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**Preclinical Evaluation of a Small  
Molecule CDK2 Inhibitor in  
Combination with Ionizing Radiation**

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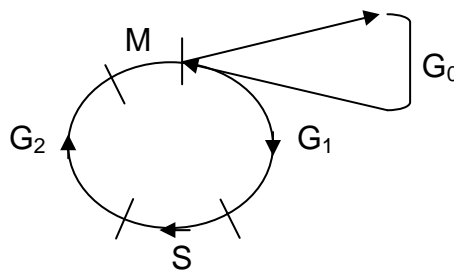
This project would not have been possible without Pharmacia, who provided the CDK2 inhibitor PHA533533 used in these experiments.

I would like to declare that, except where assistance was previously mentioned, this report is entirely my own work.

## Section 1: Introduction

### 1.1 Cell Cycle

During the cell cycle, the two major stages of activity are S-phase (synthesis) and M-phase (mitosis) (Figure 1). The cell duplicates its DNA during S-phase, while during M-phase the two sets of chromosomes separate into two daughter cells. The gap phases preceding both S-phase and M-phase –  $G_1$  and  $G_2$  respectively – ensure that cellular division is advancing correctly, allowing time for accumulation of critical cellular components like nutrients and growth factors. Unless stimulated by mitogens, most cells in the body are not actively replicating and therefore remain out of the cell cycle in quiescence ( $G_0$  phase).



**Figure 1:** The cell cycle

There are numerous internal checkpoints throughout the cell cycle, allowing the cell to monitor the completion of one phase before proceeding to the next (McDonald and El-Deiry 2000). Major checkpoints include the  $G_1$  checkpoint, the DNA damage checkpoints ( $G_1/S$ , S and  $G_2/M$ ) as well as a mitotic spindle checkpoint during M-phase (McDonald and El-Deiry 2000). As discussed in the following sections, proteins involved in maintaining checkpoints are often mutated in cancer and are currently being explored as targets for novel cancer therapies (Swanton 2004).

## 1.1.1 Cell cycle regulation

### 1.1.1.1 Cell cycle components - cyclin dependant kinases/cyclins/Rb-Pathway

The cyclin dependent kinases (CDK) are a family of constitutively expressed Serine/Threonine protein kinases and are at the centre of cell cycle control. CDKs become activated when bound to their cyclin subunits – a family of proteins whose expression is regulated during different phases of the cell cycle. Active CDK molecules phosphorylate specific proteins and thereby regulate cell cycle progression. Each cyclin associates with one or two CDKs while most CDKs associate with one or two cyclins (Murray 2004). Table 1 lists the major CDK-cyclin complexes and their cell cycle phases of activity.

**Table 1<sup>a</sup>:** CDK/cyclin binding partners and cell cycle activity

CDK	Cyclin binding partner	Cell cycle activity
CDK1	A	G <sub>2</sub> and M
CDK1	B	M
CDK2	E1, E2	Late G <sub>1</sub> to S.
CDK2	A	S and G <sub>2</sub>
CDK3	?	G <sub>1</sub>
CDK4	D1, D2, D3	G <sub>1</sub> and S
CDK5	p35 (non-cyclin activator)	Post-mitotic neurons
CDK6	D1, D2, D3	G <sub>1</sub> and S
CDK7	H (complex referred to as CAK)	All
CDK8	C	All
CDK9	T1, T2a, T2b	All

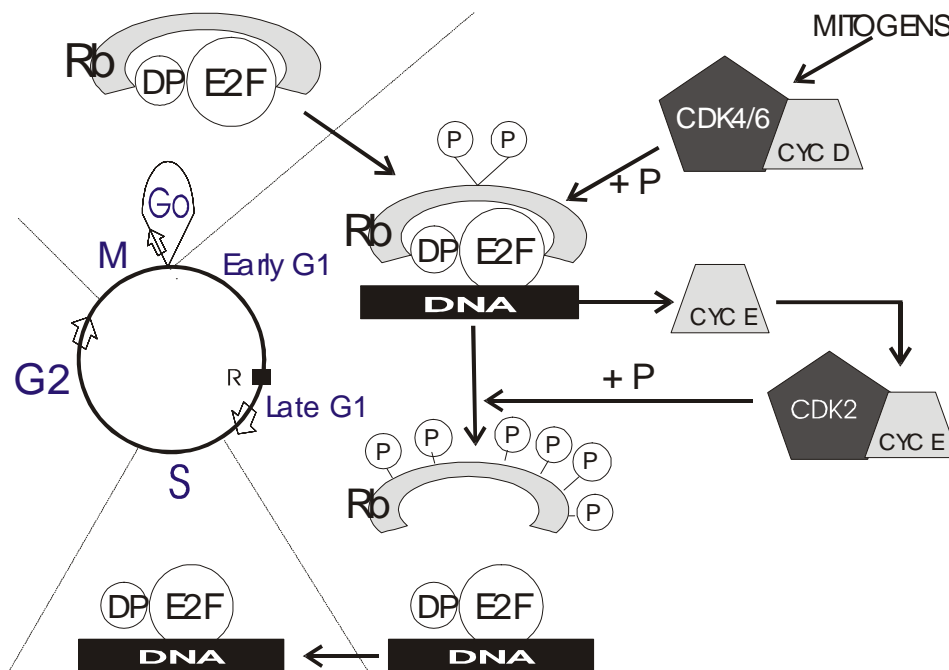
a; adapted from (McDonald and El-Deiry 2000)

A major target for CDK phosphorylation at the G<sub>1</sub> checkpoint is the retinoblastoma nuclear phosphoprotein (Rb), which arrests cells in G<sub>0</sub> and early G<sub>1</sub> by repressing the transcription of genes necessary for G<sub>1</sub> to S-phase transition (Harbour and Dean 2000). Rb exists in various stages of phosphorylation; from hypo-phosphorylated during G<sub>0</sub> (repressive) to hyper-phosphorylated (non-repressive) at the G<sub>1</sub> checkpoint. In quiescence, Rb significantly represses transcription through binding of E2F – a

transcriptional activator with binding sites in the promoters of multiple genes involved in cell cycle progression (Harbour and Dean 2000). There are various members of the Rb and E2F protein families; these members interact in many different combinations at different stages in the cell cycle (Dyson 1998). E2F proteins exist as heterodimers with members of the DP protein family, while E2F-1/DP-1 and Rb are the major players during the G<sub>1</sub>/S transition.

### 1.1.1.2 Transition through the cell cycle

Transition from G<sub>1</sub> to S-phase involves the phosphorylation of Rb by CDK4/6-cyclin D and by CDK2-cyclin E to completely disable Rb/E2F binding (Lundberg and Weinberg 1998) (Figure 2).



**Figure 2:** The Rb-pathway

Via receptor mediated signalling through the RAS/RAF/MAPK pathway, extracellular mitogenic stimulation initiates the synthesis of D-type cyclins and their assembly with CDK4/6 in the cell's nucleus (Marshall 1999). There are tissue specific expression patterns of the different D-type cyclins (D1, D2 and D3) together with CDK4

and CDK6 – yet the CDK4-cyclin D1 complex has been most heavily studied to date (Sherr and McCormick 2002). CDK4/6-cyclin D phosphorylates Rb, partially releasing the Rb-bound E2F and thereby allowing the transcription of specific cell cycle proteins. One such protein is cyclin E1, which associates with CDK2 from late G<sub>1</sub> into S-phase (Table 1, Figure 2). The CDK2-cyclin E1 complex completes the phosphorylation of Rb at a site differing to that of CDK4/6-cyclin D (Lundberg and Weinberg 1998). It has been recently shown however, that in cancer cells complete phosphorylation of Rb can be undertaken solely by either CDK2-cyclin E (Keenan et al. 2004) or CDK4-cyclin D (Tetsu and McCormick 2003), rendering the alternative pathway dispensable for G<sub>1</sub> progression.

There is a juncture in late G<sub>1</sub> from when cellular proliferation will occur independently of mitogenic stimuli: this is called the Restriction point ('R') (Figure 2) and is often used to separate early and late G<sub>1</sub> (Malumbres and Barbacid 2001). It is believed that 'R' demarcates the time from when Rb phosphorylation has overcome a certain threshold, CDK4-cyclin D is no longer required and the cell commits to the cell cycle. It is widely accepted (although not entirely) that the restriction point and the G<sub>1</sub> checkpoint are synonymous (Swanton 2004).

In late G<sub>1</sub>, E2F also promotes the transcription of cyclin A2, which then forms a complex with CDK2 during S-phase (McDonald and El-Deiry 2000). Before entering G<sub>2</sub>, the CDK2-cyclin A2 complex binds with E2F and then phosphorylates DP-1 to prevent further DNA binding (Krek et al. 1995). Towards the end of S-phase and throughout G<sub>2</sub> and M-phase there is an increase in activity of CDK1-cyclin A and CDK1-cyclin B, both of which are involved in the induction of mitosis (Jackman and Pines 1997). CDK1-cyclin A drive the cell through G<sub>2</sub> in preparation for mitosis while CDK1-cyclin B1 is the only active mitotic CDK-cyclin complex.

CDK-cyclin activity is terminated when cyclin levels decrease. All known cyclins are targeted for destruction by the proteasome following the addition of a chain of ubiquitins (Murray 2004). For example, active CDK2-cyclin E autophosphorylates cyclin E on threonine 380 at the beginning of S-phase, targeting it for ubiquitin mediated degradation (Clurman et al. 1996).

### 1.1.1.3 Activation and inhibition of CDK proteins

The CDK catalytic core is found in the cleft between its two lobes (De Bondt et al. 1993). When cyclin binds the CDK, conformational changes allow for CDK activation (Morgan 1995). A structure called the T-loop is initially displaced, exposing the catalytic core for ATP and substrate attachment. Moreover, T-loop displacement allows access to its conserved Threonine residue (T<sub>160</sub> on CDK2), which requires phosphorylation by CDK7-cyclin H (the CDK-activating kinase [CAK]) to become catalytically competent (Morgan 1995). Deactivation of CDK2 can be achieved through phosphorylation of the conserved threonine (Thr14) and tyrosine (Tyr15) residues (or equivalent residues on other CDKs), located in the glycine rich loop that forms the roof of the ATP binding site (De Bondt et al. 1993).

Throughout the cell cycle, CDK activity is inhibited by two different families of cyclin dependent kinase inhibitors (CKI), the INK4 (Inhibitors of CDK4) family and the CIP/KIP family (Sherr and Roberts 1999). The INK4 family, which consists of p16<sup>INK4a</sup>, p15<sup>INK4b</sup>, p18<sup>INK4c</sup>, p19<sup>INK4d</sup>, only bind to and inhibit cyclin D-dependent kinases (CDK4 and CDK6) to prevent Rb phosphorylation and maintain the G<sub>1</sub> checkpoint. The CIP/KIP inhibitors - p21<sup>CIP1</sup>, p27<sup>KIP1</sup>, and p57<sup>KIP2</sup> - are more broadly acting, inhibiting the activity of cyclin A- and E-dependent kinases, binding both the cyclin and CDK molecules when inhibiting the CDK-cyclin complex. Both p21 and p27 are potent inhibitors of CDK2 (Toyoshima and Hunter 1994; Harper et al. 1995).

CDK4-cyclin D complexes sequester p27 and p21 in proliferating cells (Sherr 1996). This promotes G<sub>1</sub> transition in two ways: while p27 (and p21) binding promotes assembly and stability of the CDK4-cyclin D complex (LaBaer et al. 1997), it also relieves CDK2-cyclin E of inhibition, allowing for increased activity and unperturbed G<sub>1</sub> transition. Furthermore, active CDK2-cyclin E phosphorylates p27, targeting it for ubiquitin mediated degradation (Vlach et al. 1997).

p53 is a transcription factor induced in response to cellular stress like DNA damage and hypoxia, and regulates gene expression that leads to cell cycle arrest or apoptosis (Levine 1997). Cell cycle arrest can ensue through transactivation of the CIP/KIP CKI, p21<sup>CIP1</sup>, by wild type p53, causing G<sub>1</sub>/S checkpoint arrest through the inhibition of CDK2-cyclin E (Dulic et al. 1994).

### 1.1.2 Cell cycle mutations in cancer

Most human tumours have genetic defects associated with cell cycle control (Senderowicz 2003). Whether these defects are tumour suppressor or oncogenic mutations, they eventually lead to uncontrolled and unchecked cellular proliferation, a hallmark of human neoplasms (Sherr and McCormick 2002). Mutations involving G<sub>1</sub> checkpoint associated proteins often contribute to uncontrolled tumour proliferation; the two major aberrant pathways are the p53 and Rb pathways.

Mutations of the tumour suppressor gene encoding p53 is the most common genetic aberration in human tumours, mutated approximately 50% of the time (Levine 1997). Thus, p53-induced expression of p21 in response to DNA damage is impaired, resulting in the deactivation of the G<sub>1</sub> DNA-damage checkpoint through failure to inhibit the CDK2-cyclin E complex.

The Rb pathway (cyclin D1/CDK 4/p16<sup>INK4A</sup>/Rb) is also frequently deregulated in human tumours (Sherr 1996) and is similarly capable of abrogating the G<sub>1</sub> checkpoint. Common mutations include either overexpression of cyclin D1, repression of p16<sup>INK4</sup> function or direct inactivation of the Rb tumour suppressor (although less common). Table 2 summarises the Rb-pathway mutations commonly seen in selected human neoplasms.

**Table 2<sup>a</sup>:** Rb pathways mutation in cancer

<b>Cancer type</b>	<b>INK4a loss</b>	<b>CDK4-cyclin D1 overexpression</b>	<b>Rb loss</b>
Small cell lung cancer	15%	5%	80%
Non-small cell lung cancer	58%		20-30%
Pancreatic cancer	80%		
Breast cancer	30%	>50% cyclin D1	

a; adapted from (Sherr and McCormick 2002)

### **1.1.2.1 CDK2 related cell cycle mutations**

Mutations involving CDK2 and its cyclin/CIP-KIP regulators are less frequently described and believed to be less common than of other CDKs (Malumbres and Barbacid 2001). Nevertheless, tumour types wherein these alterations manifest may be optimal targets for CDK2 inhibition per se. For example, in breast cancer patients it has been discovered that increased levels of cyclin E and decreased levels of p27 are both strongly predictive of heightened mortality (Porter et al. 1997). However, these changes may in fact be a result of altered post-transcriptional mechanisms, rather than a mutated chromosome.

## **1.2 Ionizing Radiation (IR) and Cancer Treatment**

Ionizing radiation (IR) is a major form of cancer therapy. Surgery has been replaced by radiation for long term control of many cancers (including head and neck, bladder, skin, prostate and cervix), regardless of whether the intent is either curative or palliative (Steel 2002b). 50% of patients will receive radiotherapy as part of their treatment.

Cell killing induced by IR is due to the generation of free radicals and resultant DNA damage (McMillan and Steel 2002). IR produces free electrons, which break down water molecules into hydrogen ions (H<sup>+</sup>) and highly reactive free radicals with an unpaired electron (OH<sup>•</sup>). These free radicals react with DNA to create single stranded (SSB) and double stranded (DSB) breaks. An increase in radiation dose leads to an increase in the incidence of DNA DSBs, identified as the critical lesion for radiation induced cell killing in most cell types (McMillan and Steel 2002). Exposure to IR also activates numerous cellular pathways, including cell cycle arrest, DNA repair, apoptosis and proliferation.

Cellular radiosensitivity varies throughout the cell cycle according to a conserved pattern. Based on early studies done with chinese hamster cells, it was discovered that cells are most resistant to IR during mid-late S-phase, most sensitive during G<sub>2</sub>/M-phase, while are moderately radiosensitive during G<sub>1</sub> (Steel 2002a).

### 1.2.1 The cellular response to DNA damage

The DNA-damage response to IR is elicited as follows: *sensor* molecules recognise DNA damage (probably through direct DNA interaction) and signal downstream to the *transducers*, a group of proteins, which include kinases. Through complex transducer interactions, the signal is amplified and a response is elicited from the *effector* molecules; the outcome being cell cycle arrest, and followed then by either DNA repair or apoptosis (Fei and El-Deiry 2003).

This sequence of events is primarily undertaken at the three major cell cycle DNA-damage checkpoints: G<sub>1</sub>/S, S and G<sub>2</sub>/M (Iliakis et al. 2003). Although each checkpoint has its own distinct set of protein interactions and signaling cascades, several of the major proteins/pathways involved are conserved between them.

Central to the DNA damage response are two related and highly conserved tyrosine kinases – ATM (Ataxia Telangiectasia Mutated) and ATR (Ataxia Talangiectasia Rad3 Related). They are both members of the PI3K-related kinases and are believed to act as transducer proteins, immediately downstream of the DNA damage sensors (Shiloh 2001). Individuals with a mutant ATM gene have Ataxia Telangiectasia (A-T) – a disease which causes cerebellar ataxia, immune deficiency, significantly higher incidence of cancer and heightened sensitivity to IR (Rotman and Shiloh 1999).

Mechanisms of ATM activation are still largely unknown and are currently under investigation (Iliakis et al. 2003). The DNA-damage response elicited by ATM is, however, more clearly understood; following DNA damage, ATM is responsible for the initial phosphorylation of a number of key proteins involved in DNA repair, cell cycle arrest and apoptosis, including Mdm2, BRCA1, p53, Chk2 and Wbs1 (Zhou and Elledge 2000). ATR (Ataxia Talangiectasia Rad3 Related) is less thoroughly understood, partly due to the absence of ATR mutations in human disease (Zhou and Elledge 2000). However, expression of dominant negative ATR<sup>-/-</sup> sensitises mammalian cells to many forms of DNA damage (including UV radiation and IR), and decreases the G<sub>2</sub>/M checkpoint response induced by IR (Wright et al. 1998). Although ATM and ATR substrates largely overlap, ATM is more predominant in the response to IR, while ATR appears as backup and is more relevant in response to UV radiation or treatment with agents that interfere with DNA replication (eg: hydroxyuria) (Abraham 2001).

One major function of ATM is stabilization of the p53 transcriptional activator – an important DNA damage checkpoint protein (Fei and El-Deiry 2003). As mentioned previously, transactivation of p21 by wild type p53 causes G<sub>1</sub>/S checkpoint arrest through the inhibition of CDK2-cyclin E (Dulic et al. 1994), allowing time for DNA repair or apoptosis.

### **1.2.1.1 Apoptosis following ionizing radiation**

Several cytoplasmic and nuclear signalling pathways are involved with DNA damage-induced apoptosis. The decision to arrest or induce apoptosis is made through complex interactions between many factors, including oncogenic characteristics, cell type, DNA repair efficiency and final integration of incoming external signals and pathways (Sionov and Haupt 1999).

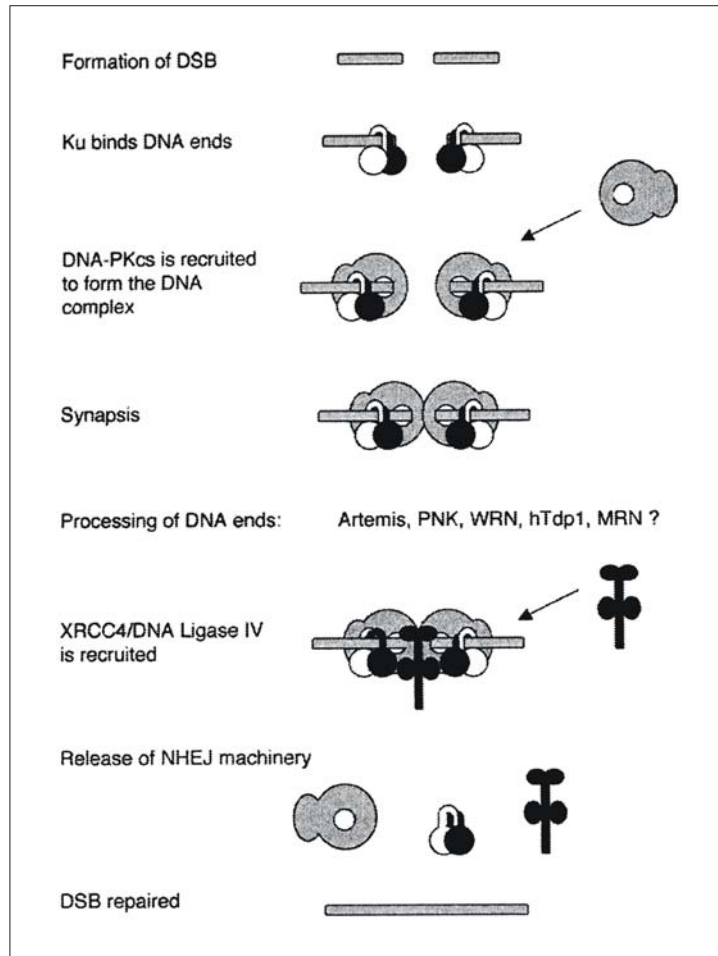
This complexity has been extensively studied in relation to the cellular response to p53 activation following IR. Once the decision has been made to kill the cell, p53 promotes the transcription of multiple proapoptotic proteins/processes (eg, Bax and Fas/Fas ligand) while at the same time represses certain survival promoting and anti-apoptotic genes (eg, Bcl-2) (Sionov and Haupt 1999). Cells which are mutant for p53 have increased resistance to IR-induced apoptosis.

### **1.2.1.2 Double stranded break repair pathways following IR**

There are two major DSB repair pathways, Non-Homologous End Joining (NHEJ) and Homologous Recombination (HR) (Karran 2000). Mammalian cells seem to rely more heavily on NHEJ than HR for DSB repair, although in late S-phase and G<sub>2</sub>, HR is the preferential repair method (Karran 2000).

NHEJ does not require existing homology between the two DNA ends to be joined. Initially, Ku protein heterodimers (Ku70:Ku80) recognise the DSB and bind to the two DNA ends (Figure 3) (Lees-Miller and Meek 2003). This facilitates the interaction of DNA-PKcs with the Ku heterodimers to form the DNA-PK catalytic complex. DNA-PK belongs to the same protein kinase family as ATM and ATR, and catalyses the rejoining procedure. Following DNA-PK formation, the two DNA ends are

joined and processed before the XRCC4:DNA ligase IV heterodimer is recruited for ligation (Figure 3).



**Figure 3:** The NHEJ DNA repair pathway (Lees-Miller and Meek 2003)

While the relationship between DNA damage checkpoints and repair is speculative, Iliakis et al. (Iliakis et al. 2003) suggests that the kinetics of the HR process are much more aligned with DNA damage checkpoint arrest, than the extremely efficient NHEJ pathway. The authors propose that following DSB, NHEJ occurs first to restore the genome continuity while HR follows to restore the sequence around the break and general DNA fidelity. The function of the checkpoints would therefore be to delay the transition and maybe align the cell cycle to a phase where optimal repair may occur.

### **1.2.2 Radiation-induced cellular signaling pathways**

IR has also been found to influence the activity of other cell signalling pathways not involved in a DNA damage response. Following IR, surviving cells may experience activation of cellular pathways that promote increased proliferation and enhanced anti-apoptotic mechanisms (Dent et al. 2003). On such pathway is the pro-proliferative epidermal growth factor tyrosine kinase receptor (EGFR) – overexpressed in many epithelial tumours – and the mitogen activated protein kinase (MAPK) pathway downstream (Mendelsohn and Baselga 2000). Ionizing radiation activates EGFR signaling, while also enhancing the cleavage of pro-TGF $\alpha$  to active TGF $\alpha$  (an autocrine EGFR ligand) in a number of cell lines (Schmidt-Ullrich et al. 1997), potentiating tumour cell proliferation.

### **1.3 Molecular Targeted Therapies**

Tumour development occurs as a result of the accumulation of genetic aberrations. These aberrations lead to altered expression of three types of genes: activation of oncogenes, inactivation of tumour suppressor genes (gatekeepers) and impairment of DNA repair genes (caretakers) (Mutch 2000). Failure of DNA repair gene-function contributes to genetic instability and mutations in the cell genome. Activation of oncogenes results in a gain-of-function and promotes tumour cell proliferation. Tumour suppressor genes generally monitor cell growth and replication and are often inactivated in cancer cells.

These mutations lead to changes in signal transduction, cell cycle control, apoptosis, invasion, angiogenesis and metastasis (Garrett and Workman 1999). Understanding of these pathways disrupted in cancer has led to the identification of specific targets for therapeutic intervention; of significance, protein kinases have been discovered as major pharmacological targets, as they play an essential role in all molecular aspects of the cell life (Cohen 2002). Several different molecular targeted therapies are either currently in clinical practice or under development.

The pioneer molecular targeted drug to reach the clinic is the small molecule kinase inhibitor Glivec (imatinib), used to treat chronic myelogenous leukemia (CML). In CML a reciprocal translocation between chromosomes 22 and 9 results in the production of the

bcr-abl fusion protein – the defining causative molecular abnormality during its early stages (Druker 2002). Bcr-abl drives pathogenesis through its enhanced tyrosine kinase activity – promoting increased proliferation, genetic instability and protection from apoptosis (Deininger et al. 2000). Glivec inhibits the tyrosine kinase activity of the abl portion of this protein, binding to ATP docking site. Although this malignancy is unique in having one major genetic mutation, its success was largely in proving the concept of molecular targeted therapy.

Herceptin (trastuzumab) is the only molecular targeted therapy for breast cancer routinely used in the clinic (Atalay et al. 2003). It is a monoclonal antibody that binds to and inhibits the activation of the tyrosine kinase HER-2 (ErbB2), an oncogenic cell surface receptor, overexpressed in 20-25% of breast tumours (Atalay et al. 2003).

Iressa (gefitinib) is a small molecule inhibitor of the tyrosine kinase EGFR (ErbB1), overexpressed in many solid tumours (Blackledge and Averbuch 2004). It recently received approval for the treatment of previously treated advanced non-small cell lung carcinoma (NSCLC) in Japan, the USA and other countries, and has shown efficacy as a single-agent as well in combination with conventional therapies (Blackledge and Averbuch 2004).

#### **1.4 The Cell Cycle as a Target for Therapeutic Intervention in Cancer**

When targeting the aberrant cancer cell cycle, there are three major objectives: to restrain proliferation, to sensitise the cells to the anti-tumour effects of cytotoxics (discussed in Section 1.5) or to take advantage of hyperproliferation to induce apoptosis (Fischer and Gianella-Borradori 2003). The cyclin dependant kinases are at the heart of the cell cycle machinery. Their intrinsic involvement with each stage of the cell cycle and its checkpoints implicate them as a suitable target to achieve the aforementioned aims of tumour suppression (Knockaert et al. 2002).

### 1.4.1 CDK inhibitors in development

Over the past decade there have been significant advances in the development of small molecule CDK inhibitors. Screening has yielded numerous compounds, yet only four molecules that target the CDK ATP-binding domain (and subsequently prevent ATP-mediated activation) have been clinically evaluated. These are Flavopiridol (FPD), 7-Hydroxystaurosporine (7HS), Roscovitine (ROS) and an aminothiazole compound (Fischer and Gianella-Borradori 2003). Considering that these compounds are competitive inhibitors of ATP, problems with kinase specificity manifest – this is because 850+ kinases discovered to date have relatively conserved ATP-binding domains. As evident in Table 3, ROS, FPD and 7HS interact with multiple kinases, making it difficult to elucidate whether certain anti-tumour properties are directly related to CDK inhibition.

**Table 3<sup>a</sup>:** In vitro potency and selectivity profiles of CDK inhibitors

<b>Kinase Inhibition IC<sub>50</sub> (uM)<sup>b</sup></b>	<b>FPD</b>	<b>7HS</b>	<b>ROS</b>
CDK1-cyclin B	0.03-0.04	0.03	2.7
CDK2-cyclin A	0.1	0.03	0.7
CDK2-cyclin E	0.1	?	0.1
CDK4-cyclin D1	0.02-0.04	0.03/3.6 <sup>c</sup>	14
CDK7-cyclin H	0.11-0.3	?	0.5
CDK9-cyclin T1	0.006-0.01	?	0.06
Other Kinases	0.45(GSK-3), 6(PKC), >15(EGFR-TPK, p60 Src, ERK-1)	0.006(CHK1), 0.004 (PKCs), 0.033(PDK1), 0.04 (PKA), 0.05(p60 Src), 0.62 (Raf-1), 0.91(MAPK)	1.2(ERK-2)

a; adapted from (Fischer and Gianella-Borradori 2003). b; ATP concentration used is usually 10 $\mu$ M. c; two different references

#### 1.4.1.1 Flavopiridol (FPD) – the pioneer

FPD was the first drug developed as a CDK inhibitor to reach clinical trials (Matranga and Shapiro 2002). It is structurally related to a compound derivative of an indigenous Indian plant, *Dysoxylum binectariferum* (Sedlacek 2001). FPD is a pan-CDK inhibitor (see Table 3), which not only mediates cell cycle arrest but promotes apoptosis and differentiation, inhibits transcription, and has anti-angiogenic properties (Senderowicz 2003).

FPD induces cell cycle arrest through different mechanisms. A number of breast and lung carcinoma cell lines experienced reversible G<sub>1</sub> and G<sub>2</sub> cell cycle arrest following FPD treatment in vitro, attributable to CDK inhibition (Kaur et al. 1992; Carlson et al. 1996). As a pan-CDK inhibitor, FPD can also prevent CDK7-cyclin H (CAK) from activation phosphorylation of CDK2 (T<sub>160</sub>) and CDK4 (T<sub>161</sub>) (Sedlacek 2001). An alternative mechanism of FPD-induced arrest occurs through direct transcriptional repression of the cyclin D1 oncogene, preventing Rb phosphorylation by the CDK4-cyclin D1 complex (Carlson et al. 1999).

FPD induces apoptosis in various cell lines. A panel of 7 NSCLC cell lines all underwent apoptosis after 72-hour FPD exposure, regardless of p53 status or whether the cells were actively cycling (Shapiro and Harper 1999). Haematopoietic (Parker et al. 1998) and head and neck (Patel et al. 1998) carcinoma cell lines are also quite sensitive, yet mechanisms of FPD-induced apoptosis are still unclear. In addition, FPD can act as an anti-angiogenic agent, as it inhibited hypoxia-induced endothelial growth factor expression in human monocytes (Melillo et al. 1999).

A phase I study of FPD as monotherapy found that pan-gastrointestinal (GIT) symptoms – including nausea/vomiting and diarrhoea – were the dose limiting toxicities (Senderowicz et al. 1998). The same dosing regime of 72 hour continuous infusions every 2 weeks was applied to three different phase II studies (Stadler et al. 2000; Schwartz et al. 2001; Shapiro et al. 2001). The only positive result was a 6% response rate (2/35) for the metastatic renal cancer patients (Stadler et al. 2000). Accompanying the GIT symptoms seen in the phase I study were increased incidents of asthenia and vascular thromboses. These results led to a different phase I dosing regime of 1hr infusions (for 1, 3 or 5 consecutive days) every three weeks (Tan et al. 2002). The dose limiting toxicity this time was neutropenia, while GIT symptoms were still present with reduced severity. 12 patients had stable disease for  $\geq 3$  months. To date, one phase II study on patients with mantle-cell lymphoma has been conducted using this regime – 1hr infusion for 3 consecutive days, every 3 weeks (Kouroukis et al. 2003). Following treatment, 82% of patients experienced either a partial response or stable disease for an average of 3.3 months. GIT symptoms were the major toxicities.

#### 1.4.1.2 7-Hydroxystaurosporine (7HS)

As evident in Table 3, 7HS (or UCN-01) has a wide array of kinase targets. Similar to FPD, it induces cell cycle arrest and apoptosis, yet also inhibits  $\text{Ca}^{2+}$ -dependent Protein Kinase C isoenzymes (PKC) and abrogates the DNA damage  $\text{G}_2/\text{M}$  checkpoint (Senderowicz 2002). It is an analogue of the staurosporine class of kinase inhibitors, which generally have multi-kinase inhibitory activities. However, the anti-proliferative and apoptosis-inducing capabilities of 7HS are believed to be anti-PKC independent (Wang et al. 1995).

7HS induces a  $\text{G}_1$ -block in HNSCC and A431 squamous cell carcinoma lines in vitro (Akiyama et al. 1997; Patel et al. 2002), which is associated with hypophosphorylated Rb and elevated levels of p27 and p21. These results were also reproduced in an HNSCC HN12 xenograft model (Patel et al. 2002). Cell cycle effects resulted in decreased activity of  $\text{G}_1$  CDK molecules (CDK2/CDK4), albeit not through direct binding of 7HS to the CDK ATP docking site. Direct mechanistic involvement of CDKs in the anti-tumour effects of 7HS still needs to be fully elucidated.

7HS is also capable of abrogating the  $\text{G}_2/\text{M}$  DNA-damage checkpoint, a feature likely to sensitise cells to conventional cytotoxics (discussed in Section 1.5) (Swanton 2004). Potent inhibition of the Chk1 and Chk2 kinases by 7HS may elicit checkpoint abrogation, as seen in HeLa and HCT-116 colon carcinoma cells (Graves et al. 2000; Yu et al. 2002). As a result, Chk1/Chk2 cannot phosphorylate and deactivate Cdc25C following DNA damage. Active Cdc25C is responsible for promotion of  $\text{G}_2/\text{M}$  transition by removing inhibitory phosphorylations from CDK1.

One phase I study with 7HS has been fully completed (Sausville et al. 2001). The compound was administered as a 72-hour continuous infusion every 2 weeks. Dose limiting toxicities were hyperglycemia with nausea/vomiting, metabolic acidosis and pulmonary dysfunction. One partial response was observed (~8 months), while one patient with anaplastic large cell lymphoma is still disease free 4 years later. A phase II dosing/scheduling regime was recommended (Sausville et al. 2001). However, due to slow clearance and an extremely long half life (447-1176h), short term infusions (1-3hr) are now also being examined (Fischer and Gianella-Borradori 2003).

### **1.4.1.3 Roscovitine (ROS)**

Roscovitine is a purine analogue, the only compound of this class to have reached clinical evaluation. A major point of distinction between ROS and FPD/7HS is its enhanced selectivity for CDK1 and CDK2 inhibition, compared with other CDK molecules (McClue et al. 2002). It also appears to have fewer multi-kinase interactions.

ROS displays marked anti-proliferative effects *in vitro* on both cancer cells (Mgbonyebi et al. 1998) and normal human fibroblasts (Alessi et al. 1998). The latter effect was completely reversible and associated with G<sub>1</sub> arrest through inhibition of CDK2 activity. This feature has also been observed using *in vivo* models, where growth inhibition followed ROS treatment in many colorectal and human uterine carcinoma xenografts (McClue et al. 2002). Potent inhibition of CDK2-cyclin E activity was identified as a major outcome, while rapidly proliferating cells were selectively targeted.

ROS induces apoptosis in many cancer cell lines (McClue et al. 2002; Mihara et al. 2002; Wojciechowski et al. 2003). In the MCF-7 breast cancer cell line the main apoptotic wave is preceded by cell cycle arrest at the G<sub>2</sub>/M checkpoint (Wojciechowski et al. 2003). In another breast carcinoma cell line, MDA-MB-231 cells also underwent cellular events associated with apoptosis when exposed to ROS (Mgbonyebi et al. 1999). In a panel of HNSCC cells *in vitro* (Mihara et al. 2002), those which underwent apoptosis in the absence of cell cycle arrest (4/11 lines) were associated with increased expression of Bcl-X<sub>s</sub>, a proapoptotic protein from the Bcl-2 family.

The first phase I studies of ROS has delivered preliminary pharmacokinetic information (Benson et al. 2002). No toxicities were recorded, the drug had good bioavailability (unlike FPD and 7HS) and was very well tolerated by all patients.

## **1.5 Combining Molecular Targeted Drugs and Conventional Therapies**

Combination therapy, involving conventional therapies and molecular targeted drugs is a promising approach to cancer treatment. Increased cell killing without increased host toxicity, evasion of drug resistance, and biochemical synergy create the rational foundation for the use of combination therapy over monotherapy (Shah and Schwartz 2000). In addition, potential drawbacks associated with the use of specific

molecular targeted therapy as a single agent include the inability of these drugs to completely inhibit their target, as well as the redundancy of aberrant signalling pathways in late-stage cancer (Arteaga 2003).

In the case of Gefitinib, mechanistic rationale supports its use in combination with IR. IR leads to an increase in activity of the EGFR and MAPK pathway in certain tumour cell lines, potentiating tumour proliferation (Schmidt-Ullrich et al. 1997). Thus, by inhibiting EGFR with Gefitinib, IR-induced cellular proliferation can be curbed, while optimal cell killing can be achieved.

The cell cycle effects of CDK inhibitors can also be exploited when used in combination with conventional chemotherapy. In the case of paclitaxel (an M-phase specific drug), administration following FPD on the NSCLC line A549 resulted in cytotoxic antagonism, while the reverse sequence caused greater than additive cell killing (Bible and Kaufmann 1997). This may be explained by the cell cycle effects of FPD. The arrest of cells in G<sub>1</sub> following FPD treatment is expected to decrease the number of cells in M-phase, where paclitaxel exerts its cytotoxic effects (Schwartz et al. 2002).

## **1.5.1 Combining CDK inhibition with radiotherapy**

### **1.5.1.1 FPD and radiation**

FPD has been shown to enhance the radiosensitivity of OCA-I ovarian cancer cells in a dose dependent manner (Raju et al. 2003). This study found that FPD treatment resulted in decreased levels of Ku70/Ku80 proteins (required for NHEJ), suggesting impaired DSB repair capacity. Furthermore, it was proposed that the FPD-induced G<sub>2</sub>/M arrest also contributed to radiosensitivity, as the cells shifted out of the radioresistant S-phase and into the radiosensitive G<sub>2</sub> phase.

A second study found that FPD potentiates IR-induced apoptosis in the colon cancer line HCT-116 and the gastric cancer line MKN-74 (Jung et al. 2003). In vitro, the highest apoptotic levels were observed when IR preceded FPD administration (by 24h) in both cell lines. This was confirmed in vivo, where HCT-116 xenografts responded more effectively to the combination when irradiated before FPD administration.

### **1.5.1.2 7HS and radiation**

It has been suggested that abrogation of the G<sub>2</sub>/M DNA-damage checkpoint sensitises cells to the effects of DNA-damaging agents, as a result of premature and lethal mitosis (Lau and Pardee 1982). 7HS potently abrogates the G<sub>2</sub>/M checkpoint in p53-mutant cell lines, including human lymphoma CA46 cells (Wang et al. 1996) and several colorectal cell lines (Playle et al. 2002). However, while 7HS enhanced the cytotoxicity of IR in the CA46 cells (Wang et al. 1996), only 2 out of the 5 colorectal lines used (HT29 and SW486) showed increased cytotoxicity (Playle et al. 2002). In both the HT29s and SW486s, a decrease in cell yield following the combined treatment correlated with increased levels of apoptosis. It was proposed that the other 3 colorectal lines had ineffective G<sub>2</sub>/M checkpoints (perhaps due to dysfunctional DNA repair machinery).

### **1.5.1.3 Roscovitine and radiation**

A recent study (Maggiorella et al. 2003) showed that ROS can enhance radiosensitivity of p53<sup>-/-</sup> cancer cells. Using the breast cancer cell line MDA-MB231, it was found that ROS radiosensitises in vitro and in vivo (xenograft models). Similar to FPD (Raju et al. 2003), decreased clonogenic survival did not correlate with increased IR-induced apoptosis. Rather impairment of DNA double stranded break repair was observed, evident by decreases in Ku-DNA binding as well as DNA-PK activity.

## 1.6 PHA533533 – A Novel Small Molecule CDK2 Inhibitor

PHA533533 is a small molecule CDK inhibitor. As with FPD, 7HS and ROS, this drug targets the ATP-binding pocket of the catalytic site of the kinase. Table 4 indicates the biochemical IC<sub>50</sub>s of PHA533533 for difference kinase targets.

**Table 4<sup>a</sup>:** General in vitro potency and selectivity profiles of PHA533533

<b>Kinase complex</b>	<b>Biochemical IC<sub>50</sub> (μM)</b>
CDK2-cyclin E	0.071
CDK2-cyclin A	0.037
CDK1-cyclin B	0.308
CDK4-cyclin D1	>10
CDK5-p25	0.084
Gsk3b	0.690
Erk2	1.200
Other kinases	>100× selectivity Vs additional 31 kinases

a; (Mercurio et al. 2004)

In contrast to the small molecule CDK inhibitors previously described, PHA533533 is highly selective for CDK2. Potency for CDK2 inhibition compared with CDK1 and CDK4 is respectively 6 times and 200 times greater (approximately). However, the CDK5-p25 IC<sub>50</sub> is very similar to that of CDK2-cyclin E, indicating that due to conserved kinase ATP-binding domains, it is difficult to be entirely selective for any one protein.

## 1.7 Project Aims

Recent papers have shown that the use of novel small molecule CDK inhibitors in combination with IR results in anti-tumour synergy (Maggiorella et al. 2003; Raju et al. 2003). These compounds have been shown to target several CDK proteins, as well as inhibiting a number of other kinases. It is important to investigate whether a more selective agent can have increased anti-tumour efficacy in combination with IR, compared with these more ‘promiscuous’ inhibitors. This study aims to investigate whether PHA533533, a novel small molecule inhibitor selective for CDK2, is capable of sensitising cancer cells to the anti-tumour effects of ionizing radiation in vitro.

## Section 2: Materials and Methods

### 2.1 Cell culture

The H460 cell line (a human non small cell lung carcinoma) was obtained from the National Cancer Institute, USA. Cells were cultured with RPMI, supplemented with 10% fetal bovine serum (FBS, JRH Biosciences), and maintained in a humidified atmosphere of 5% CO<sub>2</sub> in air, at 37°C.

The A431 cell line (a human vulvar squamous cell carcinoma) was obtained from Dr. Francesca Walker at the Ludwig Institute, Parkville, Melbourne. These cells were maintained in  $\alpha$ -MEM and 10% FBS in a humidified atmosphere of 5% CO<sub>2</sub> in air, at 37°C.

### 2.2 Drug

PHA533533 (Pharmacia, Nerviano, Italy) was dissolved in DMSO to a concentration of 10 mM and stored at -20°C

### 2.3 Sulphorhodamine B (SRB) assays

Exponentially growing A431 and H460 cells were plated in 96 well plates (Greiner) at 1500 and 1200 cells/well respectively. Cells were left to adhere overnight after which drug was added at 10 different concentrations (in replicates of 5 wells) and then incubated for 72 hours (drug, solvent and cell controls, as well as plate blanks were also included). Following incubation cells were fixed with ice-cold 50% trichloroacetic acid (final concentration 10%) for 1 hour at 4°C. Plates were then washed vigorously in water to remove all fixative, and then allowed to dry. Once dry 100  $\mu$ L SRB solution was added to each well for 30 minutes to stain the protein. To remove unbound dye, plates were washed again in water and then briefly in 1% acetic acid before being left to dry. The dye was then solubilised by adding 100  $\mu$ L 10 mM Tris to each well and placed on a shaker for 5 minutes. Optical density of each well was read at 550 nm using an automatic plate reader.

## **2.4 Protein Analysis**

### **2.4.1 Preparation of lysates**

Cells were washed in ice-cold phosphate-buffered saline (PBS), scraped into RIPA buffer (1% NP-40, 0.5% deoxycholate, 0.1% SDS in PBS) with freshly added protease inhibitors (1 mM sodium vanadate, 1  $\mu\text{g}/\mu\text{L}$  leupeptin, 2  $\mu\text{g}/\mu\text{L}$  aprotinin), and incubated on ice for 30 minutes. The lysates were then centrifuged in a Biofuge 13 centrifuge at 13000 rpm for 10 minutes at 4°C. The supernatant was then stored at -80°C. Protein levels were quantified using a BCA protein assay kit.

### **2.4.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting**

Protein lysates were denatured in loading buffer (250 mM Tris pH 6.8, 10% SDS, 30% glycerol, 10%  $\beta$ -mercaptoethanol, 0.05% bromophenol blue) by heating to 100°C for 3-4 minutes, and were then loaded into separate wells in acrylamide-bis (7% or 15%) gels in a Mini-protein II apparatus (Biorad). Electrophoresis was carried out at 150 V for 1-2 hours in running buffer (0.25 M Tris pH 8.6, 0.5% SDS, 0.19 M glycine). Proteins were then electroblotted onto polyvinylidene fluoride membrane (Immobilon-P, Millipore) at 100 V for 1 hour in transfer buffer (0.025 M Tris and 0.192 M glycine in 10% methanol) using a Biorad Mini Trans-Blot cell. Membranes were blocked in TBS-T (tris-buffered saline [TBS, 2% 1M Tris pH 7.4, 3% 5M NaCl in distilled water] and 0.1% Tween 20) with 5% powdered milk overnight and then incubated in primary antibody at room temperature for 1 hour. Primary antibodies used were p27 (Santa Cruz, California, USA, SC-528, 1:2000, polyclonal rabbit), Rb (Zymed, San Francisco, USA, 18-0159, 1:2000, monoclonal mouse) and adaptin- $\alpha$  (Transduction Laboratories, Kentucky, USA, A43920, 1:2000, monoclonal mouse). After washing the membranes thoroughly in milk/TBS-T, the membranes were incubated in the corresponding secondary antibody (Biorad, California, USA, goat polyclonal HRP-conjugated anti-mouse or anti-rabbit IgG [H+L], 1:2000) for 1 hour at room temperature. Membrane were washed in TBS-T only, and proteins were detected by enhanced chemiluminescence (ECL<sup>TM</sup> detection kit, Amersham Biosciences). Autoradiographic signals were detected by exposing

membranes to Kodak Scientific Imaging Film for between 20 seconds and 5 minutes, and developing in an Agfa Curix 60 X-ray developer.

### **2.4.3 Stripping of blots**

Membranes were incubated in stripping buffer (50 mM, Tris pH 6.8, 2% SDS, and 100 mM  $\beta$ -mercaptoethanol) at 50°C. Membranes were then washed 4 times with TBS-T, before being blocked with milk/TBS-T for >1 hour and re-probed with antibody.

## **2.5 Fluorescence activated cell sorting: DNA content**

Cells were harvested in trypsin/EDTA and washed in PBS with 2% FBS before being resuspended in 0.5 mL PBS and fixed with 5 mL ice-cold 95% ethanol. The cells were then left at 4°C for 30 minutes before being washed in PBS/2% FBS and resuspended in 10  $\mu$ g/ml propidium iodide (PI) and 0.25 mg/mL RNAase A in 38 mM sodium citrate (with 10mM Tris and 15 mM NaCl). Cells were then incubated at 37°C for 30 minutes prior to being analysed by fluorescence activated cell sorting (FACS) using the FACScaliber (Becton Dickinson). Cell cycle distribution was determined using Modfit LT (Verity Software).

## **2.6 Cell proliferation assays**

Cells were plated into 94 mm Petri dishes and left to adhere overnight. The following day the cells were treated with PHA533533 or vehicle control (DMSO) for two hours and irradiated using a  $^{137}\text{Cs}$  source (Gamacell 40, Atomic Energy of Canada) before being incubated for a further 48 hours. Following incubation, cells were harvested with trypsin/EDTA and counted on an automated cell counter (CDA-500, Sysmex corporation).

## **2.7 Clonogenic survival assays**

Exponentially growing H460 and A431 cells were treated with either PHA533533 or vehicle control (DMSO) and incubated for 24 hours. Following incubation, cells were

irradiated with a  $^{137}\text{Cs}$  radiation source and then immediately harvested using trypsin/EDTA, washed free of drug, counted and re-plated (in replicates of 4) in a drug-free medium for clonogenic survival in 35 mm Petri dishes. The plates were incubated in a 5%  $\text{CO}_2$  humidified atmosphere at  $37^\circ\text{C}$  for 10 days (H460) or 12 days (A431). At the end of the incubation period, plates were fixed with neutral formalin (40% formaldehyde, 30 mM  $\text{NaH}_2\text{PO}_4$  and 45 mM  $\text{Na}_2\text{HPO}_4$ , pH 7) and stained with 0.01% (w/v) crystal violet. Colonies consisting of  $\geq 50$  cells were counted. The survival fractions were calculated relative to the plating efficiency of untreated control cells (taken as 100%).

In several experiments conducted with the H460 cell line,  $0.5\mu\text{M}$  PHA533533 or vehicle control was added to the medium for the duration of the colony forming incubation period.

## **2.8 Statistical analysis**

Statistical analysis was performed on SigmaStat®, using an unpaired student  $t$  test.

## Section 3: Results

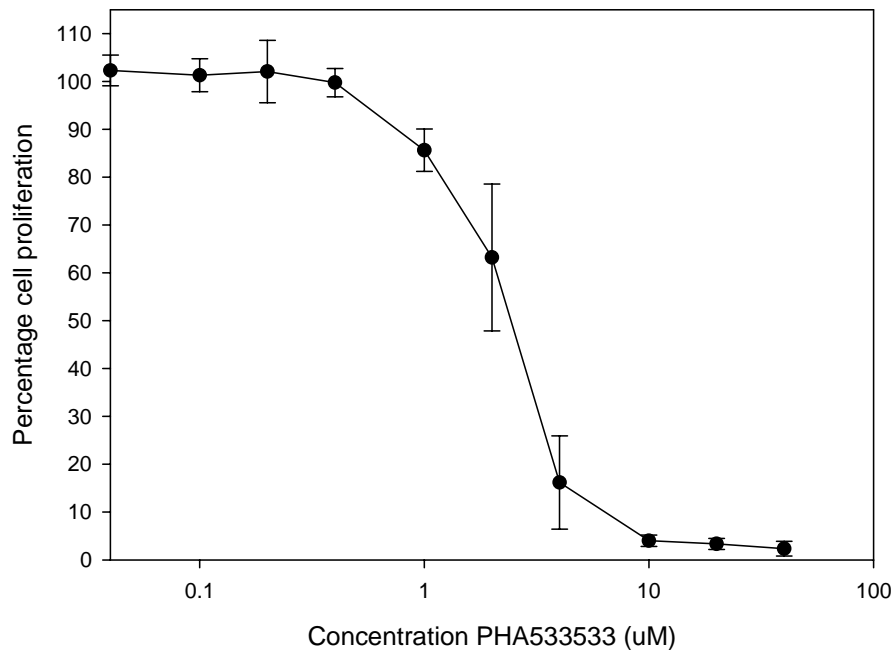
Experiments were performed using two human cell lines, H460 (non-small cell lung carcinoma) and A431 (vulvar squamous cell carcinoma). These two cell lines were chosen since:

- current treatment for both squamous cell carcinomas and NSCLC involves radiotherapy
- the lines are derived from different tissues of origin
- both lines are wild-type for Rb (Akiyama et al. 1997; Adachi et al. 1998)
- they have different known important cell cycle protein mutations (H460 = p53<sup>+/+</sup>; A431 = p53<sup>-/-</sup>) (Akiyama et al. 1997; Adachi et al. 1998)

### 3.1 The H460 Cell Line

#### 3.1.1 PHA533533 inhibits proliferation of H460 cells

Sulphorhodamine B (SRB) assays were conducted to determine the IC<sub>50</sub> (defined as the PHA533533 concentration which induces 50% growth inhibition in treated cells compared to untreated control cells). Figure 5 demonstrates that the drug effectively inhibits growth of H460 cells, and the IC<sub>50</sub> was found to be 2.3 μM. The gradient of this curve is very steep between 1 μM and 4 μM, where a slight change in concentration has a dramatic effect on cellular proliferation, suggesting a narrow therapeutic window. Concentrations greater than 10 μM completely inhibited proliferation.



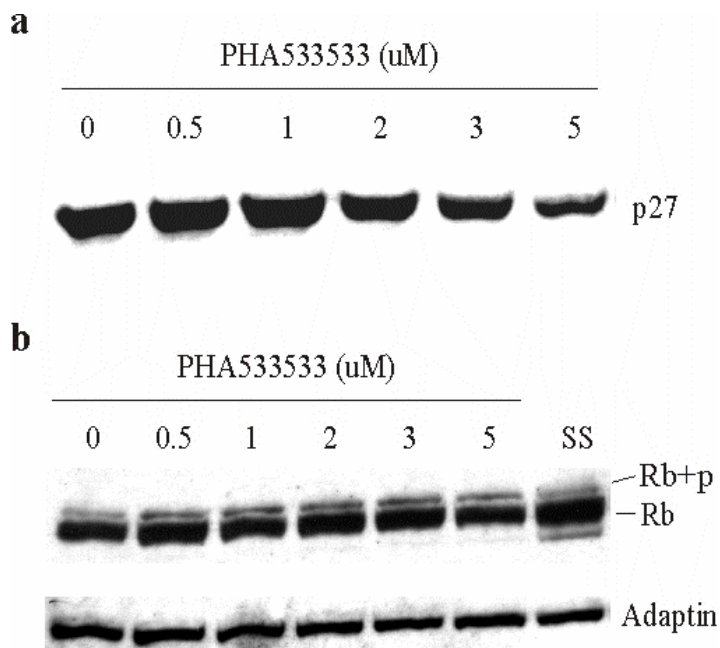
**Figure 5:** Cell proliferation dose-response curve for H460 cells treated with PHA533533. Cells were treated with PHA533533 (0.04  $\mu\text{M}$  - 40  $\mu\text{M}$ ) for 72 hours in 96 well plates. The cells were then stained with SRB before being solubilised with 10 mM Tris and read in a plate reader at 550 nm. Data shown are mean from three independent experiments: error bars,  $\pm$  SE.

### 3.1.2 The effect of PHA533533 on CDK2 substrate activity

To determine whether PHA533533 inhibits CDK2 in H460 cells, the effect of PHA533533 on the activity of CDK2 substrates p27 and Rb (retinoblastoma protein) were analysed by western blot. Levels of p27 were assessed because this protein is phosphorylated and targeted for ubiquitin-mediated degradation by the CDK2-cyclin E complex (Vlach et al. 1997). As a result, p27 levels are expected to rise following CDK2 inhibition. The phosphorylation status of Rb was also determined, since Rb is similarly phosphorylated by CDK2-cyclin E (in late G<sub>1</sub>) (Malumbres and Barbacid 2001). This allows for release of the E2F transcriptional activator from Rb, promoting transition into S-phase. As we expect PHA533533 to inhibit CDK2, we also expect it to inhibit Rb phosphorylation (levels of hypo-phosphorylated Rb should increase while levels of hyper-phosphorylated Rb accordingly decrease). The cells were exposed to increasing

concentrations of PHA533533 for 24 hours and results of the p27 and Rb western blots are shown in Figure 6.

Levels of p27 decreased with increasing concentrations of PHA533533 (Figure 6a). This effect appears at 2  $\mu$ M PHA533533, with significantly reduced p27 levels evident by 5  $\mu$ M. This result is in contrast to what was expected from CDK2 inhibition.



**Figure 6:** Western blot analysis of CDK2 substrate levels in H460 cells treated with PHA533533. The cells were plated in 25-cm<sup>2</sup> petri dishes and treated with increasing concentrations of PHA533533, or were serum starved (*lane SS*) for 24 hours. Total protein extracts of the cells were obtained as described in “Materials and Methods”. **a**, membrane was probed with p27 antibody. **b**, membrane was probed with Rb antibody, stripped, and then re-probed with adaptin- $\alpha$  as a loading control.

As seen in Figure 6b, the serum starved sample (SS) appears to have a faint upper band (hyper-phosphorylated Rb) and a thick middle band (hypo-phosphorylated Rb). This is consistent with G<sub>1</sub> checkpoint arrest induced through withdrawal of nutrients, and also illustrates how the bands should appear following CDK2 inhibition. However, no change in the phosphorylation status of Rb was demonstrated at any concentration of PHA533533 (0.5  $\mu$ M – 5  $\mu$ M) in this cell line. At 0  $\mu$ M it does appear that the upper band is faint (similar to the SS sample), however, as the band below is not correspondingly

thicker, it is likely not to be a real effect. Finally, absolute levels of Rb also remained constant following treatment with PHA533533. The loading control used was adaptin- $\alpha$ , and showed even loading of the samples. Adaptin- $\alpha$  is a subunit of the AP-2 protein, a member of the clathrin coated vesicle family involved in vesicle cargo and protein recruitment for endocytosis (Robinson 2004). At 112 kDa, it was a suitable loading control for Rb (110 kDa) western blots.

In summary, the effect of PHA533533 on CDK2 substrate levels is not supportive of CDK2 inhibition in the H460 cell line.

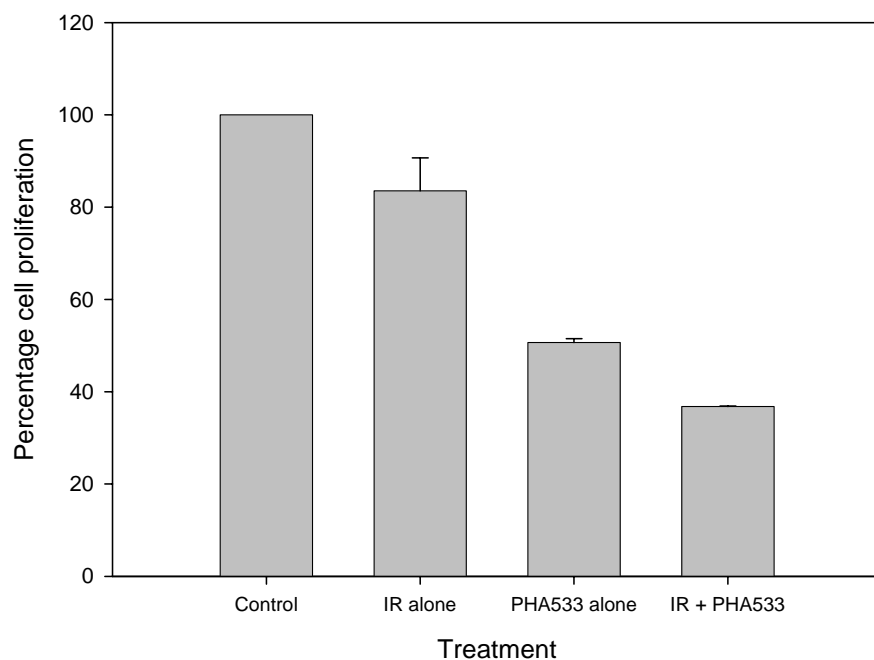
### **3.1.3 PHA533533 does not influence cell cycle distribution**

Cell cycle analysis was also used to ascertain whether PHA533533 was inhibiting CDK2 in tumour cells. As CDK2-cyclin E phosphorylates Rb in late G<sub>1</sub> to allow for transition into S-phase, it was expected that CDK2 inhibition by PHA533533 would increase the G<sub>1</sub> cell population in a dose dependent manner, at the expense of the S-phase and G<sub>2</sub> populations. Cells were treated with increasing concentrations of PHA533533 for different time periods, after which they were harvested and stained with PI/RNAase before DNA content analysis on the FACS Caliber. Several experiments were conducted to investigate H460 cell cycle distribution following treatment with PHA533533. Cells were exposed to concentrations ranging from 2  $\mu$ M to 5  $\mu$ M, for 8 to 48 hours. No change in cell cycle distribution was observed for any sample (data not shown). These results were unexpected and may suggest that PHA533533 is arresting cells across all phases of the cell cycle.

### **3.1.4 Anti-proliferative effects of PHA533533 in combination with IR are not greater than additive**

To determine whether there was any anti-proliferative synergy between PHA533533 and IR, proliferation assays were conducted. Cells were incubated with either 3  $\mu$ M PHA533533 or vehicle control for 2 hours, and then treated with 1.5 Gy radiation before incubation for a further 48 hours. Cells were then harvested and counted on an automated cell counter. 3  $\mu$ M PHA533533 was used because the growth inhibition assays had demonstrated that this drug concentration caused extensive anti-proliferative

effects (Figure 5). Figure 7 shows the proliferation status of all treated samples, compared to an untreated control (100% proliferation). Proliferation of cells treated with PHA533533 alone was reduced to 51%, while treatment with only IR lowered proliferation to 84%. These two figures combine to a percent proliferation of 35%, very similar to the efficacy of cells treated with both PHA533533 and IR together (37%). These results suggest that the combined anti-proliferative effect was not greater than additive.

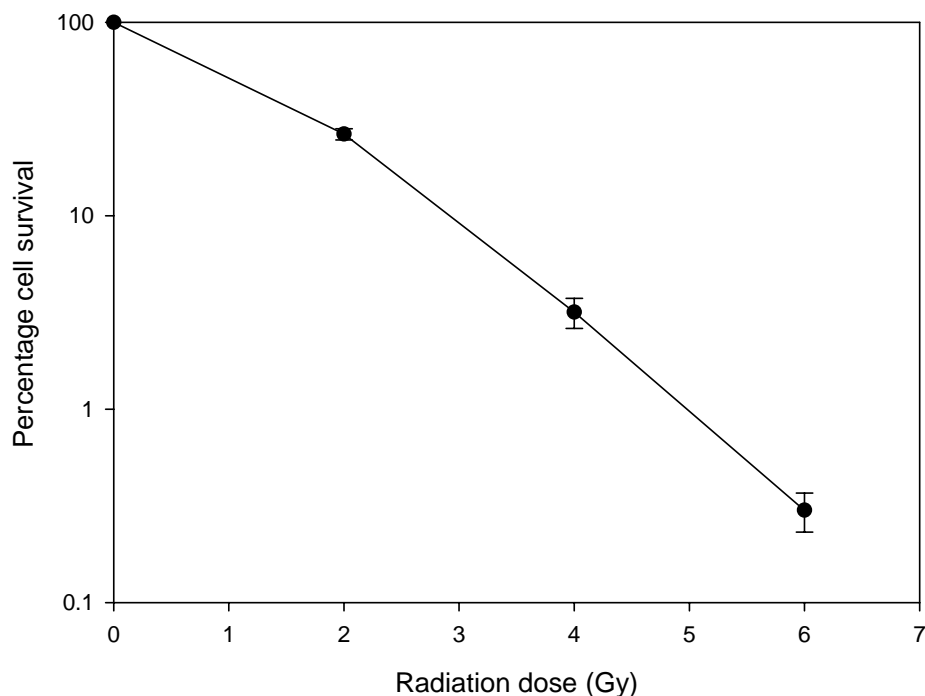


**Figure 7:** Cellular proliferation following treatment of H460 cells with IR and PHA533533. Triplicate samples of cells were either treated with DMSO (control), IR alone (1.5 Gy), PHA533533 alone (3  $\mu$ M) or IR + PHA533533 (1.5 Gy + 3  $\mu$ M). After 48 hours, total cell number of each sample was counted on an automated counter, and the average of three samples was calculated for each treatment. Percentage data was calculated against an untreated control (100%). Data shown are mean from two independent experiments.

### 3.1.5 PHA533533 does not sensitise cells to IR-induced cell killing

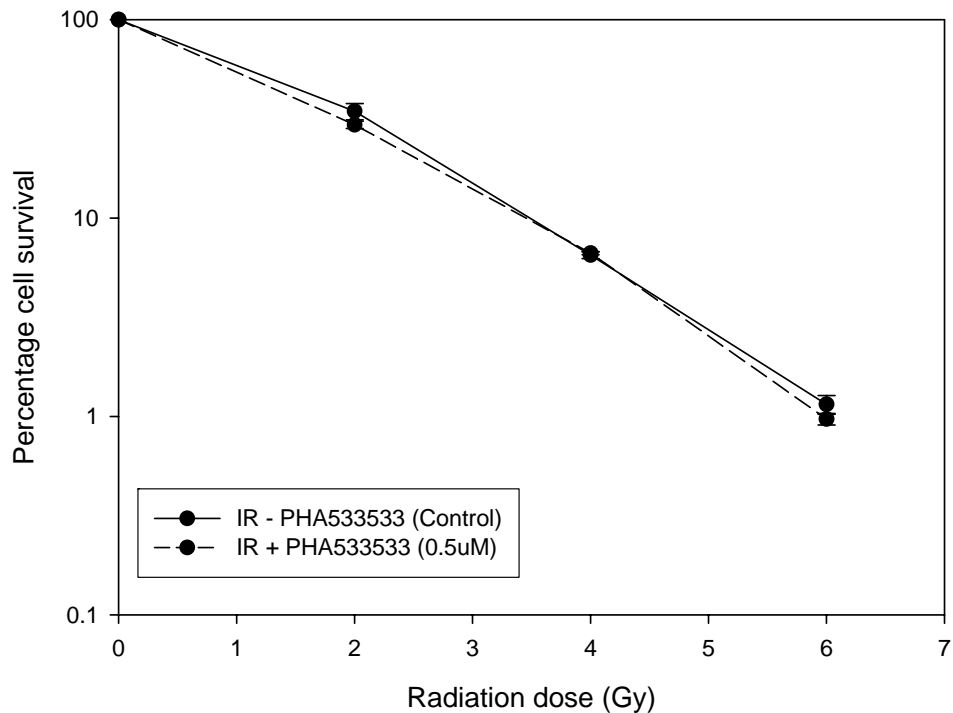
Clonogenic survival assays were used to determine whether PHA533533 increases the radiosensitivity of tumour cells. Initially, clonogenic assays were conducted to measure the effects of radiation alone on H460 cells. Figure 8 shows the radiation-response curve for 2, 4 and 6 Gy, indicating that radiation alone caused a dose-dependent

reduction in cell survival. The 3 doses of radiation used in this experiment were routinely used for all subsequent H460 clonogenic assays.



**Figure 8:** Clonogenic survival assay showing response curve of H460 cells to IR. Cells were irradiated with 2, 4 or 6 Gy. The cells were then removed with trypsin, plated in specific numbers in medium and incubated. 10 days later, the colonies were fixed, stained and counted, and the survival curve was constructed against the untreated control. Data shown are mean from four independent experiments: error bars,  $\pm$  SE.

The effect of PHA533533 on cell survival after IR was then investigated. Several different regimens of combining the drug and radiation were tested. In one experiment, the cells were exposed to 0.5  $\mu$ M PHA533533 for 24 hours before irradiation, and then plated in medium with 0.5  $\mu$ M drug for the complete incubation period (10 days). Although the 72-hour  $IC_{50}$  was 2.3  $\mu$ M, 0.5  $\mu$ M PHA533533 was chosen because a 10-day exposure to 1  $\mu$ M completely inhibited cell proliferation, resulting in no colonies. Incubation in 0.5  $\mu$ M drug resulted in colonies fewer in number and smaller in size than the untreated controls, yet failed to enhance radiation induced cell killing, as shown in Figure 9.



**Figure 9:** Clonogenic survival assays showing failure of PHA533533 to sensitise H460 cells to IR. Cells were treated with 0.5  $\mu\text{M}$  PHA533533, irradiated with 2, 4 or 6 Gy, and then plated in medium containing 0.5  $\mu\text{M}$  PHA533533 for the 10-day incubation period (control samples were treated accordingly with vehicle control). The colonies were fixed, stained and counted. The curve representing the effect of PHA533533 + IR was normalised; (the colony number after drug alone was used as 100% survival). Data shown are mean from two independent experiments.

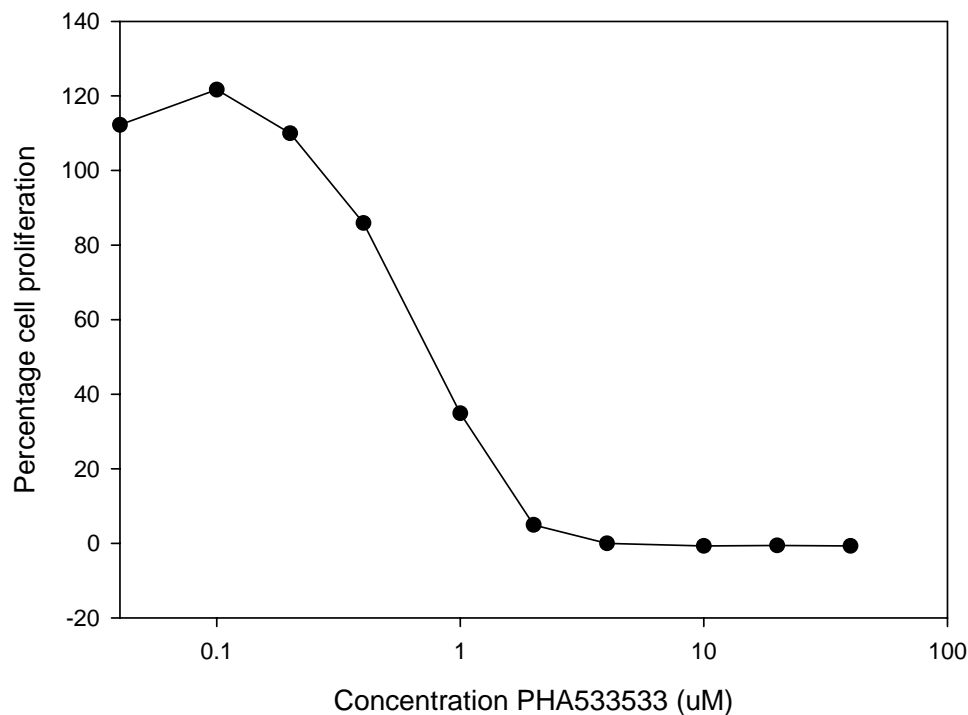
Previous experiments using ROS and FPD showed synergy with IR when the drug was given at a dose greater than the  $\text{IC}_{50}$  (Maggiorella et al. 2003; Raju et al. 2003). In these studies the drug was only administered for 24 hours, before the cells were irradiated and re-plated in a drug free medium for the colony forming incubation period. This regimen was consequently applied to H460 experiments, where cells were treated with 3, 5 and 7  $\mu\text{M}$  PHA533533 (greater than the  $\text{IC}_{50}$  of 2.3  $\mu\text{M}$ ). Colonies produced from the drug-treated cells were again slightly smaller and fewer in number in the drug treated plates than the controls, but again, no radiosensitisation was observed (data not shown).

Following the failure of PHA533533 to sensitise H460 cells to the anti-tumour effects of IR, a different cell line (A431) was then used to either confirm the H460 results or determine whether the effects of PHA533533 + IR were cell-line specific.

## 3.2 The A431 Cell Line

### 3.2.1 PHA533533 inhibits proliferation of A431 cells

A431 cells were more sensitive than H460 cells to the anti-proliferative effects of PHA533533. The  $IC_{50}$  was determined to be  $0.9 \mu\text{M}$  (Figure 10), as compared with  $2.3 \mu\text{M}$  for the H460 line.

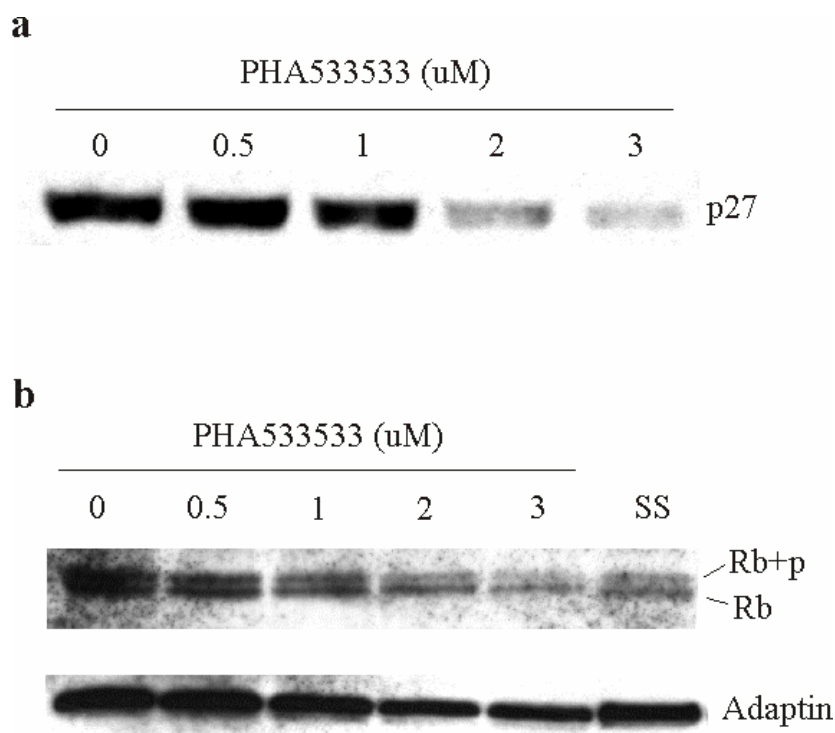


**Figure 10:** Cell proliferation dose-response curve for A431 cells treated with PHA533533. Cells were treated with PHA533533 ( $0.04 \mu\text{M} - 40 \mu\text{M}$ ) for 72-hours in 96-well plates. The cells were then stained with SRB before being solubilised with 10 mM Tris and read in a plate reader at 550 nm. Data shown is from one experiment.

Similar to the H460 data, the A431 dose-response curve also has a steep gradient, in this instance between  $0.2 \mu\text{M}$  and  $2 \mu\text{M}$ . At concentrations exceeding  $4 \mu\text{M}$ , cellular proliferation was completely inhibited, while below  $0.2 \mu\text{M}$  there is a slight increase in proliferation – reasons for this are unknown.

### 3.2.2 PHA533533 alters CDK2 substrate activity

As seen in Figure 11a, incubation of A431 cells with increasing concentrations of PHA533533 for 24 hours resulted in decreasing levels of p27, with significant loss at concentrations of 2  $\mu$ M and 3  $\mu$ M. This is the same effect that was demonstrated in the H460 cell line.



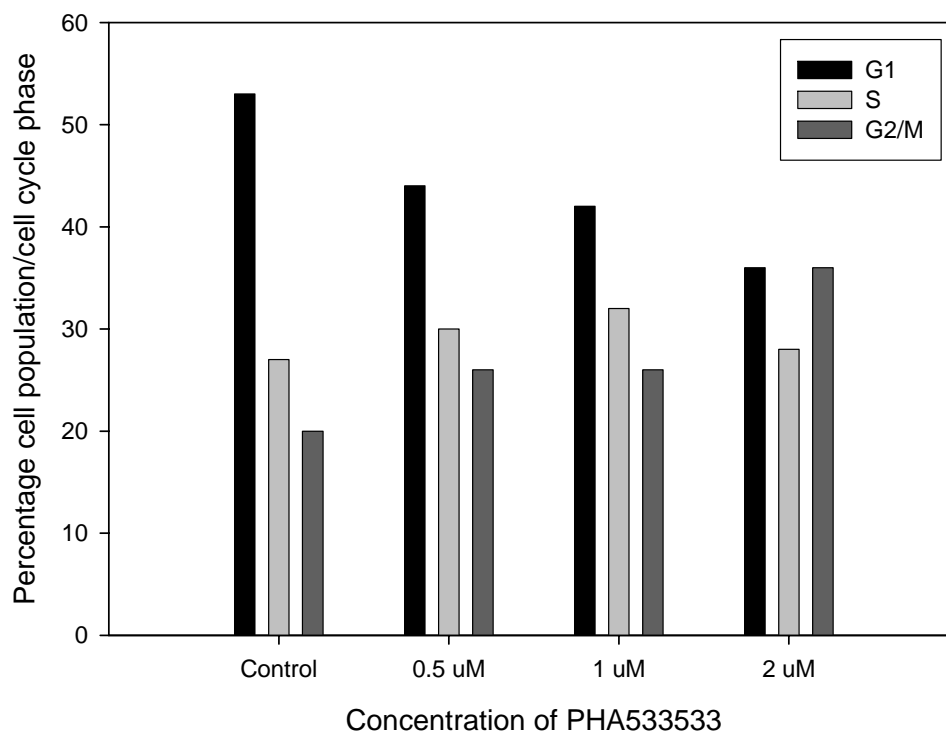
**Figure 11:** Western blot analysis of CDK2 substrate levels in A431 cells treated with PHA533533. The cells were plated in 25-cm<sup>2</sup> petri dishes and treated with increasing concentrations of PHA533533, vehicle control (DMSO) or were serum starved (lane SS) for 24 hours. Total protein extracts of the cells were obtained as described in “Materials and Methods”. **a**, membranes were probed with p27 antibody. **b**, membranes were probed with Rb antibody, stripped, and then re-probed with adaptin- $\alpha$  as a loading control.

As seen in Figure 11b, levels of hypo-phosphorylated Rb (lower band) are greater than hyper-phosphorylated Rb (upper band) in the serum starved sample (SS), indicating G<sub>1</sub> cell cycle arrest induced through withdrawal of nutrients. Cells treated with 2  $\mu$ M and 3  $\mu$ M PHA533533 also demonstrate a high ratio of hypo- to hyper-phosphorylated Rb, indicative of G<sub>1</sub> cell cycle arrest induced through CDK2 inhibition. Furthermore, levels

of total Rb decreased with increasing drug concentrations. These Rb western blot results differ dramatically from the H460 experiments, where no effect of PHA533533 was seen.

### 3.2.3 PHA533533 alters cell cycle distribution in a dose dependent manner

Cells were exposed to increasing concentrations of PHA533533 (0.5  $\mu$ M – 2  $\mu$ M) for 48 hours and then analysed for DNA content on the FACS Caliber. Figure 12 illustrates A431 cell cycle redistribution following the drug treatment. A dose dependent decrease in the G<sub>1</sub> cell population corresponded with an increase in the G<sub>2</sub> cell population (S-phase levels remained relatively constant). The results suggest that in this cell line, PHA533533 does not induce a G<sub>1</sub> arrest.

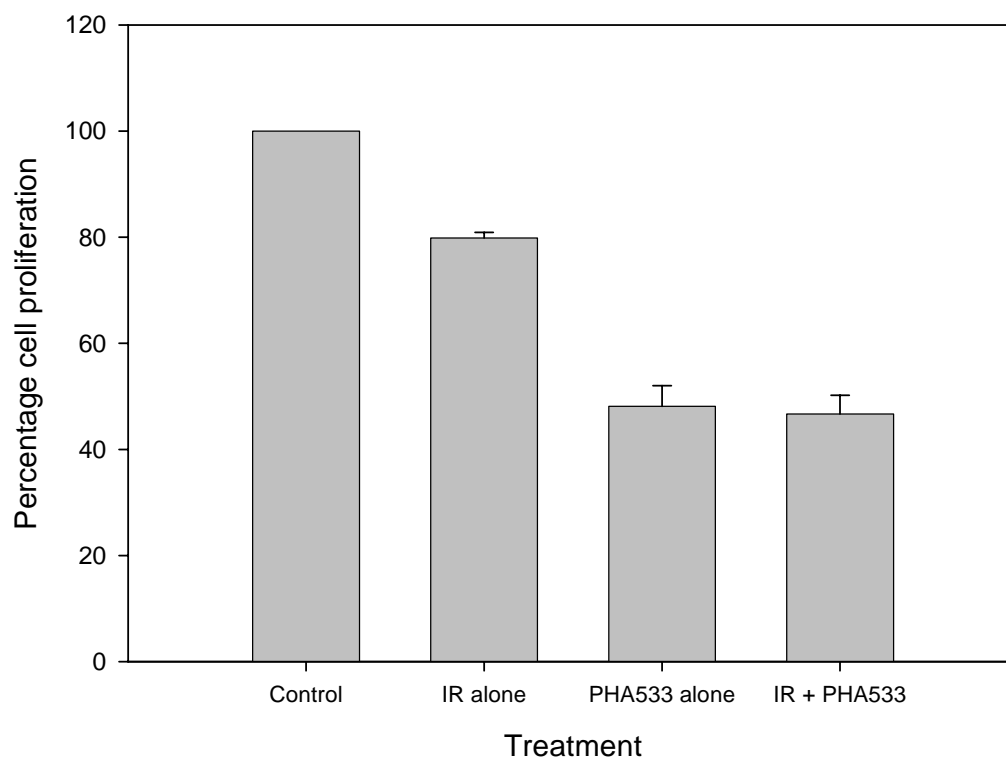


**Figure 12:** A431 cell cycle redistribution following treatment with PHA533533. Following 48-hour exposure to various concentrations of PHA533533, cells were fixed with 95% ethanol for  $\geq 15$  minutes, washed, and then exposed to propidium iodide/RNAase solution before analysis on the flow cytometer. Data shown are a representative experiment of three independent experiments.

These results are in contrast with the H460 data, where no change cell cycle distribution was seen following treatment with PHA533533.

### 3.2.4 PHA533533 in combination with IR does not demonstrate enhanced anti-proliferation

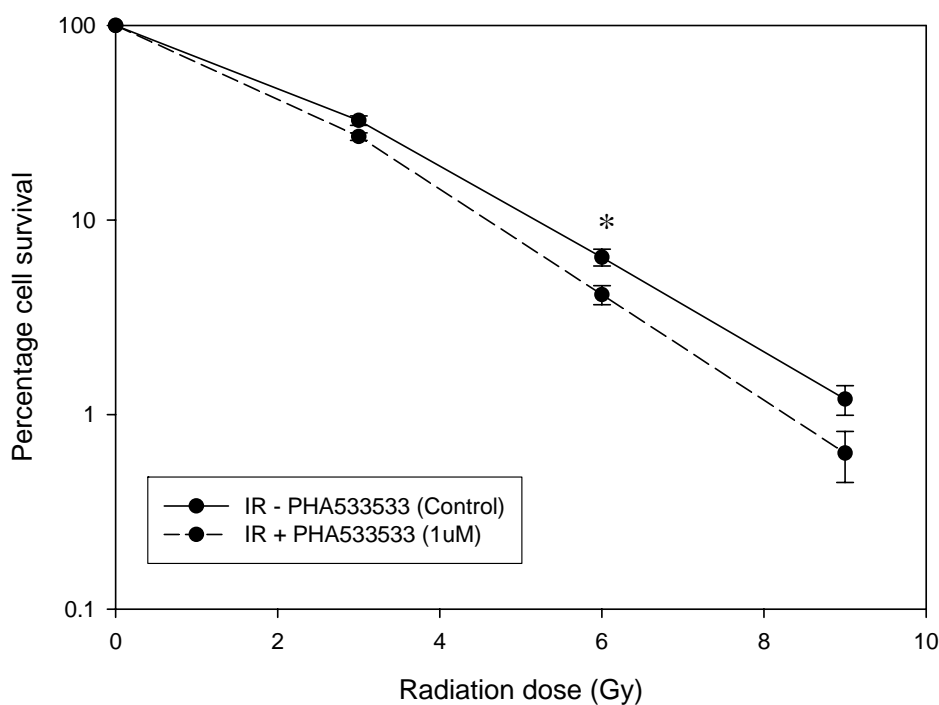
Cell proliferation assays were conducted on the A431 cell line, using 0.7 $\mu$ M PHA533533 together with 3 Gy radiation. These doses were chosen since they individually inhibited proliferation to a similar extent as those doses used in the H460 experiments. Figure 13 illustrates that proliferation of A431 cells with the combined treatment (47%) equaled that of cells given drug alone (48%), indicating no enhanced anti-proliferation. This outcome contrasts with that of combining PHA533533 and IR on H460 cells, where the anti-proliferative effect was additive.



**Figure 13:** Cellular proliferation following treatment of A431 cells with IR and PHA533533. Triplicate samples of cells were either treated with DMSO (control), IR alone (3 Gy), PHA533533 alone (0.7  $\mu$ M) or IR + PHA533533 (3 Gy + 0.7  $\mu$ M). After 48 hours, total cell number of each sample was counted on an automated counter, and an average of three samples was calculated for each treatment. Percentage data was calculated against an untreated control (100%). Data shown are mean from two independent experiments.

### 3.2.5 PHA533533 enhances IR-induced cell killing

A431 cell survival in response to IR had already been investigated in the lab using clonogenic assays, and the results indicated that these cells are more radiosensitive than H460 cells (Solomon et al. 2003). To determine the effect of PHA533533 on A431 cell survival following IR, clonogenic assays were performed as per studies described previously using FPD and ROS (Section 3.1.5) (Maggiorella et al. 2003; Raju et al. 2003). PHA533533 was administered at a dose of 1  $\mu$ M (greater than the  $IC_{50}$  0.9  $\mu$ M) for 24 hours before irradiation (3, 6, and 9 Gy), and cells were then re-plated in a drug free medium for a 12 day incubation. The results shown in Figure 14 indicate that PHA533533 enhanced IR-induced cell killing in this cell line, with a significant difference evident at 6 Gy. This is again in contrast with the H460 data, where PHA533533 did not sensitise the cells to IR-induced cell killing.



**Figure 14:** Clonogenic survival assays demonstrating radiosensitisation of PHA533533 on A431 cells. Cells were treated with 1  $\mu$ M PHA533533 (or DMSO as vehicle control), irradiated with 3, 6 or 9 Gy, and then plated in medium for 12 days incubation. The colonies were fixed, stained and counted. The curve representing the effect of PHA533533 + IR was normalised; (as in the colony number after drug alone was used as 100% survival). Data shown are mean from three independent experiments: error bars,  $\pm$  SE. Statistical analysis by unpaired student *t* test, \* $P < 0.05$

## Section 4: Discussion

Previous studies have provided evidence of anti-tumour synergy between novel CDK inhibitors and IR, including work with FPD, 7HS and ROS (Wang et al. 1996; Maggiorella et al. 2003; Raju et al. 2003). Therefore, it is important to investigate whether a more pure CDK2 inhibitor – like PHA533533 – is similarly capable of synergising with IR, and to determine how this effect may compare with the other more broadly acting compounds.

### **PHA533533 inhibited phosphorylation of Rb in the A431 cell line, consistent with CDK2 inhibition**

Since CDK2-cyclin E phosphorylates Rb in late G<sub>1</sub>, inhibition of CDK2 is also expected to inhibit Rb phosphorylation, as seen on two human colon cancer cell lines following treatment with ROS (a CDK1 and CDK2 inhibitor) (Whittaker et al. 2004). This effect was demonstrated following treatment with PHA533533 on the A431 cell line, as high doses of PHA533533 increased the ratio of hypo- to hyper-phosphorylated Rb, indicative of CDK2 inhibition and G<sub>1</sub> cell cycle arrest.

Moreover, the PHA533533-induced dose dependent decrease of *total* Rb in A431 cells has also been shown following treatment with ROS on colon cancer cells (Whittaker et al. 2004). This effect is further supported by other experiments conducted on cells treated with tamoxifen, staurosporine and ROS (Dou 1997; Fattman et al. 1998). These papers describe certain forms of apoptosis (induced in these cases by molecular targeted drugs), which promote internal cleavage of the Rb protein into two smaller fragments.

In contrast, H460 cells showed no change in Rb phosphorylation or total Rb levels, perhaps indicating a cell line specific effect of PHA533533.

### **Decrease in p27 levels following treatment with PHA533533 is inconsistent with CDK2 inhibition**

Following CDK2 inhibition, p27 levels are expected to rise as CDK2 is no longer capable of phosphorylating p27 and targeting it for ubiquitin mediated degradation (Vlach et al. 1997). This effect has been demonstrated on a number of different cancer

cell lines following treatment with ROS (Zhang et al. 2004). However, in both the A431 and H460 cells used here, PHA533533 instead caused a *decrease* in levels of p27. This outcome is similar to that obtained in another study, where human prostate cancer cells (DU-145) treated with increasing concentrations of ROS also resulted in a reduction of total cellular p27 (Wartenberg et al. 2002). These results may imply a cell line specific effect of CDK2 inhibition, or alternatively, that CDK2 inhibition may not be the only cell cycle effect of PHA533533.

One possible explanation for the decrease in p27 levels may be a PHA533533-induced S-phase delay. Transformed and non-transformed cells both exhibit conserved patterns of p27 expression throughout the cell cycle: via post-transcriptional mechanisms other than CDK2 phosphorylation, p27 accumulates as cells enter G<sub>1</sub> from M-phase, and then subsequently decreases throughout S-phase transition (Hengst and Reed 1996).

### **Cell cycle analysis following PHA533533 treatment was inconsistent with CDK2 inhibition**

As phosphorylation of Rb by CDK2-cyclin E releases E2F and promotes G<sub>1</sub> to S-phase transition, CDK2 inhibition is expected to arrest cells in G<sub>1</sub>. However, neither cell line demonstrated this effect. H460 cell cycle analysis failed to show a change in cell cycle distribution following PHA533533 administration, suggesting that the drug is arresting cells evenly across all phases of the cell cycle. On the other hand, the A431 cells accumulated in G<sub>2</sub> in a dose dependent manner, with a corresponding decrease in the G<sub>1</sub> population. The A431 data correlates with studies done on several cell lines following treatment with ROS (Wartenberg et al. 2002; Whittaker et al. 2004), however, these effects may not be due to CDK2 inhibition as ROS also inhibits CDK1.

### **Many different factors may contribute to the unexpected and varied cell cycle effects induced by PHA533533 on both cell lines**

There are many possible explanations for the unexpected effects PHA533533 had on CDK2 substrate levels and cell cycle analysis in both the A431 and H460 cell lines. Firstly, the small molecule kinase inhibitors bind to the ATP-docking site in a protein's catalytic pocket, a domain which is relatively conserved between the 850+ kinases

(Knockaert et al. 2002). This presents problems with target specificity, as indicated by the abundance of non-CDK proteins inhibited by FPD, ROS and 7HS (Table 3, Section 1.4.1). Therefore, although PHA533533 was developed as a selective inhibitor of CDK2, it does have activity (albeit less potently) against a range of other known kinases (Table 4, Section 1.6). This being the case, it is difficult to know whether it binds any of the other hundreds of kinases not tested for, and if so, whether these interactions significantly impact on cellular processes (Knockaert et al. 2002). Inhibition of other kinases by PHA533533 may therefore have influenced the cell cycle markers investigated in this study.

Secondly, CDK2 phosphorylates substrates other than Rb, including E2F, NPAT, histone H1 and BRG1 – and each are potentially capable of affecting cell cycle progression (Zhao et al. 1998; Shanahan et al. 1999; Malumbres and Barbacid 2001). Therefore, the effect of CDK2 inhibition by PHA533533 could influence cellular processes and cell cycle regulation through mechanisms not directly related to Rb phosphorylation and G<sub>1</sub> transition. For example, unregulated E2F-1 in S-phase positively influences an apoptotic pathway (Dyson 1998). As CDK2-cyclin A deactivates E2F/DP-1 in S-phase (previously described in 1.1.1.2), inhibition of this regulatory complex may promote tumour apoptosis (Chen et al. 1999).

Finally, the combination of genetic mutations unique to each tumour (Mutch 2000) may account for differences between the H460 and A431 results. Rb phosphorylation and cell cycle distribution analyses were not consistent between the two cell lines following treatment with PHA533533, nor too were the IC<sub>50</sub>s similar. This may relate to A431 cells being mutant for p53, while H460 cells are wild type, or it could result from other unknown mutations. Discrepancies between cell line responses are not unique to PHA533533. In one particular study (Zhang et al. 2004), three different cell lines treated with either ROS or FPD demonstrated very different cell cycle distribution patterns, while all displaying similar changes to the levels of p27. This may indicate that the effects of novel CDK or other kinase inhibitors may be cell line specific.

**By targeting only a single CDK protein, PHA533533 may be at a disadvantage against its more ‘promiscuous’ counterparts FPD, 7HS and ROS.**

Results observed following treatment by PHA533533 in combination with IR were positive in the A431 cells, while disappointing in the H460 cells. Although anti-proliferative synergy was not observed on either cell line, 1  $\mu$ M PHA533533 sensitised the A431 cells to IR-induced cell killing, with a statistically significant outcome at one radiation dose (6 Gy). In vitro results demonstrated with FPD and ROS in combination with IR (Maggiorella et al. 2003; Raju et al. 2003) were however more dramatic.

Arteaga (Arteaga 2003) proposes that the advantage of multi-targeted therapeutics is that molecular-specific agents with a short in vivo half-life are incapable of inhibiting their target persistently and completely. Moreover, in late stage cancer additional genetic alterations and redundant aberrant pathways may counteract the impact of a drug that targets a single molecule. This may explain why PHA533533 did not synergise with IR in vitro in the same manner as the other more ‘promiscuous’ CDK inhibitors.

In support of this hypothesis, the redundancy of G<sub>1</sub> associated proteins in cancer cells has been investigated to some extent. A recent paper (Tetsu and McCormick 2003) used anti-sense oligonucleotides and small interfering (si) RNA techniques to impair CDK2 function in numerous cancer cell lines. Surprisingly, loss of CDK2 did not change the proliferation status of any cells. It was found that CDK2 is unnecessary for G<sub>1</sub>/S-phase transition because tumour cells generally have an aberrant Rb-pathway. As a result, CDK4 activity increases and becomes capable of phosphorylating Rb at CDK2 conserved sites. It has also been shown that in the absence of CDK4-cyclin D1 in certain cell lines, CDK2-cyclin E alone is capable of completely phosphorylating Rb to release E2F (Keenan et al. 2004). Perhaps this indicates a general redundancy of G<sub>1</sub>-related proteins and the need to inhibit more than one such protein to generate a significant cell cycle inhibitory effect.

In vivo targeted disruption of mouse genes encoding several CDK and cyclin proteins has also been investigated (Malumbres et al. 2000). Knockouts of CDK4, cyclin D1/D2, cyclin A1 and members of CIP/KIP and INK4 families of CDK inhibitors do not result in lethality. Most recently, it was discovered that CDK2 knockout mice are also viable (Berthet et al. 2003). These mice are slightly smaller in size and sterile, indicating

that CDK2-cyclin E2 is important for germ cell development. Mouse embryonic fibroblasts (MEFs) from these animals proliferate without CDK2 but are delayed during S-phase entry, a trait rescued by ectopic CDK2 expression. Although CDK1 knockouts have not been investigated, cyclin A2 and B2 inactivation causes embryonic lethality (Malumbres et al. 2000).

These experiments may explain why a compound highly specific for CDK2 may not be such a successful anti-tumour drug compared with compounds that inhibit a number of targets, like FPD, 7HS and ROS. Alternatively, due to the necessity of G<sub>2</sub>/M-related cyclin molecules in embryonic viability (Malumbres et al. 2000), and noting the significant anti-tumour efficacy of ROS as a monotherapy as well as combined with IR, may implicate CDK1 as a more suitable target.

### **Conclusions**

The CDK2 inhibitor PHA533533 shows marked anti-proliferation against both A431 and H460 cells in vitro. However, results obtained from CDK2 substrate and cell cycle analyses indicate that it is difficult to know whether these effects are due to CDK2 inhibition, or whether PHA533533 is influencing other cell cycle processes. Furthermore, although the anti-proliferative properties of PHA533533 and IR failed to act synergistically, PHA533533 did manage to sensitise the A431 cells (but not the H460 cells) to IR induced cell killing. These results indicate that the effects of PHA533533 are cell line specific and may relate to the different mutations in different cell lines.

### **Future Directions**

The promising in vitro findings with the A431 cell line suggest that further investigation into this interaction is required. It would be interesting to discover whether the p53 mutation contributed to the ability of PHA533533 to sensitise A431s to IR, compared with the p53 wild type H460s. This could be done using isogenic cell lines or siRNA techniques. Additionally, it is important to investigate the efficacy of the combined therapy on an in vivo model. As seen in other studies (Solomon et al. 2003), factors characteristic to the in vivo environment may be important in the interaction between molecular targeted drugs and radiation.

However, in regard to recent papers which describe the redundancy of CDK2 and other cell cycle proteins, perhaps the way of the future is with more 'promiscuous' broadly acting compounds, rather than a pure single-kinase inhibitor.

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