for PKC activity. Figure 53A demonstrated that although the histone H1 phosphotransferase peaks were shifted 2 fractions to the left, a calcium-dependent histone H1 phosphotransferase peak was abrogated by Taxol pretreatment relative to cells treated with fMLP alone. The calcium-independent peak remained relatively unchanged indicating that neutrophil pretreatment with Taxol resulted in the inhibition of a PKC isoform that is distinct from the calcium-

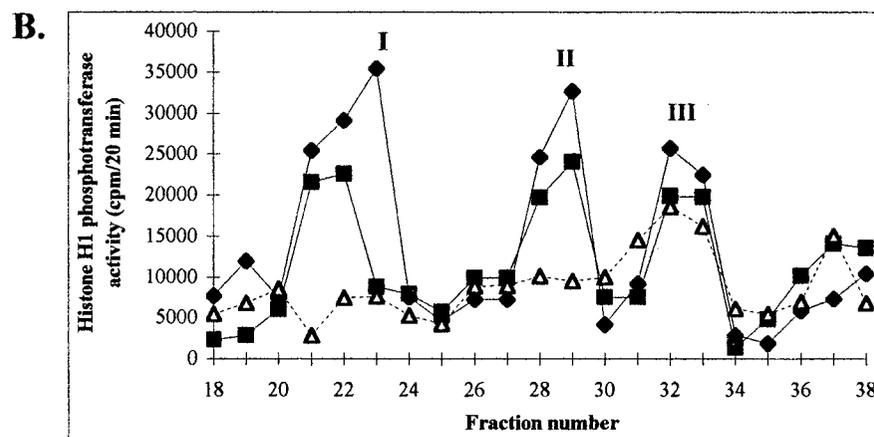
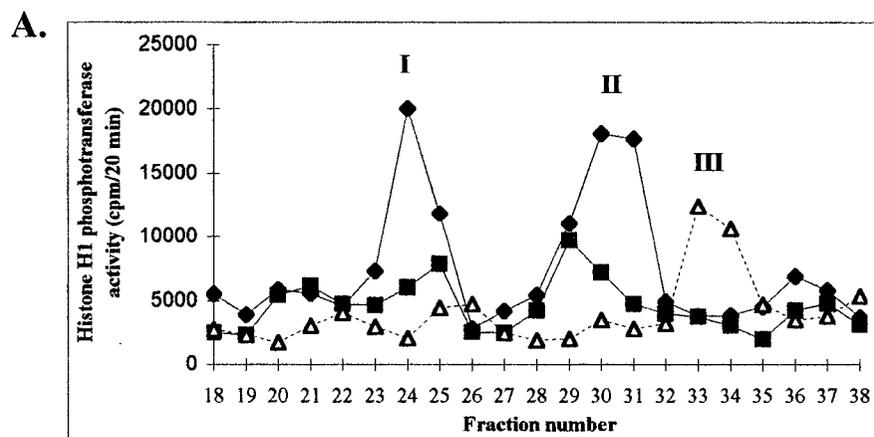
independent isoform of PKC inhibited in CPPD crystal-induced neutrophils. It is possible that Taxol also inhibited a calcium-dependent PKC isoenzyme that may be activated in response to CPPD crystal incubation, but this is unlikely given the relative 'total' A.U.C.'s of the calcium-dependent and -independent phosphotransferase peaks were approximately the same. This was not investigated further in this study. Taxol pretreatment of cells stimulated with PMA did not result in an inhibition of PKC (Figure 53B), which is similar to the finding with AGM-1470 pretreated cells. Therefore both AGM-1470 and Taxol pretreatment of neutrophils resulted in the inhibition of fMLP-induced PKC activity.

The effects of AGM-1470 pretreatment on fMLP- and PMA-induced activation of PKC in neutrophils was assessed via analysis of histone H1 phosphotransferase activity of MonoQ fractions obtained from extracts of neutrophils treated with fMLP or PMA, with and without AGM-1470 pretreatment (Figure 54). The results show that the PKC phosphotransferase peak that eluted in fractions 25-28 induced by fMLP and PMA (Figures 54A and 54C, respectively) was probably a non-conventional, calcium-independent isoform. PMA also appeared to induce a calcium-independent histone H1 phosphotransferase activity which exceeded the activity of the fractions assayed with the PKC cofactors (fractions 22 and 32), and therefore may represent the activity of some isoforms that are not induced following CPPD crystal incubation. For example, I have shown in other systems that the peak eluting in fractions 32-35 under the same separation conditions to represent PKM, the active form of the catalytic subunit resulting from proteolysis of PKC. Pretreatment of fMLP-induced neutrophils with AGM-1470 resulted in the inhibition of PKC activity, whereas none of the activated peaks in the PMA-induced profile were significantly inhibited by AGM-1470 incubation. This latter result, together with previous data showing that PMA rescues the anti-proliferative effect of AGM-1470 in growth factor induced HUVEC indicates that AGM-1470 inhibits PKC indirectly at a step upstream of PKC. Taken together, the observations illustrated in Figures 52 and 56 show that AGM-1470 effectively inhibits PKC indirectly in neutrophils induced by CPPD crystals and fMLP.



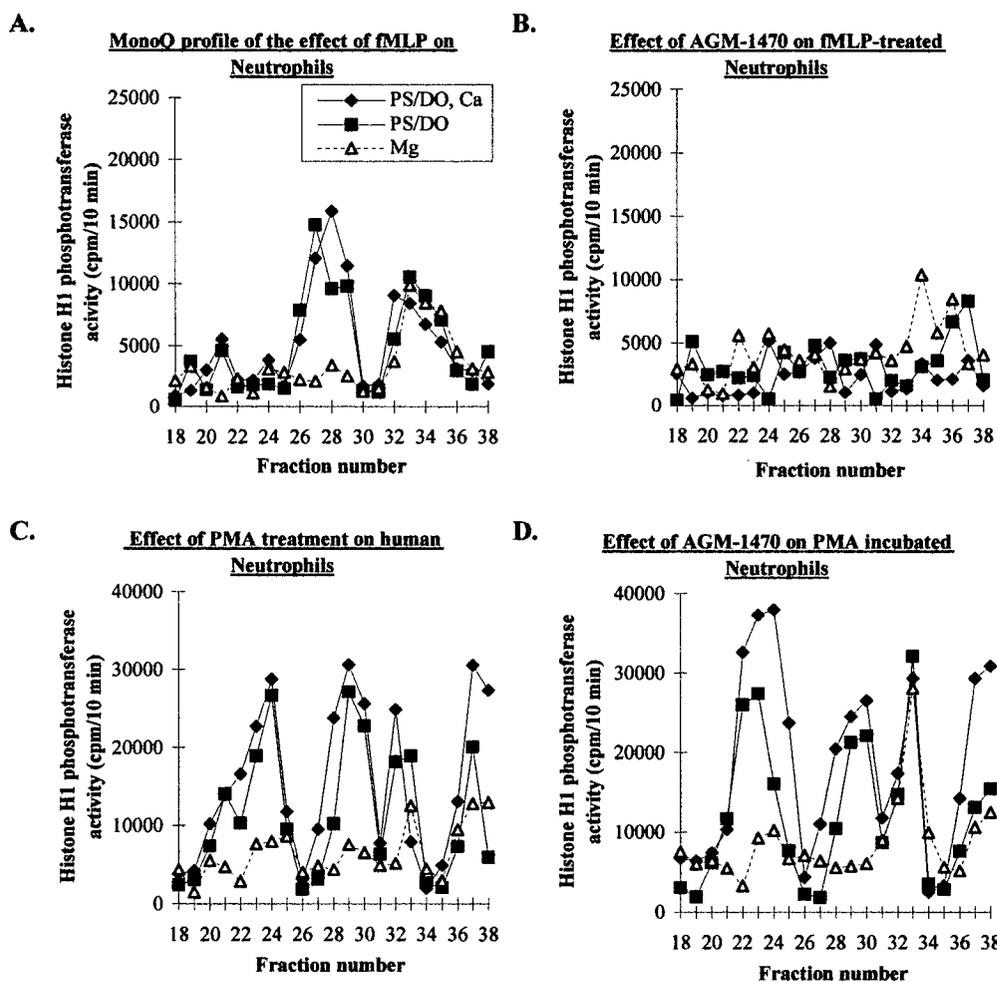
**Figure 52.** AGM-1470 inhibits CPPD(T) crystal induced activation of the p70 S6 kinase. Cytosolic extracts from neutrophils treated with opsinized CPPD(T) crystals (50 mg/ml) for 2 min with or without pre-incubation of 5  $\mu$ M AGM-1470 (AGM), the PKC inhibitor Compound 3 (cmpd3; 10 nM) or 25  $\mu$ M Taxol, were fractionated over a MonoQ anion-exchange column (with a linear 0-800 mM NaCl gradient) and assayed for S6-10 peptide phosphotransferase activity for p70<sup>S6K</sup> activity determination as described in Experimental Methods. P70<sup>S6K</sup> activity within the S6 kinase immunodetected peak was determined and is illustrated in (A). Error bars represent the S.D. of n=3 experiments. The p70<sup>S6K</sup> peak fractions were pooled and subjected to SDS-PAGE for Western blotting with the affinity-purified antibody that recognizes the N-terminal region of p70<sup>S6K</sup> (Kinetek Pharmaceuticals, Inc.). Immunodetection (top panel) was conducted for control cells, (1<sup>st</sup> lane); CPPD and AGM-1470 incubated cells, (2<sup>nd</sup> lane); CPPD and Compound 3 (cmpd3) incubated, (3<sup>rd</sup> lane); or CPPD-treated without, (4<sup>th</sup> lane), or with 25  $\mu$ M Taxol, (5<sup>th</sup> lane).

fMLP



PMA

**Figure 53.** Taxol pretreatment inhibits fMLP but not PMA stimulated PKC activity. MonoQ fractionation was conducted on detergent-solubilized extracts from neutrophils ( $5 \times 10^6$  cell/ml) stimulated with fMLP ( $1 \mu\text{M}$ , 1 min) (A), or PMA ( $0.5 \mu\text{M}$ , 5 min) (B) following pretreatment with  $25 \mu\text{M}$  Taxol for 30 min. PKC activity was determined by analyzing histone H1 phosphotransferase activity (cpm/20 min) in the presence of the calcium-dependent PKC cofactors (PS/DO,  $\text{Ca}^{2+}$ ; closed diamond) and calcium-independent PKC cofactors (PS/DO; closed square), or magnesium without cofactors (open triangle) as described in Figure 32.



**Figure 54.** The effect of AGM-1470 on fMLP- and PMA-induced PKC activity in neutrophils. MonoQ fractionation was conducted on detergent-solubilized extracts from neutrophils stimulated with fMLP (1  $\mu$ M, 1 min) (A), or PMA (0.5  $\mu$ M, 5 min) (C); and AGM-1470 (0.5  $\mu$ M, 1 hour) pre-incubated cells with fMLP (B) or PMA (D). PKC activity was determined by analyzing histone H1 phosphotransferase in the presence of the calcium-dependent PKC cofactors (PS/DO, Ca<sup>2+</sup>; closed diamond) and calcium-independent PKC cofactors (PS/DO; closed square), or magnesium without cofactors (open triangle) as described in Figure 34. AGM-1470 pre-incubation inhibited fMLP-stimulated histone H1 phosphotransferase activity and the PKC-stimulated peak in fractions 25 - 28 (Figure 34), but had no effect on neutrophils stimulated with PMA.

Effect of AGM-1470 and Taxol on CPPD crystal, fMLP, or PMA induced MAP kinase activity

Activation of MAP kinase has also been shown to be mediated by PKC (Toker *et al.*, 1994, Malarkey *et al.*, 1996; Ueda *et al.*, 1996; Zou *et al.*, 1996; Liao *et al.*, 1997) and therefore it is possible that activation of neutrophils mediated by the PKC pathway may occur through MAP kinase. I tested this possibility and the ability of AGM-1470 and Taxol to inhibit MAP kinase activity in neutrophils stimulated with CPPD crystals, fMLP and PMA. I first sought to determine whether AGM-1470 would inhibit MAP kinase activation induced by CPPD crystals and fMLP, and whether Compound 3 would affect the activity of MAP kinase stimulated by CPPD crystals. Figure 55 illustrates an experiment where MAP kinase was immunoprecipitated from neutrophil homogenates of cells incubated with CPPD crystals and AGM-1470, Compound 3 or Taxol, or fMLP and AGM-1470. Additionally, cells were treated with CPPD crystals, fMLP, AGM-1470 and compound 3 independently as controls (Figures 55A and C). Immunoprecipitates were incubated directly in a kinase buffer containing  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  and the MBP substrate as described in 'Experimental Methods.' Samples were then resolved by SDS-PAGE and the location of the phosphorylated MBP was determined using autoradiography, and the MBP bands were excised from the gel and analyzed by Cerenkov counting (Figure 55D). The results demonstrate that Taxol effectively inhibited the MAP kinase activity resulting from CPPD crystal incubation in neutrophils (shown in more detail later). Furthermore, Compound 3 did not inhibit MAP kinase indicating that in CPPD crystal treated cells, PKC and MAP kinase lie on distinct pathways. AGM-1470 pre-incubation did not result in inhibition of MAP kinase in neutrophils treated with CPPD crystals. Western analysis of the immunoprecipitated MAP kinase proteins with the anti-ERK1-CT antibody that has an affinity for both isoforms demonstrated that AGM-1470 had no effect on CPPD-induced reduction in band mobility of the SDS-PAGE separated MAP kinase protein associated with activation of MAP kinase, whereas Taxol inhibited the band shift, consistent with the MBP phosphotransferase results (Figure 55B).

Following MonoQ fractionation of lysates of neutrophils incubated with CPPD crystals, fMLP or PMA, with or without AGM-1470 pretreatment, multiple peaks of MBP

**Figure 55.** Selective inhibitory effect of AGM-1470 pretreatment of neutrophils on the fMLP versus CPPD(T) stimulated MAP kinase activity. MAP kinase immunoprecipitates from 500  $\mu$ g total protein extracts were incubated with [ $\gamma$ - $^{32}$ P]ATP and MBP substrate for 30 min and subjected to SDS-PAGE. Phosphorylated MBP was transferred to nitrocellulose and subject to autoradiography. Results of the autoradiography demonstrating MAP kinase activity are shown in (A), lane 1, control; lane 2, CPPD; lane 3, AGM-1470 (5  $\mu$ M), one hour incubation; lane 4, AGM-1470 pre-treatment followed by CPPD incubation for 2 min; and lane 5, CPPD and Taxol as a positive control, and in (C), lane 1, CPPD; lane 2, Compound 3 (cmpd3; 10 nM); lane 3 CPPD and Compound 3; lane 4, fMLP (1  $\mu$ M); lane 5, fMLP and AGM-1470. Western analyses with the anti-Erk1-CT antibody (Upstate Biotechnology, Inc.) of the results illustrated in (A) are shown in (B). MBP bands were excised from the membrane and phosphorylation was determined by Cerenkov counting (D). The results are representation of 2 independent experiments.

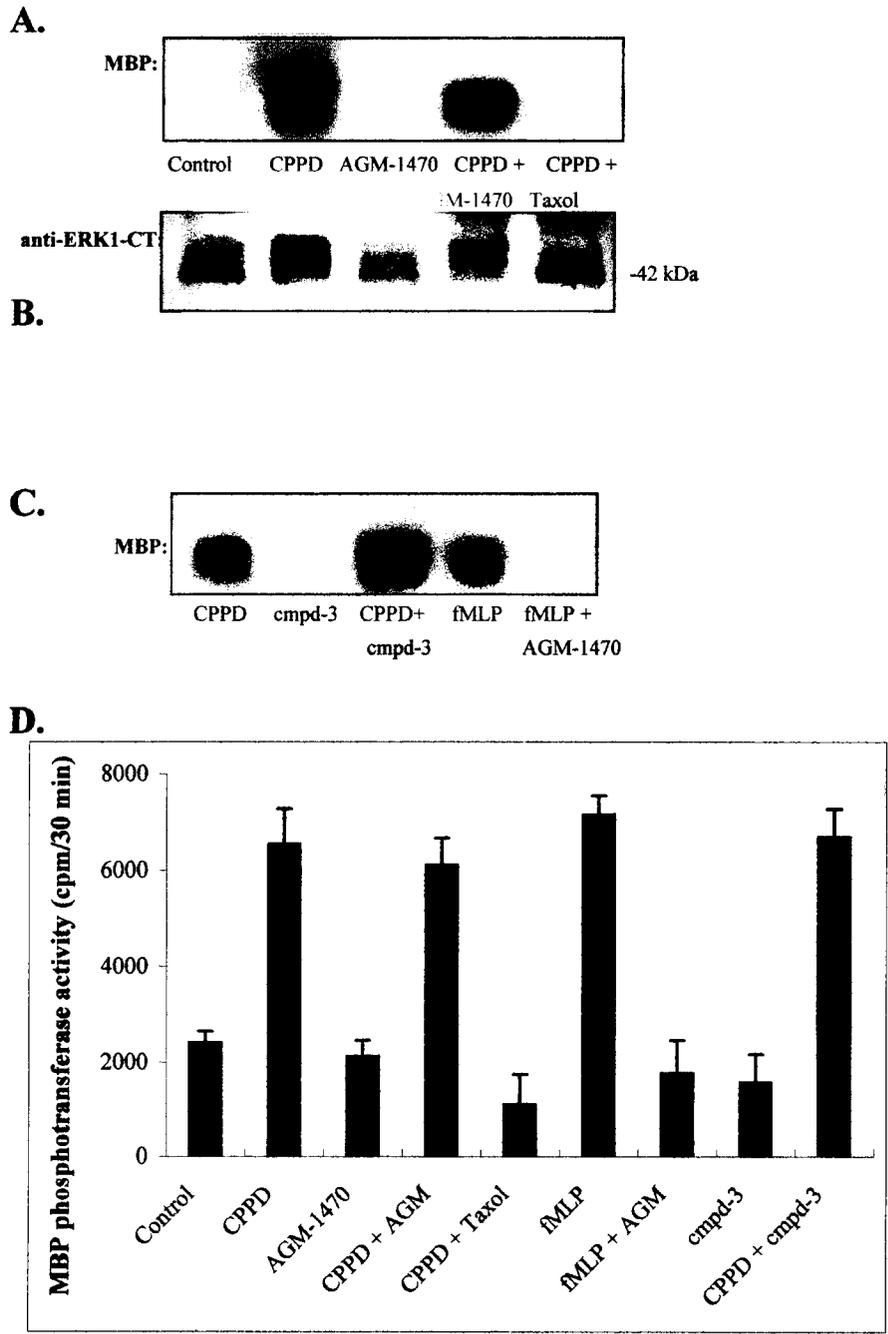
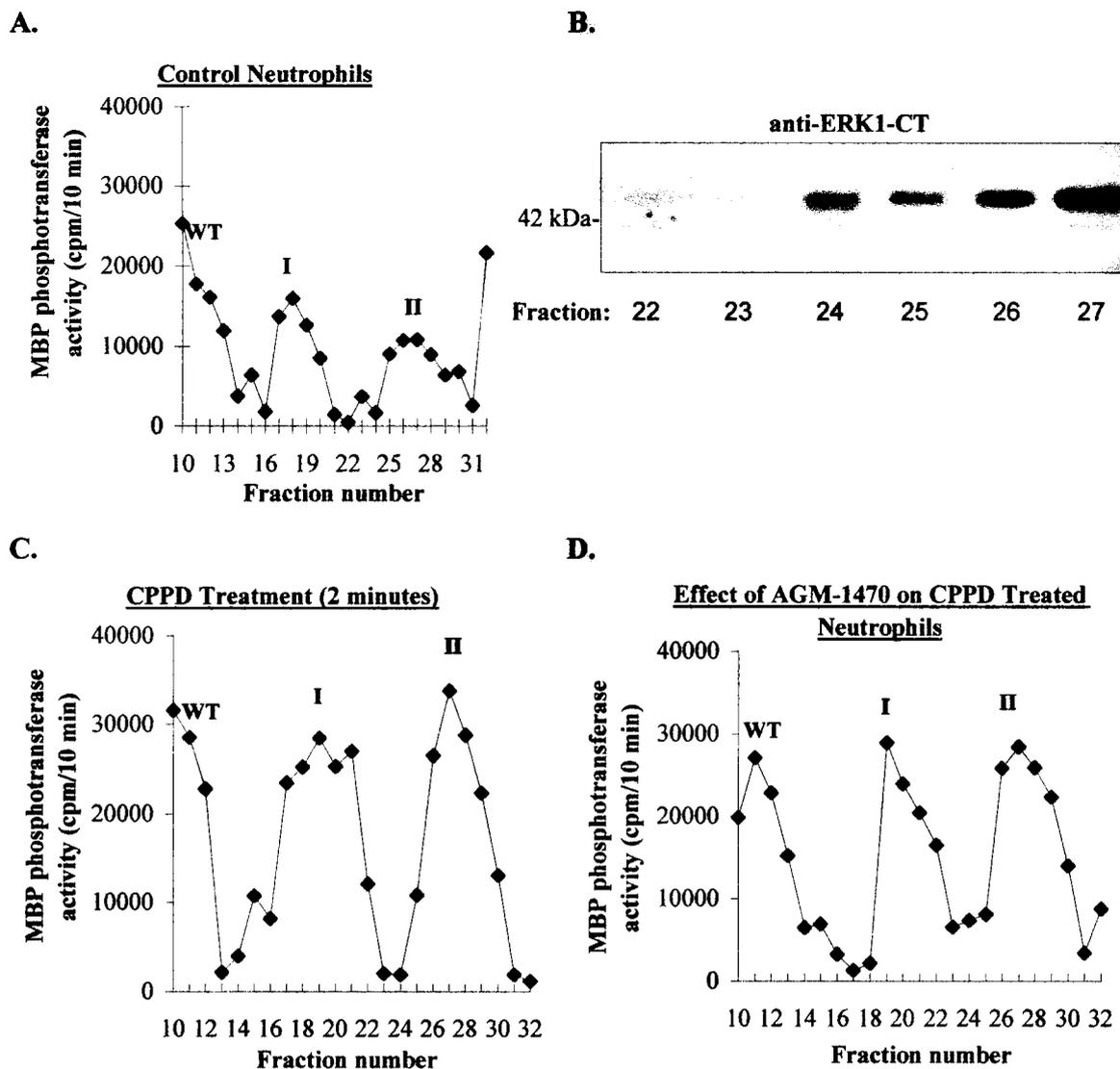


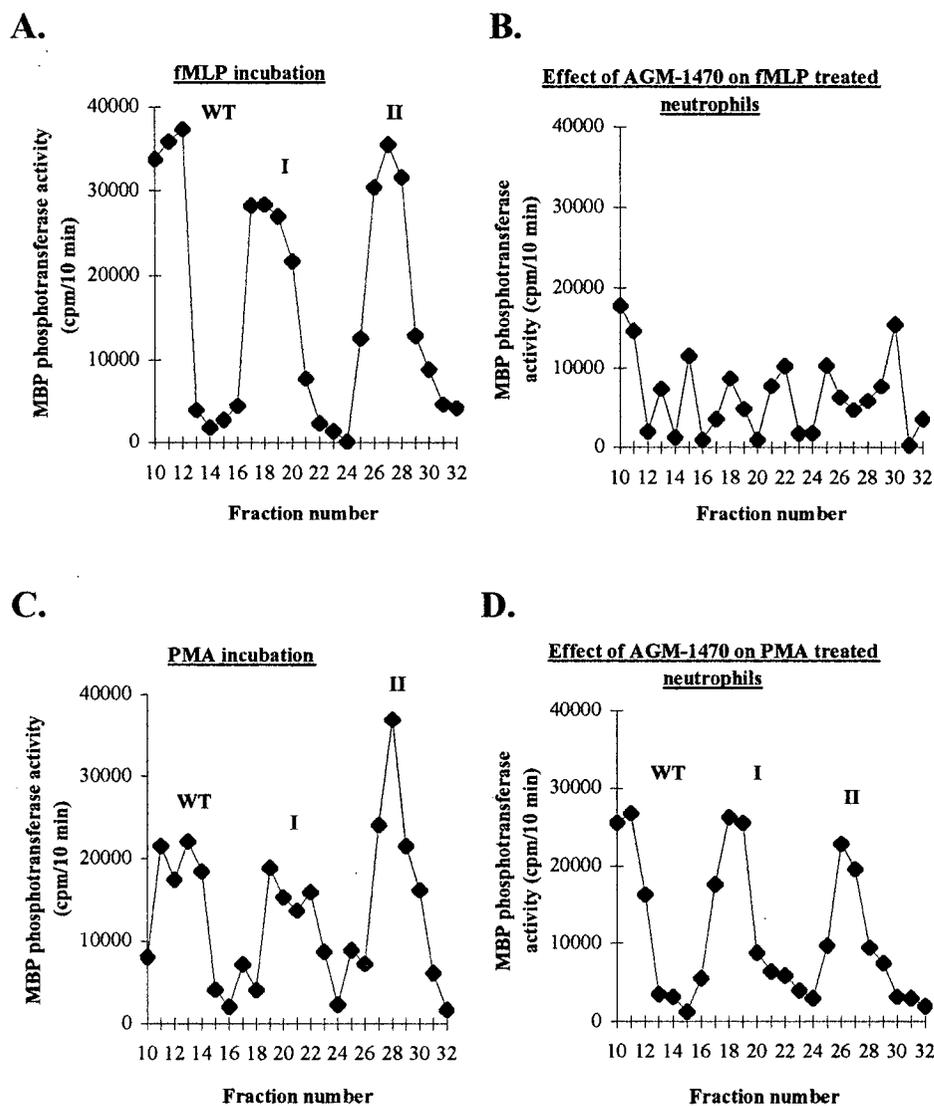
Figure 55.

phosphotransferase activity were eluted (Figures 56-58). The identities of the first and second peaks are unknown (Figures 56 and 57), but similar to MAP kinase activation experiments assayed with this method previously (Hei *et al.*, 1993), immunoblotting with the anti-ERK1-CT antibody revealed that MAP kinase was detected in the fractions of the second peak (fractions 24-28) as illustrated in Figure 56B. Figure 57C shows the CPPD crystal-induced activation of the MAP kinase associated MBP phosphotransferase peak that exceeded the activity in the second peak of the control (Figure 56A). MAP kinase activation induced by CPPD crystal incubation was not inhibited by pretreatment with AGM-1470 (Figure 56D). This is consistent with the MAP kinase associated MBP phosphotransferase activity results obtained from immunoprecipitated protein. Figure 57 demonstrates that AGM-1470 effectively inhibited the MAP kinase associated MBP phosphotransferase activity induced by fMLP and PMA. I propose that the results presented in Figure 57 indicate that AGM-1470 selectively inhibited fMLP- and PMA-induced MAP kinase activity in neutrophils, but not CPPD crystal-induced MAP kinase activity.

The effect of Taxol on MAP kinase activity in response to CPPD crystal treatment was similarly investigated. CPPD crystals induced a large increase in MAP kinase activity as described by MonoQ phosphotransferase activity peaks in fractions 24-28 in Figure 58A. Taxol (28  $\mu$ M) pretreatment of neutrophils inhibited the activation of MAP kinase associated with CPPD crystal treatment (Figure 58A). Both ERK1 and ERK2 forms of MAP kinase were immunoprecipitated from neutrophil homogenates using agarose conjugated antibodies to the C-terminus end of MAP kinase. These immunoprecipitates were then Western blotted using anti-phosphotyrosine antibodies (Figure 58B). Film exposure times were extended so that MAP kinase in non-activated cells could be visualized (control, lane 2; and Taxol control, lane 4) and the effect of Taxol on control levels could also be studied. Figure 58B shows bands at 42 and 44 kDa, the molecular masses of ERK2 and ERK1, respectively. CPPD crystals increased the degree of tyrosine phosphorylation of both ERK1 and ERK2 as shown in Lane 1 (Figure 58B). The increase in tyrosine phosphorylation of ERK1 and ERK2 associated with MAP kinase activation induced by CPPD crystals were not observed following Taxol pretreatment (28  $\mu$ M) of



**Figure 56.** MonoQ chromatography of CPPD crystal-stimulated MBP kinases and the result of AGM-1470 pre-incubation. Neutrophil ( $5 \times 10^6$  cells/ml) extracts from control (A); CPPD (50 mg/ml) treated (C); and CPPD crystal treated following 60 min AGM-1470 ( $5 \mu\text{M}$ ) pretreatment (D), were fractionated over a MonoQ anion-exchange column (with 0-800 mM linear NaCl gradient) and assayed for MBP phosphotransferase activity as described in Experimental Methods. (B), Eluent fractions 22-27 from neutrophils were subjected to SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-Erk1-CT antibody (UBI). AGM-1470 did not inhibit the MAP kinase activity induced by CPPD crystals. WT corresponds to wash through material that did not bind to the MonoQ column. Results are representative of three independent experiments.



**Figure 57.** MonoQ chromatography of fMLP and PMA stimulated MBP kinases and the result of AGM-1470 pre-incubation. Neutrophil ( $5 \times 10^6$  cells/ml) extracts fMLP treated cells ( $1 \mu\text{M}$ , 1 min) (A); fMLP and AGM-1470 pretreated cells (B); PMA ( $0.5 \mu\text{M}$ , 5 minutes) (C); and PMA and AGM-1470 pretreated cells (D), were fractionated over a MonoQ anion-exchange column (with 0-800 mM linear NaCl gradient) and assayed for MBP phosphotransferase activity as described in Experimental Methods. AGM-1470 selectively inhibited the MAP kinase activity induced by fMLP and PMA, but not CPPD crystals.

**Figure 58.** Inhibitory effect of Taxol pretreatment on plasma-opsonized CPPD(T) crystal-induced MAP kinase activity in neutrophils. **(A)** MAP kinase activity is described as MBP phosphorylation in fractions from MonoQ separated neutrophil detergent-solubilized homogenates. Control (open circle), Taxol (25  $\mu$ M, 5 min pretreatment) control (closed square), 2 min CPPD(T) crystal incubation without (open square) or with 5 min Taxol (28  $\mu$ M) pretreatment (closed circle). **(B)** Inhibitory effect on the increased tyrosine phosphorylation of ERK1 and ERK2 forms of MAP kinase in opsonized CPPD(T) crystal stimulated (2 min) neutrophils. MAP kinase immunoprecipitates from neutrophil incubations (detergent-solubilized extracts) were Western blotted using antiphosphotyrosine antibodies. Lane 1, CPPD(T) crystal treatment; Lane 2, control (without crystals); Lane 3, CPPD(T) + Taxol (28  $\mu$ M); Lane 4, Taxol control

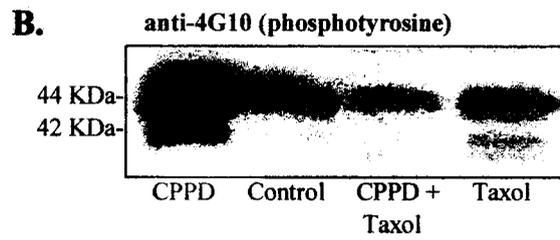
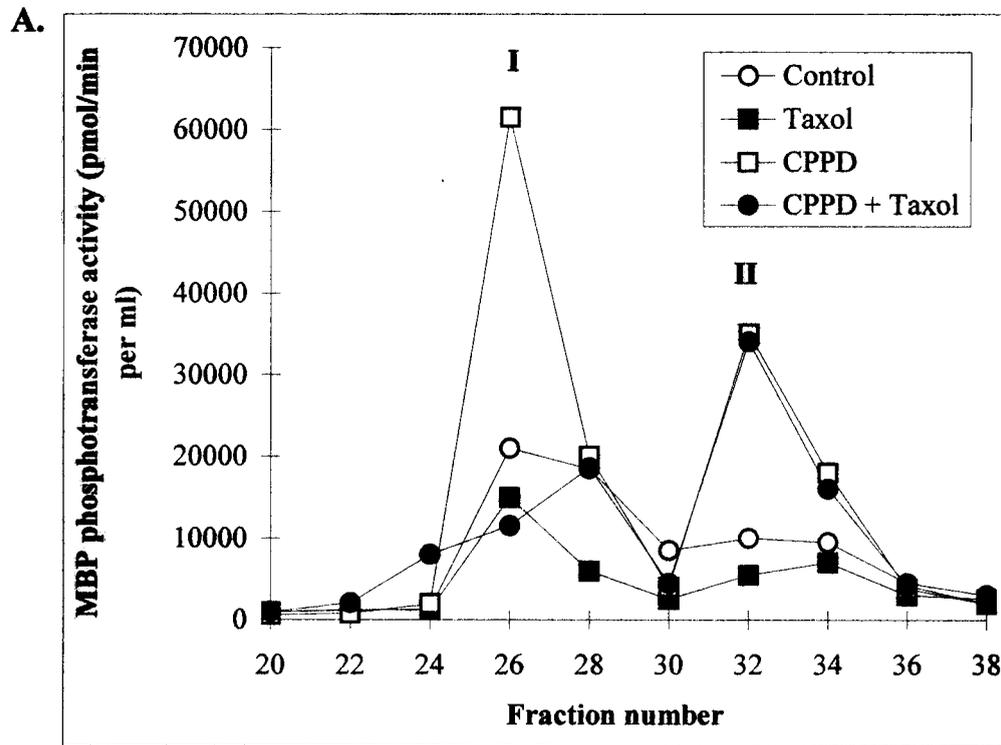


Figure 58.

neutrophils as seen in Lane 3. Taxol had no inhibitory effect on control levels in Figure 58B.

In another experiment, ERK1 and ERK2 forms of MAP kinase were immunoprecipitated from neutrophil homogenates and incubated directly in a buffer containing [ $\gamma$ - $^{32}$ P]ATP and MBP (as described for the MAP kinase assay in 'Experimental Methods'). I have previously shown that only ERK1 remains in the active state under these conditions. Samples were then resolved by SDS-PAGE and the location of the phosphorylated MBP was determined using autoradiography. Samples from CPPD-neutrophil incubations showed increased ERK1 activity (denser bands) which was inhibited by pretreatment of neutrophils with Taxol (data not shown). The bands corresponding to ERK1 were excised from the gels and analyzed by Cerenkov counting (Figure 58C) which showed the increased activity of ERK1 in neutrophils stimulated by CPPD crystals. Taxol pretreatment of neutrophils inhibited this CPPD crystal-induced increased activity of ERK1 to control levels (Figure 58C).

Figure 59 illustrates independent experiments where MAP kinase MBP phosphotransferase activity was immunoprecipitated with the anti-ERK1-CT antibody from neutrophils stimulated with each of the three agonists and pretreated with AGM-1470, Taxol, or Compound 3. The earlier observation that AGM-1470 selectively inhibited fMLP- and PMA-induced MAP kinase activation, but was ineffective with CPPD crystal stimulated cells was represented in Figures 59A and B. Figure 59B demonstrates that Taxol inhibited CPPD-induced MAP kinase activity, but had minimal to no effect on fMLP- or PMA-stimulated MAP kinase activation. Figure 59B represents the mean and standard deviation of three independent experiments. Taken together, the results presented in this chapter indicate that Taxol, but not AGM-1470 inhibited CPPD crystal-induced MAP kinase activity independent of PKC activation, whereas, AGM-1470 inhibited MAP kinase activity induced by fMLP and PMA, but Taxol had no effect under these conditions.

**Figure 59.** The effects of AGM-1470, Taxol or Compound 3 pretreatment on agonist-induced MAP kinase activity in neutrophils. Neutrophils ( $5 \times 10^6$  cells/ml) +/- AGM-1470 (5  $\mu$ M, 1 hour), Taxol (28  $\mu$ M, 30 min) or Compound 3 (10 nM, 5 min) was incubated with plasma-opsonized CPPD(T) crystals (50 mg/ml) for 2 min, or fMLP (1  $\mu$ M) for 1 min, and detergent-solubilized extracts were prepared. MAP kinase was immunoprecipitated with a polyclonal antibody that immunoprecipitated ERK1 and ERK2 forms of MAP kinase, but only the activity of ERK1, similar to the protocol described in Figure 55. Immunoprecipitated MAP kinase was incubated with [ $\gamma$ - $^{32}$ P]ATP and MBP for 30 min at 30°C. Samples were then separated by SDS-PAGE and MBP bands, detected by autoradiography, were excised from the membrane and phosphorylation was determined by Cerenkov counting. The results of the Cerenkov counting are indicated and the values represents the mean +/- the S.D. of n=3 experiments and are proportional to the degree of Cerenkov counting detected when a respective agonist (CPPD, fMLP or PMA) was analyzed (shown with a 100 % bar, the S.D. not exceeding 6 %). Control, AGM-1470, and Taxol (alone) were proportional to the MBP phosphotransferase activity detected when cells were stimulated with CPPD.

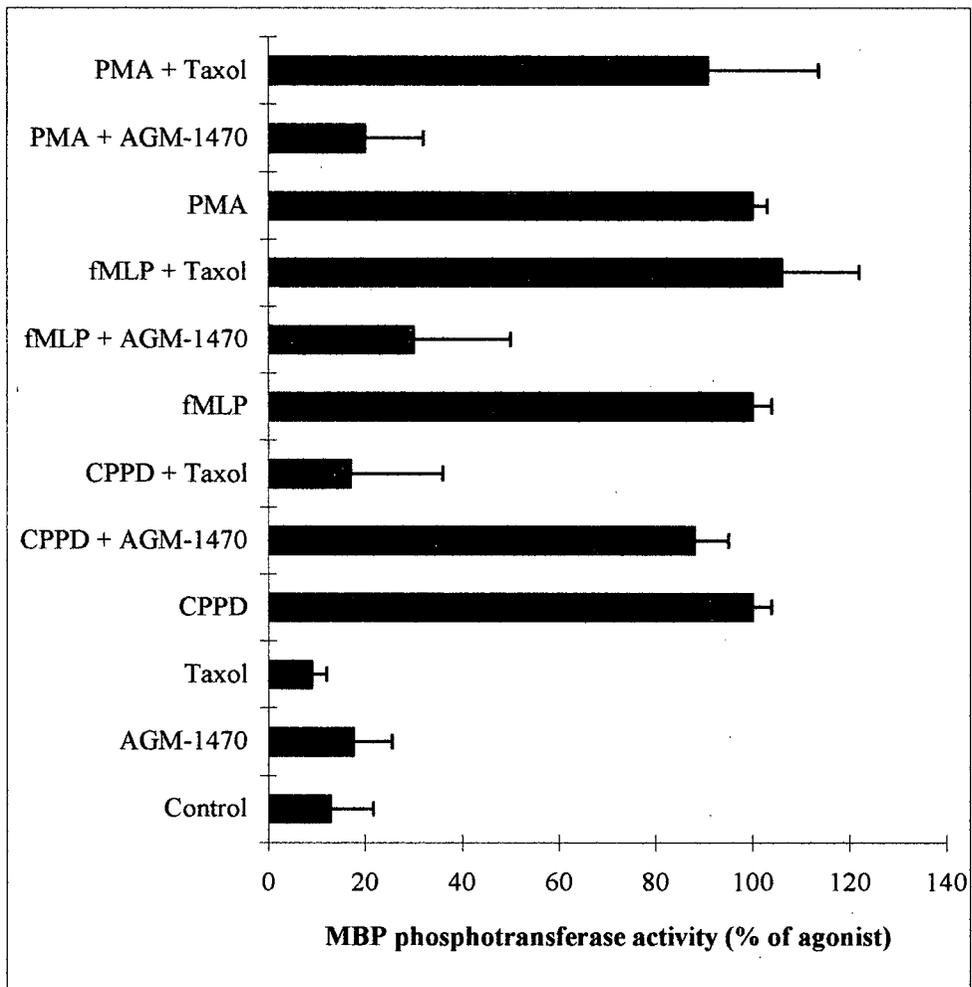


Figure 59.

Wortmannin inhibits fMLP but not PMA-induced neutrophil respiratory burst: AGM-1470 and Taxol inhibit MAP kinase and neutrophil activation independent of PI 3-kinase

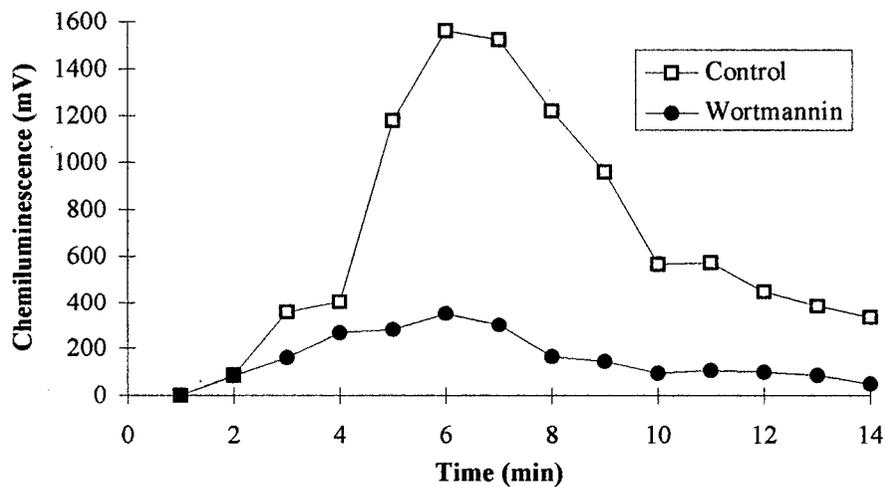
Recently, it was demonstrated that PI 3-kinase is activated in crystal-stimulated neutrophils (Jackson *et al.*, 1997b), and that two specific inhibitors of PI 3-kinase, wortmannin and LY294002, were shown to completely suppress plasma coated CPPD crystal-incubated neutrophil activation at concentrations lower than the known IC<sub>50</sub> of these inhibitors for PI 3-kinase. Additionally, the role of PI 3-kinase activity in chemoattractant stimulated neutrophils has previously been reported (Arcaro and Wymann, 1993; Okada *et al.*, 1994). To the best of my knowledge, the involvement of MAP kinase in PMA stimulated neutrophils has not been reported, although this has been anticipated. PI 3-kinase has been implicated in the activation of MAP kinase (Nishioka *et al.*, 1995; Karnitz *et al.*, 1995). The data presented in this report indicate a role for MAP kinase in neutrophil respiratory burst in response to fMLP and PMA incubation, which was inhibited by AGM-1470 pretreatment, and that inhibition of MAP kinase by Taxol probably contributed to its ability to inhibit CPPD-induced neutrophil activation. Therefore, it would be meaningful to determine if the MAP kinase activity and the neutrophil activation inhibitory effect of AGM-1470 were mediated through PI 3-kinase.

To determine if PI 3-kinase activity is necessary in the mechanism of inhibition of neutrophil activation by AGM-1470, I first analyzed the effects of wortmannin treatment on fMLP- and PMA-induced chemiluminescence. Figure 60 illustrates the consequence of wortmannin pretreatment on fMLP-treated (Panel A, control) and PMA-treated (Panel B, control) neutrophils. As indicated earlier, wortmannin inhibited fMLP-induced neutrophil respiratory burst as measured by chemiluminescence, but no effect was observed in cells stimulated with PMA. The observation that AGM-1470 inhibited neutrophil activation in response to PMA treatment, and wortmannin did not, indicates that AGM-1470 may inhibit the activation of neutrophil respiratory burst and MAP kinase activity independent of PI 3-kinase. To verify this I next examined the effects of AGM-1470 and Taxol on PI 3-kinase activity in cells stimulated with CPPD crystals or fMLP. To this aim, immunoprecipitation studies were conducted on crude neutrophil lysates with the monoclonal anti-p85 PI 3-kinase antibody and the activity of the immunoprecipitates was assessed using

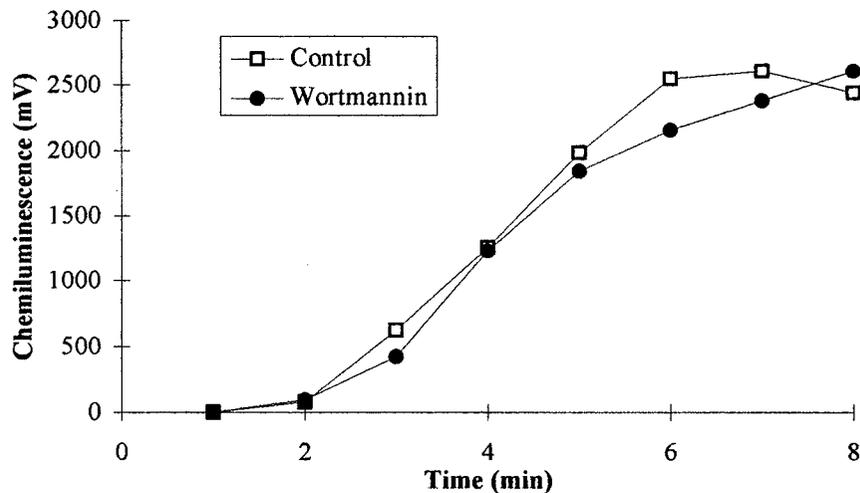
phosphatidylinositol as the substrate. The results of the radiography of the TLC separated phosphorylated substrate, phosphatidylinositol-3 phosphate (PI-3P), is shown in Figure 61. The spots were excised and counted for [ $\gamma$ - $^{32}$ P]ATP incorporation, and the results are illustrated in Figure 61. Activation of PI 3-kinase in response to CPPD crystals and fMLP treatment was demonstrated as [ $\gamma$ - $^{32}$ P]ATP incorporation in these samples was significantly greater than control cells, and CPPD crystal-treated cells pre-incubated with wortmannin demonstrated negligible activity. Pretreatment with AGM-1470 did not inhibit PI 3-kinase activation in response to either CPPD crystals or fMLP. These results indicate that neither AGM-1470 nor Taxol inhibited PI 3-kinase activation in response to either agonist, nor an effector upstream of it, and that the inhibitory effect of AGM-1470 or Taxol on neutrophil respiratory burst responses and MAP kinase activity were distinct from the PI 3-kinase pathway. Further, I propose that activation of PI 3-kinase and MAP kinase pathways are independent of each other in response to CPPD crystals, fMLP and PMA incubation of neutrophils.

## fMLP

A.

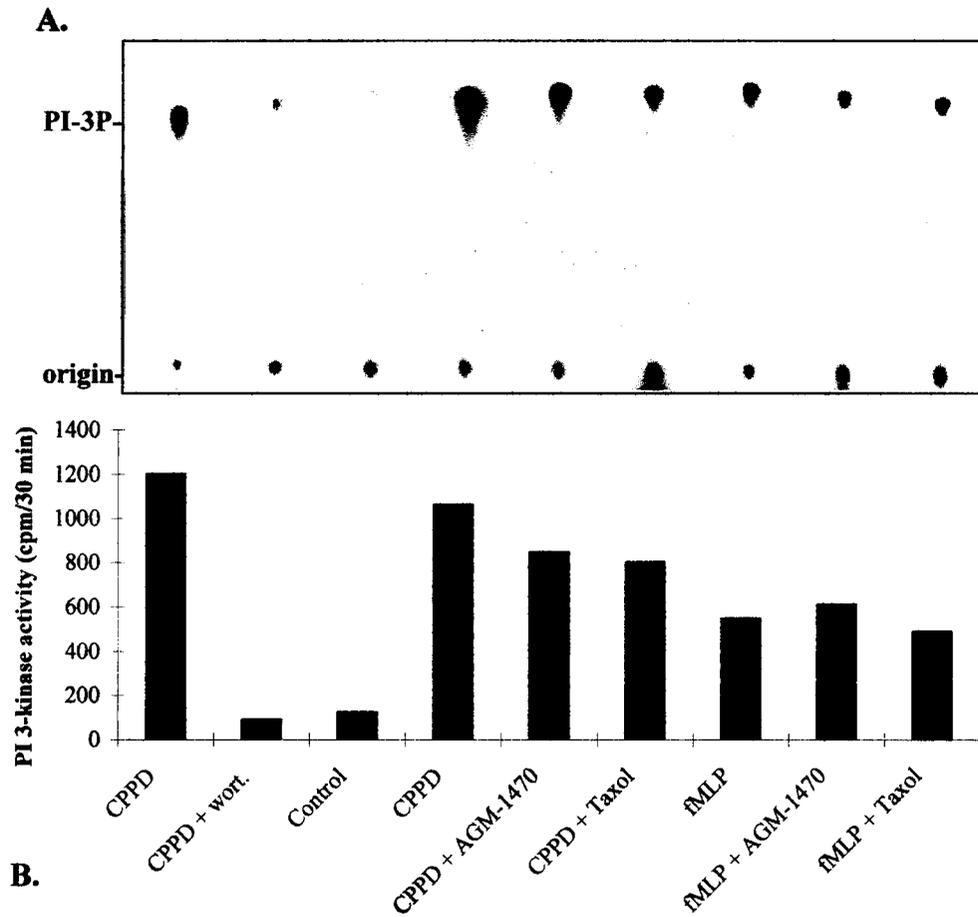


B.



## PMA

**Figure 60.** Effect of wortmannin on fMLP versus PMA induced chemiluminescence. Luminol-enhanced chemiluminescence was monitored in neutrophils ( $5 \times 10^6/\text{ml}$ ) incubated with fMLP ( $1 \mu\text{M}$ , 1 min) with and without wortmannin ( $10 \text{ nM}$ ) for periods of 1 to 15 min (A), and with PMA ( $0.5 \mu\text{M}$ ) with and without wortmannin for 1 to 8 min (B). The control represents the luminol-enhanced chemiluminescence monitored in neutrophils treated with the agonist (fMLP; top panel, PMA; bottom panel) alone. The results are representative of two independent experiments.



**Figure 61.** Effect of AGM-1470 on CPPD versus fMLP-induced PI 3-kinase activity. (A), PI 3-kinase activity was monitored in the presence of CPPD and fMLP with and without AGM-1470, and wortmannin with anti-PI 3-kinase immunoprecipitates from cells in Figure 60A. (B), PI 3-kinase activity was quantitated by scintillation counting of the PI-3P spot.

## DISCUSSION

### P70 S6 kinase

Agents that activate neutrophils do so via one or a combination of two pathways; receptor and non-receptor mediated (Burt and Jackson, 1993). The receptor linked pathways mediated by opsonized particles occur through specific receptors for C3b or the Fc domain of the IgG. Stimulation by inflammatory microcrystals such as MSUM and CPPD is currently under investigation, as they have no apparent specific receptor on the neutrophil membrane. It seems that the pathways leading to crystal induced activation are different for uncoated crystals versus serum/plasma/IgG opsonised crystals. Most studies on crystal induced neutrophil activation have employed uncoated crystals under the belief that surface absorbed proteins seriously compromise neutrophil activation levels. This is less likely in my studies of neutrophil activation since it was demonstrated that both uncoated and coated MSUM crystals cause large and rapid increases in  $[Ca^{2+}]_i$  (Burt and Jackson, 1993). Furthermore, increases in tyrosine phosphorylation levels have been reported for neutrophils activated by both uncoated and plasma-coated CPPD(T) crystals (Burt and Jackson, 1988). A time course study with neutrophils incubated with plasma coated CPPD crystals for 1, 2, 5, 10 and 15 min demonstrated that there was a marked and transient activation of cytosolic p70<sup>S6K</sup> activity following two minutes of crystal incubation. It has previously been shown that both uncoated and plasma-coated CPPD crystals activated neutrophils and that plasma precoating amplifies neutrophil responses to CPPD (Burt and Jackson, 1993). Plasma precoating of CPPD crystals enhanced S6 kinase activity at least two-fold over uncoated CPPD crystals. Although PI 3-kinase and PKC have previously been observed to be activated in neutrophils, this is the first documented observation of neutrophil activation of p70<sup>S6K</sup>. Studies pertaining to p70<sup>S6K</sup> have been primarily conducted in response to mitogens in various cell lines, such as EGF and insulin (Ballou *et al.*, 1991; Petritsch *et al.*, 1995). Swiss mouse 3T3 fibroblasts showed a robust and rapid p70<sup>S6K</sup> activation in 2.5 and 5 min following insulin and EGF treatment, respectively (Cheatham *et al.*, 1994; Petritsch *et al.*, 1995), similar to that observed here. Perhaps more pertinent to neutrophils, Tsai *et al.*

(1993) suggested a vital role for  $p70^{S6K}$  in mast cells relative to stimulation of the Fc receptor, where IgE was shown to stimulate  $p70^{S6K}$  at 30 min, presumably through the FcεRI. This activity was suppressed by rapamycin, but rapamycin did not inhibit 5HT (5-hydroxytryptamine) release.

To investigate crystal-induced activation of  $p70^{S6K}$ , I fractionated cytosolic extracts from neutrophils incubated with crystals by anion exchange chromatography on MonoQ. Compound 3 was recently reported to inhibit  $p70^{S6K}$  purified from rat skeletal muscle (Alessi, 1997). I am certain that the presence of Compound 3 in the S6 kinase reaction buffer did not affect the activity of  $p70^{S6K}$  in neutrophils, since our original studies which did not contain the inhibitor did not produce an enhancement in the S6-10 and MPB-NT phosphotransferase activities compared to activities observed in samples where the inhibitor was present. I was not able to immunoprecipitate the activity of  $p70^{S6K}$  with the anti-S6 kinase antibody distributed by Santa Cruz Inc., although I have immunoprecipitated  $p70^{S6K}$  from various rat tissues in experiments where the effects of insulin were studied by myself. The results presented in this study demonstrated that another S6K antibody does immunodetect the enzyme, even in its activated and multiphosphorylated state. Phosphotransferase activity of the fractions was resolved using three different substrates of the S6 kinase: MBP-NT, S6K-10 peptide and MBP. Assays with the MBP-NT peptide included the PKC inhibitor, Compound 3, since MBP-NT is a substrate for both  $p70^{S6K}$  and PKC. Extracts from neutrophils treated for two min with plasma coated CPPD crystals resolved a major peak at approximately 0.4 M NaCl for each substrate. Western analysis of the MonoQ fractions with the anti-S6K-NT antibody revealed the major immunoreactive protein coeluted with the phosphotransferase activity and migrated as approximately 70 kDa protein on SDS-polyacrylamide gels.

Previous work has established that  $p70^{S6K}$  is activated through phosphorylation (Mukhopadhyay *et al.*, 1992; Sabatini *et al.*, 1994; Ferrari and Thomas, 1994; Petritsch *et al.*, 1995; Pullen *et al.*, 1998).  $p70^{S6K}$  migrates differently in an SDS-polyacrylamide gel depending on its state of phosphorylation, with the highly phosphorylated and active form migrating more slowly than the dephosphorylated inactive enzyme (Petritsch *et al.*, 1995;

Han *et al.*, 1995). In cells not treated with crystals (control), no activation was observed and the p70<sup>S6K</sup> was present as a hypophosphorylated species. Following 2 min neutrophil incubation with plasma-coated CPPD crystals, p70<sup>S6K</sup> activity was robust, and the higher phosphorylated states of the kinase were immunoblotted. Hei *et al.* (1993) performed similar immunological studies on MonoQ fractions with antibodies generated against the C-terminus and subdomain III, and the major immunoreactive protein coeluted with the p70<sup>S6K</sup> activity identical to our results. Since these results indicate that stimulated p70<sup>S6K</sup> activity from neutrophils can be quantitatively measured, this protocol was utilized throughout this study to investigate the effects of crystal incubation of neutrophils on this kinase.

The immunosuppressive compound, rapamycin, blocks the phosphorylation and activation of p70<sup>S6K</sup> in mammalian cells following activation by EGF, v-Src, phorbol esters, calcium ionophore, heat shock, cycloheximide, interleukin-2 or insulin, and leads to either abolition or suppression of cell growth depending on the cell line used (Jurivich *et al.*, 1991; Kuo *et al.*, 1992; Price *et al.*, 1992; Chung *et al.*, 1994; Ferrari and Thomas, 1994; Pullen *et al.*, 1998). Although rapamycin can inhibit the phosphorylation of p70<sup>S6K</sup>, it does not block the activation of other mitogen-activated kinases such as the ERK-encoded MAP kinases, p90<sup>rsk</sup> or p74<sup>raf1</sup> (Kuo *et al.*, 1992; Chung *et al.*, 1994). Furthermore, rapamycin can block PKC-dependent and independent pathways, although its inhibitory effect is thought to be exerted on an upstream component of the pathway other than p70<sup>S6K</sup> itself (i.e. FRAP; Pullen *et al.*, 1998). Rapamycin does not inhibit PI 3-kinase *in vivo* or *in vitro* (Chung *et al.*, 1994; Ferrari and Thomas, 1994).

Rapamycin-pretreated cells showed a significant reduction in p70<sup>S6K</sup> activity, and was comparable to the control. The experiments were conducted on three separate occasions and the results that are illustrated were reproducible. Although it has been suggested that there are two distinct kinase signalling pathways that converge to activate p70<sup>S6K</sup>, rapamycin has been shown to block both (Chung *et al.*, 1994; Han *et al.*, 1995). Han *et al.* (1995) have suggested that phosphorylation of p70<sup>S6K</sup> was responsible for the mobility shift observed in Western blots following activation of this enzyme and that rapamycin may inhibit this phosphorylation. It is therefore possible that the activation of p70<sup>S6K</sup> by plasma

coated CPPD crystals may be due to the phosphorylation of p70<sup>S6K</sup>, since a mobility shift was observed and the activation was rapamycin-sensitive.

Mitogen-dependent p70<sup>S6K</sup> activation has been shown to be mediated by phosphatidylinositol 3-kinase (PI 3-kinase) by inhibition of both kinases with the PI 3-kinase inhibitors wortmannin and LY294002 (Chung *et al.*, 1994; Cheatham *et al.*, 1994; Downward, 1994; Han *et al.*, 1995; Ui *et al.*, 1995; Chou and Blenis, 1996). Since reports have implicated PKB as a target of PI 3-kinase (Cross *et al.*, 1995; Bos, 1995), it was assumed that PKB was upstream of p70<sup>S6K</sup> as part of the PI-3-kinase mediating pathway for S6 kinase activation (Bos, 1995). Our findings that wortmannin and LY294002 inhibit crystal-induced activation of PKB, but did not affect the activation of p70<sup>S6K</sup> further links the activation of PKB through PI-3-kinase, but makes this the first report of a non-proliferative, non-mitogenic system of p70<sup>S6K</sup> activation in human cells. Additionally, this is the first report of PI 3-kinase activation that did not contribute to the activation of p70<sup>S6K</sup>. Pullen *et al.* (1998) subsequently demonstrated that the Thr-229 site of p70<sup>S6K</sup> that is necessary for its activation, is phosphorylated directly by PDK1 (phosphoinositide-dependent protein kinase1; Alessi *et al.*, 1997). PKD1 is also responsible for the phosphorylation and regulation of PKB (Cohen *et al.*, 1997), and it has been demonstrated that the Thr-308 site of PKB contains significant identity to the Thr-229 site of p70<sup>S6K</sup>. Regulation of p70<sup>S6K</sup> by PDK1 is independent of PKB (Pullen *et al.*, 1998), and inhibition of p70<sup>S6K</sup> by wortmannin is probably a result of PDK1 requiring the product of PI 3-kinase for its activation. Given that the neutrophil system is non-proliferative, and the method of opsonized crystal stimulation is unknown, I propose that alternative pathways mediating p70<sup>S6K</sup>, and even MAP kinase, are in play, and may repress the pathway linking PI 3-kinase to p70<sup>S6K</sup>.

Studies pertaining to mitogen activation of p70<sup>S6K</sup> have implicated other mediators of this kinase. These include FRAP, which is not highly wortmannin sensitive (Brown *et al.*, 1995), PDK1, and PKC, which lies downstream of PLC $\gamma$  (Massagué *et al.*, 1995; Chou and Blenis, 1996; Kardalidou *et al.*, 1994; Chung *et al.*, 1994; Pullen *et al.*, 1998). Complete suppression of crystal-induced p70<sup>S6K</sup> activity was observed following Compound 3

preincubation, indicating that in this system PKC is a mediator of p70<sup>S6K</sup> activation. Therefore, I report here that crystal induced activation of p70<sup>S6K</sup> is not only independent of PI 3-kinase, but is PKC-dependent. Further, I showed that crystal activation of ERK1 and ERK2 are independent of p70<sup>S6K</sup>. These kinases lie on distinct signalling pathways, and activation of MAP kinase in neutrophils following crystal treatment is wortmannin- and Compound 3-insensitive.

### MAP kinase

MAP kinases are a family of serine/threonine protein kinases activated as an early response to many stimuli including growth factors, cytokines, integrin-matrix association or exposure to cellular stresses. MAP kinases are thought to be key intermediate proteins functioning in a variety of signal transduction networks. Dissecting the pathways subsequent to MAP kinase activation and elucidating their physiological roles depending on the agonist and cell type, is as difficult as distinguishing the events leading from the receptor to MAP kinase induction. MAP kinases phosphorylate a variety of proteins *in vivo* including Rsk1, Rsk2, MAP kinase-activated protein kinase-2 (MAPKAPK2), c-Fos, PHAS-1, PLA<sub>2</sub>, c-Jun and p62<sup>TCF</sup> (Blenis, 1996; Pelech and Charest, 1996). Furthermore, agonists of MAP kinase and neutrophil activation are mediated through various receptors, second messengers and other kinases. Activation of a down-stream target subsequent to MAP kinase activation is also regulated by the integration of other signals from MAP kinase-dependent and independent pathways. Therefore, my results indicate that neutrophil oxidative and degranulation responses can be mediated through MAP kinase signalling pathways, but these responses are probably regulated through a network of pathways that converge in the vicinity of the mediators of neutrophil activation downstream of MAP kinase.

Human neutrophils incubated with Taxol have been observed to contain microtubules that are located in bundles (Roberts *et al.*, 1982) and the normal microtubule-associated functions of the neutrophil become disrupted in the presence of the drug. Microtubules function in maintaining the architecture of the cell, organize membrane traffic within the cell, and are involved in cell migration, phagocytosis, secretion, and most

notably, mitosis (Kelly, 1990; Oliver *et al.*, 1982). Microtubules have been suggested to be a component of the intracellular signalling mechanism leading to neutrophil activation (Wang *et al.*, 1990; Ravindra and Aronstam, 1993). Several of these microtubule-associated mechanisms within neutrophils are involved in particulate-induced neutrophil responses. It is therefore reasonable to conjecture that Taxol inhibits neutrophil activation-associated responses by stabilizing microtubules and hence disrupting the MAP kinase events associated with microtubule function. However, because not all MAP kinase is associated with the nucleus, and microtubules, it is possible that Taxol may inhibit MAP kinase by another non-specific route (Jackson *et al.*, 1997a). I have demonstrated almost full suppression of MAP kinase by Taxol in response to CPPD crystals, as well as inhibition of the upstream kinase PKC.

Both uncoated and plasma opsonized CPPD crystals have recently been shown to activate MAP kinase, with peak activation occurring within 5 min of CPPD crystal incubation of neutrophils. Neutrophil oxidative and degranulation responses also peaked at approximately 5 min following crystal incubation, and it was observed that like CPPD crystal-induced MAP kinase activity, neutrophil activation was inhibited with Taxol pretreatment (Jackson *et al.*, 1997a). This inhibition of MAP kinase and neutrophil activation by Taxol in response to CPPD crystal incubation, and reports supporting the involvement of MAP kinase in chemoattractant-stimulated neutrophil responses (Meyer *et al.*, 1996; Jackson *et al.*, 1997b; Krump *et al.*, 1997), further confirms a role for MAP kinase in the induction of neutrophil activation associated responses. Induction of neutrophils by IgG, plasma and serum opsonized CPPD crystals results in a pleiotropic effect, and the link between the large cationic charge of the crystals, or the charge of the peptides bound to them, and the resulting signal transduction events is difficult to ascertain. Therefore, whether there exists an association of MAP kinase activation and neutrophil respiratory burst and degranulation responses associated with more physiological, non-disease state conditions, such as in response to chemoattractants and chemokines, has yet to be determined. This study is the first to demonstrate an association between agonist-dependent MAP kinase activation and stimulation of neutrophil oxidase responses.

Although ERK1 and ERK2 were activated in response to each agonist analyzed in this report (Figures 55 and 56), only fMLP and PMA, but not CPPD crystal-induction of these MAP kinases was inhibited by AGM-1470 pretreatment. This correlation between down-regulated MAP kinase activation and abrogation of neutrophil oxidative response by AGM-1470 implies that MAP kinase may lie upstream in one of the signal transduction pathways leading to neutrophil activation induced by fMLP and PMA. The observation that both MAP kinase and neutrophil activation associated with CPPD crystal treatment, were not affected by AGM-1470 pretreatment, also indicates that the function of MAP kinase in the signalling pathway leading to oxidative response is dependent upon the agonist. This was further confirmed with the observation that Taxol inhibited both CPPD-induced MAP kinase activation and neutrophil oxidative and degranulation responses. Since AGM-1470 inhibits CPPD crystal-induced PKC activity, but not neutrophil activation, we propose that it is possible that both PKC and MAP kinase activity are necessary for oxidative responses, and that AGM-1470 fails to inhibit CPPD crystal-induced neutrophil activation since it does not inhibit a mediator of the MAP kinase pathway stimulated in response to CPPD treatment. For instance, we demonstrated that Compound 3 inhibited PMA-induced PKC activity, but not MAP kinase or PKC activation, whereas Taxol will also not inhibit MAP kinase or neutrophil activation. The data presented in this study clearly shows, for the first time, that CPPD crystals activate both ERK1 and ERK2 and a rapamycin sensitive p70<sup>S6K</sup> within two min of crystal-neutrophil incubation. The physiological roles of these enzymes in neutrophil signal transduction are not known.

#### AGM-1470 as a potential anti-arthritis compound

AGM-1470 is a more potent and less toxic synthetic analog of fumagillin now in clinical phase III studies as an antitumour and anti-angiogenic agent, the first anti-angiogenic compound to be submitted for clinical studies. It has been proposed that AGM-1470 functions as an anti-angiogenesis agent by inhibiting the proliferation of growth factor-induced endothelial cells (Antoine *et al.*, 1994; Kusaka *et al.*, 1994; Ito *et al.*, 1996), probably because of its effect on inducing a G1 phase block in the cell cycle (Antoine *et al.*, 1994, 1996; Hori *et al.*, 1994). AGM-1470 also suppresses vascular endothelial growth

factor (VEGF) levels (Oliver *et al.*, 1994), a potent mediator of vascular permeability and angiogenesis (Rak *et al.*, 1995; Hanahan and Folkman, 1996). Various tumour cell lines are sensitive to AGM-1470 at concentrations at least 10 times greater than that which will inhibit endothelial cell proliferation (5 pg/ml, Antoine *et al.*, 1994), while other cancer cell lines required as much as 200,000 to 1 million time greater amounts of the drug (Yanase *et al.*, 1993; Yamaoka *et al.*, 1993; Antoine *et al.*, 1997), indicating a high affinity of the drug for specific molecules within endothelial cells. These properties of AGM-1470 make it amenable as a potent anti-arthritic agent, since both VEGF and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) are cytokines that have been associated with chronic inflammatory synovitis and rheumatoid arthritis (Arend and Dayer, 1990; Fava *et al.*, 1994). VEGF also enhances the activity of TNF- $\alpha$  which exacerbates collagen-induced arthritis (CIA) and is a chemoattractant for neutrophils (Brahm *et al.*, 1992; Oliver *et al.*, 1995). VEGF is expressed in synovial macrophages in patients with rheumatoid arthritis (RA), and the VEGF receptor flt-1 is concentrated in pannus microvasculature which is usually absent in the non-diseased state (Fava *et al.*, 1994; Koch *et al.*, 1994). VEGF levels have also been observed to increase in patients with RA compared to healthy controls (Oliver *et al.*, 1995), and is a chemoattractant for monocytes and stimulates endothelial cells to release collagenase. Furthermore, excess neovascularization has been observed in RA related pannus (Folkman *et al.*, 1989). Therefore, the inhibition of symptoms such as VEGF and receptor expression and neovascularization are significant indicators in the development of novel compounds to suppress arthritic-like diseases. AGM-1470 has recently been investigated as a new treatment option for rheumatoid arthritis. Suppression of collagen- and complete Freund's adjuvant (CFA)-induced arthritis in rats has been observed following AGM-1470 treatment (Peacock *et al.*, 1992, 1995; Oliver *et al.*, 1994, 1995). In CIA and CFA synovitis models, AGM-1470 effectively prevented pannus formation and reduced VEGF expression and neovascularization in the area of the joint where arthritis was induced in both preventative and suppressive protocols (Peacock *et al.*, 1992, 1995; Oliver *et al.*, 1994, 1995). Furthermore, histologic sections revealed significant loss of destruction of bone/cartilage (Peacock *et al.*, 1995). Combination therapy with cyclosporin (Oliver *et al.*, 1995) or Taxol (Oliver *et al.*, 1994), two compounds that have demonstrated efficacy in reducing arthritis,

demonstrated reduction in CIA symptoms more effectively than single-agent treatments with negligible immunosuppression.

#### The role of PKC in neutrophil signalling

The mechanism(s) of action of AGM-1470 is not yet clear other than its ability to inhibit proliferation by inducing a G1 phase block due to suppression of cyclin D expression, as was observed in HUVEC and WiDr human tumor cells following incubation with the drug (Hori *et al.*, 1994). I have demonstrated that AGM-1470 effectively inhibits PKC activity in HUVEC stimulated with VEGF and bFGF. This inhibition of PKC in endothelial cells is significant, since it was demonstrated that PKC was bimodal in growth regulation, where if cells were in early G1 phase, PKC activation lead to potentiation of DNA synthesis, whereas if cells were in late G1 or early S phase, PKC activation resulted in the inhibition of DNA synthesis (Zhou *et al.*, 1993). PKC has also been implicated in hematopoietic differentiation and activation of  $O_2^-$  generation in neutrophils (Ambruso *et al.*, 1990; Devalia *et al.*, 1992; Levy *et al.*, 1990), and has been shown to phosphorylate a component of the NADPH oxidase complex leading to its activation and generation of superoxide anions (Jones, 1994). Neutrophil NADPH oxidase can be activated by PLD (Watson *et al.*, 1994) as a result of PKC activation (Nishizuka *et al.*, 1992; Lopez *et al.*, 1995). The role of PKC in respiratory burst and  $O_2^-$  release has been closely studied in human myeloid HL-60 cells, which have been accepted as an *in vitro* model in retinoid-induced differentiation to neutrophil phenotypes (Niedel *et al.*, 1980; Collins *et al.*, 1987; Bunce *et al.*, 1996). Following differentiation to granulocyte-like cells, RA-induced HL-60 cells develop the capacity to produce a respiratory burst and release  $O_2^-$  comparable to that observed in human neutrophils (Thomas *et al.*, 1988; Yamaguchi *et al.*, 1998; Bertolaso *et al.*, 1998). Arachadonic acid, thought to interact synergistically with diacylglycerol to activate PKC (Lester *et al.*, 1991) was shown to activate as well as enhance PMA-stimulated  $O_2^-$  generation in a time- and dose-dependent manner in RA-induced HL-60 cells (Bertolaso *et al.*, 1998). Further, the same group demonstrated the ability of PKC inhibitors staurosporine (100 nM), Ro 31-8221 (1  $\mu$ M) and sphingosine (100  $\mu$ M) to inhibit PMA

stimulated  $O_2^-$  generation to greater than 93% in retinoic-acid-treated HL-60 cells (Mayer *et al.*, 1996), further supporting a role for PKC in  $O_2^-$  generation.

The results presented in this thesis illustrate that the neutrophil activation agonists CPPD crystals, fMLP and PMA, activated PKC as observed in MonoQ fractionated histone H1 phosphotransferase assays in the presence of PKC cofactors (Figures 50, 51, 53 and 54). One of the PKC isoforms observed in the PKC fractionated peak, as detected via Western analysis with an antibody directed to the catalytic subunit of PKC, appeared to be the calcium-independent PKC $\epsilon$  based on the apparent molecular masses of the immunoreactive proteins (Hug and Sarre, 1993). Confirmation that PKC was activated was assessed following the analysis of p70<sup>S6K</sup> activity (Figure 52). This PKC-dependent S6 kinase activity was inhibited following pretreatment with AGM-1470 at the same concentration that inhibited CPPD crystal-induced PKC activity, confirming that PKC was also inhibited. These results indicate that, similar to experiments conducted with growth factor-induced HUVEC cells where PKC activity was observed to be inhibited by AGM-1470, PKC was also inhibited in neutrophils when coincubated with CPPD crystals and fMLP. The observation that AGM-1470 did not inhibit PMA-induced PKC activity was not surprising since it was observed that HUVEC cells stimulated with growth factors and pretreated with AGM-1470 (5 nM) were rescued from the anti-proliferative effect of AGM-1470 following 30 min PMA treatment (1  $\mu$ M), but little effect was observed in cells pretreated with PMA followed by incubation with AGM-1470. Taken together, these observations would indicate that AGM-1470 inhibits PKC indirectly, at a step upstream of PKC.

Although AGM-1470 pretreatment inhibited PKC activated in neutrophils stimulated with CPPD crystals and fMLP, neutrophil oxidative responses were not affected in cells treated with CPPD crystals. Further, PMA-treated neutrophils pre-incubated with AGM-1470 were observed to display significantly reduced chemiluminescence and superoxide anion generation, although PKC activity remained up-regulated. These observations indicate that AGM-1470 affects another mediator of neutrophil activation besides PKC, because AGM-1470 did not affect CPPD crystal-induced neutrophil activation but inhibited PKC. This effect is likely mediated by an enzyme in a signal transduction pathway that is distinct from but parallel to the PKC signalling pathway. This does not exclude the role of

PKC in neutrophil oxidative and degranulation responses, as support for its function in neutrophil activation is well documented, as discussed earlier, but it is possible that the effector downstream of PKC requires the activity of one or several other signalling pathways distinct from PKC. In a cell-free system from human neutrophils, it has been demonstrated that more than one protein kinase participates in the activation of NADPH oxidase (McPhail *et al.*, 1995) including PKC (Jones, 1994), PI 3-kinase (Ding *et al.*, 1995) and MAP kinase (Torres *et al.*, 1993).

#### MAP kinase and neutrophil activation

ERK1 and ERK2 are phosphorylated on threonine and tyrosine leading to their activation by dual specificity kinases MEK1 and MEK2 (Cobb and Goldsmith, 1995). The involvement of tyrosine phosphorylated kinases in agonist-induction of neutrophils is well established (Nacceche *et al.*, 1990; Burt *et al.*, 1993; Rollet *et al.*, 1994). Plasma opsinized crystal-stimulated neutrophil chemiluminescence, superoxide generation, and tyrosine kinase activity has been shown to be inhibited by the protein-tyrosine kinase inhibitors Lavendustin C and 2,5-dihydroxycinnamate (Burt *et al.*, 1993). The chemotactic peptide fMLP, the chemokines interleukin-8 (IL-8), GM-CSF, TNF- $\alpha$ , and the chemoattractant C5a have been reported to activate MAP kinase in neutrophils (Grinstein and Furuya, 1992; Raines *et al.*, 1992; Torres *et al.*, 1993; Vietor *et al.*, 1993; Worthen *et al.*, 1994; Avida *et al.*, 1996; Krump *et al.*, 1997). CPPD crystals, fMLP, IL-8 and C5a have been observed to activate neutrophil respiratory burst and degranulation responses associated with neutrophil activation, and PMA was shown in this study to induce chemiluminescence and superoxide anion generation (Mayer *et al.*, 1996). The possible association of MAP kinase pathway with neutrophil activation in response to fMLP stimulation has previously been suggested (Grinstein and Furuya, 1992; Torres *et al.*, 1994; Worthen *et al.*, 1994), and wortmannin, a PI 3-kinase inhibitor that inhibits CPPD crystal-induced neutrophil chemiluminescence and superoxide anion generation (Jackson *et al.*, 1997b) has been demonstrated to inhibit both IL-8-induced neutrophil MAP kinase activation and degranulation responses (Krump *et al.*, 1997). These studies indicate that MAP kinase activation in response to various neutrophil agonists may result in the activation of neutrophils, although it is likely that alternate

pathways also exist that, when activated, contribute to the total oxidative and degranulation responses (Mayer *et al.*, 1996; Krump *et al.*, 1997).

fMLP-induced activation of MAP kinase has been described previously where it was suggested that fMLP-induced oxidase activation is stimulated through the MAP kinase pathway (Grinstein and Furuya, 1992; Torres *et al.*, 1993). I therefore speculated that Taxol inhibition of fMLP-induced neutrophil responses might be a result of inhibition of MAP kinase activity by this drug. Studies described here, however, indicated that 28  $\mu\text{M}$  Taxol inhibited fMLP-induced chemiluminescence by 40% and PMA-induced chemiluminescence by only 10% (Jackson *et al.*, 1997b). The data presented in this manuscript demonstrated that AGM-1470 inhibited fMLP- and PMA-induced chemiluminescence and superoxide anion generation by approximately 46 % and 54 %, respectively. Even at higher concentrations of AGM-1470 (10  $\mu\text{M}$ ), slightly below the concentration (50  $\mu\text{M}$ ) where cell death was detectable, neutrophil activation was not inhibited any further. It was previously demonstrated (Jackson *et al.*, 1997b) that the PI 3-kinase inhibitor wortmannin inhibited CPPD crystal-induced chemiluminescence, superoxide anion generation and degranulation, but a threshold of inhibition was also apparent. These observations indicate that neutrophil responses such as oxidase activation, are mediated by distinct pathways that each 'contribute' to the total response. Further, I propose that one of these pathways is mediated through MAP kinase.

Although wortmannin and Taxol inhibited CPPD crystal-induced neutrophil chemiluminescence, AGM-1470 did not at the concentration that resulted in the inhibition of fMLP- and PMA-induced chemiluminescence. Therefore, the signalling pathway leading to neutrophil activation that is inhibited by AGM-1470 is distinct from that which is mediated by Taxol or wortmannin. Figure 60 shows that wortmannin inhibited fMLP- but not PMA-induced chemiluminescence, and that AGM-1470 and Taxol did not inhibit fMLP- or CPPD-crystal-induced PI 3-kinase activity (Figure 61). I propose that both AGM-1470 and Taxol probably inhibits the signal transduction pathway leading to neutrophil activation that is independent of PI 3-kinase. I demonstrated that the PKC pathway, leading to the activation of p70<sup>S6K</sup>, is distinct from that of the PI 3-kinase pathway in neutrophils. Therefore, any contribution of PKC towards neutrophil activation may be independent of PI

3-kinase. From studies where it was shown that PKC $\zeta$  activated MAP kinase signal transduction through interaction with PIP<sub>3</sub>, a product of the phosphorylation of PIP<sub>2</sub> by PI 3-kinase, and that MAP kinase and MEK activities were wortmannin sensitive (Toker *et al.*, 1994; Nishioka, *et al.*, 1995; Serve *et al.*, 1995; Karnitz *et al.*, 1995; Liao *et al.*, 1997), it was suggested that in certain cell lines, MAP kinase can be activated via PI 3-kinase. These results indicate that the neutrophil oxidative response mediated by PI 3-kinase is independent of MAP kinase. Because it is well established that neutrophil activation can be mediated through the PI 3-kinase signal transduction pathway, and that this pathway is probably independent of MAP kinase signalling, we propose that neutrophil oxidative responses are regulated by multiple and distinct pathways that utilize MAP kinase or PI 3-kinase, and that AGM-1470 functions to inhibit neutrophil activation in a MAP kinase-dependent, PI 3-kinase-independent manner that requires PKC activity. The data would also indicate that Taxol can inhibit neutrophil oxidative responses through MAP kinase, independent of PI 3-kinase, but the role of PKC in regulating this inhibition is not conclusive as this is the first report of Taxol inhibiting this enzyme (when induced by CPPD crystals or fMLP). The observation that Taxol downregulated a different PKC isoform stimulated by fMLP compared to CPPD crystals supports the original hypothesis that independent of PKC, Taxol regulates MAP kinase, perhaps by stabilizing microtubules and therefore disrupts MAP kinase activation events associated with microtubule function (Jackson *et al.*, 1997a). The observation that a calcium-dependent isoform of PKC was inhibited by Taxol in neutrophils stimulated with fMLP does not exclude the possibility that the abrogation of this alternate PKC isoenzyme resulted in the inhibition of neutrophil chemiluminescence although MAP kinase was not affected. But as was just discussed, this seems unlikely. I propose, given this evidence together with the observations that PKC and PI 3-kinase were not downregulated by either AGM-1470 or Taxol in cells stimulated with PMA, and that Taxol did not affect MAP kinase activation induced by the same agonist, that a third signal transduction pathway leading from a receptor to a mediator of neutrophil oxidative response exists that is inhibited by Taxol. Whether this pathway is exclusively affected by Taxol cannot be determined by these results, and therefore this and the role of the calcium-dependent PKC isoform in fMLP-induced chemiluminescence requires further

investigation. This is also the first report demonstrating that PMA-stimulated neutrophil respiratory burst occurs independently of the PI 3-kinase pathway. Given the finding by Pullen and associates that p70<sup>S6K</sup> is phosphorylated and regulated by PDK1 (Pullen *et al.*, 1998), it is possible that this other pathway bifurcates at PI 3-kinase and is regulated through the PDK1, which may itself be regulated by PKC. Because both the calcium dependent and independent isoforms of PKC contains a threonine phosphorylation site with significant homology to the Thr-308 site of p70<sup>S6K</sup> (unpublished observation, CT), PDK1 may regulate PKC itself, which may be affected by AGM-1470 and Taxol. This hypothesis is now under investigation by myself.

#### Chapter summary

The signalling pathways that are induced following the binding of various agonists to different receptors on leukocytes leading to respiratory burst oxidase production of O<sub>2</sub> and degranulation have not been completely elucidated, and is the subject of recent biochemical investigation, and this study. The use of various agents as pharmacological probes have provided novel insights into the biochemical nature of signal transduction molecules and pathways that lead to such diseases as cancer, arthritis and crystal-induced inflammation when they are abrogated, or abnormally activated. These agonists have also been utilized to identify putative pharmacological targets for therapeutic intervention against leukocytes and other cells. This study has utilized the anti-angiogenesis inhibitor AGM-1470 and anti-tumor agent Taxol, to further dissect the pathways leading to neutrophil activation in response to various agonists. I have demonstrated that AGM-1470 suppresses fMLP- and PMA-stimulated neutrophil activation and further established this drug as a prototypic agent for a new class of potential anti-arthritis therapy. I have also demonstrated that AGM-1470 indirectly inhibits PKC activation in response to CPPD crystals and fMLP, and effectively inhibits fMLP- and PMA-induced MAP kinase activity without affecting PI 3-kinase activity. I have also shown that Taxol effectively inhibited neutrophil activation when stimulated with all of the agonists investigated here, and that the mechanism of inhibition in CPPD crystal-induced cells is probably mediated by MAP kinase down regulation, but that another route is also affected by this drug via its influence on microtubules. Therefore, I

propose, given our findings, that at least three pathways exist leading to the activation of neutrophil oxidative responses. One is mediated by PI 3-kinase as has been proposed elsewhere, and another mediated in parallel with MAP kinase where a down-stream effector probably also requires the activity of PKC. The third is inhibited by Taxol, but the mediators of this pathway are presently obscure. This hypothesis would explain all previous findings that various agonists activate neutrophil oxidative and degranulation responses to a limited degree. Likewise, inhibitors of these responses have not been able to show complete inhibition, indicating that the total response is a cumulative one composed of an integration of numerous signals probably initiated from various receptors.

Until very recently, the mechanisms in which AGM-1470 inhibits neovascularization, endothelial cell proliferation and CIA pannus formation, and other arthritis-like symptoms, were elusive (Yanoaka *et al.*, 1993; Hori *et al.*, 1994; Kusaka *et al.*, 1994; Antoine *et al.*, 1994, 1996; Oliver *et al.*, 1995; Peacock *et al.*, 1995). It has been demonstrated that AGM-1470 incubation with endothelial cells resulted in cell cycle arrest in early G1 phase (Antoine *et al.*, 1994) as a result of the inhibition of expression of cyclins A, D and E, and pRb phosphorylation. Work done by Abe *et al.* (1994) implied that the arrest in G1 is not a result of perturbations of early signalling events, since the addition of AGM-1470 three hours after growth factor stimulation of quiescent cells still resulted in cell cycle arrest. The results discussed in 'Chapter 4' indicated otherwise, where it was demonstrated that AGM-1470 had an irreversible effect, showing that the compound probably binds via a strong hydrogen and/or covalent bond to an effector in the vicinity of the cell membrane. It was recently reported that fumagillin covalently binds the methionine aminopeptidase, MetAP-2, and the authors have suggested that this is the physiological target of the drug. Their argument is based on the knowledge that MetAP-2 levels are greatly induced upon mitogen stimulation, and expression correlates with cell growth (Sin *et al.*, 1997; Borman, 1997). Further, because methionine peptidases effect posttranslational processing required for protein myristoylation, inhibition of MetAP-2 may prevent the myristoylation of a signalling component that probably acts in early signaling in response to mitogens. The authors acknowledged that the model remains speculative, as no link between this model and a G1 block was provided. This report is the first to propose a

mechanism of AGM-1470 that is consistent with previous studies. I believe that the inhibition of PKC is significant in endothelial cells, and probably neutrophils. Additionally, I have demonstrated that AGM-1470 inhibits MAP kinase activity and neutrophil respiratory burst responses induced by fMLP and PMA, indicating that inhibition of MAP kinase by AGM-1470 is functional in this mechanism. Also, it has been shown that prolonged MAP kinase activity is essential for cyclin D expression in endothelial cells (Hori *et al.*, 1994). Given that AGM-1470 inhibits MAP kinase activity, which would result in loss of cyclin D expression and abrogation of Cdk4 activity which is responsible for the phosphorylation of pRb (Sher, 1993; Abe *et al.*, 1994), it follows that a G1 block would result. Should inhibition of MetAP-2 result in the prevention of translation of a protein required for proper functioning of an effector upstream of PKC, this would provide a link between the finding that AGM-1470 binds to MetAP-2, provided that MetAP-2 is the physiological target. This has yet to be determined, but the data presented here provides further insight into the mechanism of AGM-1470 and the molecular signalling in stimulated neutrophils that leads to respiratory burst activation. It provides a possible explanation for how AGM-1470 inhibits angiogenesis; namely, by its inhibitory effect on PKC and MAP kinase.

#### Thesis summary

The aim of this study was to utilize three potential therapeutic compounds presently undergoing clinical studies, to investigate the role of cell cycle dependent intracellular transducing proteins in various diseases known to have signalling pathways that overlap. The compounds fostriecin, AGM-1470, and Taxol have been shown to have their effects at different phases of the cell cycle, and through presently unknown mechanisms, play a role in controlling cellular proliferation, neovascularization and angiogenesis-dependent inflammation.

To gain insight into the mechanism of each compound and assess the signalling events associated with cancer, angiogenesis and inflammation, the signal transduction pathways that are modulated following treatment were investigated. Fostriecin was identified to inhibit the cell cycle checkpoints regulated by incomplete DNA synthesis and DNA damage. The result of the abrogation of these checkpoints led to eventual cell death

resembling mitotic catastrophe, preceded by chromosome condensation, nuclear lamina dissolution, and delayed DNA polyploidy. The ability of fostriecin treatment to circumvent these checkpoints appeared to be the result of the repression of cyclins A and B expression, and most notably, the inhibition of a protein phosphatase type 2A.

The ability of AGM-1470 treatment to inhibit angiogenesis-mediated endothelial cell proliferation was shown to be a function of its ability to suppress cyclin D expression, and particularly,  $\text{Ca}^{2+}$ -independent PKC activity. It was suggested that these enzymes function in the ERK pathway since ERK has been linked to cyclin D expression in endothelial cells. The antitumour and anti-metastatic effects of AGM-1470 is thought to be exerted by its anti-angiogenesis action by affecting the cellular pathway that controls the passage of endothelial cells, that is bypassed in the genesis of cancer. The cell specificity of AGM-1470 has been suggested here to be due to the unique cell cycle dependent, PKC-mediated bi-directional growth regulation associated with endothelial cells, as was so eloquently described by Zhou and associates (1993). Because AGM-1470 has demonstrated anti-arthritic efficacy *in vivo*, additional studies were conducted in neutrophils, where cell activation in response to certain chemokines, pathogens and crystals, have been correlated with ERK activation. AGM-1470 was shown to inhibit PKC and ERK in neutrophils, and a correlation between repression of ERK activity and neutrophil activation in response to fMLP and PMA was demonstrated.

The anticancer, anti-arthritic and potential anti-angiogenesis agent, Taxol, repressed ERK and neutrophil activation in response to CPPD crystals and fMLP. PMA-induced ERK activity was not inhibited following Taxol treatment, suggesting a Taxol-dependent pathway exists, which may lie downstream of ERK. In view of these findings, I conclude that there appears to be an alternate signalling pathway for neutrophil activation that proceeds through ERK. Wortmannin, a PI 3-kinase inhibitor known to inhibit neutrophil activation when cell are pretreated with CPPD crystals or fMLP, failed to inhibit PMA-induced chemiluminescence. This further provides evidence for the hypothesis that AGM-1470 regulates the ERK pathway and that ERK is utilized by stimulated neutrophils during oxidative response.

I also sought to gain insight into potential protein targets that may be utilized in the development of novel therapeutics. I believe that several leads were identified in this study.

The study with fostriecin demonstrated the potential value of developing chemotherapeutic drugs that inhibit cell cycle checkpoints, pathways that have already been shown to be weaker in tumour cells. Additionally, such agents would be ideal to be administered synergistically with agents that induce a cell cycle block due to DNA lesions, or as a result of inhibiting enzymes that are critical for progression through S phase including topoisomerases. Topoisomerase inhibitors have not been shown to inhibit checkpoints, whereas, phosphatase inhibitors have. The cellular and nuclear morphological effects following fostriecin treatment were explained in this thesis to result from the repression of PP2A. Therefore, the identification of the phosphatases that are regulated directly by fostriecin, provides potential valid targets for drug discovery, and gives merit to the investigation and identification of protein phosphatases as therapeutic targets.

Although ERK has already been described as being necessary for endothelial cell proliferation in response to angiogenesis factors such as bFGF and VEGF, which also requires signalling from those integrins that bind to Shc through caveolin in their juxtamembrane domains, how ERK activity is regulated remains an enigma. This study proposes that a calcium-independent isoform of PKC, probably PKC $\delta$ , mediates this pathway, and recognizes this pathway as one which is amenable to the identification of potentially non-toxic, specific drugs for the treatment of angiogenesis and related diseases. The recent identification that AGM-1470 covalently binds to MeAP-2 (Liu *et al.*, 1999), suggests that AGM-1470 may inhibit the posttranslational modification of proteins that regulate PKC and/or the MAP kinase pathway in endothelial cells. Proteins that require myristoylation that affect the ERK and integrin signalling pathways include G proteins and caveolin (Shengwen *et al.*, 1996; Galbiati *et al.*, 1999), and other PKC substrates have been identified that also require myristoylation, including MARCKS and p47-Phox (Terada *et al.*, 1988; Thelin *et al.*, 1991; Curnutte *et al.*, 1994). PKC has been shown to interact with, and even phosphorylate caveolin, mediating its ability to bind other proteins including Shc (Tang *et al.*, 1994; Naoki *et al.*, 1997). Therefore, in addition to developing high throughput drug discovery screens with PKC and MetAP-2 (to identify more effective, or potentially orally available compounds), targeting caveolin, including the caveolin myristoylation site, and the site on the integrin that binds caveolin, may prove to be instrumental. The discovery

of other proteins in the MetAP-2-PKC-ERK-cyclin D pathway would lead to the identification of potential targets, and merits further investigation. The observed effects of AGM-1470 on neutrophils also leads to the identification of potential drug targets for the inhibition of neutrophil activation associated with arthritis-associated inflammation and gouty arthritis. The two pathways that are most obvious following this study include the MAP kinase pathway, and MetAP-2. The MAP kinase pathway is mediated by many enzymes, which in neutrophils, is only now being characterized. Continued studies with compounds such as AGM-1470 and Taxol would help to further dissect this pathway in neutrophils, and therefore identify neutrophil specific targets for drug discovery. The work done with Taxol further validated the MAP kinase pathway in this paradigm. Although binding of AGM-1470 to MetAP-2 has not been demonstrated in neutrophils, its specificity for this enzyme (Liu *et al.*, 1998) suggests that it is also the target in these cells. In addition to ERK-mediating proteins requiring myristoylation (Galbiati *et al.*, 1999), components of the NADPH oxidase machinery, including p46- and p-67-Phox, not only requires PKC phosphorylation for NADPH oxidase complex association, but also require myristoylation (Thelen *et al.*, 1991; Nauseef *et al.*, 1991). Therefore, by inhibiting the translocation of the Phox proteins to the plasma membrane through the inhibition of myristoylation, the NADPH oxidase complex cannot become activated, which is required for neutrophil oxidative responses (Neuseef *et al.*, 1991). It is worth noting, that antagonists of type 1 and 2A protein phosphatases (calyculin A and okadaic acid) prevented the activation of the NADPH complex (Curnutte *et al.*, 1994), suggesting that it may be worth investigating novel phosphatase inhibitors with other indications as potential anti-inflammatory agents.

*The outcome: Questions that require resolving*

I have demonstrated that AGM-1470 mediates its antiproliferative effect in angiogenesis factor-induced primary endothelial cells, and abrogation of neutrophil activation stimulated by fMLP and PMA because of its inhibitory effect on MAP kinase and PKC. The role of these kinases is different in each system. Down-regulating MAP kinase, probably through a PKC-dependent pathway, results in the repressed expression of cyclin D which would result in an early G1 phase block in endothelial cells. Because of the limited

biochemical analysis conducted to date on AGM-1470, other effects have not been investigated. In neutrophils stimulated with fMLP or PMA, inhibition of MAP kinase and PKC results in the deregulation of at least the NADPH oxidase pathway. In angiogenesis related signalling, regulation of the MAP kinases pathway and PKC are coordinated through growth factor and integrin receptor cross-talk, and rely on, and control, specific protein expression. Given the findings described in this thesis, some important questions have developed that I feel should be addressed in the future in order to gain a more thorough understanding of how this compound affects angiogenesis signal transduction with such significant specificity and minimal toxicity. Obviously, the elucidation of the pathway that is affected by AGM-1470 will open a new field of potential biomarkers in the development of angiogenesis-related disease therapies that may be more specific to its target, as well as disease (or tissue) type, and augment the development of orally bioavailable compounds.

I demonstrated that AGM-1470 binds to the particulate fraction in human hepatocytes (AGM-1470 does inhibit hepatocyte metastasis in colon carcinoma tumor rat model). I speculated that AGM-1470, because of its specificity *in vitro* and *in vivo*, as implied by its very low  $IC_{50}$  and apparent irreversible effect, binds to a particulate fraction protein in endothelial cells. Although evidence for this recently emerged with the finding that AGM-1470 covalently binds to MetAP-2, this conjecture does require analysis, similar to that described with hepatocytes. Developing labeled AGM-1470 (i.e. biotinylated, or photoaffinity; Griffith *et al.*, 1997; Sin *et al.*, 1997) has been described elsewhere, and these analogs can be used to detect proteins in any cellular fraction that are bound to AGM-1470, directly. Renaturation of cytosolic versus membrane proteins on a membrane (i.e. nitrocellulose) can also be done and probing with these analogs would facilitate a more rapid identification of the proteins.

The importance of the biphasic growth regulatory mechanism of PKC in endothelial cells to the function of deregulating PKC in inhibiting angiogenesis was emphasized in this study. Given the observation that AGM-1470 inhibits cyclin D expression through MAP kinase deregulation, the question of whether the PKC biphasic growth-regulation and AGM-1470  $G_0/G_1$  phase specificity correlate with the expression of cyclin D and function of cdk4-cyclinD complex should be addressed. It has been shown that antibodies against cyclin D1

inhibit cell cycle progression when microinjected during Mid G1, but are ineffective near the G1-S phase boundary (Musgrove *et al.*, 1994) and that Ras is required for both the induction of cyclin D1 and the down-regulation of p27<sup>KIP1</sup> until cells pass the restriction point (Aktas *et al.*, 1997). Therefore, given the cell cycle specificity of AGM-1470, that was also demonstrated in this study, a methodical cell cycle analysis (i.e. FACS analysis) with expression analysis of cyclin D1 and determination of the Cdk4, PKC and ERK2 activity should be done in order to confirm this hypothesis and define more precisely the cell cycle specificity of AGM-1470. Also, in light of the importance of PKC in the progression of endothelial cell during angiogenesis, the expression and activity of all of the isoforms of PKC should be analyzed in endothelial cells. Given the emphasis other laboratories are putting on the development of specific isoform inhibitors as potential therapeutics, this type of study would prove invaluable to those efforts as well as dissecting the roles of different PKC isoenzymes in different systems, and in this case, in different angiogenesis-like diseases.

Methods of measuring PKC experimentally consist of those demonstrated in this report as well as with the use of inhibitors or activators of the enzyme, or the direct measurement of translocation from the cytosol to the membrane. These experiments are indirect and do not account for the activity of a majority of endogenous PKC. Since the experiments described in this study measure PKC that is activated *in vitro* with cofactors, an important corollary has emerged that may be the most significant as far as a biochemist interested in PKC signalling is concerned. Regulators of PKC have thus far remained very elusive, and the class of specific PKC kinases has not been identified. The results presented in this study indicate that AGM-1470 treatment is resulting in the inhibition of an activator of PKC, or the activation of a PKC inhibitor, such as a specific phosphatase. Therefore, AGM-1470 could be used to identify what these PKC regulators are directly. This information, resulting from relatively basic biochemical analysis with the use of AGM-1470, would open a window to an entirely new class of proteins that could be exploited as therapeutic targets, as well as a means of understanding how specific isoforms of PKC are controlled in different systems.

When breast cancer cells are blocked in early G1, cyclin D1 expression is sufficient to induce DNA synthesis (Vadiveloo *et al.*, 1996), whereas in quiescent fibroblasts blocked

with serum deprivation, cyclin D1 expression is not sufficient to induce DNA synthesis (Musgrove *et al.* 1994). As discussed above, we must determine precisely where in the cell cycle AGM-1470 induces its block. Do cells become quiescent following AGM-1470 treatment, or are they blocked in early G1 phase, perhaps due to the abrogation of the Ras-Raf-MEK1-ERK2 pathway, and cyclin D expression. This information may explain how it is that AGM-1470 is relatively non-toxic to cells, and why they do not appear to undergo apoptosis (unpublished observation, CT). It would be interesting from the signal transduction perspective to observe whether AGM-1470 treated cells are rescued when transformed with constitutively active cyclin D1, ERK2, or p65<sup>PAK</sup>, or particularly, PKC or constitutively active eIF-2 (see Figure 64). For instance, do cells transfected with constitutively active mutants cyclin D1, ERK2, p65<sup>PAK</sup>, Mek1, Shc or eIF-2 undergo DNA synthesis when concomitantly treated with AGM-1470?

ERK2 is activated by activated mutants of Ras and Raf, and is cell adhesion dependent, whereas activation of MEK is not (Chen *et al.*, 1996; Renshaw *et al.*, 1997; Zent *et al.*, 1998). Ras and Raf are still activated in suspended cells, whereas Mek1 activity is not (Renshaw *et al.*, 1997). An aspect of angiogenesis signalling that was not addressed in this study was whether AGM-1470 affects adhesion independent cell signalling. Therefore, it would be interesting to observe whether AGM-1470 inhibits proliferation in suspended cells. An analysis of the activities of Ras (GTP loading), Raf, and particularly Mek1, and p65<sup>PAK</sup> (Frost *et al.*, 1997; Tang *et al.*, 1997) would give more detailed information regarding the pathways that are affected by AGM-1470 (see Figure 62). An advantage with AGM-1470 regarding its specificity towards normal versus tumorigenic cells would be a lack of drug resistance with AGM-1470 treatment. Understanding this mechanism may shed light on the mechanism of drug resistance and can be used to develop drugs that are directed towards cancer versus normal cells. Since 23% of cancer cells have deregulated Ras function, and a major proportion of breast and colon cancers contain a mutation in the cyclin D1 locus, these cells would be resistant to AGM-1470 since it affects this pathway upstream of the PKC→Ras-Raf-MEK-ERK→cyclin D1 pathway. A more detailed study with cancer cells lines that are immortalized without function in this pathway, i.e. Ras, cyclin D1 or pRb<sup>-/-</sup> would verify this. In conjunction to the rescue experiments described above, one

might determine if cells that are transfected with constitutively expressed proteins in this pathway are resistant to AGM-1470 treatment

**Figure 62.** Signal transduction pathway cross talk involved in angiogenesis. The paradigm of signalling cross talk is central to the elucidation of those pathways that mediate the morphological machinery of endothelial cells, such as lamellipodia, stress fiber assembly as well as proliferation and survival. This scheme illustrates those proteins that have been implicated in the above processes that are also involved in signalling to parallel pathways. Induction of Ras through Shc phosphorylation at the integrin has implications in the Raf-MEK-ERK pathway in proliferation as well as mediating in conjunction with growth factor receptor (i.e. bFGF, VEGF) activity, activation of the Cdc42/Rac pathway involved in membrane ruffling and lamellipodia. Activation of p65PAK through Cdc42 and Rac has been described to affect MEK in angiogenesis, which may enhance ERK activity for endothelial cell growth. G-coupled receptors may also signal to the Raf-MEK-ERK pathway through PKC via PLC or  $G_0/G_q$  activity, as well as affect the recruitment of focal adhesion protein and stress fiber assembly through Rho. Although PLC activity results in the release of  $[Ca^{2+}]_i$  that contribute to the activity of  $Ca^{2+}$ -dependent PKC isoforms, this mechanism is not illustrated since it was shown that AGM-1470 treatment results in the repression of  $Ca^{2+}$ -independent isoforms of PKC. This does not exclude the possibility that  $Ca^{2+}$ -dependent isoforms do not contribute to the proliferation of endothelial cells, but it is argued in the text that this is unlikely. Whether  $Ca^{2+}$ -dependent PKC isoforms contribute to the survival or expression of VEGF and/or TNF- $\alpha$  from neutrophils or in the synovium was not addressed in this study.

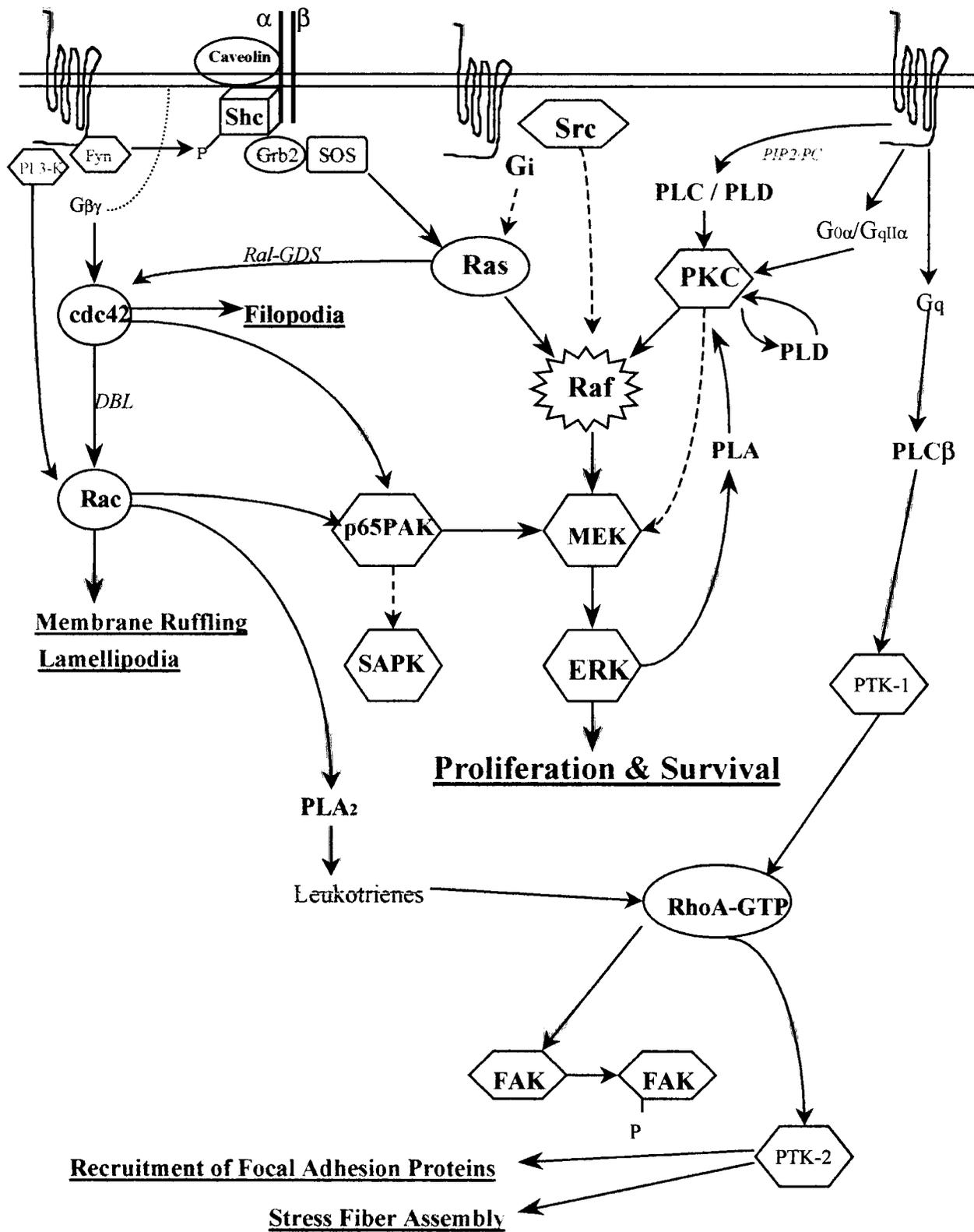


Figure 62. Signal transduction pathway cross-talk in angiogenesis.

A question that I feel is the most significant given the studies of Li and Chang (1996) who demonstrated that the human homologue of a rat eIF-2 associated protein (p67 eIF-2-BP) is a methionine aminopeptidase is whether the function of AGM-1470 is translation dependent. This cannot be determined directly as inhibitors of protein synthesis also block DNA synthesis (unpublished observation, CT). Differential expression analysis might identify proteins that are expressed in cells not treated with AGM-1470, but with bFGF and VEGF, compared to cells not treated with growth factors, or treated with growth factors and with AGM-1470. Expression of MetAP-2 or eIF-2 should be studied in the presence of AGM-1470, as well as binding studies (between these two proteins), and phosphopeptide analysis of the eIF-2 protein should be done to test this hypothesis as well as validate the eIF-2-BP (MetAP-2) as a putative target of AGM-1470. These experiments might also be conducted in fMLP and/or PMA stimulated neutrophils once the experimental conditions are established given the fragility of the primary cells. If MetAP-2 is the target, then it should be determined what protein is expressed that is required for activation of PKC and the pathway that is abrogated in response to AGM-1470 treatment. The eIF-4E protein is upregulated in breast cancer cells, implicating protein synthesis in carcinogenesis. These tumours might also be resistant to AGM-1470 treatment, and this should also be determined.

Some aminopeptidases are tripeptidases, and share a peptide binding sequence similar to that of PEPT1 peptide transporter. Other peptidases, including MetAP-2, have a peptide recognition sequence. This may be exploited in the development of orally active anti-angiogenesis drugs, or a second-generation compound that is orally bioavailable and targets MetAP-2 (a putative AGM-1470 target). By targeting a site on the MetAP-2 that is within the peptide recognition sequence and shares some homology with a peptide transporter system, such as PEPT1, then conceivably, a drug can be developed that is recognized by both, and therefore is transported following oral administration across the peptide transporter in the intestine, and maintain the specificity of AGM-1470. In addition to developing screening assays, X-ray crystallography can be used to determine sites and medicinal chemistry techniques utilized to develop compounds and a structure activity relationship for each.

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