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EFFECTS OF FLAVOPIRIDOL ON CDK2 ACTIVITY IN CULTURED CELL LINES

BY

DONALD G MATTHEWS

A Thesis
Submitted to the Faculty of Biology
in Partial Fulfilment of the Requirements
for the Degree of

MASTER OF SCIENCE

Department of Biology
Lakehead University
Thunder Bay, Ontario

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Abstract

Flavopiridol reduces cyclin-dependent kinase activity by competitive inhibition with ATP by binding to the ATP-binding pocket of these cell cycle regulators, thus competing with ATP binding and subsequent kinase activity. Flavopiridol also changes the expression levels of a wide array of other proteins, and binds to DNA and acts to inhibit transcription at therapeutically relevant concentrations. Because it was initially thought to specifically inhibit cdks, it was thought that flavopiridol would be an excellent inhibitor of cell proliferation and effective tumor suppressor. However, clinical trials have been notably disappointing as a single chemotherapeutic agent. In previous studies in this lab, we found that the proliferation inhibition of primary tumor cells treated with flavopiridol was independent of any decrease in cdk2 activity. Cdk2 phosphorylates its target proteins during G_1 and S phase transition, leading to initiation DNA replication. In this study, we examined the effects of flavopiridol on the cdk2 activity of cultured cell lines; MCF7, HeLa, DU145, Saos2 and HSF55. We found an upregulation of the cdk inhibitor $p21^{WAF1/CIP1}$ in flavopiridol-treated MCF7, HeLa and HSF55 cells. This upregulation caused cdk2 kinase inhibition in flavopiridol-treated cell lines. Partial cdk2 activity at the same concentrations was found in HeLa, DU145, HSF55 and Saos2 cell lines, while complete inhibition was found in MCF7 cells. Although apoptosis was not observed after a 24hr incubation period, the antiapoptotic protein Bcl-2 was downregulated. Our findings illustrate an alternate mechanism of cdk2 inhibition *in vitro*, through $p21^{WAF1/CIP1}$ upregulation, showing that drugs that were shown to have specific activities when assayed using purified systems may have multiple *in vivo*

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activities and these activities may differ depending on the cells used. These different effects may contribute to the variable results seen in clinical trials.

Thesis Abbreviations

ALDH-1	- Aldehyde dehydrogenase class 1
Arf	- ADP-ribosylation factor
ATP	- Adenosine triphosphate
B-CLL	- Chronic B-cell leukemia
Bak	- Bcl2-associated killer protein
Bax	- Bcl2-antagonist x protein (proapoptotic)
Bcl-2	- B cell lymphoma leukemia 2 protein (antiapoptotic)
Bcl-X_L	- Bcl-2 family member X-large segment (antiapoptotic)
BSA	- Bovine serum albumin
c-Abl	- Abelson murine leukemia viral oncogene homolog
c-Myc	- Oncogene of the MC29 avian myelocytomatosis virus
Caspase	- Cysteinyl aspartate-specific protease
CAK	- Cyclin-dependent kinase activating kinase
Cdc	- Cell division cycle
Cdk	- Cyclin-dependent kinase
Cip1	- Cyclin-dependent kinase inhibiting/interacting protein1
Cki	- Cyclin-dependent kinase inhibitor
CTD	- Carboxy-terminal domain
DDW	- Double distilled water
DLT	- Dose limiting toxicity
DMEM	- Dulbecco's modified Eagle's medium
DMSO	- Dimethyl sulfoxide
DNA	- Deoxyribonucleic acid
DU145	- Prostate carcinoma cell line (isolated from brain metastasis)
E2F	- Elongation factor 2
ECL	- Enhanced chemiluminescence
EGFR	- Epidermal growth factor receptor
*erb*B-2	- v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
ERK	- Extracellular signal-regulated kinase
et al	- And associates
Fas	- Fas receptor (transmembrane protein)
FBS	- Fetal bovine serum
G_1 phase	- Growth phase 1
G_2 phase	- Growth phase 2
HeLa	- Henrietta Lacks – Cervical cancer cell line
HIV	- Human immunodeficiency virus
HMR	- Hoechst Marion Roussel
HRP	- Horseradish peroxidase

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HSF55	- Human skin fibroblasts 55
HSV-1	- Herpes simplex virus-1
IC_{50}	- Inhibition concentration 50%
IGF-1	- Insulin-like growth factor
IL	- Interleukin
INK	- Inhibitor of cdk4
KE4	- Esophageal squamous cell carcinoma cell line
Kip1	- Kinase inhibiting protein1
M phase	- Mitotic phase (Mitosis)
MAPK	- Mitogen-activated protein kinase
MCF7	- Michigan Cancer Foundation – Breast cancer cell line
Mcl-1	- myeloid cell leukemia sequence 1
MDA-MB-468	- Breast cancer cell line - adenocarcinoma
Mdm2	- Mouse double minute 2
MEM	- Minimum essential medium Eagle
MMP	- Matrix metalloproteinase
mRNA	- Messenger RNA
MS-KCC	- Memorial Sloan-Kettering Cancer Center
MTD	- Maximally tolerated dose
MTT	- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Myt1	- Myelin transcription factor 1
NB	- Nuclei buffer
NCI	- National Cancer Institute
NF-κB	- Nuclear Factor κB
PAGE	- Polyacrylamide gel electrophoresis
PBS	- Phosphate buffered saline
PCNA	- Proliferating cell nuclear antigen
PI	- Propidium iodide
PKA	- Protein kinase A
PKC	- Protein kinase C
PMSF	- Phenylmethyl sulfonyl fluoride
pRb	- Retinoblastoma susceptibility protein
P-TEFb	- Positive transcription elongation factor b
PVDF	- Polyvinylidene difluoride
Rb	- Retinoblastoma susceptibility gene
RNA	- Ribonucleic acid
RNA pol II	- RNA polymerase II
RPA p34	- Replication protein A – 34kDa subunit
S phase	- DNA synthesis phase
Saos2	- Osteosarcoma cell line
SDS	- Sodium dodecyl sulfate
TBS	- Tris buffered saline
TBST	- Tris buffered saline + tween20
TBST-5%M	- Tris buffered saline + tween20 + 5% milk
TBST-8%M	- Tris buffered saline + tween20 + 8% milk
Thr (T)	- Threonine

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TNF	- Tumor necrosis factor
TNFR	- Tumor necrosis factor receptor
Tyr (Y)	- Tyrosine
UBC	- Ubiquitin carrier
VEGF	- Vascular endothelial growth factor
Waf1	- Wild-type p53 activating fragment 1
wt	- Wild-type
XIAP	- X-linked inhibitor

Introduction

1. The Cell Cycle

Progression through the cell cycle involves a vast array of proteins. When these proteins are working harmoniously, they facilitate the successful replication of a single cell into two daughter cells. As one can imagine, it takes an enormous amount of coordination to make sure all proliferation processes go smoothly and malfunctions are corrected in a timely manner.

1.1. Phases of the cell cycle

Due to the complexity of the cell cycle, it has been broken down into separate phases, in order to methodize the numerous processes. There is a specific progression to these phases. Each phase contains a set of events that needs to be completed before the succeeding one can be initiated. Typically, the cycle is presented as beginning upon the completion of mitosis and the formation of new daughter cells. At G_1 or the first growth phase, the cell begins the accumulation of material for cellular growth. In this stage, a multitude of signalling pathways is activated to initiate the transcription factors and regulatory proteins necessary to promote cellular growth. DNA synthesis begins during

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S phase upon accumulation of enough cellular material in G_1. Once the genome has successfully been replicated, a second growth phase (G_2) must be completed to amass the cellular content (ie organelles, proteins etc) necessary for two daughter cells before the onset of cell division. M phase (mitosis) then occurs and the cellular components are divided amongst the two new daughter cells that then begin the cycle anew (Figure 1).

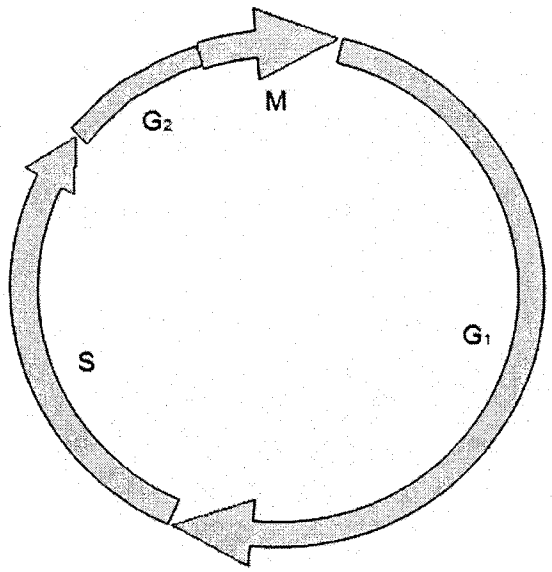

Figure 1 - The cell cycle.
A representation showing the sequential progression of phases of a typical mammalian cell: first growth phase G_1 following cytokinesis; DNA replication phase (S phase); second growth phase G_2; Mitosis (M phase). Cytokinesis follows and G_1 begins again.

The cell cycle processes are carried out in an orderly fashion. Specific mechanisms and checkpoints exist to ensure that each phase is completed before the subsequent one is initiated. One of the principal regulators of the cell cycle is the cyclin-dependent kinases (cdks). Cdks phosphorylate a variety of substrates to facilitate cell

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cycle progression. Leland Hartwell, Paul Nurse and Timothy Hunt share the 2001 Nobel Prize in Physiology or Medicine for their discovery of these key cell cycle regulators in the 1980s, based on work that originated in the early 1970s. The genetics of cell cycle regulation were initially done in two different strains of yeasts, the fission yeast *Schizosaccharomyces pombe* by Paul Nurse and the budding yeast *Saccharomyces cerevisiae* by Leland Hartwell, from which a number temperature-sensitive cell cycle mutants were isolated. These were referred to as cell division cycle (cdc) mutants and were given numbers to distinguish between the genes that were expected to have been affected (1). Paul Nurse's group transferred a human cDNA yeast expression library into one of these mutants, the *S. pombe* cdc2, and was able to rescue the defect. This human cDNA that complemented the cdc2 mutation was then identified to be a key kinase that functions in the G_2 to M phase transition in mammalian cells. This work was followed closely by David Beach's group who used similar the *Saccharomyces cerevisiae* cell cycle mutant, cdc28, to identify the human homolog. Since the gene from the *S. pombe* was the first to be published, the mammalian homolog was referred to as the cdc2. This was the beginning of the explosion in research on cell cycle regulation. In both strains of yeasts, the cdc2/cdc28 functions at the G1/S and the G2/M boundaries. In mammalian cells, this gene was first found to function only at the G2/M phase when microinjection of antibody to cdc2 arrested cells at the G2/M boundary (2). When the temperature-sensitive mutation in the mouse FT210 cell line was identified to be in the cdc2 gene product, it was used to demonstrate a sole function at G2/M (3). At that time, there was biochemical evidence that other cdc2-like kinases were present in mammalian cells and they may function at other phases of the cell cycle (4). Soon after, it was revealed by Hunt that

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these molecules require binding by other proteins known as cyclins to become active. The discovery of related protein kinases with similar attributes and functions, all with a dependence on cyclin binding to become active, led to this family of kinases to be aptly named cyclin-dependent kinases. The second mammalian cyclin-dependent kinase was later identified that have similarities to the cdc2, and this was named cdk2. Since then the cdk family has grown, with functions cell cycle progression to transcriptional control. Cdc2 has since been renamed cdk1, signifying its discovery as the initial cdk protein studied.

1.2 Cell cycle regulatory proteins – The cdk/cyclin complex

Cdk protein expression is constant throughout the cell cycle; however it is the cyclin proteins which fluctuate in concentration (5). Different cyclins bind specifically to certain cdks to initiate activation of kinase activity. The cdk/cyclin complex is also modified by other kinases and phosphatases and can be bound in an inhibitory manner by other molecules to prevent premature cdk activity. Phosphorylation is thought to cause physical changes in cdk protein structure to prevent the access of ATP to the ATP binding site and facilitate access of substrates to the cdk substrate-binding site. Inhibitory molecules bind to the cdk/cyclin complex and physically block the substrate binding site or displace the cyclin, thus decreasing phosphorylating activity. When the inhibitory phosphate groups are removed by the corresponding phosphatases, the activated cdks are then able to phosphorylate their substrates to facilitate cell cycle progression. The cdk/cyclin pairing, and thus the targets of the complex, depends on the phase of the cycle that the cell is in (Figure 2)

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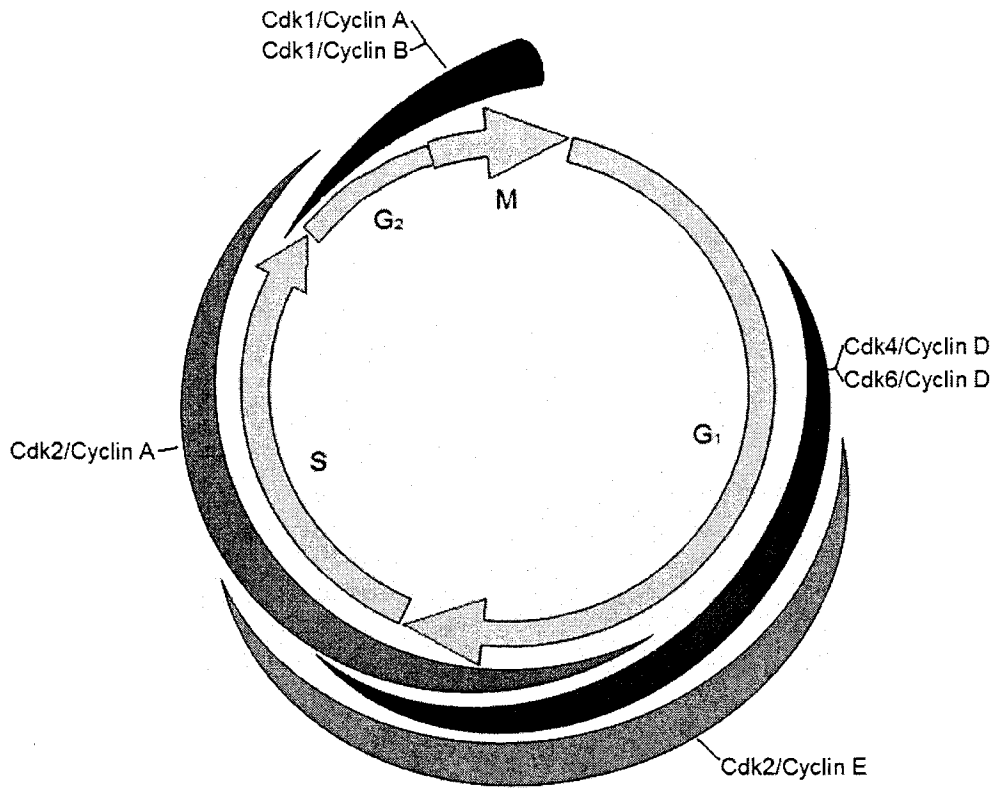

Figure 2 - Cdk regulation of the cell cycle.
Cyclins become available to their perpetually present cdk counterparts during specific phases. Once the cdk activity is no longer required, the cyclins are targeted for degradation until that phase of the cell cycle comes around again. Cdk2 is active for most of the cell cycle while bound to cyclin A or cyclin B.

G_1 progression is controlled by the pairings of cdk4/cyclin D1 and cdk6/cyclin D1. They work cooperatively to phosphorylate and subsequently activate the transcription factor family E2F (6). The E2F proteins then proceed to transcribe S phase genes (7-9). Cdk6/cyclin D1 contributes by activating the proliferating cell nuclear antigen (PCNA), which is required for DNA replication (6;10). The cyclin D-associated cdks indirectly assist in G_1/S phase transition by sequestering cdk inhibitors, such as $p27^{Kip1}$ and $p21^{Waf1/Cip1}$, preventing binding of these molecules to the complex consisting of cdk2/cyclin E (11).

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The cdk2/cyclin E complex is essential to the G_1/S phase transition (6;11). As is the case with all cdks, cdk2 is phosphorylated at threonine160 to activate and at threonine14 and tyrosine15 to inhibit its activity. Cdk2 is bound by cyclin and is phosphorylated in an inhibitory manner by the kinase Wee1 in a sequence independent manner (12). This inactivation occurs at mitosis and is maintained into early G_1. Cyclin E binds to cdk2 and as the cell progresses through mid-G_1 and nears the conclusion of G_1, the complex is activated through phosphorylation by the cdk-activating kinase (cak) complex, consisting of cdk7 and cyclin H and by dephosphorylation, phosphatases cdc25A and cdc25B remove the phosphates added by Wee1 (Figure 3) (6). Cdk2/cyclin E serves to phosphorylate DNA polymerase α, the retinoblastoma susceptibility protein (Rb), RNA reductase (responsible for DNA synthesis and repair) and activates PCNA (6). These processes enable the cell to enter S phase and begin DNA replication. Cdk2/cyclin E also phosphorylates and subsequently causes the ubiquitin-mediated degradation of cdk inhibitors, such as $p27^{Kip1}$ (13;14). A decrease in $p27^{Kip1}$ levels facilitates cdk activity.

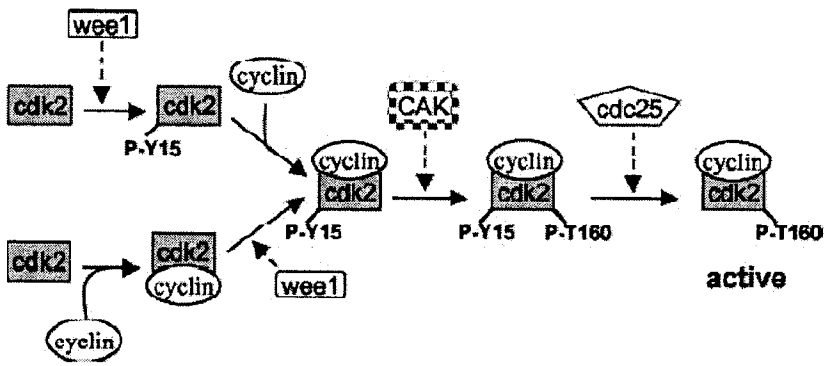

Figure 3 - Cdk2 activation pathway.
Cyclin binding promotes a conformational change in the cdk2 structure to initiate activation. Inhibitory phosphorylation of the Tyr^{15} residue by wee1 prevents activation. This phosphate group is later cleaved by the phosphatase cdc25 to initiate cdk2 activity. The cak molecule phosphorylates cdk2 at the Thr^{160} residue to promote its activation (12).

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S phase progression is facilitated by cdk2/cyclin A. Cyclin A is upregulated shortly after the upregulation of cyclin E. Once cyclin A associates with cdk2, the cdk2/cyclin A complex is activated through phosphorylation by cak and dephosphorylation by cdc25A and cdc25B, the active complex then begins to phosphorylate its specific substrates (8;15). One such substrate is the 34 kDa subunit of replication protein A (RPA p34) responsible for binding to single stranded DNA to prevent DNA replication (6;16). Rb-related proteins (p107 and p130) are also targets for cdk2/cyclin A (10;17). P107 and p130 bind to and inhibit the E2F family of transcription factors (17). Following the phosphorylation of these proteins by cdk2/cyclin A, their dissociation from E2F allows direct phosphorylation of E2F proteins by cdk2/cyclin A to occur (7;18). This modification results in the prevention of DNA binding and transcription by the E2F family proteins, consequently instigating the termination of S phase (Figure 4). Through late G1 and into S phase, the cdk2 kinase also phosphorylates histone H1 (19-21). This phosphorylation increases in the amount of histone H1 that gets phosphorylated, and also in the number of phosphates added to each molecule of histone H1.

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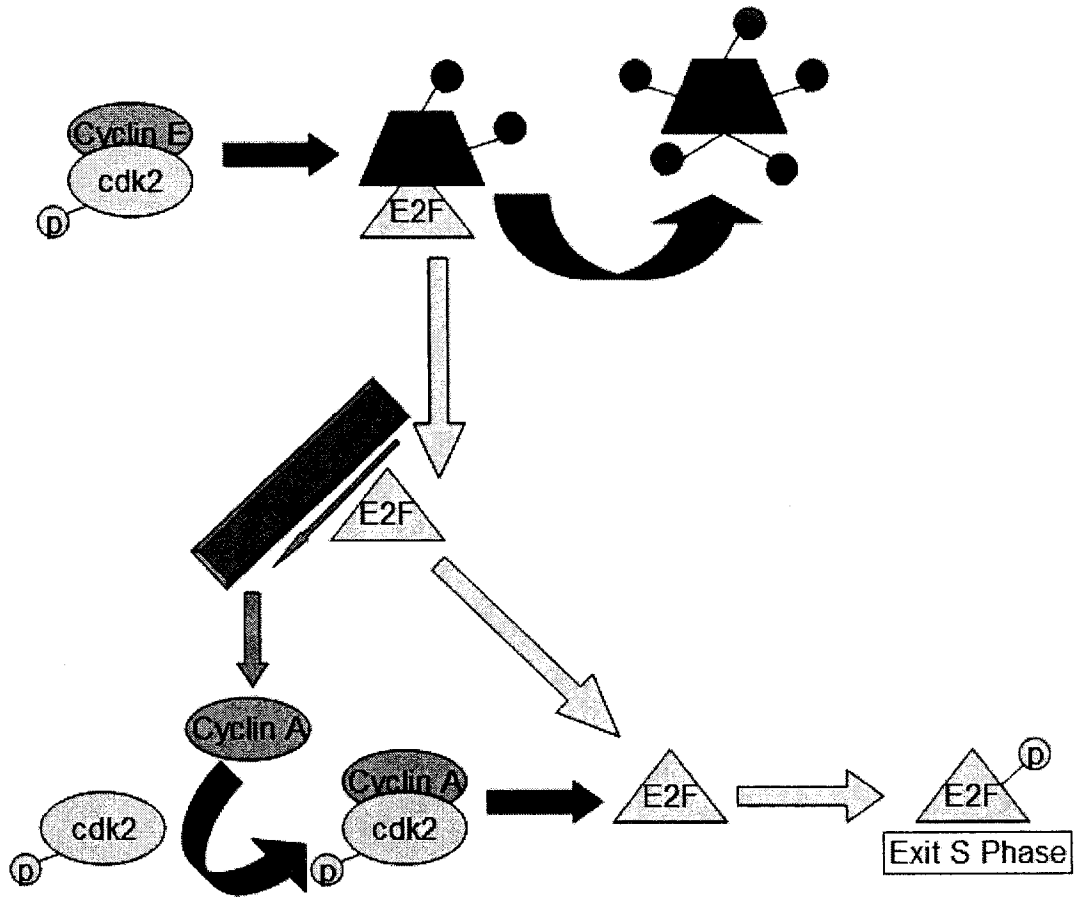

Figure 4 - Cdk2 activity in phase transition.
Cdk2/cyclin E hyperphosphorylates pRb, which releases the E2F transcription factors to initiate transcription of S phase genes. Cyclin A is upregulated and after binding to cdk2 and becoming activated in S phase, this complex phosphorylates its target proteins. E2F proteins are a target of cdk2/cyclin A to begin the termination of DNA replication and S phase.

The second growth phase, G_2, follows the completion of DNA replication. In G_2 the cell must become substantial enough to support the processes of the potential two daughter cells. When enough material (e.g. organelles, proteins etc.) has accumulated to support viability of both daughter cells, cdk1 is activated in much the same way as cdk2 and mitosis begins. Cdk1/cyclin A triggers the degradation of the nuclear membrane by phosphorylation of the nuclear lamins (22). Soon after the upregulation of cyclin A, cyclin B levels are upregulated to bind with cdk1 and assist in the progression of mitosis.

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Cdk1/cyclin B is responsible for the direct hyperphosphorylation of histone H1 and the activation of topoisomerase II which are required for chromosome condensation during prophase and segregation (6;10;23;24). Finally, cdk1 activity stabilizes microtubule formation and facilitates the segregation of chromosomes during early anaphase (10;25-27). After the completion of telophase and the degradation of G_2/M phase cyclins, phosphatase activity promotes the formation of two nuclei around the chromosomes. The binucleated cell can then undergo cytokinesis and the daughter cells enter the subsequent G_1 to wait for the signal to initiate the cell cycle once again.

Other members of the family of cdks (cdk 5, 7, 8, 9) are not involved in cell cycle progression directly, but serve to regulate the activity of other cdks and also act as transcription regulators (28-30). The cdk2 was initially identified by the PSTAIR motif that is shared with cdk1 and later members were identified by their association with cyclins. Cdk5 is activated by associating with p35 as cells exit the cell cycle and differentiate into neurons (31). Cdk7 and cyclin H form the cdk-activating kinase (cak) complex which phosphorylates other cdks. Cdk9 binds with cyclin T to form the positive transcription elongation factor b (P-TEFb), which is responsible for phosphorylating a number of targets, including the C-terminal domain (CTD) of RNA polymerase II (pol II) (29;30). Cdk8/cyclin C also phosphorylates RNA pol II. Without this modification, it is observed that RNA pol II cannot polyadenylate mRNA tails properly after transcription leading to instability and subsequent degradation (29).

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1.3 Regulation of cdk activity by proteins

1.3.1 By cyclin availability

Cdk molecules are found at constant levels throughout the cell cycle, so it is the oscillating nature of the cyclin proteins that dictates when each cdk/cyclin complex will become active and carry out its respective responsibilities. The production of each cyclin protein is controlled by numerous transcriptional regulators to ensure that they are only available to their complimentary cdks at the appropriate times. The downregulation of cyclins is initiated by phosphorylation and ultimately caused by ubiquitin-mediated proteolysis and the cyclin molecules are tagged for degradation by a variety of regulatory proteins depending on the cyclin to be degraded (27;32). The degradation of cyclin B at the end of mitosis is guided by the ubiquitin carrier protein (UBC) E2-C (33). E2-C facilitates the ubiquitination of cyclin B by the cyclin-specific ubiquitin ligase E3-C which is part of the larger cyclosome protein that is activated by cdc2 kinase. Degradation of cyclin B is then carried out by the 26S proteasome complex. Cyclin E degradation is mediated through cdk2 and glycogen synthase kinase 3 phosphorylation of the cyclins' serine72, threonine372, threonine380 and serine384 residues which facilitates binding of the ubiquitin ligase, SCF^{Fbw7} (34). When cyclin E is not bound to cdk2, target residues are accessible for ubiquitination by the Cullin-3 (Cul-3) ubiquitin ligase.

1.3.2 By protein inhibitors

The KIP/CIP family of ckis mainly inhibit cdk2/cyclin complexes by physically binding to the entire complex, preventing its activity (8;10;11;35). These proteins can also inhibit cdk1 complexes, but to a lesser extent (8;10). The proteins in this family are

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$p21^{WAF1/CIP1}$, $p27^{KIP1}$ and $p57^{kip2}$. $p21^{WAF1/CIP1}$ is a transcriptional product of p53 and has been found to not only inhibit cdk2, but also PCNA which halts DNA replication. In contrast, $p27^{KIP1}$ prevents cak-activation of cdk2 (10). Of all the ckis, $p57^{kip2}$ is the only one required for embryonic development (36). Loss of $p57^{kip2}$ function results in the abnormal development of several tissues within the embryo. These inhibitory proteins also promote cyclin D-associated cdk (cdk4 and cdk6) activity, presumably to ensure G_1 phase completion before the phase transition can commence (Figure 5) (11).

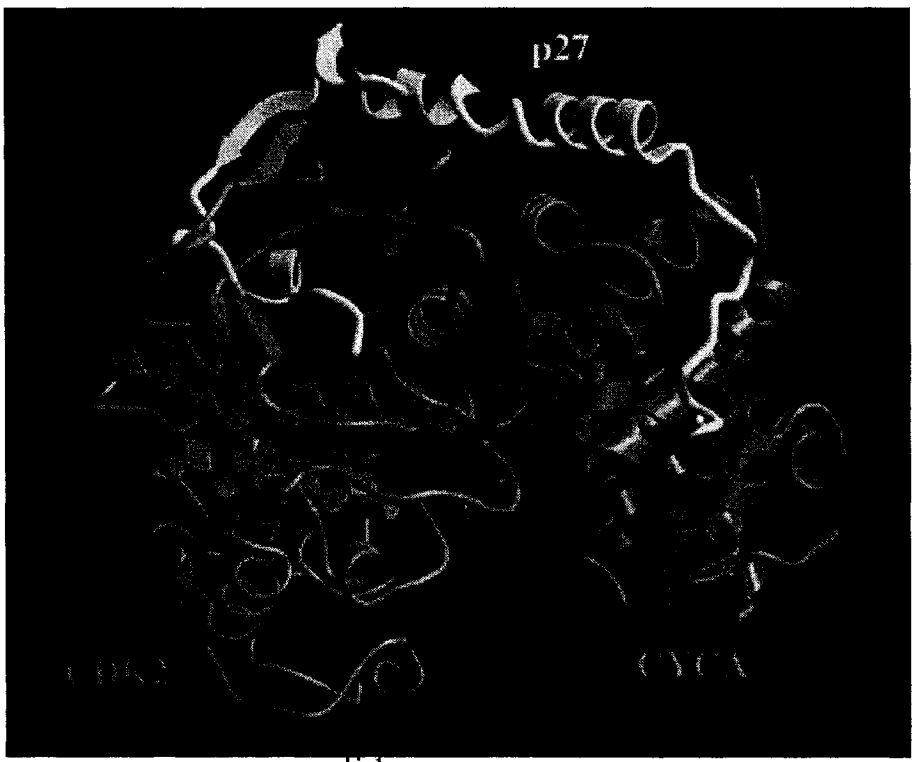

Figure 5 – Crystalline structure of $p27^{kip1}$ binding to the cdk2/cyclin A complex. $p21^{waf1/cip1}$ and $p27^{kip1}$ bind both cyclin and cdk molecules. Inhibition is due to the prohibition of ATP binding in the catalytic cleft of the cdk2 enzyme (37).

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The INK family, $p16^{ink4a}$, $p15^{ink4b}$, $p18^{ink4c}$, $p19^{ink4d}$ and $p14^{arf}$, inhibit cyclin D-associated cdks by competing with cyclin D for binding with the cdk (10;11). $P16^{ink4a}$ was the first of this family to be examined and due to its effects on cdk4/cdk6. Rb is no longer phosphorylated and subsequently sequesters E2F transcription factors once again to provide G_1 arrest (10).

1.3.3 Summary

The elaborate mechanisms required to regulate the cell cycle are central to maintaining timely cell cycle progression. Many safety features are present to keep these cell cycle regulators active or inactive throughout each phase. Cyclins are tightly regulated by transcription and degradation factors. Cdks are controlled by a multitude of activating and deactivating phosphorylation and dephosphorylation steps. Ckis are present to prevent inopportune cdk activation. Other measures are in place to counter any aberrant protein function. However, even with all of these cellular safeguards, things can still go awry. When this occurs, cells can begin to divide faster with an abbreviated G_1, enter mitosis without adequate DNA replication, or cause superfluous DNA replication leading to polyploidy, among a host of other possibilities, which may lead to tumor development.

1.4 Apoptosis

Apoptosis is the cellular process of undergoing programmed cell death. Apoptosis is initiated when a cell cannot effectively and efficiently repair a malfunction in the cellular machinery. The main tumor suppressor gene of mammalian cells is the transcription factor p53. It is responsible for promoting cell cycle arrest or, if the

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deviation cannot be remedied, instigating apoptosis. Cell cycle arrest and apoptosis is performed through numerous pathways. The activation of p53 is a part of many of these pathways. In a normal cell, p53 mRNA is always present with a very long half-life and a high turnover of translated p53 protein, keeping it at a very low intracellular level (28;38). P53 is prevented from becoming activated by Mdm-2, which binds to its acidic domain, translocates it from the nucleus and tags it for ubiquitin-mediated degradation (39;40). Upregulation, stabilization and activation of p53 is carried out by a variety of proteins, the majority of which are activated by DNA damage or malady. For example, the proliferation-promoting protein c-Myc upregulates transcription and enhances stabilization of p53, while c-Abl binds to, and enhances the transcriptional ability of p53 (40).

Activation of p53 leads to the upregulation of other proteins that promote a number of outcomes, including cell cycle arrest, apoptosis and autoregulation of p53. The latter of these outcomes is realized in the upregulation of Mdm-2 by p53 for the function of inhibiting its own actions as it becomes no longer required (40). Cell cycle arrest can occur by the p53-mediated upregulation of ckis such as $p21^{WAF1/CIP1}$ and $p27^{KIP1}$, the transcription factor inhibitor Rb, 14-3-3σ which serves to inhibit the cdk1 phosphatase activator cdc25C and the inhibitory kinase Wee1. Downregulation of PCNA by p53, through which DNA polymerase δ and cyclin D1 production is are reduced, as well as an increase in the growth suppressor gene Gadd45, along with a host of other mechanisms will also lead to cell cycle arrest (40).

Finally, if the repair mechanisms cannot adequately mend the DNA, apoptosis can be initiated by p53. The Bcl-2 apoptosis regulatory family form heterodimers to

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create a proapoptotic/antiapoptotic equilibrium in normally functioning cells (Bcl-2 and Mcl-1 are antiapoptotic, while Bax is proapoptotic). This equilibrium is influenced in favor of apoptotic protein expression in response to p53 activity (40). For apoptosis to occur, an upregulation of proapoptotic genes, combined with a downregulation of antiapoptotic genes initiates cell death. This imbalance in Bcl-2 family proteins results in cytochrome *c* release, which in turn activates the caspase-mediated apoptosis cascade. Various stimuli have been found to prevent apoptosis in favor of cell cycle arrest, such as the X-linked inhibitor of apoptosis (XIAP), which serves to inhibit caspase activity to abort apoptosis (Figure 6) (41).

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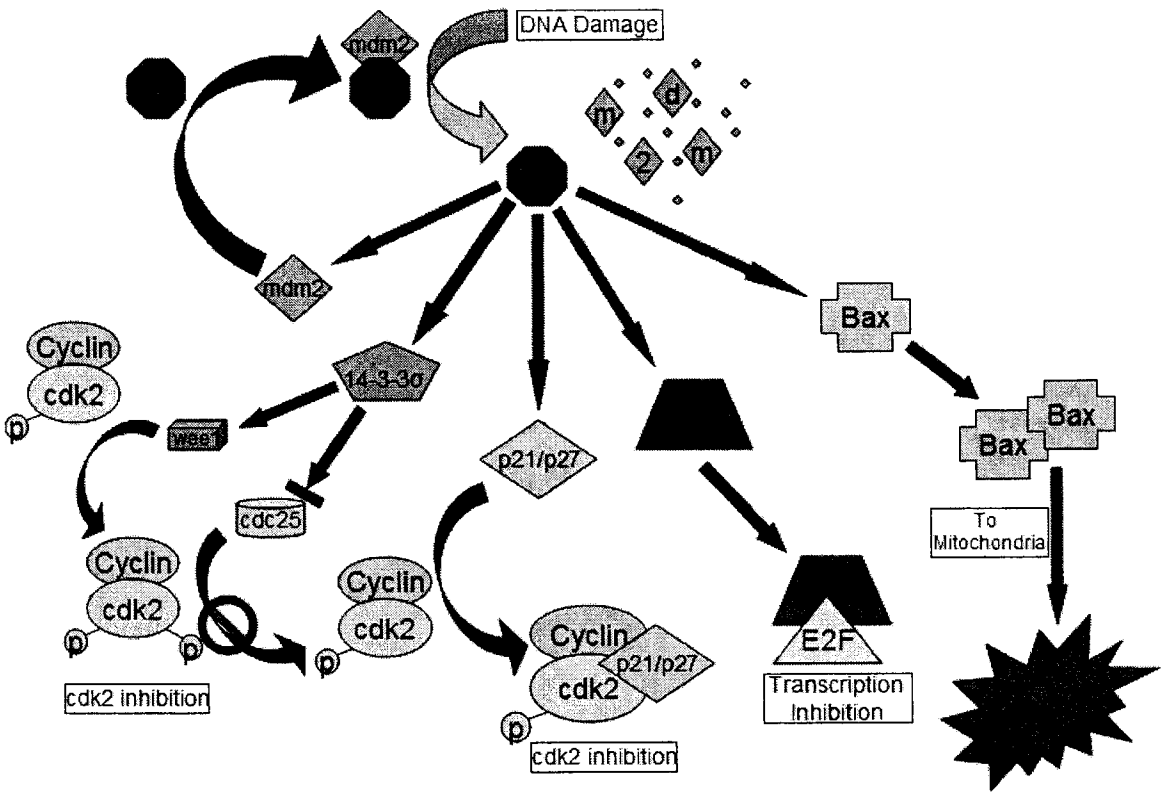

Figure 6 – p53 activity cascade.
Activation of the transcription factor p53 is caused by DNA damage, which initiates mdm2 degradation. Activation of p53 leads to a variety of cell cycle arrest pathways, transcriptional inhibition and even apoptosis. The KIP/CIP family of ckis are upregulated in an attempt to cease activation of DNA replicating genes and facilitate inhibition of cdk function. The E2F family of transcription factors are inhibited by the newly formed functional pRb. Apoptosis is initiated through the upregulation of proapoptotic factors such as Bax.

1.5 Development of cancer

The manifestation of a tumorigenic cell can be described using the analogy of an out of control automobile. Loss of cell cycle regulation, combined with the inability to promote apoptosis is similar to a car without brakes that has also lost the ability to steer, so not only is the car unable to stop but it cannot steer to avoid injuring surrounding motorists. Such is the case with a tumorigenic cell. The cell has lost the ability to

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maintain control of cellular proliferation as well as the capacity to avoid overall damage to the host organism by inducing apoptosis.

A potential tumor begins with the loss of cell cycle regulatory protein function within a single cell. An exceptionally large number of stimuli are able to initiate cancer growth, from the foods we eat, to the molecules in the air we breathe, to the sunlight that shines on our faces. If the stimulus has the ability to damage DNA, it has the potential to mutate key cell growth regulatory genes such as p53, $p21^{WAF1/CIP1}$ or pRb and thus initiate the first step in promoting unchecked cell proliferation. The p53 protein was one of the first tumor suppressors identified, and has been referred to as the "Cellular Gatekeeper" and "Guardian of the Genome" (42). The majority of human cancers are found to be p53-deficient and it is the mutations to this crucial safeguard protein, or induced irregularities that enhance degradation of p53 that can prove to be consequential to the host cell as it continues to proliferate without its safety net. In the majority of instances, cells are successful in arresting growth and repairing damaged DNA, but only one unsuccessful repair of a critical gene is required to initiate the process of tumorigenesis. This can then lead to other mutations within the cell that promotes proliferation, eventually resulting in a mature cancer. If DNA cannot be repaired, the cell commits apoptosis in order to protect the host organism (42). Loss of p53 function can have grave repercussions to an organism due to the inability of a cell to effectively initiate apoptosis if the need arises. Once these mutations are established and goes unrepaired, the cell is able to proliferate and form a tumor. Other mutations during this growth can occur due to loss of fidelity in DNA replication, truncated or superfluous genome duplication. Any cell in the body, which is capable of replicating, is capable of

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becoming a tumor as long as the unrepaired mutations prevent cell cycle arrest and apoptosis.

The novel unchecked cell now quickly divides and forms a tumor in the case of tissues, as opposed to hematopoietic cells that float freely in the blood or collect in the bone marrow. The cells within the tumor are also subjected to various mutational stimuli by outside sources and by internal errors caused by incorrect DNA duplication. Once again, a lot of these mutations are effectively corrected, although to a lesser extent than in cells with growth arresting capabilities. The tumor will continue to grow until other mutations occur that enables cells to degrade the basement membrane which acts as a barrier to its growth (43). One such mutation includes an increased ability to secrete matrix metalloproteinases (MMPs). Metastasis is the newfound ability of tumor cells to spread throughout the body. During metastasis, cells will become lodged in capillaries in various parts of the body. Once the cells adhere to their new location, they begin to break down the basement membrane of the surrounding tissue and initiate invasion of that tissue.

There are many different types of cancers. The cell type dictates what type of cancer will result. Hematopoietic cells lead to development of lymphomas and leukemia, while epithelial cells result in carcinomas and sarcomas. Nerve cells and other terminally differentiated cells (meaning they no longer proliferate) rarely become cancerous, although they can undergo dysfunctional apoptotic pathways that result in an increase in apoptosis. Neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and dementia with Lewy bodies are a result of increased caspase-mediated apoptosis in

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nerve cells (44). The number of diseases of this nature is small, relative to the number of cases of cancer found each year.

Although cancers arise as a result of mutations in genes that lead to the loss of regulation of cell growth, these mutations have to occur in particular parts of a large genomic sequence (45). Most mutations in the body are inconsequential to the organism since about 90% of the human genome does not have any known functions and other mutations are silent. It should also be emphasized that mutations are a normal process of nature and are essential for human evolution when coupled with natural selection.

2. Chemotherapy

2.1 Development of modern chemotherapeutics

Chemotherapeutics have been used for thousands of years in an effort to inhibit the spread of cancer. All of the earlier agents were plant-derived, as are a substantial number of modern medicines. Certain types of ginseng (*Alpinia officinarum*) and red clover (*Trifolium pratense*) were among the earliest plants used to treat various types of cancers (46). It was during the 1960's that nitrogen mustards, derivatives of the active agent of mustard gas used in WWI, became the first modern day cancer therapeutics due to their gene expression inhibition properties (47). Many of today's chemotherapeutics, such as taxol and etoposide are semisynthetic agents isolated from plant products. Some plants use poisons to deter animals and insects from eating them and it is these agents that scientists isolate and use to poison cancer cells. Modifications to these drugs are made to make them more soluble, less readily metabolized or adopt other features that increase their efficacy to cancers.

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2.2 Flavopiridol

2.2.1 History of Flavopiridol

In the early-mid 1980s, a group of chemicals known as flavonoids was investigated for their kinase inhibitory activity. The bioflavonoid quercetin was studied by Graziani *et al* in 1983 to determine the mechanism of its kinase inhibition (48). In an effort to treat rheumatic diseases, Naik *et al* of the Centre for Basic Research of Hoechst India Limited attempted to isolate natural compounds from *Dysoxylum binectariferum*, a plant indigenous to India (49). The compound being analyzed was an alkaloid called (+)-*cis*-5,7-dihydroxy-2-methyl-8-[4-(3-hydroxy-1-methyl)-piperidinyl]-4H-1-benzopyran-4-one that was isolated from the stem bark of *Dysoxylum binectariferum* via methanol extraction and was discovered to make up 0.9% of the dry weight of the plant (6;49). Upon isolation and analysis of the extraction product, it was found that it is structurally related to Rohitukine, an alkaloid found in another species, *Amoora rohituka* (Figure 7) (6;49). Rohitukine and a variety of related compounds were screened for epidermal growth factor receptor (EGFR) kinase and protein kinase A (PKA) inhibition as well as cytotoxicity in certain tumor cell lines by Sedlacek *et al* of Hoechst Marion Roussel (HMR) in Germany in 1989 (6;50;51). As a result of these trials, a compound was discovered that had high cytotoxicity in relation to its low IC_{50} on xenografted human tumors onto nu/nu mice (6). This compound was (-)-*cis*-2-(2-chlorophenyl-5,7-dihydroxy-8-[4-(3-hydroxy-1-methyl)-piperidinyl]-4H-1-benzopyran-4-one hydrochloride (flavopiridol, L86 8275, HL275, NSC 649890) and was targeted for further testing (Figure 7)

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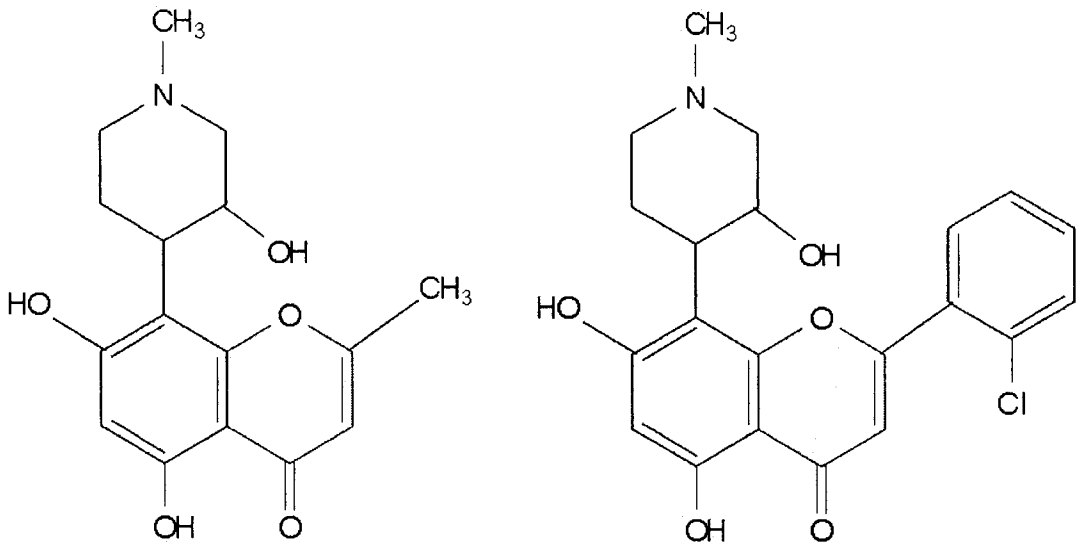

Rohitukine Flavopiridol

Figure 7 – The structure of chemotherapeutic agents rohitukine and flavopiridol. Large similarities exist between the two chemicals as flavopiridol is a derivative of rohitukine. A methyl group of rohitukine is replaced by the chlorinated benzene ring of flavopiridiol.

Testing involved its kinase inhibitory effects compared to other flavonoids (quercetin and genistein) as carried out by Kaur and his associates at the National Cancer Institute (NCI) in Bethesda, Maryland in collaboration with HMR in Germany and Hoescht India Limited in 1992 (52). The results showed that flavopiridol inhibits cell growth of the breast carcinoma cell line MDA-MB-468 by a factor of 60 and 400 times more than quercetin and genistein respectively, as measured via the colorimetric MTT cell viability assay (6;52). These results were verified by Czech *et al* in 1995 (53). In 1993 and 1994, Worland and Losiewicz, respectively, measured flavopiridol's effects on cdk1 activity in MDA-MB-468 cells. They found that flavopiridol's inhibition of this chief cdk was more than 250 times more effective than quercetin or genistein and interferes with the activating phosphorylation of cdk1 (54;55). These experiments stimulated interest in flavopiridol's cell cycle influence. Further work over the past

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decade has attempted to unravel the details of the mechanisms of action of flavopiridol. These effects range from binding to DNA (56), various cdks (8;15;57-61), cyclin regulation (15;62;63) and apoptotic regulatory protein expression (7;15;64-69).

2.2.2 Structure of Flavopiridol

Flavopiridol directly competes with ATP for its binding site of the cdks (6;58;62;70-73). In its natural state, cdk2 possesses a structure known as the t-loop that physically impedes ATP binding to its catalytic cleft (74). Conformational changes in response to cyclin binding causes the t-loop to move away from the catalytic cleft and the phosphorylated tyrosine 15 residue moves to take its place and block access to the ATP-binding site. The phosphatase cdc25 then dephosphorylates this residue, exposing a site suitable for ATP binding (74). Binding of cyclin to cdk2 also causes the threonine 160 residue to extend out, presumably for easier access by cak (74).

Sedlacek's and his associates' screening of a number of derivatives of Rohitukine resulted in the consensus that flavopiridol is the most active in inducing cytotoxicity and inhibiting protein kinases of murine and human tumor cell lines (6). Other derivatives with slightly altered chemical structures show nearly the same activity. A substitution of the chlorophenyl group with an ethyl or propyl group displays a 36-fold decrease in kinase inhibition (58). Removal of the chlorine from the phenyl group (compound L868276), results in a 10-fold decrease in potency (6). Also, it is the (-)cis isomer that proves to be the most potent enantiomer of flavopiridol (6).

To determine how flavopiridol binds to cdk2, de Azevedo Jr. *et al* (1996) at the University of California, Berkeley, determined the x-ray structure of its deschlorophenyl

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derivative L868276 in complex with cdk2 and compared it to the binding of ATP to cdk2 as determined by De Bondt *et al* (58;75). The results concluded that L868276 orients itself to cdk2 in the catalytic cleft in a manner similar to ATP binding (58). The binding of L868276 to cdk2 consists of hydrophobic interactions, hydrogen bonding and van der Waals forces and the average difference in distance between the cdk2/ATP complex's C^{α} atoms in comparison to cdk2/L868276's C^{α} atoms is 0.46Å (not including the most highly flexible regions, residues 36-47 and 150-164) (58). The benzopyran ring of L868276 was found to occupy essentially the same space in the catalytic cleft as the purine ring of ATP, although there is a slight rotational difference in the same plane (60°) (58). De Azevedo Jr. *et al* discovered that L868276 possesses 56 contact points with cdk2 compared to ATP's 79 contacts although L868276 makes more hydrophobic and van der Waals contacts with its benzopyran ring (34 contacts) than ATP does with its adenine ring (26 contacts) (58). L868276 displays 5 of the hydrogen bonds identified in the ATP interaction with cdk2 (58). Seven van der Waals interactions were between the piperidinyl ring of L868276 and cdk2 in addition to the 10 van der Waals contacts with the phenyl ring (58).

It is these 10 van der Waals contacts by the phenyl ring that are hypothesized to make L868276 (and thus flavopiridol) specific to cdks, as these interactions take place just outside of the ATP binding pocket and although these residues are conserved across cdks, they are not found in other kinases such as PKA (58). These contacts outside the pocket are due to the 60° rotation of the inhibitory molecule (58). The difference between L868276 and L868275 (flavopiridol) lies in the chlorinated phenyl ring of flavopiridol, which increases the inhibition of cdks by a factor of 6-10 (15;58). This is

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assumed to be due to the additional contacts made by the chlorine atom (58). These additional contacts made outside the ATP binding pocket may increase flavopiridol's specificity to cdks compared to L868276 (Figures 8,9).

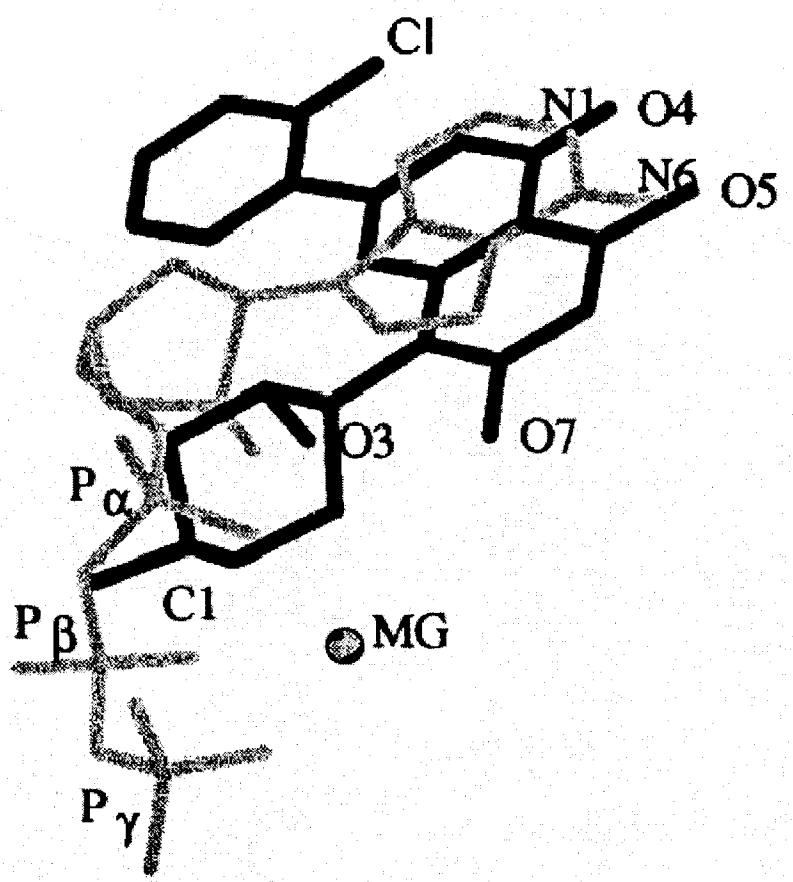

Figure 8 – Flavopiridol's structural similarity to ATP.
Flavopiridol is outlined in black. ATP is outlined in grey. Flavopiridol and ATP posses similar ring structures with a positional rotation of 60° (58).

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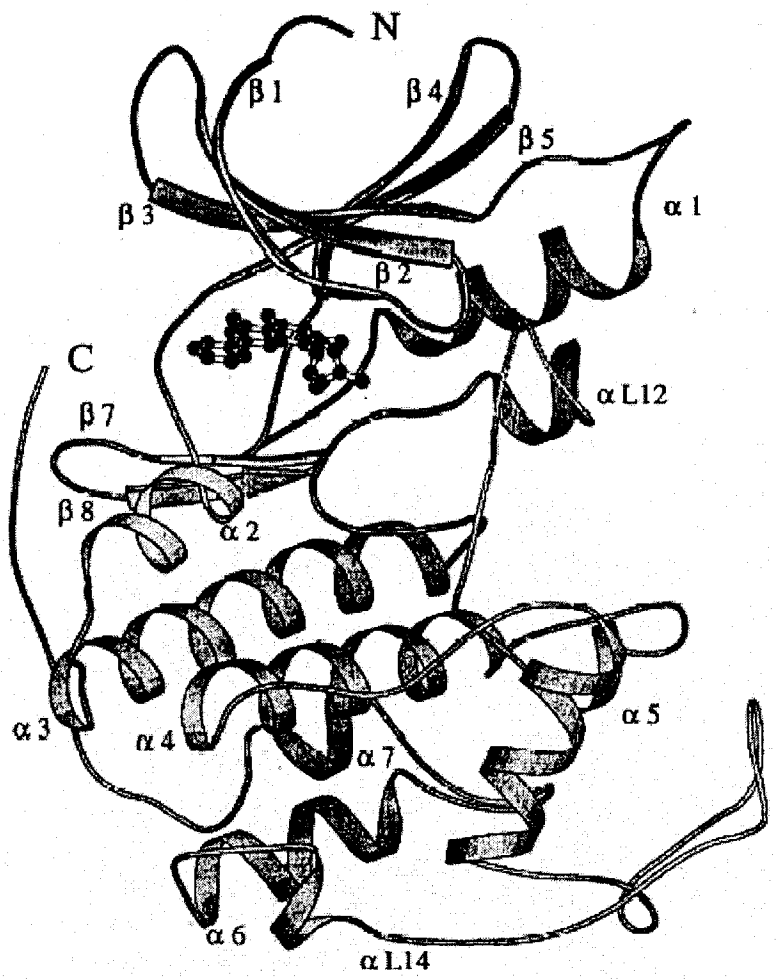

Figure 9 - Crystallography of cdk2 binding by flavopiridol.
Flavopiridol takes the position inside the ATP binding pocket of cdk2, preventing cdk2 from carrying out its kinase activities (58).

2.2.3 Action of Flavopiridol in vitro

Flavopiridol's effects on cell cycle components have been extensively studied over the past decade and a half. Initially identified as an inhibitor of the protein kinases EGFR and PKA, many other functions have since been revealed, most notably its propensity to inhibit the critical cell cycle protein family of cdks. The cytotoxicity of

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flavopiridol has also been an early distinction of this chemotherapeutic drug. Since then, flavopiridol has been identified as affecting cyclin regulation, Bcl-2 family proteins, DNA, transcription regulation and a variety of other kinases.

The 1990s - One of the initial experiments performed on flavopiridol was by Kaur *et al* of the NCI. From their experiments they concluded that flavopiridol reversibly inhibits cell growth at multiple stages of the cell cycle. Flavopiridol's cdk inhibition was found to be far greater than that of fellow cdk inhibitors, quercetin or genistein (52). Kaur's associate at the NCI, Worland *et al*, published an article a year later in 1993 on the effect of flavopiridol on the phosphorylation of cdk1. They theorized that flavopiridol interferes with the phosphorylation of cdk1 and thus inhibits its activity and subsequently arrests cellular activity in G_2 (55).

The following year (1994), another member of the NCI team in Bethesda, Losiewicz published a paper with other main contributors on the direct effect of flavopiridol on cdk1. The main contribution of this article was the observation that flavopiridol directly competes with ATP for cdk1 binding at the concentration range of 100-400nM (54). The NCI soon after began phase I clinical trials, headed up by Senderowicz.

Meanwhile in Germany in 1995, the associates of the original founders of flavopiridol, Czech and Sedlacek of the Research Laboratories of Behringwerke AG were continued their studies and published a report detailing the effects of flavopiridol on tumor growth, EGFR tyrosine kinase and PKA in various xenografted tumors (53). Flavopiridol was found to be a potent inhibitor of both EGFR tyrosine kinase and PKA in the micromolar range, while displaying a high antiproliferative effect *in vivo*.

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Xenografted tumors of the lung, colon, breast and ovary were xenotransplanted onto nu/nu CD1 mice and results showed that the growth of these tumors was significantly inhibited by flavopiridol treatment.

In 1996, de Azevedo Jr. *et al* illustrated the precise orientation of flavopiridol binding to cdk2 via x-ray crystallography and discovered that it is very similar to ATP binding (58). This same year saw Bible and Kaufmann of the Mayo Medical School in Minnesota describe flavopiridol's ability to induce apoptosis in noncycling cells (76). They also suggested that RNA and protein synthesis is the main causes of its cytotoxicity (76). Back at the NCI, Carlson *et al* were studying the G_1 arresting abilities of flavopiridol. They observed the inhibition of cdk4 and cdk2 as well as a decrease in Rb phosphorylation prior to G_1 arrest (57). They also noted the significant decrease in cyclin D1 levels, the gradual decrease in cyclin E and A and the lack of change in cdk2 or cdk4 levels in MCF7 and MDA-MB-468 breast carcinoma cells in response to flavopiridol (57).

König *et al* of the Memorial Sloan-Kettering Cancer Center (MS-KCC) in New York City were the first researchers to suggest that flavopiridol contributes to downregulation in protein and mRNA levels of the antiapoptotic protein Bcl-2 in chronic B-cell leukemia cells (B-CLL) (67). The level of Bcl-2 protein in B-CLL cells is typically relatively high (67). In contrast Byrd *et al* of the Walter Reed Army Medical Center in Washington DC and Parker at the NCI found that flavopiridol induces caspase-mediated apoptosis in chronic lymphocytic leukemia cells without affecting Bcl-2 levels (65;72). Byrd *et al* also determined that apoptosis is induced independently of p53 (65).

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More research out of the NCI showed a decline in cyclin D1, Rb and p107 in esophageal cancer cells treated with flavopiridol (63).

University of Chicago-based Chien *et al* determined the effects of flavopiridol on urothelial cell lines with defective p53, Rb and p16. He found that after 24 hours of treatment, all cells arrested at the G_2/M phase transition (77). Motwani *et al* of the MS-KCC demonstrated a synergistic effect of flavopiridol when co-administered with paclitaxel, a drug that induces mitotic arrest, with respect to induction of apoptosis (59;78). These results also suggested that these synergistic effects only occur if flavopiridol is utilised for treatment after paclitaxel, to treat the cells and not given for treatment before paclitaxel (59;78). An interesting observation by Schnier *et al* of the University of California, Davis, showed that flavopiridol bound well to aldehyde dehydrogenase class 1 (ALDH-1) molecules and thus cells with high ALDH-1 may show increased resistance to flavopiridol cytotoxicity (79). Verification of Carlson's observation that flavopiridol decreases the amount of cyclin D1 in MCF7 cells was made in 1999. He supplemented this data with observations that cyclin D1 protein and mRNA levels decrease within 6 hours of initial treatment (using 300nM flavopiridol) followed by a decline in cyclin D3 levels but no change in cyclin D2 levels (62). Shapiro *et al* of the Dana-Farber Cancer Institute at Harvard Medical School found that flavopiridol causes an increase in p53 protein levels in A549 cells although the apoptosis that is induced, is p53-independent, as A549 cells possess mutated p53 (80).

2000 - A molecule implicated in the metastasis of breast cancer is *c-erb*B-2, has been found to be significantly downregulated in flavopiridol-treated breast cancer cell lines by Li *et al* of the Wayne State University School of Medicine in Detroit (81). It was

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also found that flavopiridol modestly reduces the levels of Bcl-2 and increases the levels of Bax in the same cells (81). Of note in this paper, it was suggested that flavopiridol inhibits the secretion of MMPs, subsequent metastatic invasion and therefore may inhibit metastasis of breast cancer cells (81). Later in 2000, Li *et al* demonstrated the downregulation of Bcl-2 in prostate cancer cells by flavopiridol and it was suggested that this may be the mechanism for flavopiridol-induced apoptosis (82). However, Achenbach *et al* of the Institute of Molecular Biology and Tumor Research in Marburg, Germany showed that apoptosis is independent of Bcl-2 downregulation. Their paper proposed that there are multiple flavopiridol-induced mechanisms to promote caspase-mediated apoptosis through caspase 8 activation, and that this apoptotic pathway is largely independent of Bcl-2 presence in human lung carcinoma cells (64). Kitada *et al* of the M.D. Anderson Cancer Center in Houston discussed the effects of flavopiridol on B-CLL samples. They found that flavopiridol decreases levels of antiapoptotic factors Mcl-1 and XIAP in nearly all samples and Bcl-2 in approximately half of the 49 B-CLL samples analyzed (66). Flavopiridol's cdk inhibiting effects were being explored in neurodegenerative research. Its cdk inhibiting properties were being tested in murine nerve cells for the prevention of apoptosis in stroke victims, thereby reducing the damage done during the stroke (83;84). They proposed that overactive cdk kinases are at least partially responsible for neuronal cell death during a stroke and this damage was reduced upon treatment with flavopiridol 24hr after reperfusion (resumption of blood flow). Motwani *et al* demonstrated the formation of chromosome abnormalities as the result of flavopiridol treatment of MDA-MB-468 cells treated with the microtubule inhibitor paclitaxel. Their results illustrated a preferential initiation of apoptosis in these polyploid

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cells as a result of flavopiridol treatment and also prevents further reduplication of cellular DNA (59). It was proposed by Bible *et al* that flavopiridol's cytotoxicity in noncycling cells is due to its ability to bind to DNA in A549 human lung cancer cells (56).

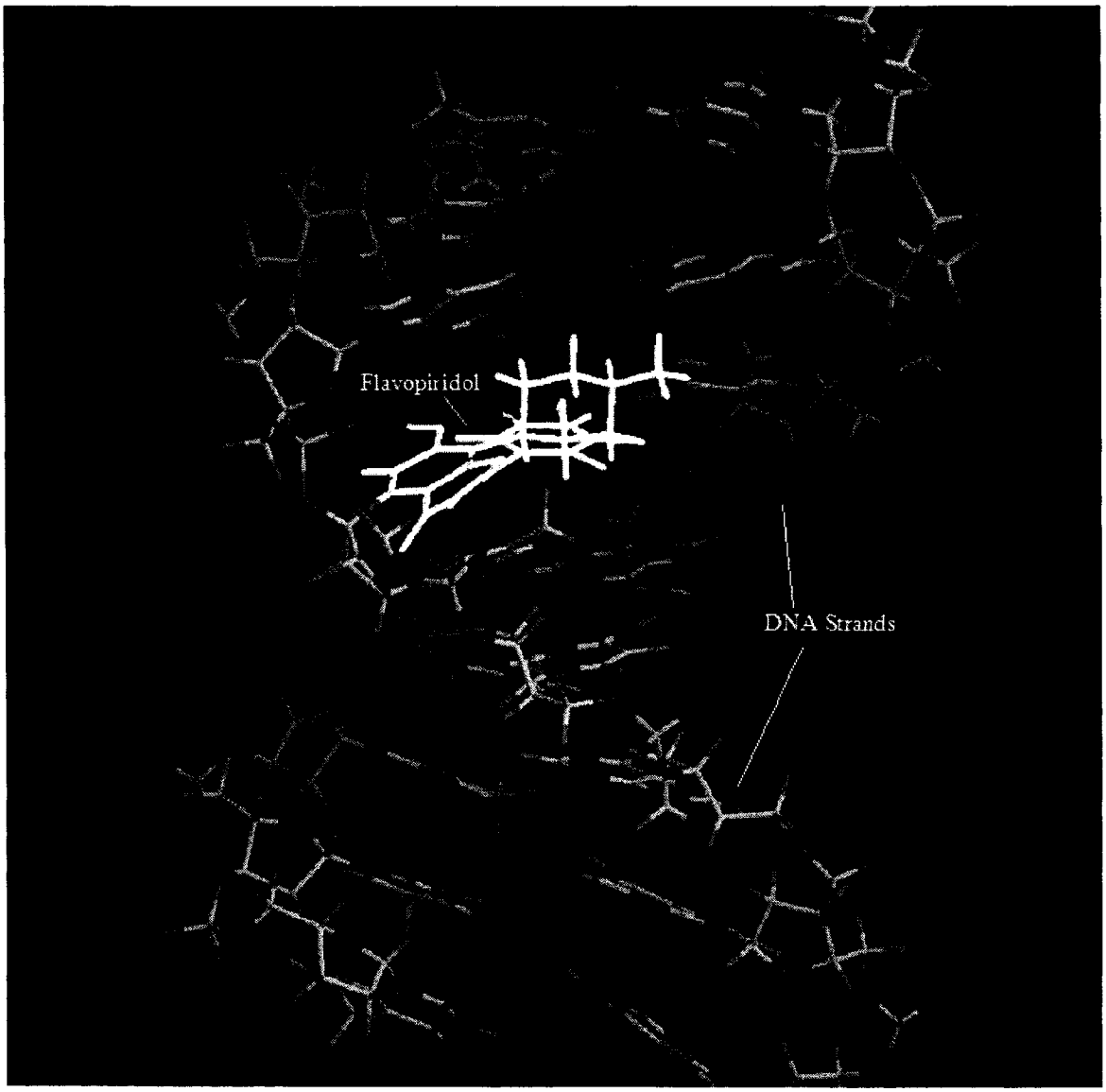

Figure 10 - Flavopiridol binding to DNA - Expanded view.
Flavopiridol takes up residence in the minor groove of double stranded DNA (56).

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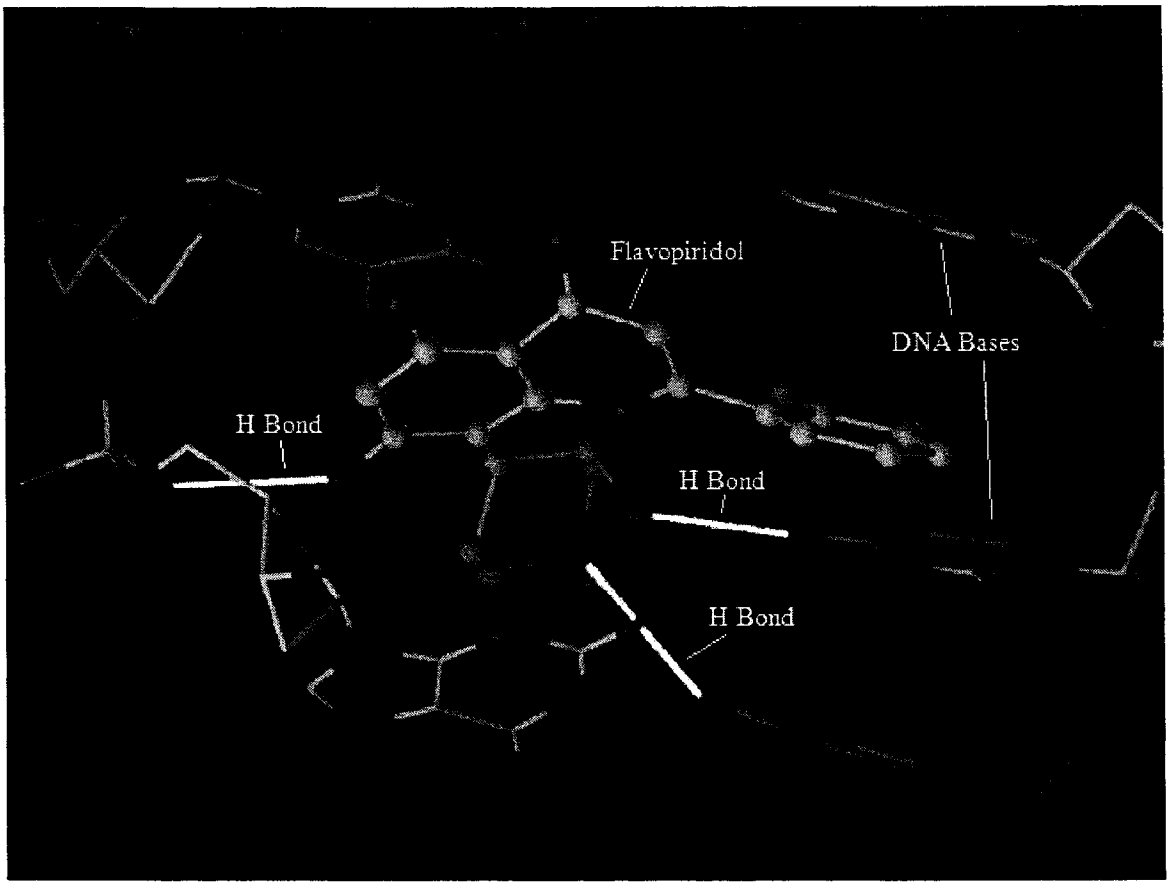

Figure 11 - Flavopiridol binding of DNA - Hydrogen bonding.
A closer view of flavopiridol forming hydrogen bonds with DNA bases (56).

2001 - Using DNA microarrays, Lam and associates from the NCI in 2001 found that flavopiridol inhibits the expression of a broad range of genes, similar to those obtained with some transcription inhibitors (85). This was verified by the work of Price at the University of Iowa, who found that 3nM of flavopiridol inhibits the activity of P-TEFb. This level of flavopiridol is a lower concentration than is necessary to inhibit most other cdks, other than cdk9/cyclin T and implies some role for flavopiridol in transcription inhibition (86). A link between transcriptional inhibition and HIV proliferation was made as well. The HIV protein Tat requires P-TEFb to increase the activity of RNA pol II in order for the virus to transcribe its viral mRNAs (86). Li *et al* of the MS-KCC in New York City observed in an experiment with the Rb deficient cell

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line Saos2 that flavopiridol (100nM) induces expression of $p21^{WAF1/CIP1}$ and inhibition of cdk2, but was not observed in Rb-restored Saos2 transformed cell lines (70). They found that the levels of Bcl-X_L, Bax, cyclin D1, cyclin A, and cdk2 are not affected by flavopiridol in either Rb-deficient or -restored cell lines (70). Li *et al* also demonstrated the synergistic effect of flavopiridol and doxorubicin (70). Pepper of Llandough Hospital in Birmingham in the UK illustrated the effects of flavopiridol on some Bcl-2 family proteins in B-CLL. Pepper *et al* observed that flavopiridol (5-100nM) decreased the expression of Bcl-2 and Mcl-1 while there was no change in Bax expression (68).

2002 - In 2002, Matranga and Shapiro of the Harvard Medical School analyzed the enhanced flavopiridol-induced apoptosis of NCI-H661 non-small cell lung cancer cells arrested in early S phase by hydroxyurea (71). In addition, they illustrated the synergistic cytotoxic effects of pretreatment with gemcitabine or cisplatin (71). Gojo *et al* of the University of Maryland demonstrated that flavopiridol decreased the levels of Mcl-1 protein and mRNA and increased inhibition of RNA polymerase phosphorylation (87). They also showed that Mcl-1 played an important role in flavopiridol-induced apoptosis of multiple myeloma cells (87). During this time, caspase-mediated apoptosis of human leukemia cells via the synergistic effects of treatment with flavopiridol and the histone deacetylase inhibitor, suberoylanilide hydroxamic acid were researched by Almenara *et al* of the Virginia Commonwealth University in Richmond (69). Blagosklonny, at the Brander Cancer Research Institute at the New York Medical College discovered an interesting paradox regarding an increase in p53 accompanied by a decrease in $p21^{WAF1/CIP1}$ upon flavopiridol treatment (88). It was this downregulation that

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he purported was the reason for flavopiridol's protection of p21-sensitive cells from paclitaxel (88).

2003 - In January 2003, Ma *et al* of the H. Lee Moffitt Cancer Center and Research Institute in Tampa, Florida detailed the apoptotic effects of flavopiridol on H1299 lung carcinoma cells with respect to E2F1 and Mcl-1 levels. They discovered that in these cells, apoptosis was mainly, but not entirely, reliant on an increase in E2F1 and a decrease in Mcl-1 proteins observed after treatment with 200nM flavopiridol for 12 hours (7). Pepper *et al* illustrated their results demonstrating a relationship between the apoptotic cell death of B-CLL and activation of p38 MAP kinase and partial inhibition of ERK activity (89). Litz *et al*, colleagues of Almenara, at the Virginia Commonwealth University, found flavopiridol induces mitosis preferentially in small cell lung cancer cells that are pretreated with aphidicolin and consequently these cells were arrested in G_1/S transition (90). They also suggested a connection between flavopiridol treatment and mitochondrial dysfunction (90). Jiang *et al* of the Harvard Medical School echoed the observation that flavopiridol preferentially caused apoptosis in cells during S phase (18). They suggested that this was a direct result of flavopiridol's inhibition of cdk2/cyclin A and the resultant inability to phosphorylate E2F1 in an inhibitory manner in late S phase (18). Without a downregulation of E2F1 activity, Mcl-1 transcription continued to be inhibited thus decreasing the overall concentration of antiapoptotic proteins (18).

2004 - Entering the year 2004, researchers had begun to broaden the scope of research examined with flavopiridol. Due to flavopiridol's apparent downregulation of antiapoptotic proteins such as Mcl-1, Bcl-2, cyclin D1 and vascular endothelial growth

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factor (VEGF), Takada and Aggarwal of The University of Texas in Houston examined flavopiridol's effect on NF-κB, a nuclear transcription factor that regulates these proteins (91). They found that flavopiridol suppressed the ability of tumor necrosis factor (TNF) to activate NF-κB in under 6 hours at a 100nM concentration (91). They suggested it was this effect that contributed to flavopiridol's anti-inflammatory effect and its ability to alter the immune response and affect cellular growth (91). Another colleague of Almenara at the Virginia Commonwealth University, Rosato, illustrated the effects of flavopiridol in conjunction with sodium butyrate. Rosato *et al* reported that flavopiridol countered the upregulation of $p21^{WAF1/CIP1}$ by sodium butyrate (92). In 2004, they published a paper further examining this phenomenon. This effect was demonstrated in Jurkat cells and it was suggested that this interference of $p21^{WAF1/CIP1}$ upregulation led to mitochondrial damage and caspase-mediated apoptosis (93). Sato *et al* from the Juntendo University School of Medicine in Tokyo, Japan provided data supporting the use of flavopiridol, in low doses (<1nM), as a radiation sensitizer and showed a modest decrease in Bcl-2 levels in an esophageal squamous cell carcinoma cell line (KE4) at the surprisingly low concentration of 50pM (94).

Recent Developments - In the most recent months, the emphasis of study on flavopiridol has shifted to transcription inhibition. Because so many cell cycle proteins and mRNAs have been found to be downregulated upon flavopiridol treatment, a closer look at its effect on transcription factors was being elucidated. Blagosklonny, who had previously observed the unexpected inverse regulation of p53 and $p21^{WAF1/CIP1}$ with flavopiridol treatment, attributed it to a global transcriptional inhibition (28). In a review article published in December of 2004, he proposed that the potency of flavopiridol's

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transcriptional inhibition was a major component of its influence on cellular processes. It is through Mdm2 transcription inhibition that p53 was activated, which in turn, upregulated $p21^{WAF1/CIP1}$. However at 200-400nM flavopiridol, $p21^{WAF1/CIP1}$ transcription was inhibited as well, causing the contrasting results in protein levels (28). At lower doses (50nM-100nM), $p21^{WAF1/CIP1}$ was upregulated (95), which may have been due to p53 activation having a stronger influence than the inhibition of $p21^{WAF1/CIP1}$ transcription by flavopiridol (28;95).

Other fields have also found uses for flavopiridol. Virologists have suggested that flavopiridol may play a role in viral replication inhibition due to some viruses' reliance on cdk activity (96-98). Flavopiridol is being researched as a plausible treatment for viruses like HIV-1, HSV-1, adenoviruses, papillomaviruses and any other virus that rely on cdks or cellular division (97). In a recent publication (2005), flavopiridol was been implicated in a possible treatment for Alzheimer's and Parkinson's diseases, and amyotrophic lateral sclerosis due to the diseases' reliance on cdk activity resulting in nerve cell apoptosis (99). Even though the original and still centrally intended application of flavopiridol is cancer, its pleiotropic effects are being evaluated for treatment in other diseases.

2.2.4 Preclinical Trials

The majority of preclinical trials consist of treatments of xenografted tumors on murine animals and beagle dogs. Infusional and bolus treatments of flavopiridol showed promising results. Flavopiridol is effectively glucuronidated in the liver and efficiently excreted (100). The mean total body plasma clearance of murine animals is

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approximately 22.6 mL/min/kg (100). In beagle dogs that received 72 hour continuous infusion of flavopiridol, it was shown that the dose-limiting toxicity (DLT) is diarrhea with a maximally tolerated dose (MTD) of 26 mg/m^2/day (100). Pharmacokinetic studies on humans showed that the plasma serum level could be maintained at concentrations of 300-400nM, but its clearance can vary by up to 10-fold between individuals (101). Preclinical trials also demonstrated flavopiridol's efficacy in prostate cell xenografts (102).

2.2.5 Clinical Trials

The study of chemotherapeutic agents in clinical trials is imperative to the advancement of knowledge of a potential cancer therapy. A novel therapeutic agent is ready for clinical trials once it has demonstrated promise in combating tumor development and shows an acceptable toxicity in animals. The initial stage of clinical trials is phase I. These studies consist of a small number of patients being treated with the experimental drug to determine any side effects, to identify a safe dosage range and an effective delivery method. Phase II differs from phase I trials in that a larger and homogeneous group of patients is used to further evaluate side effects of the drug and to determine the safe dosing. Finally, phase III studies are then developed to verify the agent's effectiveness compared to other establised chemotherapeutic agents and to collect data from the larger group size to identify the proper doses to be used, as well as the avoidance, or treatments, of potential side effects.

Flavopiridol was introduced into phase I clinical trials in 1997 by the NCI. As expected, the DLT in humans who are given a 72 hour continuous infusion of

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flavopiridol is diarrhea (103;104). Chloride secretion induced by flavopiridol was observed to occur in epithelial cells *in vitro* and this was suggested to be the cause of the gastrointestinal side effect (100). It was determined using human liver microsomes that flavopiridol could be metabolized into glucuronides and that this metabolism was inversely proportional to the incidence of diarrhea, thus inferring that breakdown of flavopiridol in the liver prevents increased toxicity in the intestines (104). Antidiuretics (cholestyramine and loperamide) were given prophylactically to patients receiving infusional flavopiridol and the MTD increases from 62.5 to 78mg/m^2/day x 3 (103). Cholestyramine has been found to bind to flavopiridol, preventing toxic action in the intestines (103;104). This association was a possible explanation for the increased clearance of flavopiridol with use of diarrhetics (101). A wide range of clearance rates was found in patients treated with flavopiridol, from 50mL/min to 3000mL/min (101). The precise mechanism responsible for diarrhea may involve flavopiridol's interactions with various endogenous secretagogues (e.g. acetylcholine or bile acids) (105). Some other side effects which occurred with flavopiridol treatment were neutropenia (decrease in white blood cells), nausea, vomiting, hypotension (low blood pressure) and proinflammatory syndrome (fatigue, fever, and localized tumor pain) (100;106). The proinflammatory syndrome may be explained by the induction of the cytokine IL-6 by flavopiridol (100;107).

Plasma concentrations observed in patients treated with 50mg/m^2/day x3 or higher were in the 300-500nM range, which is well within the range of flavopiridol activity (100). Attempts to recover flavopiridol from human plasma within 48 hours occurred with a success rate of 85-87% (108).

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Phase II trials were begun in 1999 with similar results. One study involving metastatic gastric cancer showed that patients treated with 50 mg/m^2/day for 72 hours of continuous infusion every 2 weeks develop an unexpected high incidence of vascular thrombosis (blot clotting in blood vessels) and fatigue with little inhibitory action on the primary tumor (109). An additional study with metastatic hormone-refractory prostate cancer concluded that flavopiridol had little effect at the schedule of 60 mg/m^2/day in a 72 hour continuous infusion every 14 days (110). These disappointing results led to the attempts of combining flavopiridol with other chemotherapeutic agents during treatment. This combination therapy has proven to be fruitful, as synergistic effects between flavopiridol and paclitaxel and various signal transduction inhibitors were observed (111-113). Combined therapy with flavopiridol also has the potential advantage of overcoming resistance of some cell lines to flavopiridol (107;114-116).

2.3 Other chemotherapeutics used in these experiments

The two drugs used in these experiments to give contrasting effects to flavopiridol were staurosporine and sodium butyrate. Staurosporine and its analog UCN-01 (7 hydroxystaurosporine) are small molecule, general protein kinase inhibitors that act directly on protein kinase C (PKC) as well as cdks and other kinases (68;88;95;117). It has also been illustrated that staurosporine upregulates $p27^{KIP1}$, while UCN-01 upregulates $p21^{WAF1/CIP1}$ and $p27^{KIP1}$, which is necessary for cell cycle arrest in some tissue cultured cells (67). The upregulation of $p21^{WAF1/CIP1}$ in these cells is regulated by the mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK) pathway (67). Staurosporine was reported to selectively arrest normal cells at G_1

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(118;119). This arrest was not seen in transformed cells, indicating an alteration in a G_1 checkpoint. Early trials of staurosporine in cancer treatments were not successful when it was shown to depress blood pressure.

Sodium butyrate is a short chain fatty acid, histone deacetylase (HDAC) inhibitor, known to promote apoptosis and cellular differentiation (120). The precise mechanism of apoptosis initiated by butyrate treatment is yet unknown, but has been shown to alter the expression levels of apoptotic genes. Butyrate also upregulates $p21^{WAF1/CIP1}$ via p53 independent means (121). This is the mechanism in which we are most interested in as the mechanism of $p21^{WAF1/CIP1}$ upregulation has not been fully elucidated. Butyrate is a short-chain fatty acid that is produced in the colon as a natural fermentation product of a high-fibre diet and has been suggested to be protective against colorectal cancers (122-124). Variations of butyrate with a longer half-life in the body are being tested for its use as anticancer agent. There was one report that phenylbutyrate successfully treated a patient with leukemia that was resistant to conventional chemotherapy (125). Currently, several novel HDAC-inhibitors are undergoing clinical trials.

Inhibitors of cdk molecules have been found to be proficient at inducing cell cycle arrest due to the integral roles of their kinase activities in cell cycle checkpoint regulation and this is why researchers have attempted to isolate and modify cdk-inhibitory compounds in a search for novel cancer treatments. Scientists have found a promising target in cdks and hope to manufacture a drug which inhibits cells undergoing unregulated growth and induces apoptosis in tumor cells. Drugs that promote cell cycle arrest and apoptosis through inhibition of kinase molecules in general promise to be a good place to start enhancing our knowledge of what would make an ideal cancer

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therapeutic agent. A kinase-inhibiting drug which also induces apoptosis has been found in flavopiridol, but it also has many other observed effects. Currently, its various mechanisms of action continue to be extensively researched in an effort to determine how to effectively use flavopiridol and to utilize the information for future potential chemotherapeutic agents.

3. Summary

Flavopiridol is a novel cdk inhibitor currently participating in phase III clinical trials. Flavopiridol has been found to be an inhibitor of many kinases but preferentially inhibits cdks at lower concentrations due to the partial binding outside of the ATP binding pocket of cdks. Binding inside this catalytic cleft is remarkably similar to the hydrogen bonds and van der Waals forces exhibited by ATP binding. Cdk inhibition is mainly responsible for the caspase-mediated apoptosis and cell cycle arrest of cells treated with flavopiridol although it is noted that its broad activity makes this drug a broad-spectrum chemotherapeutic agent.

Flavopiridol has been observed to promote apoptosis in treated cells. This could be a result of a variety of pathways. The change in the ratio of expression of multiple proteins of the Bcl-2 family such as a decrease in anti-apoptotic proteins Mcl-1, Bcl-X_L and Bcl-2 and an increase in the pro-apoptotic protein Bax, may be one such pathway, thus promoting apoptosis. There has been some controversy regarding the apparent downregulation of Bcl-2 in cells treated with flavopiridol. There have been a number of studies that illustrate no change in Bcl-2 protein levels in some B-CLL samples while most transformed cell lines show a dramatic decrease in Bcl-2. Another

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antiapoptotic protein, XIAP, has been found to be downregulated in a number of cell lines. It has been suggested that apoptosis is a result of an extended activation period of E2F1 as a result of the inhibition of cdk2/cyclin A. DNA is also a target of flavopiridol and although no direct damage or topoisomerase upregulation has been observed. In contrast, it has been observed in recent studies that this pathway is not vital for flavopiridol-mediated apoptosis.

Transcription factors other than the E2F family have been demonstrated as a target for flavopiridol. The activation of NF-κB, the nuclear transcription factor that regulates Mcl-1, Bcl-2, cyclin D1 and VEGF has been found to be suppressed, so it is no surprise that a downregulation of these downstream proteins has been observed. Also, the inhibition of P-TEFb by flavopiridol has been shown to hinder the transcription process. A decrease in VEGF could be a reason for the antiangiogenic effect of flavopiridol.

Flavopiridol may also contribute to the prevention of metastatic activity. Flavopiridol has been shown to significantly decrease the expression of MMPs and *c-erb*B-2, which are proteins implicated in breast cancer metastasis.

The effects of flavopiridol have not been as promising after analysis as a single agent in clinical trials. It has been suggested that flavopiridol would be more beneficial as a supplementary chemotherapeutic agent, used as a treatment after another agent, specifically a signal transduction inhibitor or a G_1/S transition inhibitor. Achievable concentrations *in vivo* are more than adequate for the activity of flavopiridol and levels in plasma can be accurately measured above 10nM. The DLT has been found to be diarrhea

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but can be alleviated with antidiuretics. Other side effects include fatigue, neutropenia, nausea, hypotension and fever.

It is all these factors that make flavopiridol a promising drug for use as a chemotherapeutic agent. Further research is needed to elucidate additional mechanisms of action and to clarify mechanisms already discovered. The research community needs to determine the exact method of growth inhibition in mammalian cells as a result of flavopiridol and whether it is through cdk inhibition or an alternate pathway.

4. Objectives

Previous experiments in our lab showed primary cancer cells that had undergone cell cycle arrest with flavopiridol, did not demonstrate inhibition of cdk2 activity. Other labs showed flavopiridol induced apoptosis in noncycling cells. Flavopiridol also has been observed to upregulate $p21^{WAF1/CIP1}$. Flavopiridol clearly elicits effects other than cdk inhibition and so the exact mechanism of cell cycle arrest *in vitro* and *in vivo* remains unclear and led to this study. The purpose of this study is to determine if cdk2 inhibition in cultured cell lines via flavopiridol is through direct kinase inhibition, or through $p21^{WAF1/CIP1}$ inhibition at clinically achievable concentrations. Cdk2 is being studied due to the strong inhibitory effect of $p21^{WAF1/CIP1}$ on this particular kinase. Presence of $p21^{WAF1/CIP1}$ bound to the cdk2/cyclin complex was analyzed to determine if this could be the means of cdk2 inhibition in the cultured cells. The level of $p21^{WAF1/CIP1}$ was then compared to the level of cdk2 activity to determine if there may be a link between the two upon treatment with flavopiridol. Cdk2 activity was examined in cells versus immunoprecipitated cdk2, both treated with flavopiridol. By contrasting

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these kinase activities, the role of $p21^{WAF1/CIP1}$ in flavopiridol-mediated cdk2 inhibition in these tissue cultured cell lines was analyzed.

5. Hypothesis

Our hypothesis is that flavopiridol's primary mechanism of action to promote cell cycle arrest is not through direct inhibition of the cdks governing cell cycle progression but through other means, such as upregulation of the cdk inhibitor $p21^{WAF1/CIP1}$.

Materials & Methods

Buffers & Solutions

Various

ATV (10X) (1L)	80g NaCl 4.0g KCl 10g Glucose 5.8g $NaHCO_3$ 2g Na_2EDTA Fill up to 1L with Double Distilled Water (DDW) Autoclave
ATV (1X) (1L)	100mL ATV 10X 900mL DDW Autoclave
MTT (5mg/mL) (40mL)	0.2g MTT 40mL DDW Syringe Filter Store at 4°C and protected from light

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Nuclei Buffer (NB) (100mL)

8.6g Sucrose (0.25M)
1.16g NaCl (0.2M)
1mL 1M Tris-HCl pH 8.0 (10mM)
0.2mL 1M $MgCl_2$ (2mM)
0.1mL 1M $CaCl_2$ (1mM)
4mL 25% TritonX-100 (1%)
Fill up to 100mL with DDW
Store at 4°C

Phosphate Buffered Saline (PBS) (10X) pH 7.4 (1L)

80g NaCl
2g KCl
2g KH_2PO_4
11.5g $Na_2HPO_4 \cdot 2H_2O$
Fill up to 1L with DDW

PBS (1X) (1L)

100mL 10X PBS
900mL DDW

Kinase Buffer (1mL)

50mM Tris (pH 7.4)
2mM $MgCl_2$
1mM DTT (dithiothreitol)
100mM NaCl
0.05mM ATP
395µL DDW

Kinase Reaction Buffer (1mL)

50mM Tris (pH 7.4)
2mM $MgCl_2$
1mM DTT (dithiothreitol)
100mM NaCl
0.05mM ATP
100nM calyculin A
0.1µg/µL H1 histone
0.05µCi/µL of γ-^{32}P-ATP
360µL DDW

Propidium Iodide (PI) (50mL)

50mL 1X PBS
50µg/mL RNAse
50µL of 5mg/mL stock PI
Makes 5µg/mL solution
Keep at 4°C and protected from light

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Western Blotting

10% Ammonium Persulfate (APS) (1mL)
100mg Ammonium Persulfate
1mL DDW

Coomassie Blue Stain (1L) 0.3g Coomassie Blue
100mL Glacial Acetic Acid
500mL 95% Ethanol
400mL DDW
Stir for about 30 minutes and filter with filter paper

Coumeric Acid (90mM) (10mL)
0.15g Coumeric Acid
10mL Dimethylsulfoxide
Store at -80°C

Destain (1L) 100mL Glacial Acetic Acid
200mL 95% Ethanol
700mL DDW

Enhanced Chemiluminescence Solutions
Solution #1 (20mL) 2mL 1.0M pH 8.5 Tris-HCl
12μL 30% H_2O_2
Fill up to 20mL with DDW

Solution #2 (20mL) 2mL 1.0M pH 8.5 Tris-HCl
88μL 90mM Coumeric Acid
200μL 250mM Luminol
Fill up to 20mL with DDW

Luminol (3-aminophthalydrazide) (250mM) (10mL)
0.44g Luminol
1mL Dimethylsulfoxide
Store at -80°C

Running Buffer (10X) (1L) 144g Glycine
30.3g Tris Base
50mL 10% SDS
Fill up to 1L with DDW

Running Buffer (1X) (1L) 100mL 10X Running Buffer
900mL DDW

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Separating Gel (12% acrylamide) (~10mL)

4.35mL DDW
2.5mL 1.5M pH 8.8 Tris-HCl
3mL 40% Acrylamide
100µL 10% SDS
5µL TEMED
50µL 10% Ammonium Persulfate

Stacking Gel (4% acrylamide) (~5mL)

3.18mL DDW
1.26mL 0.5M pH 6.8 Tris-HCl
0.5mL 40% Acrylamide
50µL 10% SDS
5µL TEMED
25µL 10% Ammonium Persulfate

10% Sodium Dodecyl Sulfate (SDS) (1L)

100g Sodium Dodecyl Sulfate
Fill up to 1L with DDW

2X SDS Sample Buffer (50mL)

1.2mg Bromophenol Blue
8mL 1M Tris-HCl pH 6.8
5mL Glycerol
20mL 10% SDS
1mL β-mercaptoethanol
16mL DDW

1X SDS Sample Buffer (50mL)

25mL 2X SDS Sample Buffer
25mL DDW

Transfer Buffer (10X) (1L) 144g Glycine
30.3g Tris Base
Fill up to 1L with DDW
Store at 4°C

Transfer Buffer (1X) (1L) 100mL 10X Transfer Buffer
200mL Methanol
700mL DDW
Store at 4°C

Tris Buffered Saline (TBS) (10X) pH 7.5 (1L)

30.3g Tris Base
87.6g NaCl
Fill up to 1L with DDW

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1X Tris Buffered Saline with Tween 20 (TBST) (1L)
100mL 10X TBS
900mL DDW
1mL Polyoxyethylene sorbitan monolaurate (Tween 20)

Tris Buffered Saline with Tween 20 and 5% Milk (TBSTM) (40mL)
40mL 0.1% TBST
2.0g Condensed Milk

Tris Buffered Saline with Tween 20 and 8% Milk (TBSTM) (40mL)
40mL 0.1% TBST
3.2g Condensed Milk

Tris-HCl Buffers
1.5M pH 8.8
1.0M pH 8.5
0.5M pH 6.8
Mix Tris Base in DDW and pH with HCl

Cultured Cell Lines

Many malfunctions can occur in the machinery of the cell cycle and these aberrations can lead to tumorigenesis. Inhibition of p53 magnifies the consequences of abnormalities in cell cycle regulatory proteins such as the cyclins, cdks and their inhibitors. Mutations in p53 are very common in tumor development. This is apparent in *in vivo* tumors as well as the tissue cultured cell lines used in research laboratories. Although *in vitro* cells do not display the exact characteristics as *in vivo*, analyses of these cell lines are the most convenient way to determine cell cycle processes in abnormal cells as well as assessing methods of treatment with various chemotherapeutic agents. In these experiments, we use a variety of cell lines with different cell cycle protein deficiencies.

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The MCF7 cell line is an adenocarcinoma isolated from the breast of a 69yr old Caucasian female. MCF7 cells have a doubling time of 29hrs as listed by the American Type Culture Collection (ATCC). MCF7 cells are known to have functional p53, $p21^{WAF1/CIP1}$, Bcl-2, Bax, XIAP cyclin E, cdk2 and pRb (57;126-128). They also possess a hypertriploid to hypotetraploid genome with approximately 82 chromosomes.

HeLa cells were derived from an aggressive adenocarcinoma of the cervix isolated from a 31yr old black woman more than 50yrs ago. This cell line was the first continuous cancer cell line available for research. HeLa cells contain functional $p21^{WAF1/CIP1}$, cyclin A, cyclin E, pRb and p53, however an overexpressed E6 protein causes rapid inhibition and degradation of p53, allowing damage to DNA to go unchecked (129).

The metastatic prostate carcinoma cell line, DU145, was isolated from the brain metastasis of a 69yr old Caucasian male. This hypotriploid (almost 3 full sets of chromosomes) cell line has wild-type $p21^{WAF1/CIP1}$, XIAP and E2F1. The tumor suppressor gene p53 is functional but suppressed by overexpression of mdm2 (130).

The Saos2 osteosarcoma cell line was isolated from an 11yr old Caucasian female. These cells are also hypotriploid, and has over 2/3 of chromosomes structurally rearranged. Saos2 cells contain no viable p53 gene and also have a defective pRb (17;129).

Normal human skin fibroblasts (HSF55) were used as a control cell line. These cells were isolated from the foreskin of a fetus and have a limited life span after the onset of senescence. As the cells progress during this life span, a retardation of growth rate begins at higher passage numbers (number of times in which the cells pass through the

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cell cycle) which is a sign of the initiation of senescence. These cells possess normal cell cycle protein expression and function. These cells also possess the distinct characteristic of contact inhibition, which most immortal cell lines do not possess. The cancer cell lines in this study do not possess this trait. Normal fibroblast cells receive signalling stimuli to promote growth arrest upon sensing physical contact from neighboring cells. Cancer cells often lack this inhibition and some cell lines display altered protein expression upon confluency of a culture dish.

We used the differences in protein function of these cell lines as a focus in our attempt to elucidate the mechanism of cdk2 inhibition by flavopiridol. To assist us in determining these effects, other chemotherapeutics with known mechanisms were used as a comparison. These drugs enable us to get a clearer picture as to the overall effect of flavopiridol in our tissue cultured cell lines.

Cell Culture

A variety of human cultured cell lines from different tissues were used in these experiments. HSF55 cells, human skin fibroblasts, were used as a control. HeLa (cervical cancer), MCF7 (breast cancer), Saos2 (osteosarcoma) and DU145 (prostate cancer) cell lines were used for their differences in cell cycle protein expression (HSF55, HeLa and MCF7 cell lines were generously donated by Dr. John Th'ng and Saos2 and DU145 cell lines were generously donated by Dr. Helga Duivenvoorden). HSF55, HeLa, MCF7 and Saos2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4500mg/L glucose (Sigma), while DU145 cells were cultured in minimum essential medium with Eagle's salts (MEM) (Sigma). All media was supplemented with 10% fetal

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bovine serum (FBS) (Hyclone), 100units/mL penicillin G sodium, 100μg/mL streptomycin sulfate and 0.25μg/mL amphotericin B (Gibco). In addition, MEM was supplemented with 0.1mM MEM non-essential amino acid solution and 1.0mM sodium pyruvate (Sigma). All cell lines were maintained in cell culture flasks (Corning) in fully humidified incubators (Sanyo & Fisher Scientific) at 37°C and 5% CO_2. All cell lines were treated with Mycoplasma Removal Agent (1:100 dilution) (ICN Biomedicals) for 2 weeks after thawing from their cryogenically frozen state to inhibit mycoplasma infection.

Cells were subcultured twice per week using 0.05% trypsin (+EDTA) (Gibco) to detach the cells from the flask surface. The separation process of the cells by trypsin was promoted by rinsing cells with ATV prior to trypsinization.

Treatment of Cells

Treatment of cultured cells was consistent throughout all experiments. Cells were seeded on day 0 and on the next day, after the cells adhered to the surface of the well, the media was aspirated and replaced with either fresh media as a control or media with 40-70nM flavopiridol (IC_{50}) (Aventis Pharmaceuticals Inc) or 5mM sodium butyrate (Sigma). The cells were then incubated for 24, 72 or 96hrs, depending on the experiment.

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Doubling Time Analysis

The rate of proliferation of each cell line was experimentally determined for the cell cultures utilized in these experiments using the Coulter Particle Counter Z1 (Beckman Coulter). Cells were trypsinized with 0.05% trypsin (+EDTA) and resuspended in medium (~5mL). One-hundred microlitres of each cell solution was put into 10mL of isoton II (Beckman Coulter) in a cuvette (Beckman Coulter), mixed and read with the particle counter. The number of particles enumerated by the counter was multiplied by the dilution factor 100 to calculate the number of cells per milliliter.

Specific numbers of cells from each cell line were seeded in each well of duplicate 6 well plates (Corning). The density of HSF55 cells seeded was 1.5×10^4 cells per well and the rest of the cell lines were seeded at a density of 2.0×10^4 cells per well. All wells were filled with 3mL of media. Each day for 6 days after initial plating of cells, one well of each plate (2 wells total for each cell line) was trypsinized and cells were counted as previously described. Two samples were taken from each well for a total of 4 samples for each cell line per day from a total of 2 wells per cell line. Each sample was read four times and then averaged. The average growth rate over the 6 days was calculated and the doubling time was approximated for each cell line.

This technique was used to determine the growth rate of cells treated with flavopiridol. Cells were plated out as previously described for each cell line into all wells of 2 six-well plates. On the following day, the media was aspirated from 2 wells of each plate and the attached cells in these wells were trypsinized and counted as previously described with the Coulter Counter to establish a reference of cell number at that point in time. Half of the remaining 4 wells on each plate were treated with flavopiridol (IC_{50})

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and half were left as controls. All media was replaced with fresh media. Three days later, the media was recovered and the cell number was calculated for both the cells suspended in the media and the cells still attached to the plate. These numbers were then used to provide a comparison between cell growth with flavopiridol and the untreated controls.

Flavopiridol IC_{50} Analysis

Standardization of MTT Assay

Cells were seeded in a 96 well cell plate (Corning) in an ascending manner, relative to cell number. The first column of wells was left empty and in each subsequent column, 500 more cells per well were seeded, with the 12^{th} column containing 5.5×10^3 cells per well. Each well contained a final media volume of 100μL.

The cells were then incubated at 37°C for 5 days and MTT assays were performed. Ten microlitres of the total media volume of MTT solution (Sigma) was added to each well and the cells were incubated for an additional 4 hours at 37°C. The media was then aspirated and 100μL of dimethyl sulfoxide (DMSO) (Fisher) was added to each well. The plates were left at room temperature for 10 minutes before being analysed with the Kinetic Microplate Reader (Molecular Devices). These readings were used to determine the number of cells to seed in each well of the 96 well plates used for IC_{50} analysis. The number of cells which produced an absorbance reading on the curve below the maximum absorbance obtained was used as the standard number of cells to be seeded. This was done to avoid confluency during the IC_{50} MTT assays.

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Utilizing the MTT Assay

The IC_{50} of each cell line treated with flavopiridol was determined via MTT assay. Flavopiridol was dissolved in 100% DMSO. One to two thousand cells were seeded into all of the wells in a 96 well plate except the first 4 wells of the first column, which were left vacant as a blank control to hold only media. Total media volume in every well was 100μL. This was carried out for each of the cell lines. After a 24 hour incubation period at 37°C, the cells were treated with a variety of flavopiridol concentrations. Stock solutions of flavopiridol, diluted in media, were made at concentrations three times that of the desired final concentration. The final concentrations of stock solutions ranged from 15nM to 300nM. Fifty microlitres of each stock solution was then aliquotted into predetermined wells. The last column of each plate was reserved for an untreated control. Fifty microlitres of media was added to the blank and control wells. Aliquots of 50μL of each stock solution were dispensed into each column, corresponding to a particular concentration. The final concentrations ranged from 5nM to 100nM. The cells were then incubated for 4 additional days and the MTT assay was performed as previously described. The absorbencies were read by the Kinetic Microplate Reader at a wavelength of 490nM minus 650nM. This wavelength was used to accurately detect the color of the dissolved formazan crystals. The IC_{50} of flavopiridol for each cell line was determined by observing at which concentration, growth was inhibited to 50% of the untreated control.

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Protein Determination

Harvesting Cell Proteins

Cells from each cell line were trypsinized from their stock culture flasks and put into 15mL tubes to be counted by the particle counter. Using the determined concentrations of these stock cell solutions, specific numbers of each cell line were plated into 100mm cell culture dishes (Corning). Two dishes were used for each cell line. For the HSF55 cell line, 3.0×10^5 cells were plated into each dish, while 4.0×10^5 cells of HeLa and DU145 were plated into each of their respective dishes. MCF7 and Saos2 cells were plated onto the cell culture dishes at a density of 5.0×10^5 cells per dish. The total volume was then made up to 10mL with the corresponding media and the cells were incubated for 24 hours at 37°C. The cells were treated as previously described and the dishes were then incubated for another 24 hours and cell protein was harvested. This was done by trypsinization of the cells from the surface of the dishes and placement of these cells into 15mL tubes. The cells were centrifuged for 7 minutes at 1200rpm to pellet the cells and the media was then aspirated. The pellets were washed with 3mL of phosphate buffered saline (1X PBS) and centrifuged for another 7 minutes at 1200rpm. This washing step was performed twice (for each pellet of cells). The PBS was aspirated from the tubes and 100μL of cold nuclei buffer (NB) with 1mM PMSF was added to lyse the cells in each pellet. The solutions of lysed cells were then put into 1.5mL microcentrifuge tubes and placed on ice. The tubes were centrifuged in a microcentrifuge (Beckman) for 30s at 14000xg.

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Protein assay

Protein Assay Dye Reagent Concentrate from BioRad Labs was used in the determination of sample protein concentration. Five microlitres of each sample was added to 800μL of double distilled water in a spectrophotometer cuvette. Two-hundred microlitres of assay reagent was then added to each cuvette and mixed. A 5 minute waiting period was then observed. After the spectrophotometer (Milton Roy) had been standardized at 595nm with a blank of 800μL of double distilled water, 5μL of NB and 200μL of assay reagent, each sample was read and absorbencies documented. A standard curve was made using samples of bovine serum albumin (BSA) in NB. Normalization of protein concentrations to 2μg/μL was done by adding additional NB. An equal volume of 2X SDS sample buffer was added to each sample to bring the concentration to 1μg/μL. Samples were then boiled for 2 minutes and placed in a -20°C freezer for storage. Before beginning the western blot procedure, samples were removed from the freezer and boiled again for 2 minutes and centrifuged for 30s at 14000xg in a microcentrifuge before aliquotting to the acrylamide gel wells.

Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The Mini-PROTEAN® II Cell gel apparatus from BioRad was used to perform these western blot procedures. A 12% separating gel and 4% stacking gel were prepared according to the BioRad instruction manual. All wells were filled with 1X running buffer before samples were loaded. Ten to sixty micrograms of each protein sample were

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loaded into each lane. One lane was reserved for a protein standard (low range, prestained SDS-PAGE standard from BioRad). The apparatus was filled with 1X running buffer and the gel was run at 100V until the sample dye front ran off the gel.

The gels were removed from the electrophoresis apparatus and cut to eliminate excess gel before placing in 1X transfer buffer for 5-10 minutes. Polyvinylidene difluoride (PVDF) membrane (Pierce) was soaked for 15-20 minutes in 100% methanol prior to transfer. Transfer proteins were transferred to the PVDF membrane by electrophoresis at 100V for 1 hour. Upon completion of the transfer process, the membrane was removed from the transfer apparatus and soaked in TBST buffer for 10 minutes. The membrane was then placed in a solution of TBST-8%M to block overnight at 4°C with agitation.

Western Blot Analysis

The membrane was incubated in a primary antibody solution in a 50mL tube on a rotator for 1.5-2.0 hours at room temperature. The primary antibody solution was made in TBST-5%M. The antibodies used were specific for; cdk2, cyclin E, p21, XIAP, Bcl-2, Bax or cyclin A (Table 1). The membrane was subsequently washed with 0.1% TBST buffer for 1 hour with gentle agitation. A secondary horseradish peroxidase-conjugated antibody solution at a concentration of 1:2000 in TBST-5%M was used appropriate to the species of the primary antibody. This incubation period was carried out in a range of 45 minutes to 1 hour with gentle agitation. Excess secondary antibody solution was then washed off using 0.1% TBST buffer solution for 1 hour.

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Table 1 - Experimental antibodies.
Antibodies used to detect cell cycle and apoptotic proteins during western blot analysis.

Antibody	Designation	Type	Concentration used	Source
p21	(Ab-3)	Mouse monoclonal	2µg/mL	Calbiochem
Cyclin A		Rabbit polyclonal	1:1000	Dr. M Bradbury
Cyclin E	(C-19)	Rabbit polyclonal	2µg/mL	Santa Cruz
cdk2		Rabbit polyclonal	1:8000	Dr. M Bradbury
Bcl-2	(N-19)	Rabbit polyclonal	1µg/mL	Santa Cruz
Bax	(B-9)	Mouse monoclonal	1µg/mL	Santa Cruz
XIAP	(2320-PC-050)	Rabbit polyclonal	1µg/mL	Trevigen
Rabbit 2°	Goat α Rabbit	HRP conjugate	1:2000	Santa Cruz
Mouse 2°	Rabbit α Mouse	HRP conjugate	1:2000	Pierce

Enhanced Chemiluminescence (ECL)

An enhanced chemiluminescence process was used for detection of the desired bands on each membrane. ECL solutions 1 and 2 were prepared and mixed. Excess washing solution was removed from the membranes by gently blotting the surface with kimwipes. The membranes were then placed in the combined ECL solutions for 1 minute before they were removed and the excess solution was removed with kimwipes. The membrane was placed protein side down on a sheet of plastic wrap and wrapped inside with all excess plastic wrap folded towards the side opposite of the protein. The wrapped membrane was then taped into an autoradiography cassette (Fisher Biotech) and was exposed to various areas of the Kodak Scientific Imaging Film - BioMax MS Film for 1, 2, 5 and 10 minute intervals. Development of the film was done in a M35A X-OMAT Processor (Kodak). The bands present on the film were compared and evaluated.

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Immunoprecipitation

Cells were seeded and treated with flavopiridol as previously described. Twenty-four hours after treatment, the cells were removed from the surface of each dish via trypsinization and lysed in cold NB containing 1mM PMSF and kept on ice. NB was used in the ratio of 300μL NB per 1.0 x 10^6 cells. The lysed cell solutions were kept on ice for 20 minutes in microcentrifuge tubes and then centrifuged for 15 minutes at 14000xg at 4°C. The supernatants were transferred to new tubes without disturbing the pellets and kept on ice. Protein assays were then performed to determine protein concentrations. The protein samples were diluted to a final concentration of 1μg/μL in 500μL.

Protein A sepharose (Amersham Biosciences) was pre-rinsed in NB 4 times just prior to use. Each solution was pre-cleared for non-specific sepharose bead binding by incubating the solutions with 30μL of 50% protein A sepharose slurry for 1 hour at 4°C on a rotator. The samples were then centrifuged for 5 minutes at 14000xg at 4°C and the supernatants were transferred to new microcentrifuge tubes and kept at 4°C. The samples were then incubated on a rotator at 4°C for 2 hours with 1μL of cdk2 antibody. Subsequently, 50μL of protein A sepharose was added to the samples and incubated for another 1 hour at 4°C on a rotator. The samples were pelleted via centrifugation for 5s and the supernatant was discarded. The pellets were washed twice with 300μL of NB buffer and the pellets were dissolved in 2X SDS sample buffer. The samples were boiled for 5 minutes to release the proteins back into solution. The samples were centrifuged for 1 minute and the supernatants removed for analysis by western blot analysis.

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Kinase Assay

Cells used for kinase assays were plated, treated with flavopiridol or sodium butyrate and lysed as previously described in the immunoprecipitation section. Once the sepharose beads with attached antibody/protein complexes were washed with NB buffer 4 times, the NB buffer was discarded, 100μL of kinase buffer was added to the beads, the contents were mixed and allowed to sit on ice for 10 minutes. The supernatants were then removed and replaced with 50μL of the kinase reaction buffer.

The slurries were mixed and incubated in a rotating 30°C water bath for 10 minutes. Forty-five microlitres of the supernatants were removed and put in new tubes containing 405μL of cold acetone, while the beads were saved in the original microcentrifuge tubes. All samples were then left overnight in a -20°C freezer. The samples in acetone were centrifuged and the supernatant removed and discarded. The pellets were then left to air dry at which point, 20μL of SDS sample buffer was added to resuspend the pellets. The samples were boiled for 5 minutes and the contents were run on polyacrylamide gels. The gels were stained with coomassie blue, destained with a destain solution and dried on a gel dryer (BioRad) to pieces of chromatography paper. The gels were then exposed to film in an autoradiography cassette for 6hrs, 12hrs, 24hrs and 48hrs.

After exposing the dried PAGE gel containing phosphorylated histone to film, the bands were individually cut out and ^{32}P activity was measured on a scintillation counter (Beckman LS6500). Scintillation vials (Skatron) were filled with Ready Safe liquid scintillation cocktail (Beckman) and a band was inserted into the solution. The vials were capped and the radioactivity was read. The radioactivity of the histones

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phosphorylated by the treated samples was compared to the control samples. A background reading was taken using a piece of the filter paper used to dry the gels on instead of a cut out band. The sample readings were then adjusted accordingly, by removing the background reading from the sample reading.

The remaining kinase reaction buffer with the sepharose beads was removed and 2X SDS sample buffer was added to each of the pellets and the slurries were mixed and boiled for 5 minutes. The supernatant was transferred to new tubes and the beads were discarded. These parts of the samples were subjected to western blotting procedures with p21, cdk2 and cyclin E antibodies as previously described.

To provide samples of direct cdk2 inhibition by flavopiridol, 3 samples of 500μg of protein were used from each cell line to isolate cdk2 via immunoprecipitation. These samples were prepared for kinase activity analysis as previously described up until the addition of the H1 kinase reaction buffer. One of each set of three samples was treated with 50μM flavopiridol, one sample was used as a negative inhibition control and one was treated with 100ng/mL staurosporine as a positive control for kinase activity inhibition. The samples were incubated at 30°C for 10 minutes as before and continued to be treated as previous kinase assays.

Flow Cytometry

Cells were plated in 100mm dishes and treated with flavopiridol as previously described for 24 hours. The media was removed and kept in a 15mL tube. Cells were trypsinized and added to the previously removed media. The cells were then pelleted via centrifugation, the supernatant removed and the pellet was washed twice with 1X PBS

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and resuspended in 1mL 1X PBS. 3mL of 95% cold ethanol was then added, dropwise while vortexing to fix the cells. The samples were then kept at 4°C overnight. The samples were then centrifuged and the supernatant was aspirated. The pellets were resuspended in a solution of the intercalating dye, propidium iodide (PI) (5μg/mL) in 1X PBS, with the volume relative to pellet size (300-1000μL). Cell cycle profile (DNA content) analyses were carried out on a FACSCalibur flow cytometer (Becton Dickinson).

Results

Measurement of growth rates of cells

Cellular proliferation rates measured for each of the cell lines used in this study were different. Thus, this experiment was necessary to optimize the conditions for MTT assay measurement, as the cells are incubated for 4 days. Confluency during the incubation period of the MTT assay is undesirable and will affect the data. Also, the effects of cytotoxic agents, such as flavopiridol, that affect cell cycle proteins may be influenced by cellular growth rates. Cell counts using the Coulter Counter showed that the MCF7, HeLa and DU145 cells lines have doubling times of roughly 24 hours, while the Saos2 cells exhibited a doubling time of approximately 44 hours. At a low passage number, the normal skin fibroblast HSF55 cell line demonstrated a doubling time of 38 hours. Growth curves presented as semilogarithmic plots are illustrated in Figure 12, and are consistent with those reported in literature.

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Contact inhibition is the process in which normal cells will cease to proliferate upon coming into physical contact with neighboring cells. Although the cancer cell lines used in these experiments do not experience contact inhibition, confluency of these cell lines may also affect growth in some cells and influence flavopiridol's effects.

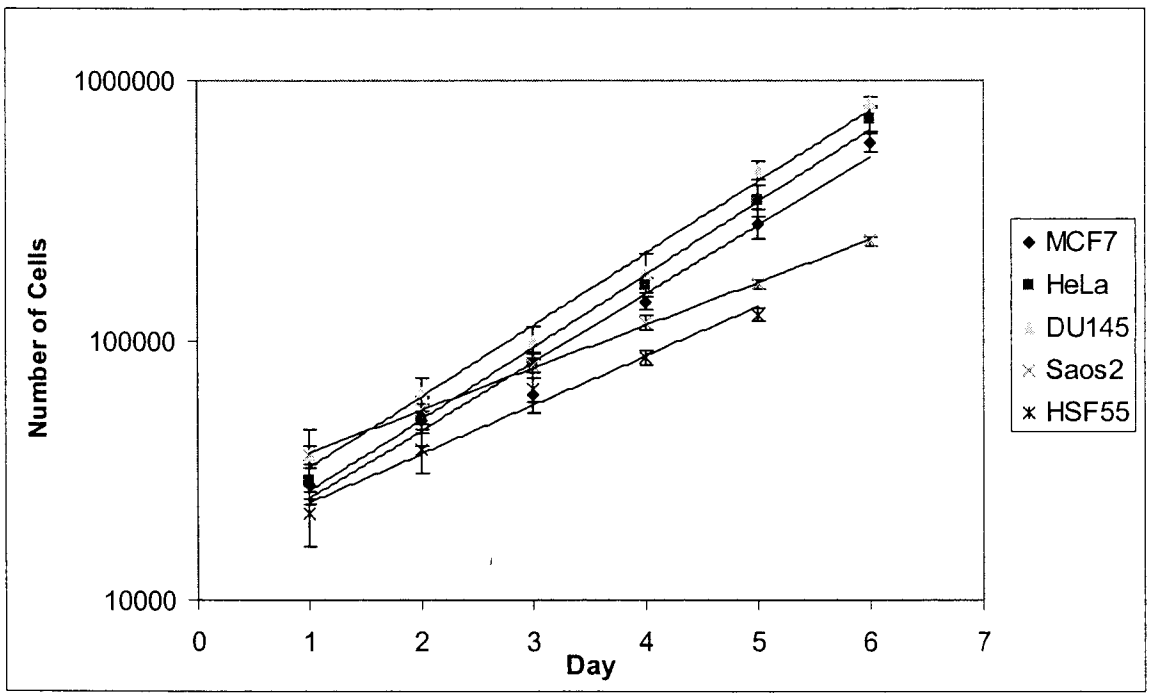

Figure 12 - Normal cell growth of tissue cultured cells.
MCF7, HeLa, DU145, Saos2 and HSF55 cell lines were plated in duplicate into 6-well plates. The cell numbers for each day were averaged from four cell counts for each of two samples from each of 2 wells for a total of sixteen counts per point on the graph. Slope of the curve indicates rate of growth (steeper slope=faster growth).

Table 2 - Cell line doubling times.
The slope of the growth curves of Figure 12 were measured and used to determine the doubling time of each cell line.

Cell Line	Doubling Time (hrs)
MCF7	27.54 +/- 1.44
HeLa	26.00 +/- 0.30
DU145	26.26 +/- 0.84
Saos2	43.94 +/- 0.82
HSF55	38.23 +/- 1.27

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Flavopiridol induces cell cycle arrest

MTT assays were performed to analyze growth inhibition by flavopiridol and to measure the IC_{50} values for each cell line after 4 days of growth. The IC_{50} values correspond to the necessary concentration of flavopiridol to reduce cell growth to 50% of normal. Before these measurements could be done, standardizations of cell numbers were performed to account for different growth rates. This was also done to determine the growth of each cell line during the 5 days after seeding, with the goal of achieving subconfluency during IC_{50} measurements. An example using HeLa cells is shown in Figure 13 and Table 3 summarizes the optimal cell numbers of each cell line. It was determined that 1000-2000 cells per well of the 96 well plate was sufficient to produce the optimal readings and avoid saturation of the wells by the end of the incubation period.

Flavopiridol treatment showed a significant decrease in cell numbers of MCF7, HeLa and Saos2 cells at 10nM (20nM for DU145 & HSF55) (Student's one-tailed *t* test, $P<0.05$). The cell population of all cell lines decreased to less than 20% of the untreated control with a treatment of 120nM. MTT assays showed the IC_{50} values to be similar with all the cell lines, ranging from 40-70nM (Figure 14, Table 3).

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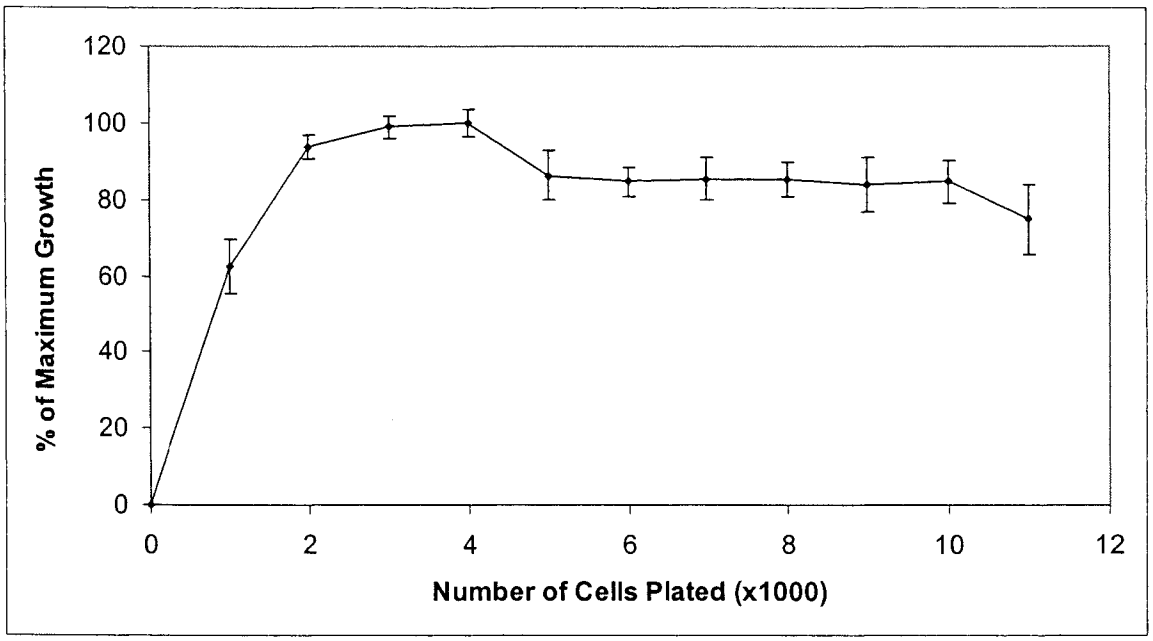

Figure 13 - Standardization of MTT assays (HeLa).
HeLa cells were plated in a 96-well plate and were incubated for 4 days before being subject to MTT assay analysis. It is determined here that the optimal density at which to plate HeLa cells for MTT assay analysis is 1000cells/well with a maximum of 2000cells/well.

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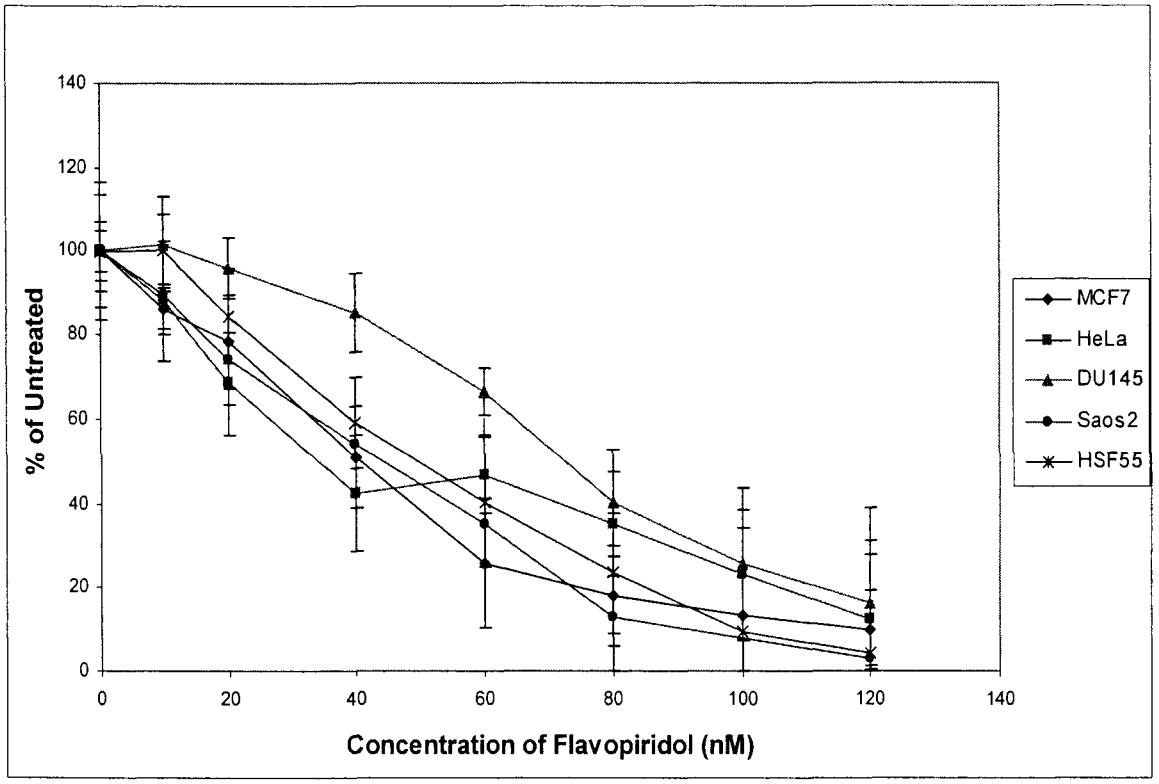

Figure 14 - Flavopiridol inhibition curves of cell line growth.
Cells for each cell line were plated at the predetermined standard number (1000-2000 cells/well) into 96-well plates. All cell lines were subjected to a range of flavopiridol concentrations (10nM-120nM) and incubated for 4 days before MTT assay analysis. There was a significant decrease in cell growth with 10nM flavopiridol for MCF7, HeLa and Saos2 cell lines and after 20nM for DU145 and HSF55 cells. Student's one-tailed *t* test ($P<0.05$).

Table 3 - MTT assay results - IC_{50} values.
Determination of IC_{50} values for each cell line upon flavopiridol treatment using the MTT assay. Included are the numbers of cells plated in each well of the 96-well plates, as determined via previous standardization.

Cell Line	Original Number of Cells Plated (Cells/Well)	IC_{50} (nM)
MCF7	1000	50.6 +/- 7.4
HeLa	1000	56.5 +/- 12.0
DU145	1000	56.2 +/- 13.8
Saos2	2000	64.1 +/- 23.0
HSF55	1000	45.5 +/- 11.6

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Induction of cell cycle arrest by flavopiridol

Cell counts showed the inhibition of proliferation that was observed during MTT assays. Panels A and B of Figure 15 show a lack of proliferation after 3 days of flavopiridol treatment in all cell lines. Normal cell proliferation is shown as a comparison after the same time period. To determine if the lack of proliferation with flavopiridol treatment was due to cell death, floating cells were counted. Cells that have undergone apoptosis would detach from the culture dish (all cell lines used in these experiments were adherent). The difference between the inherent number of floating cells in the controls and the number of floating cells in the treated samples can be attributed to apoptosis. As shown in Panel C of Figure 15, no significant difference was found between the numbers of floating cells in flavopiridol-treated cells versus the untreated controls. It is typical for cell cultures to have a baseline level of apoptotic cells, and this proportion of spontaneous apoptosis varied with each cell line. This number varied from about 10,000 for Saos2 and HSF55 to about 50,000 for DU145, and they represent about 25% of the total population. There was no significant difference between the proportions of detached cells in the untreated controls versus flavopiridol-treated cells. This showed an absence of an increase in apoptotic cells and suggested that flavopiridol may have reduced cell growth.

Cell cycle arrest was further verified by cell cycle analysis in a flow cytometer. The relative DNA content in each cell was measured after staining with propidium iodide, and a typical untreated control cell cycle profile is shown in Panel A of Figure 17. The presence of a sub-G1 peak, as shown in Panel C, indicates the presence of apoptotic cells. A decrease in S phase cells was observed in all cell lines that were treated with

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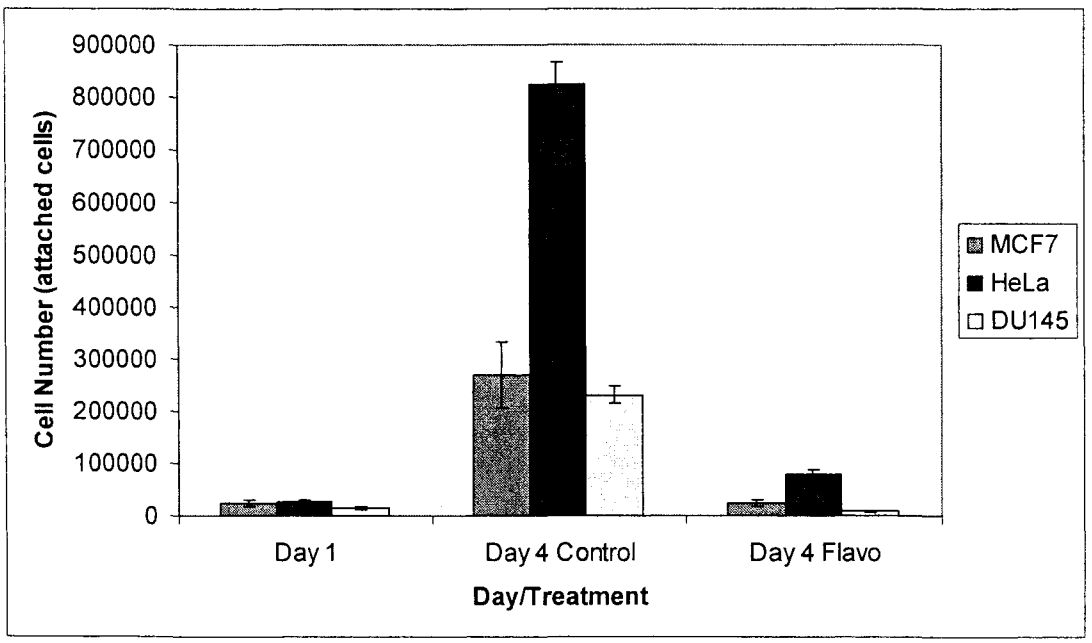
flavopiridol, accompanied by an increase in proportion of cells in G_1 and G_2/M, indicating cell cycle arrest in these phases (Figure 16). Panel B shows a representative profile of this arrest. The lack of a sub-G_1 peak, in the cell cycle profiles verified the absence of apoptosis associated with the 24hr incubation period used in these tests (Figure 17 - Panel C).

A



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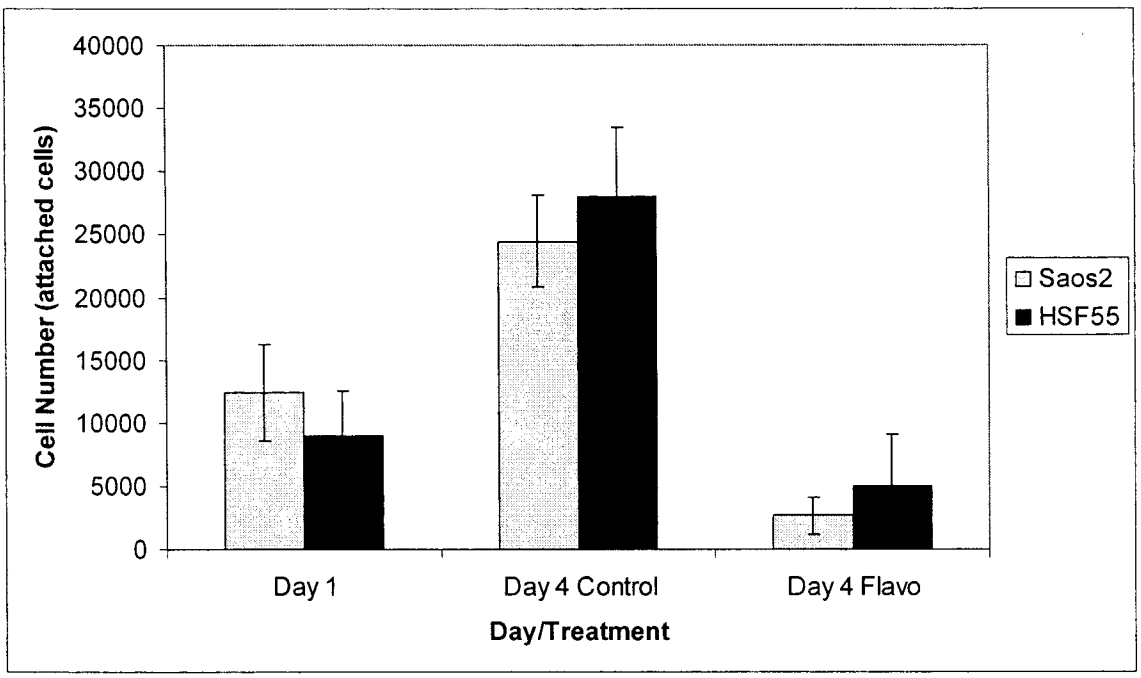
B



C

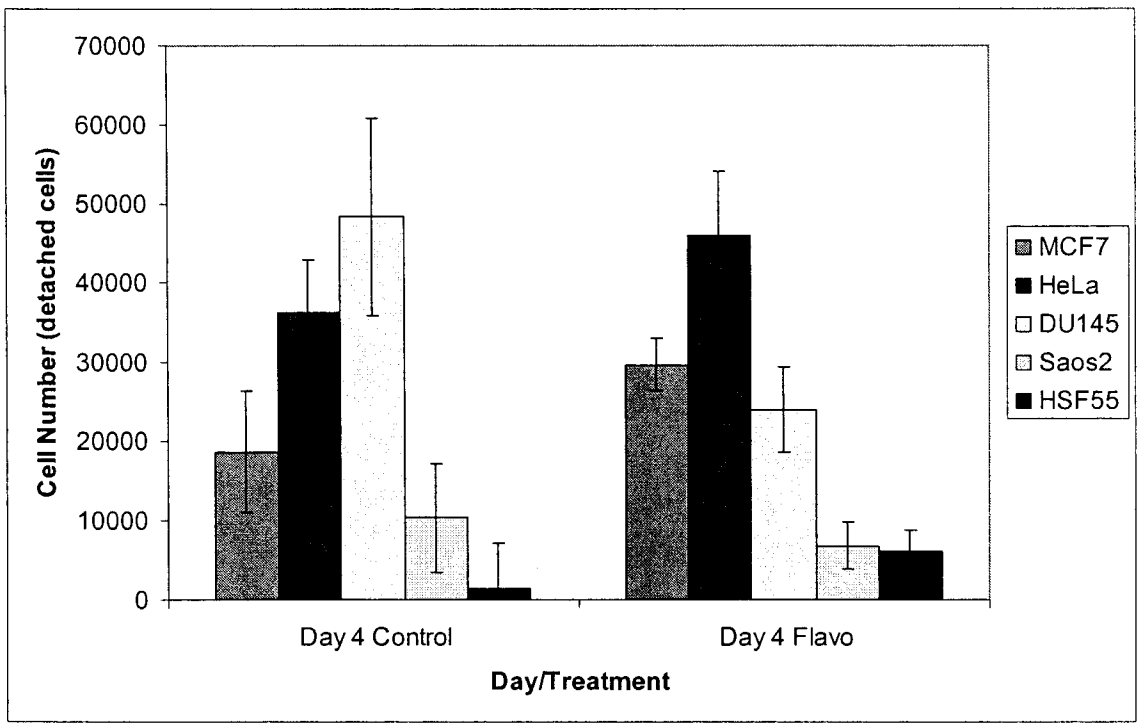


Figure 15 - Analysis of cellular growth after 4 days of flavopiridol treatment vs control.

Panels A and B show the number of cells counted 1 day after seeding, and the number of cells after 3 days incubation with and without flavopiridol. The concentrations of flavopiridol used corresponded to the IC_{50} values determined from Figure 4. Panel C shows the number of cells that were detached after the 3 day incubation period.

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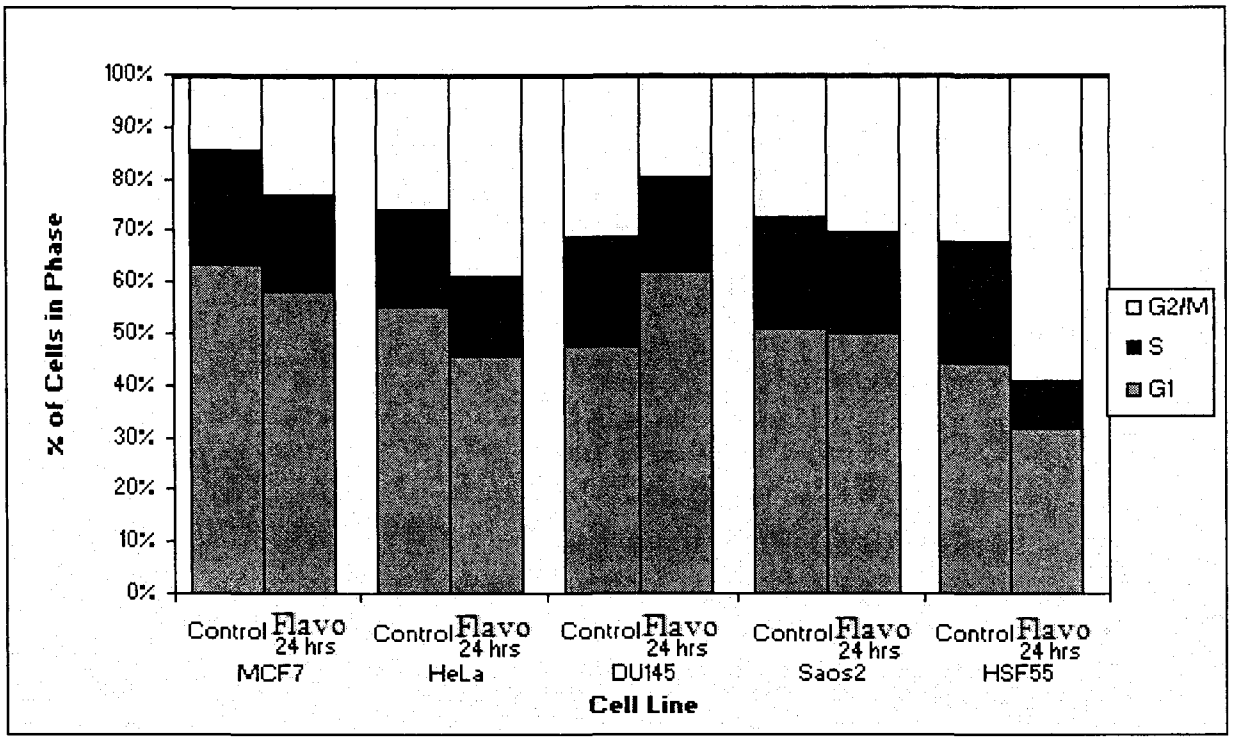

Figure 16 – Analysis of percentage of cells in S phase using flow cytometry. Decreasing percentages indicate cell cycle arrest in G_1 and G_2/M phases. The normal HSF55 skin cells show a dramatic decrease in S phase cells with flavopiridol.

Table 4 - Cell cycle profiling data using flow cytometry. A decrease in S phase cells is observed in all cell lines with flavopiridol treatment. G_1 and G_2/M peaks also fluctuate with flavopiridol treatment.

Sample	Cell Cycle Phase		
	G_1 (%)	S (%)	G_2/M (%)
MCF7			
Control	63.51	21.58	14.9
24 hours	58.49	18.5	23.01
HeLa			
Control	55.22	18.2	26.58
24 hours	45.69	15.26	39.04
DU145			
Control	47.64	20.78	31.58
24 hours	62.15	17.98	19.87
Saos2			
Control	51.18	20.84	27.98
24 hours	50.1	19.4	30.51
HSF55			
Control	44.25	23.04	32.71
24 hours	32	8.4	59.6

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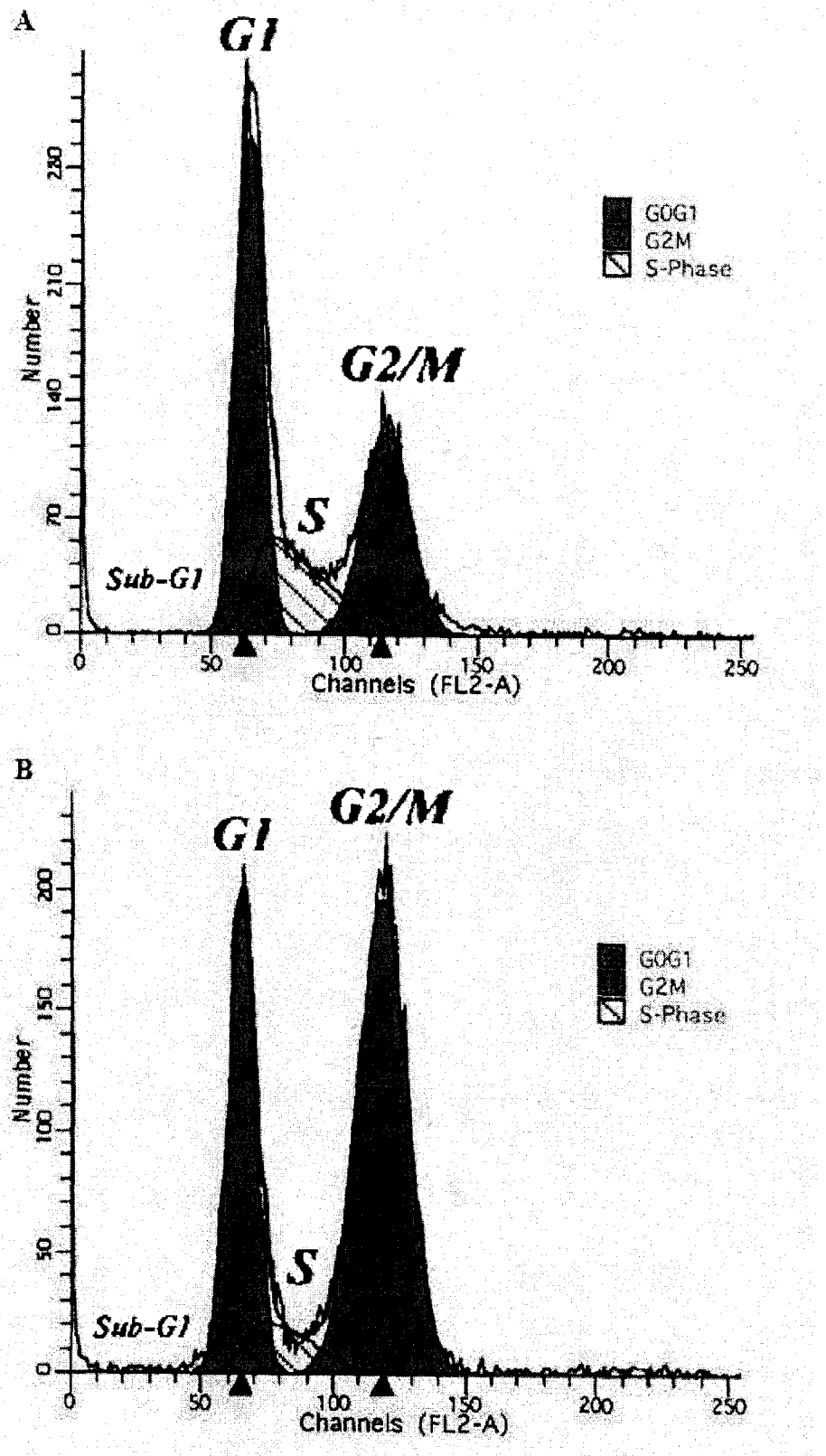

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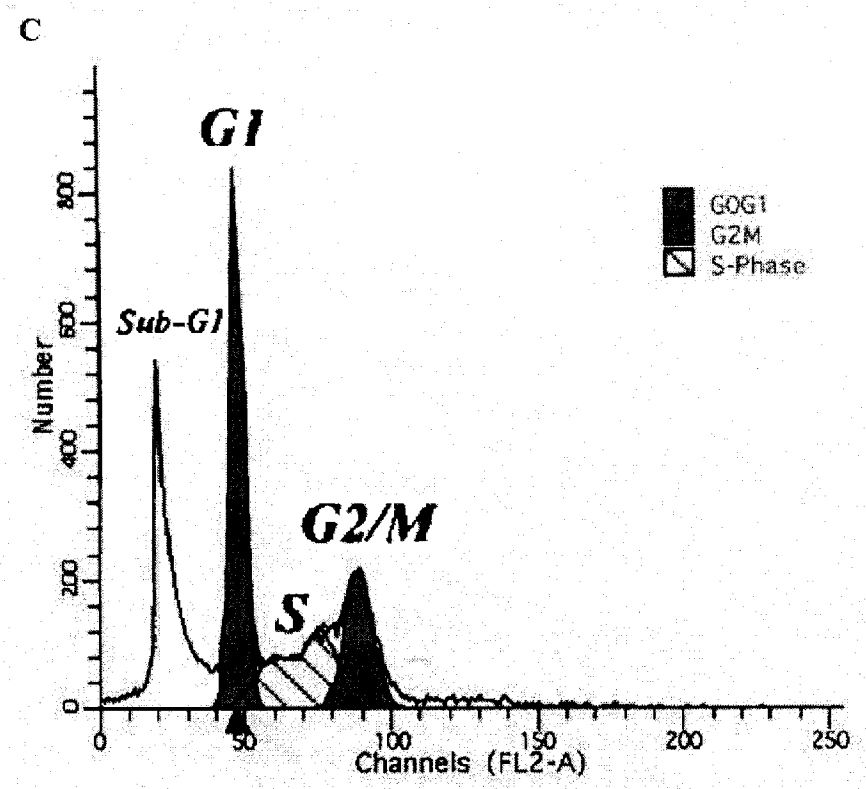

Figure 17 – Flow cytometry profiles of the cell cycle of HSF55 cells.
A. Cycling HSF55 cells demonstrating the distribution of DNA content in cells through each of the phases of the cell cycle. **B.** Cell cycle profile of HSF55 cells treated with flavopiridol for 24hrs. **C.** A typical cell cycle profile of cells that undergo apoptosis, showing the apoptotic sub-G_1 peak.

Expression of apoptotic proteins

The Bcl-2 family of proteins is the quintessential apoptotic trigger of apoptosis. While anti-apoptotic family members Bcl-2, Bcl-X_L, Boo and Mcl-1 are more abundant than pro-apoptotic proteins (when the cell is functioning normally) when these proteins are downregulated, programmed cell death is favored. In addition to this downregulation, the pro-apoptotic Bcl-2 proteins, Bax, Bak, Bad and Bcl-X_S are upregulated. Upon 24hr treatment with flavopiridol, we found Bcl-2 levels decreased in all cell lines versus untreated cells. No change was observed in Bax protein levels with the same treatment

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(Figure 18). Bax was not expressed in either DU145 or Saos2 cell lines. DU145 contains mutated Bax genes on both alleles and subsequently is unable to express the protein (131). Saos2 cells are p53-null and therefore are missing a key regulator of Bax expression (132).

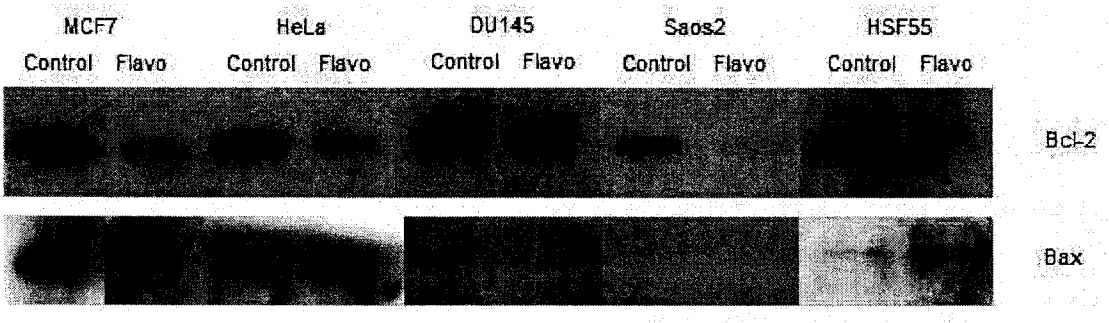

Figure 18 – Western blot analysis of apoptotic proteins from cell lysates.
After flavopiridol (Flavo) treatment at IC_{50} concentrations anti-apoptotic protein, Bcl-2, decreased in all cell lines. There was no apparent change in pro-apoptotic Bax protein levels.

Expression of cell cycle proteins

As shown by flow cytometry, incubation of cells with 50-70 nM flavopiridol induced cell cycle arrest. We found upregulation of $p21^{WAF1/CIP1}$ (Figure 19). This upregulation of $p21^{WAF1/CIP1}$ has been reported by others (28;88;95).

There was no discernible difference in expression of cdk2 in any of the cell lines examined. Similarly, cyclin E showed little difference in any cell line (Figure 20). These results showed that flavopiridol does not induce cell cycle arrest through a reduction in the proteins forming the cdk2 complex. The inhibitory effects could be through direct inhibition of the enzyme, or by changing expression levels of inhibitory proteins such as $p21^{WAF1/CIP1}$.

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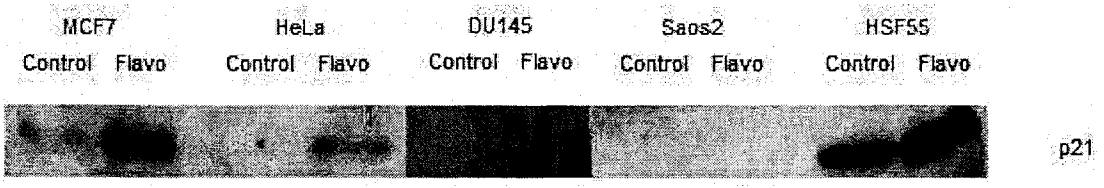

Figure 19 – Western blot analysis of the cdk inhibitor $p21^{WAF1/CIP1}$ in cells lysates. After flavopiridol (Flavo) treatment there was an upregulation of $p21^{WAF1/CIP1}$ in MCF7, HeLa and HSF55 cell lines, relative to the untreated controls. DU145 and Saos2 cells showed no increase of $p21^{WAF1/CIP1}$.

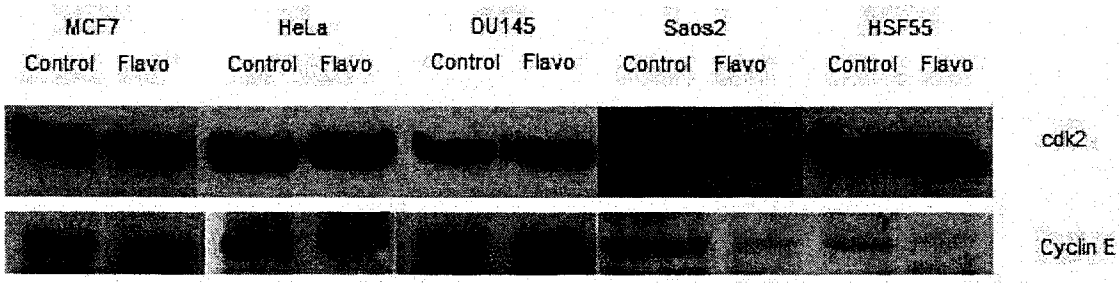

Figure 20 – Western blot analysis of G_1/S transition proteins in cells lysates. After flavopiridol (Flavo) treatment there was no change in the levels of cdk2 or associated protein cyclin E in MCF7, HeLa and DU145. Saos2 and HSF55 cells demonstrated a slight decrease in cyclin E protein levels.

Inhibition of cdk2 in cells

Determination of protein levels of cdk2 by western blot showed no differences in levels of expression from treatment with flavopiridol, and little differences in expressions of cyclin E in some of the cell lines examined. However, $p21^{WAF1/CIP1}$ was found to be upregulated in all except the Saos2 and DU145 cells. To determine if the enzymatic activity of cdk2 was inhibited, as flavopiridol was hypothesized to do, enzyme complexes were immunoprecipitated from the various cell lines and assayed for kinase activities. As a control for indirect inhibition by $p21^{WAF1/CIP1}$, the cdk2 enzyme activities were measured from cells treated with sodium butyrate. Sodium butyrate upregulates $p21^{WAF1/CIP1}$, independently of p53, to inhibit cdk2 kinase which induces cell cycle arrest

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(133;134). Autoradiography of the histone H1 substrate following *in vitro* immunoprecipitation kinase assays showed there were noticeable declines in cdk2 activity that were immunoprecipitated after 24hr treatment with flavopiridol (Figure 21). Quantitation of the phosphorylated histone H1 by scintillation counting is shown in Table 5. Cdk2 kinase immunoprecipitated from MCF7 cells showed the highest degree of inhibition when incubated with flavopiridol. The HeLa, DU145 and HSF55 cells showed partial inhibition and the cdk2 kinase from Saos2 cells did not show any inhibition by flavopiridol. When the levels of phosphorylation were quantitated, the cdk2 from MCF7 showed the highest level of inhibition by flavopiridol and by sodium butyrate, with a reduction of about 95%. However, flavopiridol treatment of the HeLa, DU145 and HSF55 cells only reduced the cdk2 activities by half, and the cdk2 from Saos2 was not inhibited. No $p21^{WAF1/CIP1}$ was observed in the cdk2 samples immunoprecipitated from DU145 or Saos2 cells. In HeLa cells, the induction of $p21^{WAF1/CIP1}$ was greater with flavopiridol treatment than with sodium butyrate, yet 5mM sodium butyrate caused a greater reduction of cdk2 activity, by about 80%, compared to the 50% induced by 50nM flavopiridol. This could be due to other cell cycle effects of sodium butyrate. The results showed that although flavopiridol had similar growth arresting effects on the cells examined, the levels of induction of $p21^{WAF1/CIP1}$ were different between cells, resulting in different degrees of inhibition of cdk2.

Western blotting for the cdk2 and cyclin E proteins after immunoprecipitation showed similar or higher levels of proteins with flavopiridol treatment, ensuring that any differences in enzymatic activities were not due to fluctuations in protein levels. Immunoblots for $p21^{WAF1/CIP1}$ showed that this inhibitor was associated with the cdk2

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complexes in cells that were treated with flavopiridol, and could be the basis behind the inhibition of cdk2 kinase, as was seen in the cells treated with sodium butyrate. In the Saos2 cells, $p21^{WAF1/CIP1}$ was not detected and there was a corresponding absence of inhibition.

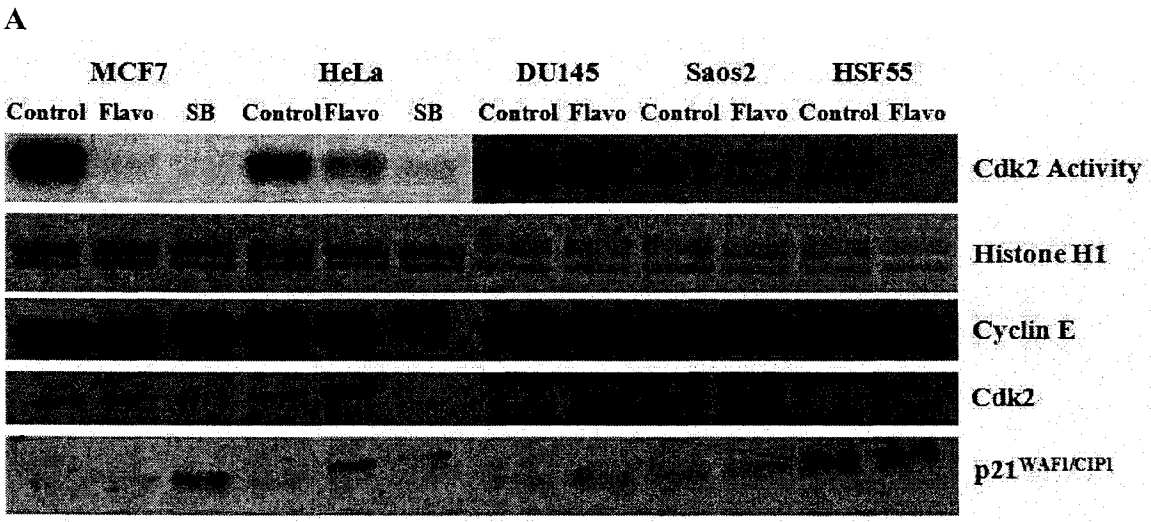

Figure 21 – Kinase assays of cdk2 activity in cells.
Untreated or flavopiridol-treated cells were lysed and the cdk2 kinase complexes were immunoprecipitated and assayed for enzymatic activities. Five millimolar sodium Butyrate (SB) was used as a positive control for cdk2 inhibition. Top row shows the autoradiogram of phosphorylated histone H1, indicating the relative levels of cdk2 activities. Second row represents coomassie-stained gels of phosphorylated histone H1 substrates that were used for autoradiogram. Bottom three rows show immunoblots of cdk2 and cyclin E of the kinase complexes that were used for the kinase assays, and the $p21^{WAF1}$ that associated with the complexes.

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Table 5 - Quantitation of histone H1 phosphorylation by cdk2 immunoprecipitated from cells treated with inhibitors.
Cdk2 activities were measured in counts per million. Background activity was removed and percent activities relative to the control for each cell line was calculated.

Sample	cpm Incorporated	% Activity of Control
MCF7		
Control	3391.89	100
Flavo	212.38	5.65
Butyrate	198.37	5.23
HeLa		
Control	1091.08	100
Flavo	595.17	53.61
Butyrate	238.48	20.25
DU145		
Control	311.13	100
Flavo	167.08	50.18
Saos2		
Control	109.06	100
Flavo	106.06	96.55
HSF55		
Control	450.03	100
Flavo	240.56	50.02

Inhibition of isolated cdk2 kinase

To determine the direct inhibitory effects of flavopiridol without any contribution of $p21^{WAF1/CIP1}$ or other inhibitory proteins, the cdk2 enzyme complexes were immunoprecipitated from each of the cell lines used in the study and flavopiridol was added to the kinase reaction buffer. To provide a positive control for direct kinase inhibition, 100ng/mL of the general kinase inhibitor staurosporine was used in the kinase reaction buffer and a decline in cdk2 activity was observed relative to the untreated control (135). The autoradiogram of histone H1 showed that the cdk2 immunoprecipitated from each cell line was more sensitive to direct inhibition of activity by 50nM of flavopiridol than staurosporine. Quantitation by scintillation counting

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showed that 50nM flavopiridol induced an 80% decline in the cdk2 activity from MCF7 cells and 100ng/mL staurosporine reduced the activity by 70%. The cdk2 from HeLa cells showed the next highest degree of sensitivity to flavopiridol with a reduction by about 65%. In the normal human HSF55 cells, flavopiridol treatment also showed a higher degree of inhibition, with a reduction by 50% for flavopiridol compared to 40% for staurosporine. The 60% reduction in enzymatic activities caused by both inhibitors was similar in DU145 cells. With Saos2 cells, the cdk2 showed the least amount of inhibition by flavopiridol, with a reduction by about 30%.

To verify that the relative amounts of proteins were equal, western blots were performed on the kinase complexes that were immunoprecipitated following enzymatic assays. As shown in the lower lanes of Figure 22, the relative levels of cdk2 and cyclin E were comparable. In the flavopiridol-treated HeLa cells, isolated cdk2 complexes showed slightly elevated levels of cyclin E and $p21^{WAF1/CIP1}$ proteins. Since the cdk2 used here was immunoprecipitated from cycling cells, the $p21^{WAF1/CIP1}$ levels were low in all of these samples as expected.

Because all of the samples were identical before kinase reaction buffer treatment, cyclin E and cdk2 protein levels remained unchanged within cell line samples. HeLa cells showed an inhibition of cdk2 activity in spite of the slightly higher levels of cyclin E. Scintillation counts from these samples were compared to histone protein amounts as were the *in vitro* samples (Table 6).

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B

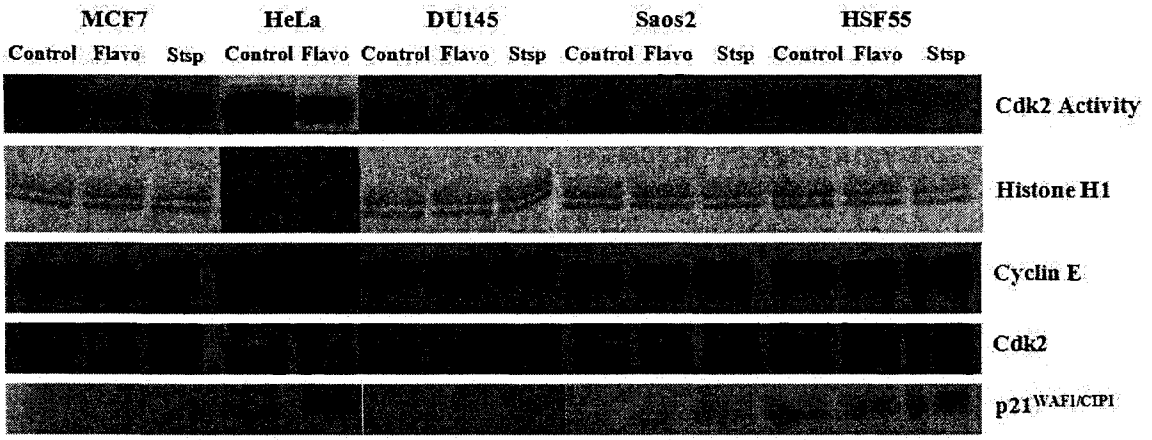

Figure 22 - Direct inhibition of cdk2 by flavopiridol.
Cdk2 kinase complexes were immunoprecipitated from cycling cells and tested for enzymatic activities in the absence of inhibitors, in 50 nM flavopiridol, and 100 ng/ml of staurosporine. Top lane shows the autoradiogram of the histones that were incubated with the cdk2 kinase, and the second lane shows the coomassie-stained histone H1. Bottom three rows show the presence of cdk2, cyclin E and $p21^{WAF1}$ that were immunoprecipitated and determined by western blot following kinase assays.

Table 6 - Quantitation of histone H1 phosphorylation by cdk2 immunoprecipitated from cells and treated with kinase inhibitors.
Cdk2 activities were measured in counts per million. Background activity was removed and percent activities relative to the control for each cell line was calculated.

Sample	cpm Incorporated	% Activity of Control
MCF7		
Control	889.67	100
Flavo	163.15	18.34
Stsp	263.24	29.59
HeLa		
Control	2984.9	100
Flavo	1030.48	34.52
DU145		
Control	115.15	100
Flavo	48.08	41.75
Stsp	43.08	37.41
Saos2		
Control	68.12	100
Flavo	47.1	69.14
Stsp	64.13	94.14
HSF55		
Control	87.17	100
Flavo	42.1	48.30
Stsp	54.12	62.09

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Discussion

Inhibition of cellular proliferation

Cellular proliferation inhibition is a known effect of flavopiridol treatment. The cell lines in these experiments demonstrated similar growth curves and IC_{50} values despite their different basal cdk2 activities. Despite the similar inhibition of growth, they did not show equivalent cell cycle arrest. Normal fibroblasts exhibited a significant decrease in S phase cells after flavopiridol treatment that coincided with an increase in G_2/M phase cells. In these cells, flavopiridol likely decreases cdk2 activity such that the cells were unable to continue past G_2/M and arrested after incubation with flavopiridol.

In contrast, the immortal cell lines showed differences in cell cycle arrest at IC_{50} concentrations although proliferation was retarded in all cell lines examined. Flavopiridol induced a decrease in the S phase populations in all cell lines but to various degrees. I found accumulations in G_2/M in MCF7 and HeLa cells, similar to HSF55 cells, although these normal cells showed a more dramatic accumulation. Flavopiridol induced a different effect in DU145 cells by causing an accumulation in G_1 phase cells. Saos2 cells showed no discernible accumulation in any specific phase, suggesting that the inhibition of growth may affect all phases equally. These differences may be a result of different cell cycle checkpoints in these transformed cells. Although flavopiridol has shown specificity to cyclin-dependent kinases, it has been found to inhibit other kinases in cells, some of which remain unidentified. In addition, some of these alternate effects include transcription factor inhibition other than that of P-TEFb, DNA binding and even

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increases in IL-6 which results in proinflammatory syndrome, a side effect of infusional flavopiridol (15;28;56;107).

Induction of apoptosis

The flavopiridol concentrations used in this study (40-70nM) were sufficient to inhibit growth by 50% and the cells were not undergoing apoptosis during the 24-72hr incubation periods. Lack of apoptosis was observed by cell counts, flow cytometry and verified by microscopy. Previous studies showed that induction of apoptosis required concentrations exceeding 200nM over 24 hours in H1299 lung carcinoma, 200nM over 3 days in A549 non-small cell lung cancer (NSCLC) and 300nM over 5 days in esophageal carcinoma (7;63;80). At the IC_{50} concentrations used in this study, it is possible that incubation periods longer than 72hr could have resulted in more apoptosis with the cell lines used. In studies previously done in this lab, it was found that primary cancer cells with very low replication rates, have reduced cell numbers to less than 10% of untreated cells over a 4-5 day period (136). Since these cells require at least 2 these cells require at least 2 weeks for one cell division, this decline in cell number is likely a result of cell death induced by flavopiridol. This suggests that in primary cancer cells another mode of action other than cdk inhibition is important in death.

A basal level of apoptosis was observed during cell growth analyses. Typically, the proportion of cells that undergo apoptosis spontaneously under regular growth conditions is under 5% (137). In my studies, the floating cells that accumulated in the culture medium over the 3-day incubation period in both flavopiridol-treated and untreated controls were counted with the Coulter Counter. The relatively high numbers

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of floating cells counted is due to the accumulation of cells that underwent spontaneous apoptosis over the 4-day incubation period without any changes of growth medium. Although the numbers of floating cells were similar in the treated and untreated samples, the proportion of floating to attached cells is strikingly higher for the treated cells. This is due to the fact that the untreated cells continued to grow, while the flavopiridol treatment inhibited cell growth, resulting in significantly fewer cells that remained adhered. This suggests that some apoptosis occurred as a result of treatment with this relatively low concentration of flavopiridol over a 3 day incubation period. Also, in the later stages of apoptosis, the cellular remnants would break up into multiple apoptotic bodies (138) which could increase the number of particles counted by the Coulter Counter.

In our experiments, we see that at 50nM, flavopiridol inhibited cdk2 activity, retarded cell growth, induced $p21^{WAF1/CIP1}$ expression and downregulated Bcl-2 protein levels. Bcl-2 forms a heterodimer with pro-apoptotic proteins of the same family (eg Bax, Bad, Bak etc). Bcl-2/Bax heterodimers prevent the assembly of pro-apoptotic homodimers. Proapoptotic homodimers form pores in the mitochondria membranes triggering the signals initiating the apoptosis cascade. A downregulation of Bcl-2 as a result of flavopiridol treatment has been widely observed in a number of studies (67;68). For example, a 50pM dose of flavopiridol was sufficient to lead to Bcl-2 downregulation in the KE4 esophageal squamous cell carcinoma cell line (94). With the reduction of Bcl-2, a vital anti-apoptotic protein is lost; leading to the induction of apoptosis. Since apoptosis is programmed, requiring the induction of multiple genes, a longer incubation period would likely be sufficient to induce the physical manifestations of apoptosis.

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Downregulation of Bcl-2 at cytostatic concentrations implies that even as cell cycle arrest is occurring, apoptotic signalling is commencing.

Because subcytotoxic concentrations of flavopiridol initiate Bcl-2 downregulation, apoptosis could be triggered in noncycling cells. Flavopiridol causes inhibition of Bcl-2 transcription at concentrations as low as 50pM in some cell lines (94). If apoptotic pathways are initiated through inhibition of transcription of some genes, this could explain the increase of apoptosis in resting cells as well as cycling cells. This downregulation of Bcl-2 has been reported to be the case in cultured cell lines (28) as well as primary cancer cells (136). A reduction in Mdm2 mRNA production by flavopiridol, induced p53 upregulation to initiate apoptotic pathways in addition to inducing cell cycle arrest (88). However, this only functions in cells with normal p53, such as the HSF55 and the MCF7 cells. Thus we suspect it is via multiple pathways that flavopiridol can cause apoptosis in noncycling cells through inhibition of transcription.

Inhibition of cdk2

Cdk binding by flavopiridol and the inhibition of cdk activity have been studied and have been well documented over the last decade (15). However, flavopiridol has also been shown to affect multiple pathways in cells to inhibit cell cycle progression and these pathways have yet to be fully elucidated. In this study, I asked the question, "Is flavopiridol's main mechanism of cdk2 inhibition due to its physical binding to cdk2 or is it through other means?" I demonstrated that a 50nM dose will cause upregulation of $p21^{WAF1/CIP1}$ in most of the cell lines used, and this could inhibit cdk2 kinase. Thus, although it is clear that flavopiridol can inhibit cdk2 activity at a cytostatic concentration,

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it is unclear whether it does solely by direct binding, as demonstrated by X-ray crystallography studies, or also through the upregulation of $p21^{WAF1/CIP1}$ that inhibits cdk2.

Since it has been reported that flavopiridol can alter gene expression, I examined its effects on the expression levels of some proteins that regulate the cell cycle to identify additional ways in which cdk2 activity may be reduced. Cyclin E expression was not changed in any of the cell lines by flavopiridol treatment, indicating that a lack of available regulatory cofactor is not relevant. Cdk2 levels themselves were not affected by flavopiridol treatment, indicating that flavopiridol causes a decrease in activity and not a decrease in protein level.

The assays of the cdk2 kinase activities suggest that the inhibition could be through $p21^{WAF1/CIP1}$ upregulation or direct cdk2 binding, but this inhibition was dependent on the cell lines used. The molecular reasons behind this cell-specific effect will require further studies. It is possible that other yet unknown protein subunits may have differential effects on flavopiridol. To address this, one would have to label the proteins in the cdk2 kinase complex with ^{35}S-amino acids in each of the cell lines and then isolate them by immunoprecipitation. The proteins would then be separated in an SDS-PAGE and the radiolabelled proteins that form the complex can be revealed by fluorography. A comparison between the bands would show if there are any subtle differences between the complexes.

An alternate hypothesis for the inhibition is that cdk2 kinase is inhibited preferentially through flavopiridol inhibition of the cak activation of cdk2. A decrease in activity of the cdk7 subunit in the cak complex would directly cause a decrease in cdk2

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activity. This could be determined by immunoprecipitating both the cdk2 and cdk7 enzymes of cells treated with varying concentrations of flavopiridol to determine the degree of inhibition of each enzyme. This would determine if cdk7 inhibition is required for cdk2 inhibition. Addition of flavopiridol directly to the immunoprecipitated cdk7 will show a direct inhibition. However, absence of effects on cdk7 would suggest that flavopiridol acts on cdk2, either directly or through upregulation of $p21^{WAF1/CIP1}$.

Measurements of cdk2 activity showed lower activity present in DU145, Saos2 and HSF55 cells and a higher activity in MCF7 and HeLa cells. There were different levels of immunoprecipitated cdk2 proteins and this may be a reflection of the cell cycle rates of the cells. In the slower growing cells, the cdk2 activities were lower, yielding counts of less that 500cpm. This was especially true with Saos2 cells. With readings this low, any inhibition of activity would be muted. These low activities lead to lower and less precise activity readings, but can be overcome by harvesting more cells to yield higher cdk2 protein. However, we found a consistent decrease in cdk2 activity in all these samples with flavopiridol treatment, except in Saos2 cells. More studies will need to be done on the Saos2 cells to determine if the cdk2 kinase complex is resistant or if the absence of inhibition is due to the lack of sensitivity of the assays done in this study. Should the cdk2 from Saos2 cells show resistance, it may be due to a mutation in the enzyme that renders it unable to bind to flavopiridol. Cloning and sequencing of the cdk2 gene would then reveal any variation in the sequence.

The effects of flavopiridol on cdk2 can be examined in MCF7 and HeLa cells as the kinase assays yielded high activities. Treatment of these cells with flavopiridol showed a distinct difference in the responses of the cdk2 kinases from MCF7 and HeLa

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cells. Flavopiridol suppressed enzymatic activity by about 95% in MCF7 cells, and this was as effective as the inhibition produced by sodium butyrate through upregulation of $p21^{WAF1/CIP1}$ (Table 5). This suggested that the mechanism of inhibition may be through this cki. However, it is possible that flavopiridol can directly inhibit cdk2, as was shown by an 80% decline in activity (Table 6). Since the concentration of flavopiridol used in both experiments was the same (50nM), the inhibition was expected to be more effective when added directly to the kinase. When added to cells, flavopiridol would bind to other molecules in the cells such as lipids, RNA and DNA and to other cdks, thus limiting the amounts available to inhibit cdk2. When added directly to isolated cdk2, the inhibitor would not be diluted out by other molecules and should have a direct inhibitory effect on the cdk2 kinases. With cdk2 isolated from HeLa cells, flavopiridol was not as effective an inhibitor. When cdk2 was immunoprecipitated from cells that were incubated with flavopiridol, the reduction in activity was only by about 50%, even though there was an upregulation of $p21^{WAF1/CIP1}$. Although a greater level of inhibition was seen when added directly to cdk2, there was only a 65% reduction in activity. It is not clear why the cdk2 kinase has such different response between the 2 cell lines. It is possible that some differences in the amino acid sequence of the enzymes may contribute to their differences in response to inhibition by flavopiridol.

Synergistic effects of flavopiridol

In the grand scheme of medical applications, why does it even matter how cdk2 is inhibited, as long as it inhibits cell growth, which was the initial goal of developing flavopiridol as an anticancer agent? The mechanisms of a drug's activity and not just its

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results are the crucial elements in understanding how to use it effectively and to determine if it has potential unexpected side effects. If flavopiridol were most effective when binding to cdk2 directly as it was originally intended, then the large array of other mechanisms of this cdk inhibitor in a tumor would hinder flavopiridol's potency, and the rate at which the body clears the drug would also affect its anticancer effects. Pharmacokinetic studies showed a variation of up to 10-fold can exist between different individuals (101). A high clearance rate would reduce its ability to inhibit the cdk2 in the tumors. Being familiar with a chemical's mode of action enables us to apply it in situations where it is most likely to be effective, for instance in conjunction with a particular drug.

It has been shown that flavopiridol has synergistic effects with a wide variety of other chemotherapeutic agents when used in a sequence-dependent manner. It has been suggested that either paclitaxel, topotecan, doxorubicin and etoposide should be given as the initial treatment preceding flavopiridol (15;139). After damage to the cell has occurred with the primary agent, flavopiridol could then inhibit the transcription of repair enzymes, causing the cells to initiate apoptosis. 5-FU, trastuzumab and cisplatin are some drugs that do not require this sequence-dependency with flavopiridol (15;61;139;140). The precise reason why these compounds are not sequence-dependent with flavopiridol is not yet known. Currently flavopiridol is co-administered with other chemotherapy agents in clinical trials. It has been reported that flavopiridol could be used as a radio-sensitizer in radiation therapy (94).

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Conclusions

The concentrations of flavopiridol found to reduce proliferation of each cell line to 50% of the untreated control were not cytotoxic over the incubation periods used in this study (1-3 days). It was my intention to avoid inducing apoptosis in order to isolate flavopiridol's effects on cdk2 inhibition and cell cycle arrest without the interference of other pathways. I believe this was achieved because even over a 3 day incubation period, apoptosis was not detected. Bcl-2 protein levels did decrease after 24hrs of flavopiridol treatment but apoptosis was still not induced after 72hrs at the IC_{50} concentrations (40-70nM).

One of the goals of this study was to determine whether the primary mechanism of flavopiridol's action to induce cell cycle arrest was through inhibition of cdk2 or through some other mechanism. It is clear that flavopiridol does not fully inhibit cdk2 activity in most of these tissue cultured cell lines at IC_{50} concentrations with the exception of the MCF7 cell line. Even when immunoprecipitated cdk2 was treated directly with flavopiridol, cdk2 was not fully inhibited in all cell lines. Therefore, the main mechanism of flavopiridol-induced cell cycle arrest is not through cdk2 inhibition in most cell lines.

It is still unclear as to whether the inhibition of cdk2 by flavopiridol is only through direct binding to the kinase's ATP pocket. Upregulation of $p21^{WAF1/CIP1}$ in MCF7, HeLa and HSF55 cells suggests that inhibition of cdk2 activity may not be reliant solely on direct inhibition by flavopiridol, but also through indirect means. The absence of *in vitro* inhibition of cdk2 and lack of upregulation of $p21^{WAF1/CIP1}$ in p53-null Saos2 cells, combined with the incomplete inhibition of the kinase in p53 mutant DU145 and

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HeLa cells suggests that $p21^{WAF1/CIP1}$ is at least partially responsible for flavopiridol-induced cdk2 inhibition and that the upregulation of $p21^{WAF1/CIP1}$ is p53-dependent in flavopiridol-treated cells.

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