Cell Cycle Regulation Changes in Drug Resistant Cancers

by

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Abstract

Cell cycle perturbation is a common event in tumorigenesis and the development of anticancer drug resistance. Acquired drug resistance is a significant hindrance in achieving effective cancer treatment. Thus, understanding of mechanisms underlying acquired drug resistance is vital to develop strategies to overcome those processes and to improve the survival rates of cancer patients. Moreover, understanding drug resistance can lead to the discovery of potential druggable targets that can contribute towards increasing success of cancer intervention. In this study, molecular mechanisms leading to acquired drug resistance in various cancer cell lines were investigated. Informed by cDNA microarray screens, the cell cycle regulators $p57^{Kip2}$, Wee1 and Stratifin (SFN) were shown to be frequently deregulated in drug resistance. Using Western blotting and qPCR, $p57^{Kip2}$ was shown to be downregulated in platinum-resistant ovarian cancer cell lines PEO1CarbR and PEO1CisR in comparison with the platinum-sensitive counterpart. $p57^{Kip2}$ downregulation was due to epigenetic silencing (hypermethylation of gene promoter region). Thus, $p57^{Kip2}$ silencing with siRNA in otherwise drug sensitive cells recapitulated platinum resistance. Interestingly, $p57^{Kip2}$ downregulation conferred collateral sensitivity towards the synthetic CDK inhibitor drug Seliciclib (CYC202). A similar approach was adopted in the study of Wee1 and SFN in taxane resistance. Wee1 and SFN were downregulated in Taxol-resistant SK-OV-3 and A2780 cell lines compared to drug-sensitive counterparts. Silencing of *Wee1* in Taxol-sensitive SK-OV-3 decreased the apoptotic response to Taxol treatment, but this effect was not seen using *SFN* silencing. I conclude that Wee1 plays an important role in response to Taxol. However, p53 status is also associated with this response. Additionally, examination of several drug resistant and sensitive pairs of cancer cell lines indicated that DNA damage checkpoint and cell cycle regulation are significant factors in mediating anticancer drug resistance. Based on the results, the ATR-Chk1-Wee1 pathway was shown to be the most deregulated pathway in drug-resistant cancer cells. I conclude that deregulation of cell cycle is

essential to the adaptive responses and enabling characteristics that result in the drug resistant phenotype.

Declaration of Originality

This thesis and the work to which it refers are the results of my own efforts. Any ideas, data, images or text resulting from the work of others (whether published or unpublished) are fully identified as such within the work and attributed to their originator in the text and the bibliography. This thesis has not been submitted in whole or in part for any other academic degree or professional qualification.

Abbreviation

53BP1	p53 binding protein 1
5-FU	5-fluorouracil
9-1-1	Rad9-Rad1-Hus1
Akt	Protein Kinase B-PKB
AP	Alkaline Phosphatase
Apaf1	Apoptotic protease activating factor 1
APC/C	Anaphase-Promoting Complex/Cyclosome
APN	Aminopeptidase N
ATLD	AT-like disorder
ATM	Ataxia-telangiectasia mutated
ATP	Adenosine triphosphate
ATR	ATM and Rad3-related
ATRIP	ATR-Interacting protein
B-CLL	B-cell chronic lymphocytic leukaemia
BLM	Blooms syndrome helicase
BP180	Bullous pemphigoid antigen-2
BRCA1	BreastCancer1
BRCT	BRCA1 C-terminus repeat
BWS	Beckwith-Wiedemann Syndrome
CABLES	CDK5 and Abl enzyme substrate
CAK	CDK-activating kinase
CAM-DR	Cell adhesion-mediated drug resistance
CDC25C	Cell Division Cycle 25-C
CDKs	Cyclin-dependent kinases
cDNA	complimentary DNA
CI	Combination Index
Cip/Kip	CDK inhibitor protein/kinase inhibitor protein
CKIs	Cyclin-dependent kinases inhibitors
CMAs	Chromatin-modifying agents
CTR1	Copper transporter 1
CYC202	Seliciclib
DDR	DNA damage response
DEAE	Diethylaminoethyl
DLBCL	Diffuse large B cell lymphoma
DMSO	Dimethyl sulfoxide
DNA-PKcs	DNA dependent protein kinase catalytic subunits
DNMTi	DNMT inhibitors
DNMTs	DNA methyltransferases
dNTP	deoxyribonucleotide triphosphate
DSB	DNA double strand break
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
egr1	Early growth response 1
elf4B	Eukaryotic initiation factor 4B

EM-DR	Environment-mediated drug resistance
EOC	Epithelial ovarian cancer
ER α	Oestrogen receptor α
ESCC	Esophageal squamous cell carcinoma
EtBr	Ethidium Bromide
ETS	Erythroblastosis virus E26 oncogene
FA	Fraction of cells affected
FANCD2	Fanconi anaemia group D2
FANCE	Fanconi anaemia group E
FCS	Fetal Calf Serum
FHA	Forkhead associated
FIGO	Federation of Gynecology and Obstetrics
FITC	Fluorescein isothiocyanate
Gadd45a	Growth arrest and DNA damage inducible gene
GOI	Gene of interest
GRE	Glucocorticoid-response element
GSH	Glutathione
HATs	Histone acetyltransferases
HCC	Hepatocellular carcinoma
HDACi	HDAC inhibitors
HDACs	Histone deacetylases
HKG	Housekeeping gene
HME1	Human mammary epithelial marker 1
HPLC	High-performance liquid chromatography
HPV	Human papillomavirus
hSMG1	Suppressor with morphological effect on genitalia
HuR	Hu antigen R
IC ₅₀	50% inhibitory concentration
ICR	Imprinting centre/imprinted control region
INK4	Inhibitor of protein kinase 4
KAP1	KRAB-associated protein 1
KID	CDK inhibitory domain
LOH	Loss of heterozygosity
LOI	Loss of imprinting
Lys	Lysine (K)
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MCL	Mantle cell lymphoma
MCPH1/BRIT1	Microcephalin
MDM2	Murine double minute 2
miRNA/miR	MicroRNAs
MM	Multiple myeloma
MMP1	Matrix metalloproteinase 1
MMR	Mismatch repair
MPF	Mitosis-promoting factor
MRN	Mre11, Rad50 and Nbs1
MRPs	Multidrug resistance-associated proteins
MRT	Malignant rhabdoid tumour
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
NADH	Nicotinamide adenine dinucleotide

NADPH	Nicotinamide adenine dinucleotide phosphate
NBS	Nijmegen breakage syndrome
NC	Nitrocellulose
NER	Nucleotide excision repair
NES	Nuclear exporting sequence
NF1	Neurofibromin 1
NHEJ	Non-homologous end-joining
OCT1	Octamer-binding transcription factor 1
OSCC	Oral squamous cell carcinoma
PBS	Phosphate Buffered Saline
PCNA	Proliferating nuclear antigen
PCR	Polymerase Chain Reaction
PI	Propidium iodide
PIKK	Phosphoinositide-3-kinase-related protein kinase
PIP	PCNA interacting protein
PKC	Protein kinase C
PLK	Polo-like kinase
PML	Promyelocytic leukaemia
PMSF	Phenylmethanesulphonylfluoride
PP2A	Protein phosphatase 2A
PS	Phosphatidyl serine
PVDF	Polyvinylidene Fluoride
qPCR	Quantitative PCR
Rbp	Restinoblastoma protein
RFC1	Replication factor C
RPA	Replication protein A
SCD	SQ/TQ cluster domain
SCF	Skp1/Cul1/F-box
SCLC	Small cell lung cancer
SEI-1	Selected with INK4A
Ser	Serine (S)
SFM-DR	Soluble factor-mediated drug resistance
SFN/14-3-3 σ	Stratifin
siRNA	Small interfering RNA/silencing RNA
SLC22A2	Solute carrier 22 family member A2
Sp-1	Stimulatory protein-1
ssDNA	single strand DNA
TBS	TRIS Buffered Saline
Thr	Threonine (T)
TLK1	Tousled like kinase 1
TNF	Tumour necrosis factor
Tome-1	Trigger of Mitotic Entry
TopBP1	Topoisomerase II binding protein 1
TRAIL	TNF-related apoptosis-inducing ligand
TSA	Trichostatin A
Tyr	Tyrosine (Y)
VEGF	Vascular endothelial growth factor
WRN	Werner syndrome helicase

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Chapter 1

General Introduction

Introduction

1. Cell cycle and its components

Cells are the building blocks of every living organism. Cells undergo division and most of them differentiate into specialized cells, which have a specific function. Cell cycle is utilised by the cell to divide into two daughter cells. This is a highly coordinated and regulated series which becomes imbalanced and leads to either cell death or tumour occurrence. Malfunctions within cell cycle regulation lead to survival of damaged or mutated cells resulting in cancer.

1.1 Cell cycle phases

The eukaryotic cell cycle is divided into four non-overlapping phases: G_1 , S, G_2 and M (Figure 1-1). In the G_1 phase, cells grow and prepare for DNA synthesis. In this phase, diploid cells contain $2n$ chromosomes. Alternatively, cells may stop temporarily from dividing and stay in the G_0 phase, which is also referred to as quiescence state. After passing the restriction point at the end of the G_1 phase, cells enter the S phase in which DNA duplication occurs. DNA content in diploid cells will be $4n$ by the end of this phase. Following S phase, cells will enter another gap, G_2 which is essential for the cell as a preparation stage before mitosis. The M phase is the mitotic phase, during which cell separate into two daughter cells (Foster 2008; Maddika et al. 2007). From here, cells that are committed to divide will enter cell cycle through G_1 while others might differentiate into specialized cells.

Figure 1-1: Cell cycle stages.

1.2 CDKs

Transition of cells from one phase to another is mediated by the expression of regulatory proteins, including cyclin-dependent kinases (CDKs). CDKs are a group of key regulator proteins, a family of heterodimeric serine/threonine protein kinases (Barbacid et al. 2005). Activation of CDKs takes place through association with its partner, the cyclins and phosphorylation of its T-loop threonine by CDK-activating kinase (CAK). Levels of CDK proteins remain stable throughout the cell cycle while cyclin expression fluctuates at different stages of the cycle (Barbacid et al. 2005; Maddika et al. 2007). Cyclins shared a conserved 150 amino acid residues termed 'cyclin box', formed by 5 helical regions which is responsible for binding to CDKs (Barbacid et al. 2005).

CDKs have diverse cellular functions. Several important CDKs in the cell cycle are CDK1, 2, 3, 4, 6, 7, 10 and 11, which associates with different cyclins and functions on different cell cycle stages. In proliferating cells, CDK4-cyclinD, CDK6-cyclinD and CDK3-cyclinC complexes play an important role in G₁ phase by phosphorylating retinoblastoma protein (Rbp). CDK2-cyclinE completes Rbp phosphorylation and is important in transition from G₁ to S phase. CDK2 then associates with cyclinA throughout S phase progression. CyclinA binds to CDK1 in the G₂ phase followed by binding of CDK1 to cyclinB in G₂ to M phase transition. CDK10 and CDK11 might play a role in mitosis but their importance is not clearly understood. CDK7 binds to cyclinH and Mat1 to form CAK, which binds and activates all cell cycle CDKs (Barbacid et al. 2005; Maddika et al. 2007).

1.3 Cyclins

Cyclins as the name implies are proteins with cyclic expression i.e. differentially expressed within the specific cell cycle phases, contrary with CDKs whose expression levels are relatively constant (Figure 1-2). Evans et al. first describe them as proteins within embryonic cell cycle which levels rise and fall (Evans et al. 1983; Hartley 2008). Further research revealed that cyclins are activating partner of CDKs, forming complexes and acts as regulatory subunits within the complex. Each specific CDK-Cyclin complex corresponds with certain cell cycle phases and promotes cell cycle progression via substrate phosphorylation (Golias et al. 2004; Hartley 2008; Stamatakis et al. 2010). Cyclical level of cyclins is a result of periodic transcription together with rapid protein turnover (Hartley 2008).

Mitogenic signal received prompts the cell to synthesize CyclinD, which forms a complex with CDK4/CDK6 and this correspond with G₁ cell cycle phase. This CDK-Cyclin complex phosphorylates Rbp, which releases transcription factor E2F. Genes transcribed by E2F are important in cell cycle progression. These genes include CyclinE, which couples with CDK2 and promotes cell cycle transition from G₁ to S phase. CDK2 then forms complex with CyclinA for S phase progression. CDK1-CyclinA/B complex is essential for entry into M phase (Hartley 2008; Stamatakis et al. 2010).

Figure 1-2: Oscillating expression of Cyclins corresponds to cell cycle phase.

Some Cyclins play a vital role in carcinogenesis. Upregulation of CyclinD isoform, CyclinD1 is found in human oesophageal cancer while CyclinD2 is downregulated in tumours such as sporadic and familial breast cancers (Golias et al. 2004). Overexpression of CyclinD1 is due to chromosomal translocations, gene amplification, disruption of normal intracellular localization and proteolysis. CyclinD also inactivates negative regulators of CDK2-CyclinE, promoting CDK2-CyclinE activity. Additionally, CyclinD1 is able to promote gene silencing via recruitment of histone deacetylases and histone methyltransferases which alters chromatin structure. Deregulation of cyclinD is found in parathyroid adenomas, papillary thyroid carcinoma, mantle cell lymphoma and breast cancer which is associated with metastasis, worse survival and tamoxifen resistance among others (Stamatakis et al. 2010). Apart from CyclinD, CyclinA is also found to be overexpressed in several cases of hepatocellular carcinoma (Golias et al. 2004). CyclinE overexpression is related with carcinogenesis and is found to be overexpressed in number of cancers which will be explained further in another chapter.

1.4 CKIs

Another regulatory proteins expressed during cell cycle are negative regulator of CDKs, termed cyclin-dependent kinases inhibitors (CKIs). CKIs are important signalling pathway components, which control cell cycle exit and essential for cell differentiation (Zhang et al. 1998). CKIs are divided into 2 families, inhibitor of protein kinase 4 (INK4) family and Cip/Kip (CDK inhibitor protein/kinase inhibitor protein) family (Collins and Garrett 2005). The characteristic of protein sequence homologies and putative CDK targets have been considered in arranging CKIs into the different families (Nam and Kim 2008). INK4 family includes p15, p16, p18 and p19 while Cip/Kip family consists of p21^{Cip1}, p27^{Kip1} and p57^{Kip2}. INK4 family members inhibit CDK4 and CDK6 while Cip/Kip family members have wider action by inhibiting CDK/cyclin complexes formed at later stages of the cell cycle (Foster 2008; Nam and Kim 2008) (Figure 1-3). Due to their function as inhibitors to the cell cycle progression, CKIs have been proposed to be tumour suppressors (Kim et al. 2005). Besides CKIs, CDK kinase activities are also regulated by phosphorylation-dephosphorylation. Wee1 and Myt1 phosphorylate adjacent threonine and tyrosine residues to inhibit CDK activity while CDC25 phosphatases reverse this process. Other regulations include folding and subcellular localization (Barbacid et al. 2005; Maddika et al. 2007).

Figure 1-3: CDK-Cyclin complexes in mammalian cell cycle. CDK-Cyclin complexes were regulated by CKIs which can be grouped into two families: broader-acting CIP/KIP family and more specifically acting INK4 family.

1.5 Cip/Kip family of CKIs

Cip/Kip family consists of $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$. $p57^{Kip2}$ is the least studied member of Cip/Kip family of CKIs. Each family member has a conserved domain at the amino-terminal of the protein termed CDK binding-inhibitory domain. The Cip/Kip family has a broader spectrum of action by inhibiting wider range of cyclin/CDK complexes compared to INK4 family which is restricted to inhibition of CDK4 and CDK6 activities (Beretta et al. 2005; Zhang et al. 1998). INK4 family members exhibit their inhibitory effects at the early phase of the cell cycle, while Cip/Kip family members inhibit cell cycle progression at several cell cycle checkpoints (Kim et al. 2005). However, their inhibitory actions are dose-dependent since at a low level, they bind to and promote the assembly of CDK-Cyclin heterodimer complexes (Pateras et al. 2009).

p27^{Kip1} is expressed in a wide variety of different epithelial and non-epithelial tissues. Its expression usually correlates with poor cancer prognosis. p57^{Kip2} on the other hand is more tissue-specific (Rosenberg et al. 2001). The importance of Cip/Kip family actions in controlling proliferation during development, differentiation and response to cellular stresses has been widely described. Biological functions of each family member are different from each other, thus different anti-proliferative signals tend to cause elevated level of only some of them. p21^{Cip1} responds to DNA damage in G₁ and G₂, causing cells to arrest as it is an important target of p53. Expression level of p27^{Kip1} is usually elevated in mitogen-starved cells and other quiescent cells. As cells enter the cell cycle, p27^{Kip1} protein level is quickly downregulated. p57^{Kip2} have the weakest CKI activity of the Cip-Kip family members. p57^{Kip2} has a significant role in regulation of the cell cycle during embryonic development and have been shown to be indispensable for embryonic development (Beretta et al. 2005; Besson et al. 2008).

1.6 Regulation of Cyclins and CKIs

Periodic degradation of Cyclins and CKIs is important in controlling the timing of CDK activation. This mechanism is promoted by the ubiquitin-proteasome system (Rodier et al. 2008). Selective recognition and degradation of protein by the 26S proteasome begins with enzymatic tagging of protein with a polyubiquitin chain. The tagging is a collaborative result of ubiquitin-activation enzyme (E1), ubiquitin-conjugation enzyme (E2) and ubiquitin ligase (E3). E3 provides specificity of target proteins (Kamura et al. 2003; Kitagawa et al. 2009). E3 ubiquitin ligases associated with tumours are categorized into several groups: - HECT, single RING, Skp1/Cul1/F-box (SCF), ECV and Cul-4. Two major E3 types involved in cell cycle control are SCF complexes and the anaphase-promoting complex/cyclosome (APC/C), the first is involved in G₁-S progression while the latter plays an important role in the anaphase and cell cycle exit from M phase into G₁ (Kamura et al. 2003; Kitagawa et al. 2009; Rodier et al. 2008).

Another mechanism of Cyclin and CDK regulation comes from MicroRNAs (miRNA/miR). miRNAs are recently discovered as the regulators of gene expression and is classified as an epigenetic event that helps in controlling gene expression. miRNAs are noncoding single-stranded RNAs with their ability to bind to the 3' untranslated region of target mRNAs resulted in mRNA cleavage or translation inhibition. Due to its ability to bind both complimentary and imperfect complimentary sequences, miRNA can regulate multiple targets. miRNAs is thought to regulate approximately more than 30% of human genes and is essential in regulating normal and pathological phenotypes (Borriello et al. 2011; Lopez et al. 2009).

2. Restriction point

Mitogenic signals are required for the cell to undergo cell division. There is a stage in the cell cycle where these signals are no longer required for the cell to divide, called the restriction point and begins at late G₁ phase. Cells that have passed this point will progress into S phase. Rbp in combination with the transcription factor E2F plays a role in restriction point; thus completion of the G₁ phase. E2F is a transcription factor crucial for expression of genes and their corresponding proteins needed for cell cycle progression from G₁ to S phase, such as cyclinE and DNA polymerase subunits. In its hypophosphorylated state, Rbp binds to E2F and renders it inactive. Mitogenic signals received by the cell at early G₁ phase will increase production of cyclinD. CyclinD activates CDK4 and CDK6 by binding to these CDKs and forms complex with them. This CDK activation promotes phosphorylation of Rbp. Hyperphosphorylated Rbp releases E2F, which in turn activates transcription of genes needed to enter S phase, including CyclinE by binding to the promoter region of those genes. In turn, irreversible inactivation of Rbp is carried out by CyclinE, which forms a complex with CDK2. This event corresponds to the restriction point (Foster 2008; Malumbres and Barbacid 2005; Sun et al. 2007) (Figure 1-4).

Figure 1-4: Restriction point between G_1 and S phase of the cell cycle. Phosphorylation of Rbp by CDK4/6-CyclinD and CDK2-CyclinE release E2F1 transcription factor subsequently results in S phase progression from G_1 .

3. Cell cycle checkpoints

There are several checkpoints within the cell cycle in which the cell cycle progression may arrest in response to stress, for example DNA damage. These checkpoints enable DNA repair or activation of cell death mechanism if the damage cannot be repaired. Cell cycle checkpoints also prevent daughter cell from inheriting faulty DNA copies. These checkpoints exist at G_1 to control entry into S phase, inter-S phase (halts S phase progression) and G_2M phase (to control entry into mitosis) (Curtin 2012) (Figure 1-5). G_1 checkpoint exists in order to ensure that DNA synthesis machinery is ready to enter S phase and the DNA template is undamaged (Gatti and Zunino 2005). DNA damage that leads to stalled replication forks or depletion of nucleotides leads to inter-S phase checkpoint activation while G_2M phase checkpoint is activated if cell suffered DNA damage that threatens DNA integrity (Gatti and Zunino 2005).

The main player regulating G₁ checkpoint is ATM(ATR)/Chk2(Chk1)-p53/MDM2-p21 pathway. p53 is directly phosphorylated by ATM/ATR upon DNA damage and can be additionally phosphorylated by Chk2/Chk1. Apart from p53, these proteins also targets MDM2, a ubiquitin ligase targeting p53 for degradation. These events result in accumulation, stabilization and increase in transcriptional activity of p53. p53 promotes transcription of CKI p21^{Cip1} thus causing G₁ arrest via inhibition of CDK2-CyclinE activity. CDK2-CyclinE inhibition also prevents cell from passing through restriction point and results in a sustained G₁ blockade (Kastan and Bartek 2004).

Contrary to ATM and Chk2 expression which are relatively constant throughout cell cycle progression, ATR and Chk1 expression and activities reaches peak nearing the end G₁ to S phase transition as a result of E2F activity (Kastan and Bartek 2004). E2F also induces CDC25A, a specialized phosphatase essential in controlling S phase progression and also entry into mitosis by removing inhibitory phosphorylation of CDK2-CyclinE, CDK2-CyclinA and CDK2-CyclinB (Karlsson-Rosenthal and Millar 2006). Regulation of CDC25A by Chk1 and Chk2 ensures appropriate level of CDC25A during cell cycle progression. DNA damage sustained during late G₁ leads to increased activity of Chk1 and Chk2, leading to downregulation of CDC25A activity which halts cell cycle progression during G₁ to S phase transition, S and G₂ phases (Kastan and Bartek 2004).

DNA polymerase α initiates DNA replication during S phase. Its recruitment onto chromatin is promoted by CDC45 protein, which is activated by CDK2. DNA damage during S phase downregulates CDK2 activity and subsequently blocks the loading of CDC45 onto chromatin, resulting in the inhibition of a new DNA synthesis complex. ATM-mediated phosphorylation of several other target proteins also leads to S phase checkpoint activation. Apart from preventing new DNA synthesis, S

phase checkpoint is important in the prevention of DNA breaks resulting from DNA adducts formation by protecting the integrity of the stalled replication forks (Kastan and Bartek 2004).

Figure 1-5: DNA damage causes activation of cell cycle checkpoints via cell cycle regulators (after Curtin 2012).

The main target of G₂M phase checkpoint is CDK1-CyclinB complex. By inhibiting CDK1-CyclinB activity, G₂M phase checkpoint prevents mitosis initiation of a cell suffering from DNA damage either during G₂ phase or during DNA damage carried over from previous S or G₁ phase. CDK1-CyclinB, which is also known as mitosis-promoting factor (MPF) promotes cell cycle progression from G₂ to M phase. Various proteins regulate MPF, including ATM/ATR-Chk2/Chk1-Wee1/SFN pathway (as negative regulators) and CDC25 phosphatases as positive regulators (Figure 1-6). Apart from these proteins, DNA-damage induced mechanisms also targets polo-like kinases (PLK) 3 and 1, which are an upstream regulators of CDC25 and/or MPF. S phase checkpoint regulators such as 53bp1 and

BRCA1 also play a role in G₂M checkpoint. This checkpoint serves as an interesting target for development of cell cycle inhibitors, since most cancers are known to have faulty G₁S checkpoint. Inhibition of G₂M checkpoint in cancer cells might sensitize them to the DNA damaging agents while sparing normal cells with an intact G₁S checkpoint (Kastan and Bartek 2004).

Figure 1-6: Regulation of CDK1-CyclinB (MPF) at G₂M cell cycle checkpoint. DNA damage causes activation of DNA damage signalling pathway and results in cell cycle arrest via downregulation of MPF activity. MPF is also regulated by CKIs.

4. Hallmarks of cancer

Hallmarks of cancer encompass several biological changes acquired during cancer development. Six of the well-known hallmarks of cancers are sustaining proliferative signalling, evading growth suppressors, resisting cell death, inducing angiogenesis, enabling replicative immortality and activating invasion and metastasis (Hanahan and Weinberg 2011; Schulz 2007). These hallmarks of cancer provide foundation for understanding the cancer biology. Information about the causes of cancer increases with research over time thus further provide us with new emerging hallmarks of

cancer. Two of these emerging hallmarks are deregulating cellular energetic and avoiding immune destruction. Tumour-promoting inflammation, genome instability and mutation are classified as enabling characteristics of neoplasia (Hanahan and Weinberg 2011).

Of all hallmarks mentioned previously, evading growth suppressors have a direct link to cell cycle deregulation. Two *bona fide* tumour suppressors are Rbp and TP53 (tumour protein 53, p53). Both control a diverse amount of cell cycle regulations. Whereas Rbp transduces signals originating from outside the cell, p53 is an intracellular stress and abnormality sensor. p53 activation can results in either cell cycle arrest or apoptotic cell death. It is a highly regulated event that depends on the severity of damage and the level of various vital components of cell growth including nucleotide pools, growth promoting signals, glucose and oxygenation. There is an underlying redundancy mechanism exists in the cell that enables compensation for the loss of p53 and Rbp function as cell proliferation regulators. Experiments in chimeric mice reveal that p53 and Rbp null mice undergo normal development and do not have abnormalities until later in life. This mechanism is important in preventing abnormal cell replication in the absence of these critical key regulators (Hanahan and Weinberg 2011).

Evading growth suppressors is also closely related with resisting cell death. This is shown by p53 role in orchestrating apoptosis. p53 is one of the prominent key players in tumorigenesis. p53 promotes apoptosis via upregulation of Bim, Noxa and Puma BH3-only proteins depending on the initiator events. The events that lead to p53 activation are chromosomal abnormalities, inadequate level of survival signals and hyperactive oncoproteins signalling which can be neutralized by antiapoptotic factors. In order to limit or evade apoptosis, cancer cells have developed several strategies with loss of p53 being the most common one. Others include overexpression of antiapoptotic regulators or

survival signals, downregulation of proapoptotic signals and bypassing the extrinsic ligand-induced death pathway. Alternatively, these strategies reflect a myriad of apoptosis-inducing signals encountered by cancer cells during their malignant transformation. The idea of apoptotic machinery and apoptotic evasion strategies has been widely accepted starting the beginning of the last decade. Since then, other forms of cell death have been incorporated as part of defensive mechanism against carcinogenesis (Hanahan and Weinberg 2011).

Genome instability has become the latest enabling characteristic of neoplasia, paving the path for the transformations of normal cells into malignant cancer cells. It is generally described as the inability of a cell to accurately pass on its genome to daughter cells caused by mutations in tumour suppressor genes and chromosome rearrangement (chromothripsis). Three mechanisms leading to genome instability are microsatellite instability, nucleotide excision repair (NER)-related instability and chromosome instability (Coschi and Dick 2012). Microsatellite is DNA sequences which have one specific base repetition in a row in a certain number of repetitions, for example ten thymidines in one sequence (Curtin 2012). Microsatellite instability results from uncorrected DNA replication errors caused by a defective mismatch repair mechanism and produces alteration of microsatellite length (Bouwman and Jonkers 2012; Curtin 2012). NER-related instability occurs with a failure of removal and replacement of UV-damaged nucleotides. Finally, chromosome instability is a result of gain or loss of either a small part of chromosome (segmental chromosome instability) or the whole chromosome (whole chromosome instability). Segmental chromosome instability also includes alteration in chromosome structure that does not change chromosome copy number, such as translocation and inversion. However, whole chromosome instability will leads to aneuploidy if sustained through continual cell division (Coschi and Dick 2012).

4.1 Cell cycle alterations in cancer

In human cancers, mutation of CDKs is infrequent (Malumbres and Barbacid 2005). However, there is a case of miscoding mutation in CDK4 leading to its resistance towards inhibition by INK4 CKIs. Translocation of CDK6 gene near the immunoglobulin loci was reported in several cases of splenic marginal zone lymphoma and B-cell lymphoma. However, alterations in upstream regulators of CDKs (such as CKIs and Cyclins) or its substrates (for examples Rbp and p53) are common. These alterations are the results of mutations or epigenetic alterations (Chen et al. 2012; Malumbres and Barbacid 2005). The G₁ checkpoint is frequently altered, thus cancer cell depends more on the S and G₂ checkpoints to avoid cell death caused by damaged DNA (Chen et al. 2012; Kawabe 2004). Within the G₁ checkpoint, CDK4/6-CyclinD-INK4-Rbp pathway is commonly deregulated in cancer. Wild-type Rbp is found in most cancers, however in some drug-resistant cancers alteration in Rbp do occurs. Mutation of one *Rbp* allele together with somatic inactivation of the other allele leads to retinoblastoma. INK4 family member p16^{INK4a} inactivation in cancer is associated with several mechanisms, such as gene deletion, point mutation and transcriptional silencing via hypermethylation. The inactivation of p16^{INK4a} results in upregulation of CyclinD-associated kinases activity, providing escape from senescence and growth advantage to cancer cells. Apart from p16^{INK4a} downregulation, overexpression of CyclinD is also common (Massague 2004; Shapiro 2006). Mechanisms of CyclinD overexpression include gene rearrangement and amplification, alternative splicing and mutations. In breast cancer, CyclinD is the main mediator for tumorigenesis originating from *Ras* or *Neu* oncogenes. In parathyroid carcinoma, CyclinD gene undergoes inversion at 11p15;q13 while translocation t11;14(q13;q32) of the gene is reported in mantle-cell lymphoma and multiple myeloma. As a consequence of the translocation, the coding region of CyclinD1 is juxtaposed to the gene of immunoglobulin heavy chain resulting in high CyclinD1 expression in lymphoid cells. Alternative splicing produces CyclinD variant that is unable to be exported out from the nucleus and contributes to the oncogenicity in the majority of mantle-cell lymphoma. Mutations at T286 residue of CyclinD1 prevents nuclear export and promotes stabilization of the protein. This

mutation is reported in endometrial carcinoma. Culmination of p16^{INK4a} downregulation and overexpression of CyclinD is associated with higher activity of CDK2-CyclinE and phosphorylation and inactivation of Rbp (Shapiro 2006). Other cell cycle deregulations in cancers are for examples, upregulation of CyclinE and expression of low molecular weight CyclinE expression, downregulation of CKI p57^{Kip2}, upregulation of Skp2 and deregulation of effector molecules such as Wee1 and Stratifin (14-3-3 σ ,SFN) which will be discussed more in other chapters.

4.2 Conventional cancer treatment

Depending on the classification of cancers, cancer treatment will include surgery, irradiation, drug treatment or a combination of the stated treatments. For localized cancers, surgery and irradiation is the treatment of choice while for leukaemias, lymphomas, locally advanced carcinomas, soft tissue cancers and metastatic cancers, drug treatment is preferable. In cancer treatment, adjuvant therapy refers to chemotherapy or irradiation following tumour removal via surgery, aimed to attack residual tumours. Alternatively, neo-adjuvant is chemotherapy given before surgery to reduce tumour size and assist in complete tumour removal. First line therapy is a standard chemotherapy regime given to cancer patients, and in case of relapse, second line therapy is given. Some cancers have high susceptibility to drugs used in chemotherapy or irradiation, however others do not respond well. Some cancers such as ovarian cancer is sensitive to its first line therapy but some do relapse and acquire resistance towards drugs used previously to treat them. Some contemporary anti-cancer drugs frequently used can be divided into several classes: drugs that binds to the DNA (for examples platinum compounds, alkylating agents), nucleoside analogs or nucleotide biosynthesis inhibitors (5-fluorouracil (5-FU), 5'-aza-deoxycytidine), topoisomerase inhibitors (topotecan), microtubule-binding agents (Taxol) and biological agents (tamoxifen) (Schulz 2007) (Table 1-1).

DNA-binding agents are further classified into several types based on their mode of action. They are radiomimetics, alkylating agents, topoisomerase inhibitors and antimetabolites (Indovina and Giordano 2010). Radiomimetics caused similar damage to DNA as ionizing radiation. Alkylating agents or crosslinking agents such as cisplatin is a molecule with two reactive groups that can react with two groups in DNA to cause intrastrand or interstrand crosslinks. Antimetabolites are agents that resemble a normal precursor or cofactor (usually for DNA synthesis) which interfere with the normal metabolic process, examples are nucleoside and folate analogs (Curtin 2012). Although these agents are widely used as chemotherapeutic agents, they possess several disadvantages. They lacked target specificity, thus also affecting normal cells especially with high proliferative nature (such as epithelia of the gastrointestinal tract and hair follicle) as well as cancer cells. This nature of non-specific target is manifested by side effects suffering by patients receiving treatments with these agents, such as gastrointestinal irritation and baldness. In several cases, some patients do not respond to treatment using these agents and some other develops resistance towards those agents after a while (Indovina and Giordano 2010).

Class of drugs	Examples	Mode of action
DNA-binding agents	Cisplatin, carboplatin	Direct binding to the DNA, reacts with DNA bases causing intrastrand and interstrand crosslink, ultimately blocks DNA replication
Nucleoside analogues/nucleotide biosynthesis inhibitors	5-FU, 5'-aza-deoxycytidine	Limiting synthesis of deoxynucleotide triphosphate precursors. Additional function of 5'-aza-deoxycytidine is as a demethylating agent via interaction with DNA methyltransferases
Topoisomerase inhibitors	Topotecan, etoposide	Binds to and inhibit enzymes (topoisomerases) involved in DNA replication. Also part of DNA-binding agents
Microtubule-binding agents	Taxol	Block assembly of or disrupt existing microtubuli or block microtubuli turnover, causing disruption in mitosis, intracellular vesicle transport and cell migration
Biological agents	Tamoxifen, interferons	A diverse group of compounds that do not directly interfere with basic cellular functions; acts on signalling pathways controlling cell proliferation and differentiation

Table 1-1: Anti-cancer drugs frequently used in treating cancers (Schulz 2007).

4.3 Drug resistance

Some cancers respond well to chemotherapy while others do not. This clinical drug resistance can be either *de novo* or acquired in origin. Primary or *de novo* resistance is a pre-existing condition of cancer present during diagnosis that leads to the failure to respond towards first line chemotherapy. *De novo* resistance can be further divided into intrinsic and extrinsic. Intrinsic *de novo* resistance is caused by random genetic mutations already presents in the cell itself that confers survival advantage to the cell upon therapy. Extrinsic *de novo* resistance is caused by extrinsic factors such as cell environment that offers protection to cancer cells from being affected by therapies. Extrinsic *de novo* resistance or environment-mediated drug resistance (EM-DR) is further sub-divided according

to the type of environmental factors involved in mediating the resistance. Soluble factor-mediated drug resistance (SFM-DR) is extrinsic *de novo* resistance facilitated by soluble factors such as cytokines, chemokines and growth factors. Adhesion of cancer cells to stromal fibroblasts or to components of extracellular matrix, which results in cancer cells being protected from therapies, is called cell adhesion-mediated drug resistance (CAM-DR). EM-DR is thought to play a major role in minimal residual disease and providing cancer cells with protective environment leading to the development of acquired resistance (Meads et al. 2009). Acquired or secondary resistance originates from natural selection of cancer cells that sequentially acquired advantageous (epi)genetic alterations which accumulates over time after therapy (Baird and Kaye 2003; Bouwman and Jonkers 2012; Meads et al. 2009). These alterations result in chemoresistance to previously utilised chemotherapy agents. Acquired resistance commonly occurs to cancers which successfully responded to the initial treatment (Baird and Kaye 2003; Bouwman and Jonkers 2012). According to oligonucleotide microarray analysis performed by Hazlehurst et al. (2003) on myeloma cell lines, acquired resistance phenotype showed higher gene expression changes in comparison with CAM-DR phenotype albeit producing similar level of protection against apoptosis induced by melphalan treatment (Hazlehurst et al. 2003; Meads et al. 2009).

4.3.1 Mechanisms of drug resistance

Failure of cancers to respond to a specific drug treatment can be attributed to two general causes: host factor and genetic or epigenetic alteration in cancer cells. These will lead to reduced drug uptake or increased in drug efflux. Host factor includes poor absorption, rapid metabolism or increased drug excretion, sub-optimal dose of drug, alteration in host-tumour environment and decreased access to cancer site caused by bulky tumour, high molecular weight drugs or low tissue penetration (Baird and Kaye 2003; Gottesman 2002). Activation of oncogenes or silencing of tumour suppressor genes are among (epi)genetic alterations acquired by cancer cells, which leads to

chemoresistance (Gottesman 2002). Additionally, tumour cells are characterized by a high degree of genomic instability and may readily have genetic alterations in genes involved in drug resistance mechanisms (Bouwman and Jonkers 2012).

According to Gatti and Zunino (2005), chemoresistance mechanisms can be broadly divided into three main groups. These mechanisms are intracellular defence mechanisms, alteration of drug-target interactions and cellular response that mediates chemoresistance. Intracellular defence mechanisms that caused chemoresistance includes alteration in metabolism and/or subcellular distribution, alteration to drug transporters such as p-glycoproteins and human multidrug resistance-associated proteins (MRPs), and upregulation of glutathione (GSH)-dependent system components. Mutations or changes in level of target proteins contribute to alteration of drug-target interactions. Finally, cellular response that mediates chemoresistance includes increased DNA repair and tolerance towards drug treatment, development of resistance towards apoptosis and cell cycle mediated drug resistance (reviewed by Gatti and Zunino 2005). Figure 1-7 summarize mechanisms of drug resistance in cancer.

Figure 1-7: Tumour cells treated with drugs will respond with either sensitivity or resistance. Sensitivity to drugs used will result in cell death while resistance towards drug treatment can either originate *de novo* or acquired after several rounds of treatment. This chemoresistance phenotype can be due to several common mechanisms.

Taxanes and platinum compounds (for example cisplatin and carboplatin) (Figure 1-8) are the most commonly used chemotherapeutic agents in cancers, especially in ovarian cancer. Thus, acquired chemoresistance towards these agents are not unheard of. Taxol and platinum compounds differ in their mode of action and resistance mechanisms that arise from these agents are different in terms of targeted cell cycle phases. Taxol affects G₂M phase by interfering with microtubule dynamics while platinum compounds induce DNA adducts formation that interferes with DNA replication machinery at G₁ and S phase (Shah and Schwartz 2001). The mode of action of these agents and mechanism of resistance are further detailed in the subsequent chapters.

Figure 1-8: Chemical structure of paclitaxel (taxane) and platinum compounds used in this study. Two platinum compounds, cisplatin (a) and carboplatin (b) were used in the study of platinum-resistant cancer while Paclitaxel (TaxolTM) (c) was utilized in Taxol-resistant cancer study.

4.3.2 Cell cycle-mediated drug resistance

Certain types of chemotherapeutic agents produce a cell cycle phase-specific effect. Some examples of these agents are Taxol that causes G₂M cell cycle arrest and camptothecins that maximally target cells in S phase (Gatti and Zunino 2005; Shah and Schwartz 2001). In combination therapy where two agents are utilized to target a certain tumour, the usage of cell cycle phase-specific agents may sometimes be ineffective. This is due to the effect of the first agent, which alters the tumour's cell cycle and produces a cell cycle condition that is not in favour of the second, cell cycle phase-specific agent. This phenomenon is called cell cycle-mediated drug resistance. To put it concisely, cell cycle-mediated drug resistance is relative insensitivity to phase-specific agents due to the position of the cell in the cell cycle (Gatti and Zunino 2005; Shah and Schwartz 2001).

Several methods can be utilized to study the cell cycle-mediated drug resistance. Shah and Schwartz (2001) have listed methods such as the isobologram, modification of isobologram method by Steel and Pecham, Webb's fractional product method, surviving fraction and Chou and Talalay's combination index method as scientific approaches to study drug synergy or antagonism (Shah and Schwartz 2001). Chou and Talalay's combination index method is employed in this study to look at

the combination effect of platinum compound with synthetic CKI drug seliciclib on drug-resistant cells. This method is further explained in Chapter 2 (Materials and Methods).

5. Epigenetic mechanisms in cells

Epigenetic mechanisms can be translated as mechanisms that results in stable inherited patterns of gene expression in somatic cells without changes in the content or sequence of genomic DNA.

Several mechanisms that are included in this category are X-inactivation in female cells, genomic imprinting, chromatin remodelling, post-transcriptional histone modification, DNA methylation and miRNAs (Feinberg and Tycko 2004; Schulz 2007). Genomic imprinting is involved in selective expression of several genes. In most cases, genes are expressed in equal strength from both alleles inherited from mother (maternal) and father (paternal). However for approximately 50 selected genes, their expression differs between the alleles either in every tissue or in selected tissue and may be qualitative or quantitative (Schulz 2007). CKI p57^{Kip2} expression is an example of genomic imprinting in action and will be explained further in another chapter.

Other epigenetic mechanisms are important in controlling gene expression. Modification of chromatin in the regulatory regions of the gene is needed in order to allow access to DNA sequence by transcription complex, which in turn initiate gene transcription. This is regulated via concerted action of DNA methylation and histone modification (Schulz 2007; Seidel et al. 2012). DNA methylation is vital in determining expression status of a gene. Generally, the promoter of an actively transcribed gene is unmethylated. Promoter methylation results in gene silencing and this phenomenon are observed in X-inactivation and in downregulation of certain genes in cancers. DNA methylation in mammals is restricted to the 5'-position of cytosine residues and only to those in CpG dinucleotides. CpG islands are DNA sequences in which higher CG contents can be observed

compared to the rest of the genome, usually around the transcriptional start site of genes and associated with housekeeping genes (Lopez et al. 2009; Schulz 2007). Methylation of CpG dinucleotides is carried out by enzyme DNA methyltransferases (DNMTs). Four main isoform of DNMT exists. DNMT1 is often known as the maintenance methyltransferase responsible for the conservation of methyl marks during DNA replication. DNMT3A and DNMT3B are involved in methylation of new sites at the sequence previously unmethylated (*de novo* DNA methylation) while DNMT3L is the catalytically inactive isoform. These enzymes are responsible in establishing DNA methylation pattern during development (Schulz 2007; Seidel et al. 2012).

Histone modification is thought to play an additional role in controlling transcription. Generally, hyperacetylation is a sign of transcriptionally active chromatin. Two opposing enzymes regulate acetylation status of a chromatin: Histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Woo et al. 2007). HDACs remove acetyl groups from the lysine residues of target proteins, in this case, histones. Apart from histones, HDACs also target other proteins such as nuclear transcription factors and cytoplasmic proteins. HDACs are classified into four classes (Class I, II, III and IV) based on the sequence homology, cellular localization and activity (Seidel et al. 2012).

5.1 Epigenetic alteration in cancers

In cancers, alteration in methylation status is commonly observed. These alterations are hypomethylation and hypermethylation and can occur within the same cell. Hypomethylation is described as diminished overall DNA methylation compared to normal status. Frequently, hypomethylation occurred throughout/globally across the genome. Global hypomethylation could lead to expression of otherwise transcriptionally silenced genes. Examples of these genes are oncogenic *HRAS*, *CyclinD2* and multidrug-resistance gene *MDR1* (Feinberg and Tycko 2004). Additionally, hypomethylation has been shown to affect chromosome instability, which is linked to

progression of carcinogenesis. In contrast, specific hypermethylation of several genes are common which results in stable silencing of the gene (Figure 1-9). Hypermethylation was shown to be as effective as mutations in inactivating tumour suppressor genes (Lopez et al. 2009; Schulz 2007). Some cell cycle regulatory genes that are silenced due to promoter hypermethylation include *RB1* which encodes Rbp, *CDKN2A* (p16^{INK4a}), *CDKN2B* (p15^{INK4B}), *CDKN1C* (p57^{Kip2}), *Wee1* and *SFN*. Promoter hypermethylation of *CDKN2A* was shown to be an early event that leads to the progression of lung cancer. Other genes involved in DNA repair, apoptosis, cell adhesion and angiogenesis are among genes that are also affected by aberrant promoter hypermethylation in cancers (Feinberg and Tycko 2004; Lopez et al. 2009).

Figure 1-9: Hypermethylated CpG islands are a common feature of cancers. Hypermethylation of promoter sequences prevents expression of downstream genes.

Several properties of DNA hypermethylation make it an attractive target for development of biomarkers. Firstly, hypermethylation of most genes is rarely found in normal cells. Hypermethylation of certain genes are acquired *en route* to carcinogenesis, thus it is cancer-specific. Secondly, methylated DNA is also chemically stable and assays developed for its detection are highly sensitive and user-friendly with wide variety of sample types that can be used to harvest genomic DNA, for example blood or small tissue biopsies. These properties also make gene hypermethylation,

a suitable candidate for screening and helping in determining early treatment strategies. Other possible usage of hypermethylation profiles are in determining tumour prognosis and cancer relapse (Lopez et al. 2009).

Apart from abnormal methylation status, alteration in acetylation pattern also contributes to the development of cancers. Several mechanisms have been identified as a contributor to the deregulation of normal acetylation status. Overexpression of HDAC isoforms are observed in a certain type of cancers. HDAC1 and HDAC3 isoforms were overexpressed in some ovarian cancers while HDAC5 and HDAC9 overexpression were found in medulloblastoma. Various cancers show loss-of-function mutations of HDAC isoform genes, which leads to their inactivation or abolished their cellular localization. Finally, HDAC abnormal recruitment to gene promoter by fusion or chimeric proteins frequently found in leukaemia, results in gene silencing and tumorigenesis via the arrest of normal haematopoietic cell differentiation (Seidel et al. 2012).

5.2 Chromatin-modifying agents

Chromatin-modifying agents (CMAs) are developed with the aim to restore normal epigenetic gene regulations. CMAs are used either singly or in combination with other CMAs. Currently only few CMAs were approved to be used clinically. There are two groups of CMAs, DNMT inhibitors (DNMTi) for example 5-azacytidine; and HDAC inhibitors (HDACi) such as Trichostatin A (TSA) with different mode of action (Figure 1-10). 5-azacytidine was approved as a treatment agent for myelodysplastic syndrome and currently in clinical trials for several other types of leukaemias (Seidel et al. 2012). Contrarily, undesirable chemical properties of TSA (instability issue and low retention level) makes it unsuitable for use in clinical settings (Seidel et al. 2012). DNMTi are incorporated into the newly synthesized DNA and works by trapping DNMT enzymes within DNA strands, thus triggers proteosomal degradation of the enzyme and inhibits further methylation of DNA (Seidel et al. 2012).

This property of DNMTi means that their effect is maximally expressed in cells with high proliferative status, for example cancer cells. DNMTi have been shown to effectively re-establish expression of a previously silenced gene. However, DNMTi lacks specificity and is less suitable for solid tumour treatment (Lopez et al. 2009). Contrariwise, HDACi acts by inhibit HDACs activity resulted in the alteration of proliferation and differentiation state of cancer cells. HDACi can be divided into several groups based on their chemical structure: benzamides, cyclic peptides, depsipeptides, hydroxamates and short-chain fatty acids. Some HDACi targets all isoforms of HDACs while others have more specific action on a certain HDACs isoform only (Seidel et al. 2012). The precise mechanisms of HDACi are yet to be established. However, promoting acetylation of chromatin through chromatin modification and structure changes in transcription factor complexes has been proposed as the mechanisms of action. This ultimately leads to re-expression of silenced genes (Lopez et al. 2009).

Figure 1-10: Chemical structure of CMAs utilized in this study. **a)** 5-Azacytidine, a DNMT enzymes inhibitor. **b)** HDAC inhibitor Trichostatin A (TSA).

6. Targeting cell cycle components in cancer

Cells rely on checkpoints, especially G_1 checkpoint to avoid oncogenesis. However, cancerous cells often have alterations in G_1 and S checkpoints. These are caused by mutations to Rbp and p53, which renders them inactive and unable to provide proper functioning checkpoints. Other alterations, for example in the level of p53 negative regulator MDM2 (murine double minute 2) also affects p53 activity. As a result, cancer cells bypass these checkpoints with a high level of genomic

instability, higher mutational rate and higher possibility of replicating damaged DNA. These events commonly favour carcinogenesis by overexpression of oncogenes such as CyclinE, CyclinD1 and silencing of tumour suppressor genes such as CKIs apart from p53 and Rbp. As a consequence of G₁ checkpoint deregulation, cancer cells rely more on G₂ checkpoint to enhance the rate of mutation which might give additional survival advantages. Thus, targeting G₂ checkpoint is a sensible way of sensitizing cancer cells specifically while sparing normal cells with intact G₁ and S checkpoints (Chen et al. 2012; Kawabe 2004). Some proteins in cell cycle have overlapping functions in cell cycle checkpoints for example p53, ATM and ATR. Therefore, proteins downstream of these sensor proteins are favourable targets for drug development, since they are more substrate-specific. These targets also preferably should not abundantly available throughout cell cycle or involved in other cellular processes as a non-redundant components (Kawabe 2004). Based on these criteria, several G₂ checkpoint-targeted drugs were developed. There is a huge interest in developing Chk1 inhibitors and other G₂ kinase inhibitors as well, for example MK1775. MK1775 is a G₂ kinase inhibitor specifically inhibiting Wee1, a negative regulator of CDK1-CyclinB (Figure 1-11). Wee1 overexpression is observed in certain types of cancers such as hepatocellular carcinoma and breast cancer (Masaki et al. 2003).

Figure 1-11: Chemical structure of Wee1 inhibitor MK1775.

Deregulation of CKIs and CDKs are also a common feature of cancers. Among CDKs, CDK2 is an attractive target for drug development, since it is vital during an early phase of the cell cycle and is often deregulated in cancer (Malumbres and Barbacid 2005). Hence, drug development aimed at

developing small molecule inhibitors resulted in agents such as seliciclib (CYC202) and PD0332991 (Figure 1-12). Seliciclib is a CDK inhibitor with potent inhibitory action against CDK2-CyclinE, with lesser activity on CDK9-cyclinT, CDK7-cyclinH and CDK1-cyclinB. Seliciclib has been shown to have antitumor activity in a broad range of cancers, either alone or in combination with other chemotherapeutic agents (Aldoss et al. 2009; Coley et al. 2007). PD0332991 is another CDK inhibitor acting on CDK4 and CDK6, specifically on cancers with Rbp-positive feature (Fry et al. 2004). PD0332991 sensitivity is inversely related with the expression of p16^{INK4a} (Katsumi et al. 2011) while p53^{Kip2} downregulation predicts sensitivity towards seliciclib treatment in carboplatin-resistant ovarian cancer (Coley et al. 2012).

Figure 1-12: Chemical structure of CDK inhibitors used in this study. a) Seliciclib (CYC202), a CDK2 inhibitor. b) PD0332991, inhibitor of CDK4 and CDK6.

Aims of study

This study is based on earlier findings that demonstrated drug resistant cancer cell lines show epigenetic silencing of several genes, including some that are cell cycle associated. The overall aim is to confirm specific epigenetic changes seen in the original microarray analysis and to do a functional analysis on various drug resistant epithelial ovarian cancer cell lines.

Three genes were chosen from the derived list: *p57^{kip2}*, *Stratifin* and *Wee1*. The first part of this work focused on *p57^{kip2}* and its association with platinum resistant ovarian cancer. From here we have gone on to look at the effects of the CDK inhibitor seliciclib in cells with silenced *p57^{kip2}*. In the next part of the study, we considered other genes that have been identified in the initial gene list and the mechanism underlying their contribution towards drug resistance. We have chosen to study *Stratifin* and *Wee1* and assess their roles in Taxol-resistant ovarian cancer.

Looking at the broader picture, alterations in cell cycle components are a common feature of cancers. Thus, we utilized western blotting as means for screening alterations of cell cycle components particularly G₂M phase in several panels of drug-sensitive and drug-resistant cancer cells. This work will serve as a basis for understanding cell cycle perturbation in drug resistance.

Finally, we chose the CDK inhibitor drug PD0332991, which has shown activity versus CDK4 and CDK6 to develop a drug-resistant melanoma cell line. This cell line harbours a defect in the INK4 family members i.e. *p16^{INK4a}*.

Chapter 2

Materials and Methods

Materials

1. Reagents

Agilent Technologies Inc., Santa Clara, CA

Mycoplasma *Plus* PCR Primer Set

Bio-Rad Laboratories Inc., Hercules, CA

BCA Protein Assay Kit

Merck & Co Inc., Whitehouse Station, NJ

Calbiochem® Annexin V-FITC kit

New England Biolabs Inc., Ipswich, MA

ATM (D2E2) (#2873) rabbit monoclonal antibody, ATR (#2790), Chk1 (#2345), Chk2 (#2662) and p53 (#9282) rabbit polyclonal antibodies

Thermo Fisher Scientific Inc., Waltham, MA

NaCl, Tris Base, Agarose, 5X siRNA buffer, DharmaFECT® Transfection Reagent, 2X Thermo-Start™

PCR Master Mix, ON-TARGET plus siRNAs (Human CDKN1C, Stratifin, Wee1 and GAPDH), siGENOME

Non-targeting siRNA Pool #1

GE Healthcare Ltd., Amersham, Herts.

Amersham Hyperfilm™ ECL

Life Technologies, Carlsbad, CA

4X NuPAGE® LDS Sample Buffer, 10X NuPAGE® reducing agent, Novex® Sharp Protein Standard, HiMark™ Pre-Stained High Molecular Weight Protein Standard, NuPAGE® Novex® Bis-Tris (4-12% and 10%) and 7% Tris-acetate gels, 20X NuPAGE® MES SDS, 20X MOPS SDS and 20X Novex® TA running buffers, Invitrolon™ PVDF membrane, 20X NuPAGE® Transfer Buffer, Novex® AP Chemiluminescent substrate (CDP-Star®), 2° (Secondary) Antibody Solution Alkaline-Phosphatase Conjugated (anti-mouse, anti-rabbit and anti-goat), TRIzol® reagent, Proteinase K (Ambion), Fetal Calf Serum (FCS) (Gibco), GlutaMAX™ 100X (Gibco)

Promega Corp., Madison, WI

ImProm-II™ Reverse Transcription System

Qiagen, Limburg, Netherlands

QuantiTect® SYBR® Green PCR kit, QuantiTect® Primer Assay (GAPDH), EpiTect® Bisulphite Kit, RT² qPCR Primer Assay for gene expression analysis (CDKN1C, Stratifin, Wee1)

Insight Biotechnology Inc., Dallas, TX

p57^{Kip2} (H-91) (SC-8298), Wee1 (C-20) (SC-325) and Survivin (FL-142) (SC-10811) rabbit polyclonal antibodies; E2F-1 (KH-95) (SC-251), p27^{KIP1} (F-8) (SC-1641) and Cyclin E (HE12) (SC-247) mouse monoclonal antibodies; XIAP (N-18) (SC-8789) and 14-3-3σ (C-18) (SC-7683) goat polyclonal antibodies

Selleck Chemicals, Houston, US

MK1775, PD0332991, Seliciclib (CYC202)

Sigma Aldrich, St. Louis, MO

RPMI-1640 medium, DMEM medium, EDTA, DMSO, Trypsin, TrisHCl, Nonidet P40, SDS, Sodium Orthovanadate, Bovine Serum Albumin (BSA) powder, Tween20, RNase-free water, GenElute DNA kit, RNase-A, β -actin (Clone AC-15) and GAPDH (Clone 71.1) mouse monoclonal antibodies, MTT reagent, 5'-Azacytidine, Trichostatin A, carboplatin, cisplatin, Taxol

Zymo Research Corp., Irvine, CA

Universal Methylated DNA standard and primers

2. Buffers and solutions

Phosphate Buffered Saline (PBS) 1X

To prepare 1L PBS (1X), 1 PBS tablets was dissolved in 100mL RO water as per instruction from manufacturer. PBS solution was autoclaved if necessary to make it sterile. Typical formulation of PBS is as follows: NaCl 8g/L, Potassium Chloride 0.2g/L, Di-sodium hydrogen phosphate 1.15g/L, Potassium dihydrogen phosphate 0.2g/L with pH of 7.3 ± 0.2 at 25°C.

MTT solution

5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide was dissolved in a sterile 1XPBS solution and filtered prior to usage if necessary. Solution was kept in cold room away from light.

Lysis buffer

To prepare lysis buffer, ice-cold Tris buffer containing 50mM TrisHCl and 150mM NaCl with pH adjusted to 7.5 was used as the basis and 1% Nonidet P-40, 0.2% sodium dodecyl sulphate (SDS), 1mM phenylmethanesulphonylfluoride (PMSF), 10µg/mL aprotinin, 10µg/mL leupeptin and 1mM of sodium orthovanadate (Na_3VO_4) were added and mixed thoroughly. Lysis buffer was kept on ice.

70% ethanol in PBS

To prepare 500mL 70% ethanol in PBS, 350mL absolute ethanol was mixed with 150mL 1XPBS solution and agitated thoroughly. Solution was kept in cold room.

SDS-PAGE Running buffer

To prepare 1L of SDS-PAGE running buffer, 50mL 20X running buffer stock was diluted with 950mL RO water and mixed thoroughly. Buffer was immediately used and discarded accordingly.

SDS-PAGE Transfer buffer

To prepare 1L of SDS-PAGE transfer buffer containing 10% methanol (per gel), 50mL 20X transfer buffer stock and measured amount of methanol was diluted with RO water to make up to 1L and mixed thoroughly. Buffer was immediately used and discarded accordingly.

Tris buffer

To prepare 200mL Tris buffer containing 150mM NaCl and 50mM Tris-HCl for protein extraction, 1.75g NaCl (FW 58.44) and 1.58g Trizma-HCl (FW 157.6) were weighed and dissolved in RO water. pH was adjusted to 7.5 and solution was sent for autoclaving. Solution was stored in cold room.

10X TRIS Buffered Saline (TBS)

To prepare 500mL 10X TBS stock solution, 12.1g Tris base and 40g NaCl were weighed and dissolved in RO water. pH of the solution was adjusted to 7.6 with HCl. To make 500mL 1X TBS working solution, 50mL of stock solution was diluted in 450mL RO water. Solutions were stored at room temperature.

PBS-/TBS-Tween20

To prepare 500mL PBS-/TBS-Tween20, 500 μ L Tween20 (final concentration 0.1% v/v) was dissolved in 500mL 1XPBS/1XTBS. Solution was kept on room temperature.

Blocking/primary antibody buffer

To prepare 10mL blocking/primary antibody buffer, 0.5g of nonfat dry milk/BSA or 0.25g dry milk and 0.25g BSA together were weighed and dissolved in 1XPBS/1XTBS per recommendation from the datasheet. 10 μ L Tween20 was added and mixed thoroughly. For primary antibody solution, a calculated amount of antibody preparation (e.g. 1:200, 1: 1000) was added into the buffer and mixed thoroughly. After initial use, primary antibody solution can be kept in 4°C or -20°C accordingly.

10X TAE

To prepare 1L 10X TAE, mixed together 48.4g Tris base, 11.42mL acetic acid glacial, 3.7g EDTA and RO water to top up to 1L. Solution was used at 1X by diluting with RO water. Solutions were kept at room temperature.

Drug solutions

Seliciclib, PD0332991, MK1775, Taxol, and TSA were diluted in sterile DMSO. Carboplatin and cisplatin were diluted in 0.9% saline. 5'-azacytidine was diluted in sterile distilled water. Drug stocks were stored in -20°C and thawed prior to use.

siRNA resuspension

SiRNAs purchased were obtained in lyophilised form and resuspended prior usage. SiRNA resuspension was carried out according to manufacturer's recommendations. SiRNA vial was briefly centrifuged to collect pellet at the bottom. To prepare 20µM siRNA stock, 5X siRNA buffer (300mM KCl, 30mM HEPES pH 7.5, 1mM MgCl₂) diluted with sterile RNase-free water to 1X concentration was used. 1X siRNA buffer consists of 20mM KCl, 6mM HEPES (pH 7.5) and 0.2mM MgCl₂ as stated in the product datasheet. Buffer was added into the vial and solution was resuspended several times. The vial was then placed on the orbital mixer at room temperature for 30 minutes before being briefly centrifuged again. siRNA was aliquoted into small volumes and stored at -20°C until use.

BSA protein standard

BSA stock solution was prepared by dissolving 25g BSA in 5mL water and stock was aliquoted into smaller tube. To make a set of BSA protein standards, stock solution (5mg/mL) was diluted accordingly using sterile RO water. Range used was 0.2, 0.5, 0.7, 1.0, 1.2 and 1.5mg/mL BSA. Solutions were stored in -20°C.

Methods

1. Cultured Cancer Cell lines

Several cell lines were utilised throughout the study and were not limited to a certain lineage. PEO1 is the parental cell line with modal chromosome number of 41, originates from a poorly differentiated ovarian serous adenocarcinoma (Langdon et al. 1988). Two drug-resistant cell lines, PEO1CarbR (carboplatin-resistant) and PEO1CisR (cisplatin-resistant) were derived from the parental cell line by step-wise incubation with carboplatin and cisplatin until a stable resistance phenotype was acquired. Maintenance of resistance phenotype was achieved by an addition of 2 μ M carboplatin and 1 μ M cisplatin respectively (Coley et al. 2006). A2780 is a cell line derived from a human ovarian carcinoma and SK-OV-3, which is a cell line derived from an ovarian adenocarcinoma. Taxane-resistant cell lines were established from these two parental cell lines utilising similar drug-resistant development protocol as platinum-resistant PEO1. A2780TaxR is a taxane-resistant counterpart for A2780 with a lower resistance level developed based on a previous study (Syed et al. 2011). Two variants of taxane-resistant SK-OV-3 with differing levels of drug resistance, SKOV3T6 and SKOV3TaxR were established from the SK-OV-3 parental cell line. The maintenance dose for A2780TaxR was 500nM Taxol, 6nM Taxol for SKOV3T6 and 10nM Taxol for SKOV3TaxR. Several cell line pairs of drug sensitive and drug-resistant counterparts were used in the other part of the study. Cell lines of ovarian cancer lineage consist of PEO1 and its drug-resistant variants PEO1CarbR, PEO1CisR and PEO1TaxR (taxane-resistant); SK-OV-3 with its drug-resistant counterparts SKOV3TaxR and SKOV3CR (carboplatin-resistant). Cell lines of breast cancer lineage were MCF7 derived from breast adenocarcinoma and its taxane-resistant MCF7TaxR (Ajabnoor et al. 2012) and T47D of ductal carcinoma with its taxane-resistant T47DTaxR (four-fold resistance to paclitaxel). Other cell lines used in this study were head and neck cancer cell line HN5 and its cisplatin-resistant HN5CisR and a cell line of melanoma origin, A375. A375 was also being used in developing a new drug-resistant cell

line using an experimental drug PD0332991 in the final part of the study (Chapter 7) and were subsequently named as A375PdR.

All drug-resistant cell lines mentioned above were established by Dr Helen M Coley, University of Surrey, Guildford apart from A375PDR, which is jointly established as part of the study. All parental cell lines utilised were purchased from American Type Culture Collection (ATCC) apart from A2780 and HN5. A2780 was purchased from European Collection of Cell Cultures (ECACC) while HN5 was kindly donated by Dr H. Modjtahedi (Institute of Cancer Research, Sutton, UK).

Cell line	Description	Drug-resistant counterpart (agent)	Chapter	Reference (where applicable)
PEO1	Ovarian serous adenocarcinoma	PEO1CarbR (Carboplatin)	3, 4	Langdon et al. 1988
		PEO1CisR (Cisplatin)		Coley et al. 2006
		PEO1TaxR (Paclitaxel)		
A2780	Ovarian carcinoma	A2780TaxR (Paclitaxel)	4, 5, 6	Syed et al. 2011
SK-OV-3	Ovarian adenocarcinoma	SKOV3T6 (Paclitaxel)	3, 4, 5, 6	Syed et al. 2011
		SKOV3TaxR (Paclitaxel)		
		SKOV3CR (Carboplatin)		
MCF7	Breast adenocarcinoma	MCF7TaxR (Paclitaxel)	4	Ajabnoor et al. 2012
T47D	Ductal carcinoma	T47DTaxR (Paclitaxel)	4	-
HN5	Head and neck cancer	HN5CisR (Cisplatin)	4	-
A375	Melanoma	A375PDR (PD0332991)	7	-

Table 2-1: Summary of cell lines utilised in this study together with the associated chapter.

2. Cell cryopreservation and resuscitation

Cells were cryopreserved in liquid nitrogen as a long-term storage and essential especially for a precious cell lines and cell lines that were still developing a drug-resistant phenotype. In order to cryopreserve a cell line, cells were seeded in a T25 flask until near confluence. Complete medium containing 10% FCS, 1X Glutamax and 10% DMSO was prepared prior to the procedure. Cells were trypsinized and pelleted before an addition of a DMSO-containing media. Cells were slowly resuspended and aliquoted into cryopreserve vials. Vials were labelled and put into an isopropyl alcohol container. The container was then left at -80°C for at least 24 hours before transferred into a long-term storage containing liquid nitrogen.

To resuscitate cells from liquid nitrogen storage, vial containing cells was quickly thawed in a waterbath. Thawed cells were then pipetted into a T25 flask containing complete medium and left inside the incubator. The medium was changed the next day if necessary to aid in recovery of the cells.

3. Viability assay

Figure 2-1: MTT chemical structure.

Cytotoxicity testing using MTT has been commonly employed to estimate cellular viability. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) (Figure 2-1) is a hydrogen acceptor, which react with oxidized substrates or appropriate coenzymes including NADH and NADPH. This assay was utilised to assess cellular reductive capacity (due to mitochondrial activity), which correlates with cell viability (Vistica *et al.*, 1991). MTT, a water-soluble yellow tetrazolium dye is

reduced by live cells to a purple formazan product, which is insoluble in aqueous solution. This product can be resolubilized in a suitable solvent (e.g. dimethyl sulfoxide (DMSO)) and its absorption can be read using spectrophotometry at $\lambda 540\text{nm}$ (Mosmann 1983). Viability assay using MTT was used as a way to look at the cell's viability after the drug treatments, and to estimate IC_{50} values. The IC_{50} value is a drug dose needed to cause a 50% reduction in cell viability relative to untreated control cells, obtained from the dose response curve (Twentyman and Luscombe 1987).

The MTT reagent was prepared by dissolving 5mg/mL MTT in sterile PBS, stored at 4°C . 200 μL of cell suspension were seeded in flat-bottomed 96-well microtitre plates using a density of 2×10^4 cells/mL up to 4×10^4 cells/mL (according to doubling times of the particular cell line in question) and left overnight in standard cell culture incubation conditions (with humidity). The following day, drug dilutions were prepared with RPMI-1640 medium containing 10% FCS. Drugs were dispensed at the appropriate concentrations. Untreated cells in the plate acted as a control and medium without any drug dilution was dispensed into the control wells accordingly. The drug-treated plates of cells were incubated for 72 hours (to allow for approximately three cell doublings to take place) followed by the addition of 25 μL MTT reagent into each well. An incubation period of three to four hours was necessary for the formation of formazan crystals before the media containing MTT reagent was aspirated. 200 μL of DMSO was dispensed into the wells to solubilise the purple formazan crystal product. Absorbance of the colour produced was read at 540nm using Multiskan RC microplate reader (Thermo Electron Corp., Finland) within 30 minutes after DMSO addition. The dose-response curves were plotted using percentage of cell viability compared to control (y-axis) versus log drug concentration (x-axis). From these plots, the IC_{50} value was calculated, which is referred to as the concentration of drug that causes 50% cell death relative to untreated controls.

4. Isobologram analysis

Isobologram analysis was selected as a method to assess interaction between two different drugs. In this study, isobologram analysis was carried out to assess the effect of seliciclib in combination with carboplatin, a drug commonly utilised in ovarian cancer treatment on PEO1 parental and its carboplatin-resistant variant PEO1CarbR cell lines. Interaction between the drugs was calculated with the aid of CalcuSyn software program (Cambridge Biosoft, Cambridge, UK). This program utilizes Combination Index (CI) described by Chou and Talalay, based on the multiple drug-effect equation derived from enzyme kinetic models. CI values of <1.0 indicate synergism, CI equals to 1 indicate additive effect, and CI >1 indicates antagonism (Chou and Hayball, 1996, Coley *et al.*, 2007).

200µL per well cell suspension were seeded into the flat-bottomed 96-well plate at the density of 3×10^4 cells/mL and left for 24 hours before the drug treatment. Duplicate plates were seeded for each cell line used for single drug and combination treatment each different plate. Six dilutions of each drug with constant ratio design were prepared in the drug dilution medium and dispensed into the wells. For the single drug treatment plate, additional media without drug were dispensed into the wells to enable equal comparison to the combination drug treatment plate. Plates were incubated for 72 hours before being subjected to the cytotoxicity assay as described previously. Viability fraction of control and drug-treated cells were calculated for each drug concentration. Fraction of cells affected (FA) value for each drug dose was then calculated using the formula $1 - \text{viability fraction}$. FA values were subsequently used as an input to calculate CI. The following table (Table 2-) contains recommended symbols to further explain the extent of synergism or antagonism in CI analysis of drug combination experiment:

Range of CI	Symbol	Description
<0.1	+++++	Very strong synergism
0.1-0.3	++++	Strong synergism
0.3-0.7	+++	Synergism
0.7-0.85	++	Moderate synergism
0.85-0.90	+	Slight synergism
0.90-1.10	±	Nearly additive
1.10-1.20	-	Slight antagonism
1.20-1.45	--	Moderate antagonism
1.45-3.3	---	Antagonism
3.3-10	----	Strong antagonism
>10	-----	Very strong antagonism

Table 2-2: Range of CI, symbols and description suggested for CI analysis of combination drug treatment.

5. Preparation of whole cell lysate

This method was employed to harvest protein from cells, untreated and drug-treated at a particular dose. Floating cells in the media were also collected. Cells were grown in T25 flask, T75 flask or a 6-well plate according to the experimental settings and needs. Generally, cells were harvested by the time the untreated controls reached approximately 80-90% confluence. By the end of the incubation period, cells were washed with PBS, trypsin was added (or cells subjected to harvesting using a sterile plastic scraper) and the flask incubated at 37°C to dissociate cells. The harvested cells were then pelleted via centrifugation at 600g for 5 minutes. The cell pellet was then washed twice with ice-cold PBS. Lysis buffer was prepared as described before. The buffer should be freshly prepared each time and used within approximately half an hour. This is because of a PMSF short half-life at room temperature. Lysis buffer was added to the pellet while disaggregating it with a pipette tip. The disaggregated pellet was left on ice for 10 minutes before being transferred into a microfuge tube. The cells were further lysed using a 23-gauge needle before being left on ice again for 20 to 30 minutes. Following incubation the tube then centrifuged at 1000g for 10 minutes at 4°C to remove

nuclei and unbroken cells. The supernatant was transferred into a new tube, labelled and stored at -70°C.

BCA protein assay kit was used in order to determine the protein concentration in the sample. This assay was based on Bradford protein assay principle. In this assay, BSA protein standards were prepared beforehand ranging from 0.2mg/mL to 1.5mg/mL. Protein samples were diluted either with 1/10 or 1/20 dilution with deionised water in duplicate with a total of 50µL per well in a 96-well plate. Working solution consisting of a mixture of Reagent S (contains SDS) and Reagent A (20µL reagent S per 1mL Reagent A) was made and added to the standards and samples. Addition of Reagent B to the mixture triggered a colorimetric reaction that was quantified by using plate a reader at 690nm after a brief incubation time. Measurement of protein standards was translated into a standard curve which was then utilised to determine the concentration (µg/mL) for each whole cell lysate sample.

6. SDS-PAGE

SDS-PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis) is an electrophoresis procedure using polyacrylamide gel. Electrophoresis is a way of separating small-sized biological samples (protein, DNA and mRNA) and PCR amplicons based on their molecular weight with the aid of electric current. To assist in visualising protein size, a marker is placed and ran alongside protein samples. The distance the protein travelled was tracked with the aid of a dye marker. The polyacrylamide percentage can be modified to optimize visualisation of the protein of interest. Before preparing samples, a known amount of protein samples were calculated so that each well contained the same amount of protein. The amount of protein per well (µg/well) was calculated from protein concentration derived using the BCA protein assay. Between 20µg to 50µg of protein

per well was sufficient for the majority of western blotting analyses. This was determined based on the performance of primary antibody and the amount of protein of interest in the sample. To prepare a sample, protein lysate was mixed with 4X NuPAGE® LDS Sample Buffer at 1X concentration which also acts as a tracking dye, 10X NuPAGE® reducing agent (1X concentration) and deionised water to top up to the final volume. Protein mixture was then heat-denatured at 70°C for 10 minutes, cooled on ice and loaded into the well of the gel. To help determine molecular weight of protein of interest, the Novex® Sharp Protein Standard or HiMark™ Pre-Stained High Molecular Weight Protein Standard was loaded in a separate well and ran together with protein samples. NuPAGE® Novex® Bis-Tris (4-12% or 10%) or 7% Tris-acetate gels combined with either 1X NuPAGE® MES SDS or 1X MOPS SDS or 1X Novex® TA running buffer were employed based on molecular weight of the protein of interest. Novex® Mini Cell (XCell SureLock™ Electrophoresis Cell) electrophoresis apparatus was employed to run electrophoresis at 150V constant for 90 minutes or until the dye was on the edge of the gel.

7. Western Blotting

Western blotting is a procedure of transferring the protein from a gel onto a membrane. After the transfer process, the membrane was incubated with antibodies and developed with a film to visualise band of the desired protein. Membranes of choice were either Polyvinylidene Fluoride (PVDF) or nitrocellulose (NC) membrane. Both membranes have different properties and pore size. The choice of membrane depends on the properties of desired protein. Protein on the gel was transferred onto the membrane facilitated by electrical current. A sandwich of gel, membrane, blotting papers and sponges were set in a transfer device XCell II™ Blot Module and ran at 35V constant for 2 hours in 1X NuPAGE® Transfer Buffer. The membrane was then washed using 1X TBS- or 1X PBS-Tween20 (0.1%) (TBS-/PBS-T) washing solution and transferred into blocking solution consists of 5% milk or BSA or the mixture of the two in TBS-/PBS-T to block non-specific binding of

antibodies. Membrane blocking was done with agitation at room temperature for minimum of 1 hour or overnight in the cold room. Following blocking, the membrane was washed and incubated with primary antibody, which will specifically bind to the protein of interest. Primary antibody solution was prepared similarly as the blocking solution. The primary antibody dilution and incubation time were determined based on optimization. After a certain period of time, membrane was washed trice, 5 minutes each with washing solution and incubated with secondary antibody solution available commercially for 1 hour. The secondary antibody that will bind to the primary antibody was tagged with molecules such as alkaline-phosphatase (ALP) to allow visualisation on film. Following incubation, membrane was washed as before and Novex® AP Chemiluminescent substrate (CDP-Star®) was added onto the membrane for at least 3 minutes before being exposed to the hyperfilm. Chemical reaction between ALP and the substrate produces a signal that can be captured on film. Intensity of the signal was proportional to the level of targeted protein in the protein mixture. After exposure, the developed film was labelled and stored at room temperature.

Figure 2-2: Schematic diagram of SDS-PAGE and Western blotting procedures. 1) Samples are separated using SDS-PAGE along with a marker. 2) Proteins are then transferred to the membrane from the gel with an aid of an electrical current. 3) After the transfer procedure, the membrane is blocked with a blocking agent to prevent non-specific binding of the antibody. 4) After a period of time, the membrane is subjected to incubation with the primary and secondary (5) antibodies. The secondary antibody is tagged with an enzyme. 6) The substrate is later added to the membrane after the end of the antibody incubation period. The substrate reacts with the enzyme tag producing a chemiluminescent signal that is captured on the hyperfilm (7). The signal on the film corresponds with the position of the target protein on the membrane.

Stripping is a process to remove an antibody's signal attached to the protein on the membrane (blot) in order to enable re-probing with different antibodies. This method is done by using heat and detergent, or by using a low pH solution. After stripping procedure, the blot can be reused again for detection of another protein of interest. The blot was washed with PBS once for 5 minutes and incubated with 1mM sodium azide in PBS for a minimum of 2 hours at room temperature or

overnight in the cold room with gentle agitation. The blot was subsequently washed as before and can be used again for another round of antibody incubation.

8. Densitometry

Densitometric analysis was utilised to make a quantitative analysis of selected western blotting results. The chemiluminescent signals seen on the hyperfilm obtained from Western blotting experiments were scanned and analysed with QuantiScan software (Version 3.0) (BioSoft, Cambridge, UK). The data obtained were presented as bar charts on the appropriate figures.

9. Alcohol fixation for DNA analysis

Cells were grown in T25 flask at a density of 3 to 4×10^4 cells/mL. Cells in an early exponential phase of growth (approximately 12 hours after seeding) then were treated with predetermined concentration of drug for a set of time (24 hours and/or 48 hours). For drug-treated cells, floating cells in the media were also collected. By the end of incubation time, cells were dissociated using trypsin; media was added and centrifuged at 600g for 4 minutes to get the cell pellet. Pellet was washed with PBS once before being resuspended in a new PBS solution. 70% ethanol in PBS was then added to the resuspended cells slowly whilst vortex-mixing. The cell suspension was left at 4°C for at least 24 hours for fixation. Cell suspension was washed post-fixation with PBS and centrifuged as before. Cell pellet was then resuspended in PBS followed by staining process. To stain the cells, 5µg/mL propidium iodide (PI) and 1mg/mL Ribonuclease A were used followed by incubation at 37°C for at least 30 minutes in the dark. Ribonuclease A aids in the removal of unwanted RNA and PI intercalates with DNA in the sample. After staining, the cells were subjected to flow cytometry analysis using Beckman Coulter Epics XL flow cytometer (Beckman Coulter (UK) Ltd., High Wycombe, UK).

10. RNA isolation

RNA isolation was carried out using TRIzol® reagent and phase-separation technique. Similarly with the cell lysate procedure, cells can be grown in T25 or 6-well plate accordingly. Medium was aspirated and cells were lysed directly in the flask using 1mL of TRIzol® per 1×10^7 cells. The lysate was passed through a pipette several times before being transferred into 2mL microfuge tube. The homogenised samples were then incubated for 2 to 3 minutes at room temperature to complete dissociation of nucleoprotein complexes. 0.3mL of chloroform was added to the sample and tube was vigorously shaken for 15 seconds. The lysate was left at room temperature for 2 to 3 minutes before centrifugation at 10,000g for 10 minutes at 10°C. After centrifugation, the aqueous solution should separate into 3 phases; the bottom red phase, thin white intermediate layer and upper clear phase, which contains RNA. The upper clear phase was transferred carefully into a new tube and isopropanol was added to the solution (0.5mL per 1mL TRIzol® used) and mixed by inversion before being left for 10 minutes at room temperature. After incubation, the lysate was centrifuged at 8,000g for 10 minutes at 4°C. White pellet may appear if the RNA concentration was high in the sample. The supernatant was removed into waste and the pellet washed with 75% ethanol with vortex mixing. Resuspended pellet was centrifuged again at 8,000g for 5 minutes, supernatant removed and the pellet was dried by opening the tube cap. Precaution should be taken as excess drying of pellet will result in difficulty in dissolving the pellet. The RNA pellet was then dissolved in RNase-free water by mixing gently with pipette. The solution was incubated at 55 to 60°C for 10 minutes before being stored at -80°C. Total RNA yields (ng/μL) and purity was verified using Nanodrop NP-100 spectrophotometer (Labtech International Ltd., East Sussex, UK). Appearance of a single peak on the graph suggest pure sample while an additional peak suggests residual chemical contamination from extraction procedures.

11. Reverse Transcriptase (RT) reaction

RNA extracted from samples can be quantified as a marker for specific gene expression. This was carried out by converting RNA into cDNA and subjected them to quantitative analysis by PCR. The RT reaction was done using ImProm-II™ Reverse Transcription System. For the initial denaturation and primer combination step, RNA (up to 1µg), random primer (0.5µg/reaction) and nuclease-free water to add up to 5µL were pipetted inside 1.5mL microfuge tube and heated at 70°C for 5 minutes. The tube was then immediately placed on ice for at least 5 minutes. Each microfuge was shortly centrifuged and put on ice. Reaction mixture comprised of ImProm-II™ 5X Reaction Buffer (used at 1X), dNTP mix (final concentration 0.5mM of each dNTP), magnesium chloride (final concentration 3mM), recombinant ribonuclease inhibitor RNasin® (20U) and nuclease-free water to top up to 14µL were added into the microfuge tube containing experimental RNA-random primer mix. If there were several samples, a master mix containing all components mentioned previously should be prepared and dispensed to the separate samples accordingly. Finally, ImProm-II™ Reverse Transcriptase (1U/20µL reaction) was added into each tube followed by mixing and short spin. Annealing was done at 25°C for 5 minutes followed by extension step (42°C, 1 hour) and enzyme inactivation (70°C, 15 minutes). The end product was cDNA which were then labelled and stored at -20°C. The cDNA concentration (ng/µL) and its quality was verified using Nanodrop NP-100 spectrophotometer. cDNAs generated were used in quantitative real-time PCR to look at the level of gene expression.

12. Quantitative real-time PCR (qPCR) assay

QuantiTect® SYBR® Green PCR kit was utilised for qPCR assay following recommended procedures by the manufacturer of primers. The reaction was prepared in a 96-well plate with duplicate reaction for each sample. Each sample has to be prepared in two sets, a set is for the gene of interest (GOI) and another is for the housekeeping gene (HKG), either GAPDH or β -Actin. Master mix for each reaction was 2X QuantiTect® SYBR® Green PCR master mix (use at 1X), gene-specific RT² qPCR

Primers (10 μ M stock for each primer, variable amount) and nuclease-free water to top up to 24 μ L reaction. Master mix was dispensed into an optical plate (MicroAmp™ Optical 96-well reaction plate) (Applied Biosystem, Life Technologies Ltd.) followed by an addition of template cDNAs. Template concentration was determined based on optimization and then diluted so that the final volume of template cDNA for the reaction was 1 μ L. The plate was then overlaid with an adhesive film (MicroAmp™ Optical Adhesive Film Kit) (Applied Biosystem, Life Technologies Ltd.). The plate was centrifuged at 3,000rpm for 3 minutes to remove bubbles before being placed into the thermal cycler ABI 5700 Real Time PCR System (Applied Biosystem, Life Technologies Ltd.). Two-step cycling program was employed as recommended by the primer's manufacturer. The first step is an initial activation for the HotStart DNA polymerase at 95°C for 15 minutes. This is followed by a repetitive cycle of template denaturation (95°C, 15 seconds) and primer annealing (55 to 60°C based on the primer selected, 1 minute) for 40 cycles. This system produced a quantitative analysis of target genes GOI and housekeeping gene HKG expression level in a form of a C_T value for each sample. C_T values of samples were employed in the data analysis using $\Delta\Delta C_T$ method. In this analysis, expression levels of GOI were normalised to the HKG by calculating the differences between the GOI and the HKG C_T values (ΔC_T). Then the differences of ΔC_T values for each pair-wise set of control and experimental samples were calculated ($\Delta\Delta C_T$). Finally, the fold-change value was calculated using the formula $2^{(-\Delta\Delta C_T)}$. Positive value indicates an increase in gene expression while negative value represents gene downregulation in experimental samples compared to control.

13. Short interference RNA (siRNA) transfection

The siRNA transfection method was employed to transfect a gene-specific siRNA into the selected cell lines of interest in order to assess the effects of (artificial) gene knockdown. The cells were seeded into a 6-well plate with a density of 1.0 to 5x10⁵ cells/well depending on the cell line selected and left overnight. Transfection procedures were carried out based on the manufacturer's

recommendation with slight modification. Two separate tubes were prepared in a similar volume. Tube 1 is a diluted siRNA consists of a calculated concentration of siRNA, 5X siRNA Buffer (used at 1X) and serum-free medium (SFM). Tube 2 is a diluted transfection buffer DharmaFECT® reagent in SFM. The solutions were left in room temperature for 5 minutes before being gently mixed together and left for a further 20 minutes. After overnight incubation, cells in plate were washed with sterile PBS twice before being treated with siRNA mixture for 4 hours. Complete medium with fetal calf serum (FCS) final concentration of 10% was added to the well after 4 hours and cells were incubated overnight. Medium in the well was then replaced with a new complete medium on the second day. Cells were harvested 72 hours post-transfection for subsequent analysis. Silenced cells can be harvested for protein and/or mRNA either directly from the 6-well plate, or by harvesting and seeding the cells into a T25 flask and incubated until it reached approximately 90% confluence. To do a drug-response analysis on the silenced cells, cells were seeded in a T25 flask and left overnight. Drug treatments were carried out using a predetermined concentration for 48 hours before being subjected to Annexin V-FITC and/or fixed for DNA analysis.

siRNAs utilised were Human CDKN1C (p57^{Kip2}), Human Wee1, Human SFN, Human GAPDH and Non-targeting siRNA Pool #1 (as negative control). Human Wee1 and Human SFN siRNAs were ON-TARGET *plus* SMARTpool® consists of a mixture of four different siRNAs targeting one specific gene. Human CDKN1C and GAPDH siRNAs were ON-TARGET *plus* siRNA consists of single siRNA targeting specific gene. siRNA sequences are as follows (Table 2-3):

Gene	Target sequence
Human CDKN1C (p57 ^{Kip2})	GAGCCAAUUUAGAGCCCAA
	CUGAGAAGUCGUCGGGCGA
Human Wee1	CGACAGACUCCUCAAGUGA
	GAUCAUAUGCUUAUACAGA
	AAUAUGAAGUCCCGUAUA
	AAUAGAACAUCUCGACUUA
Human SFN	AGACUGAGCUCCAGGGCGU
	CGAGACAACCUGACACUGU
	CGACAAGAAGCGCAUCAUU
	CAAGACCACUUUCGACGAG
Human GAPDH	GACCUGACCUGCCGUCUAG

Table 2-3: Target sequence for siRNAs used in the study.

2.16 Annexin V-FITC assay

The Annexin V-FITC assay was employed in flow cytometry analysis to aid in analysis of cell death. Annexin V is an anticoagulant that binds phosphatidyl serine (PS) in presence of calcium with a rapid high affinity binding. It acts as an early marker of apoptosis. PS in viable cells is situated on the cytoplasmic surface of the cell membrane. Early apoptosis induction brings rapid changes to the cell membrane causing PS to be exposed on the cell surface. Translocation of PS will be followed by nuclear breakdown, DNA fragmentation and other molecules of apoptosis-related. The function of Fluorescein isothiocyanate (FITC) in this assay is to enable analysis using flow cytometry or fluorescence microscopy. FITC acts as a conjugate to Annexin V. To distinguish between viable cells, early apoptosis and late apoptosis and late apoptosis or necrosis stages, PI is incorporated in the assay. As stated previously, PI is able to intercalate with DNA. DNA staining in samples indicates the disintegration of the cell membrane. Based on the properties of both Annexin V and PI, determination of cell death can be made. Viable cells will not bind any of the dye whereas early apoptotic cells binds to Annexin V-FITC but not PI. Necrotic cells were both PI- and FITC-positive. . Cell staining using Annexin V-FITC Apoptotic Detection kit can be performed following three

methods; Rapid and Conventional Annexin V binding (with or without binding buffer) and Annexin V binding to adherent cells. Conventional Annexin V binding was the method of choice in this study. Cells were grown in a T25 or a 6-well plate according to the experimental settings. At the end of the experiment, cells were harvested with trypsin. Floating cells in the medium were also collected. Cell suspension was then washed with PBS before resuspension with ice-cold 1X binding buffer prepared earlier. To prepare 1X binding buffer, 5X Binding buffer stock was diluted with deionised water and left on ice. Care has to be taken in washing and resuspension step in order not to introduce artefact. Staining process was carried out by an addition of Annexin V-FITC (0.5µg/mL) followed by 30 minutes incubation at room temperature in the dark. Following incubation, tubes were centrifuged at 1000g for 5 minutes at room temperature to collect cells. Binding buffer was removed and cells were gently resuspended in a new cold 1X Binding Buffer followed by an addition of PI (0.6µg/mL). Samples were kept on ice away from light. Samples were analysed by flow cytometry immediately using Beckman Coulter Epics XL flow cytometer analyser. The following figure (:) is an example of flow cytometer's cytogram of a sample treated with drug and analysed with Annexin V-FITC:

Figure 2-3: Typical cytogram readout of flow cytometer.

14. DNA extraction from cell lines

DNA extraction and purification was carried out to harvest the DNA from parental and drug-resistant cell lines for subsequent analysis of DNA methylation. The method was modified from GenElute Blood Genomic DNA kit. Cells were grown in a T25 flask until it reached approximately 90% confluence. Cells were then harvested using trypsin or PBS and collected in a 2mL microfuge tube. Cells were resuspended in 500µL PBS. RNase A (2µg/µL sample) was added directly and sample incubated for 2 minutes at room temperature. Proteinase K was then added to the sample (2µg/µL sample). To lyse the sample, Lysis solution C was added and sample vortexed for approximately 15 seconds until homogenous before further incubation at 55°C for 10 minutes. Binding column was prepared by an addition of column preparation solution into the column followed by centrifugation at 12,000g for 1 minute. Flow-through was discarded into waste. After incubation, 95-100% ethanol was added into the lysate and vortexed until homogenous (5 to 10 seconds) and loaded into the column. The column was then centrifuged at a minimum of 6,500g for 1 minutes and flow-through discarded. The step was repeated for the rest of the lysate. The column was placed into a new 2mL collection tube and washed with centrifugation ($\geq 6,500g$ for 3 minutes) twice using Wash solution. DNA was then eluted using Elution Solution that was pipetted directly into the centre of the column followed by 5 minutes incubation at room temperature for better elution efficiency before being centrifuged. The eluate containing DNA was labelled and stored at 2-8°C (short-term) or -20°C (long-term). DNA in the eluate was quantified with the Nanodrop NP-100 spectrophotometer. Purified genomic DNA was used in methylation PCR analysis.

15. Agarose gel electrophoresis

Agarose gel electrophoresis is principally similar to SDS-PAGE. In this study, agarose gel electrophoresis was utilised as a validation method for DNA bisulphite conversion and for detecting mycoplasma contamination on the cell cultures. 2% (w/v) agarose was used and the gel was

prepared by dissolving the agarose in 1XTAE. 1XTAE was also utilised as a running buffer. Agarose gel powder were weighed and mixed with 1XTAE before being dissolved in the microwave. The gel mixture was left to cool down slightly; Ethidium Bromide (EtBr) was added and thoroughly mixed before being poured into a gel casting apparatus that was assembled earlier. After the gel has solidified, running buffer was poured over the gel. Samples were mixed with a loading dye and loaded into the well. Samples were run alongside a marker at 100V for approximately 40 minutes or until the dye was at the edge of the gel. To analyze the gel, GeneGenius gel documentation and analysis system (Syngene UK, Cambridge, UK) was employed. The picture of the gel was captured and printed out. The gel was discarded accordingly.

16. Mycoplasma PCR

In some cases, mycoplasma infection can affect cultured cell growth. Cells become slower in growth or die without showing any symptoms of bacterial infection or other contamination. Some cell lines were particularly easily affected by mycoplasma. PCR was utilised to determine mycoplasma infection using a specific primers available in Mycoplasma *Plus* PCR Primer Set. In preparation for mycoplasma detection, cells need to be cultured without antibiotic for several days. With this kit, cell culture supernatant or cells itself can act as a template for mycoplasma detection. Cell extract was the choice of template for this study because it offers higher sensitivity of detection compared to cell culture supernatant. To prepare cell extract, cells were grown in a T25 flask until approximately 90% confluence. Cells were trypsinized and collected in 1.5mL microfuge tube. Cells were then centrifuged to collect cell pellet and washed twice with PBS carefully to prevent loss of cells. The cell pellet was resuspended with sterile water and boiled at 95°C for 10 minutes followed by brief centrifugation. StrataClean resin from the kit was thawed and resuspended by vortexing until its pellet disappears. Resin was then added into the sample and tube was flicked gently before being centrifugated for approximately 5 to 10 seconds to pellet the resin. The supernatant was

carefully transferred into a new tube by avoiding the resin pellet. Supernatant was used directly as a template in PCR and stable for several days at 4°C. 2X Thermo-Start™ PCR Master Mix (used at 1X), samples, primers supplied and nuclease-free water with or without internal control topped up to 50µL reaction was prepared and dispensed into the PCR tube. Thermal cycler was programmed as per recommendation of the kit manufacturer. Details of the optimized program are as stated (Table 2-4):

Segment	Temperature (°C)	Times (minutes)	Number of cycles
1	94	2	1
	50	2	
	72	2	
2	94	1	40
	50	1	
	72	2	

Table 2-4: PCR setting the thermal cycler to detect mycoplasma sequence amplification

Following PCR reaction, samples were analyzed with agarose gel electrophoresis as explained previously. The gel was photographed and discarded accordingly. The presence of amplicon with a size of 874bp is consistent with mycoplasma infection regardless of species. Internal control sample yields 1Kbp band. Further enzymatic digestion of the amplicons with *Sau3A* I can be done to determine the species of mycoplasma if needed.

Mycoplasma PCR detection was done routinely and as part of troubleshooting process alongside experiments.

17. Demethylation treatment

Downregulation of gene expression is a regular event in carcinogenesis. One factor contributing to this event comes from epigenetic modification including hypermethylation of gene's promoter region. In laboratory setting, the effect of hypermethylation towards gene expression can be investigated through demethylation treatment to cell lines. In this study, demethylation treatment was carried out on A2780TaxR taxane-resistant cell line based on method employed by Sato et al. (2005). Demethylation treatment was done to assess the level of Wee1 and Stratifin expression pre- and post-treatment. Cells were seeded in T25 flasks and treatment was carried out using demethylating agent 5-aza-2'-deoxycytidine (1 μ M) and/or the HDAC inhibitor Trichostatin A (1 μ M). The difference in toxicity leads to a varying incubation time for each agent. Cells treated with 5-aza-2'-deoxycytidine was incubated for 4 days while cells treated with Trichostatin A was incubated for 24 hours and the medium was changed the next day. For cells that received a combination treatment, cells were treated alternately for every passage. Cells were treated for several passages before being harvested for protein and mRNA samples. These samples were subjected to analysis using western blotting or qPCR accordingly.

18. Statistical analysis

Statistical analysis values were expressed as mean $\pm$ standard deviation, calculated with GraphPad PRISM® (GraphPad Software, Inc., USA) and Microsoft Excel software. Student's Paired T-Test was performed where applicable and results were expressed as p values. Additionally, one-way ANOVA with Tukey's test for multiple comparisons was also performed where applicable.

Chapter 3

p57^{Kip2} in Ovarian Cancers

Introduction

1. Cancer of the ovary

Structurally, the ovarian surface is lined with epithelial cells, which are contiguous with the peritoneal mesothelium. Ovarian cancer that originates from malignant transformation of this epithelium lining is called epithelial cancer of the ovary (Cannistra 2004). Epithelial ovarian cancer (EOC) can be subtyped based on different histologic features; serous, mucinous, endometrioid, clear cell, and undifferentiated carcinoma (Cannistra 2004; Nam and Kim 2008) (Table 3-). The average risk for women to get EOC in their lifetime is approximately 1 in 70 and the median age for patients with EOC is 60 years. Several risk factors of this malignancy include a genetic predisposition of ovarian cancer, breast cancer or both and nulliparity. Oral contraceptive usage, pregnancy and lactation are associated with reduced risk. There is a suggestion of acquiring EOC during nulliparity state as a result of uninterrupted ovulation which leads to repeated stimulation of the epithelial lining of the ovaries and consequences in malignant transformation. However, women who have undergone tubal ligation seem to be at a lower risk for developing EOC, however the exact mechanism remains unclear (Cannistra 2004).

EOC has the highest fatality-to-case ratio of all gynaecological malignancies. It is also a relatively common malignancy in women, rated as the 5th in the US (Jemal et al. 2004; Nam and Kim 2008). The 5 year survival expectancy for these patients is only 20-40% (Nam and Kim 2008). Most patients with an early-stage EOC do not exhibit specific symptoms or are asymptomatic, while almost 80% of EOC cases are diagnosed during their advanced stage. Several markers such as the serum CA-125 level apart from physical examinations have been used in detecting EOC. However, lack of symptoms and insufficient sensitivity and specificity of serum CA-125 measurement makes EOC diagnosis difficult. The majority of patients in advanced stage of the disease often relapse after treatment with only 10-30% of them having long-term survival. This relapse is caused by development of resistance

towards platinum-based therapy which is the first line of treatment for EOC following cytoreductive surgery (Cannistra 2004; Joo et al. 2009; Nam and Kim 2008; Rosenberg et al. 2001). Apart from an advanced tumour stage, some subtypes of EOC such as high-grade or clear-cell histologic findings have been associated with inferior survival of EOC patients (Cannistra 2004).

The International Federation of Gynaecology and Obstetrics (FIGO) have established guidelines for staging of EOC. The stages are divided into I, II, III and IV with higher rates of long-term survival for patients at the early stage of the disease (stage I and II is as high as 80-95%). With advancement of the disease, the long-term survival rates decrease to only 10-30% (Cannistra 2004).

Histologic type	Features
Papillary serous	The most common type of EOC; may contain psammoma bodies and often associated with elevated CA-125 level; histologic features identical to those of primary peritoneal serous cancer
Endometrioid	Sometimes associated with endometriosis, a separate primary uterine cancer within similar histologic features, or both; may occur within early-stage disease in younger patients, although advanced disease is also possible
Mucinous	May be associated with pseudomyxomaperitonei (rarely); CA-125 level may not be markedly elevated; like clear-cell ovarian cancer, relatively resistant to chemotherapy; not typically associated with <i>BRCA1</i> or <i>BRCA2</i> germ-line mutations; differential diagnosis includes metastatic disease from primary cancer of the appendix, especially if ovarian involvement is bilateral
Clear-cell	The most chemoresistant type of ovarian cancer; characterized by hobnail-shaped tumour cells with cleared out cytoplasm; sometimes associated with endometriosis, humorally mediated hypercalcemia, or both

Table 3-1: Common histologic types of EOC (Cannistra 2004).

1.1 Current treatment of EOC

After initial examination and confirmation of staging, cytoreductive surgery is a method chosen as a primary treatment of EOC followed by platinum-based chemotherapy to eradicate residual disease. Drugs of choice are a combination of paclitaxel and carboplatin in high-risk patients. Platinum analogs such as carboplatin and cisplatin exert their effect by forming a cross-link with DNA interstrand and intrastrand, termed drug-DNA adducts. Platinum analogs also introduce DNA strand breaks. Formation of adducts and strand breaks interfere with protein binding which is essential for intracellular process such as transcription and DNA replication. As an addition, formation of DNA lesions can be recognized by high mobility group and mismatch repair protein which subsequently leads to apoptosis initiated by p53 (Cannistra 2004; Li et al. 2009; Shah and Schwartz 2001). The taxanes are a group of chemotherapeutic agents that act as a microtubule polymerizes. They exert their effect by promoting microtubule assembly, binding and stabilizing microtubules and promoting tubulin polymerization. Because of their action, there is a shift in equilibrium, which results in the failure of assembly because of inadequate concentration of tubulin. Taxanes also interfere with mitotic spindle assembly thus compromising segregation of chromosomes resulting in G2M arrest and apoptosis. Additionally, paclitaxel has been associated with downregulation of CDK4 which brings about G₁S arrest (Cannistra 2004; Shah and Schwartz 2001).

Some patients diagnosed with EOC receiving treatment may face disease relapse, particularly if the malignancy is diagnosed during its late stage. Generally not curable, recurrent EOC is treated with approaches which aim at palliation of symptoms and prevention of complications, such as intestinal obstruction. There are patients with a short remission in which the cancer is recurrent in less than six months after 1st line chemotherapy. Usually this group of patients has a cancer that is resistant to platinum treatment. With this group, treatments are based on alternative agents such as liposomal doxorubicin, topotecan, gemcitabine, paclitaxel, oral etoposide and vinorelbine (Cannistra 2004).

1.2 Cell cycle changes in EOC subtypes

D'Andrilli et al. (2008) have summarized features of cell cycle changes in each of the EOC subtypes. The change is not restricted to only one component of the cell cycle and this implies the effect of cancer is as a result of cumulative defects. CyclinE upregulation is found in serous, clear cell and undifferentiated carcinomas while increased p16 expression can be seen in serous, mucinous and endometrioid EOC subtypes. P53 overexpression is a feature of serous carcinoma while its downregulation is consistent with endometrioid and clear cell carcinomas. Other cell cycle changes in serous EOC include CyclinD1 overexpression and downregulation of p27^{Kip1} and p21^{Cip1}. In endometrioid carcinoma, alterations in the p15 and p16 level and increased c-myc expression can be seen. Clear cell carcinoma, which is associated with poor survival of EOC patients also features CyclinA downregulation and upregulation of p21^{Cip1} (D'Andrilli et al. 2008).

1.3 Acquired drug-resistance phenotype in EOC

As stated earlier, many EOC patients have recurrent disease after chemotherapy and surgical management. The cancer does not respond well following treatment with platinum-based agents. This is due to the development of a drug-resistant phenotype within the malignancy. These patients have short median survival (2-3 years) with a lower survival percentage (Baird and Kaye 2003; Gifford et al. 2004).

Several mechanisms have been proposed to explain the development of drug-resistant EOC. Some broad mechanisms include drug inactivation, decreased accumulation of DNA adducts, defective DNA damage recognition, enhanced nucleotide-excision repair, and impaired apoptotic responses (Gottesman 2002; Li et al. 2009) which become parts of alterations gained by the cancer cell itself (Baird and Kaye 2003). These alterations enable the cancer cell to increase drug efflux, evade

apoptosis, activate detoxification systems and in some cases, alteration of drug targets inside the cells reduced the ability of the drug to bind and cause effect. Generally, some of the mechanisms result in increased drug efflux from cancer cells while others result in reduced drug uptake by the cells. Inadequate drug exposure caused by insufficient dose, poor drug distribution or impaired diffusion to cancer cells may also give rise to drug-resistant phenotype (Baird and Kaye 2003). Other mechanisms include compartmentalization of drug inside the cells, alteration in membrane lipids and altered cell cycle checkpoints (Gottesman 2002).

Several epigenetic changes within the cell may play a role in the rise of EOC drug resistant phenotype (Lopez et al. 2009). Hypermethylation of CpG islands at the promoter of several genes renders them inactive. Alternatively, treatment with drug may induce promoter methylation by means of DNA adduct formation. Distortion of DNA strands with adducts increases accessibility of DNA to DNA-methyltransferase (DNMT) enzymes and/or other auxiliary methylation machinery components thus promoting DNA methylation (Li et al. 2009). Some of the genes whose expression is affected by promoter hypermethylation in EOC includes *hMLH1*; a DNA mismatch repair gene (Baird and Kaye 2003; Gifford et al. 2004), *TGF β 1*; involved in various cell adhesion process and maintains the extracellular matrix structure (Kang et al. 2010), *SFRP5*; an extracellular signalling molecules that acts as an antagonist to Wnt signalling pathway (Su et al. 2010), *Stratifin* (14-3-3 σ); which induces G₂ arrest as a response to a DNA damaging agents (Kang et al. 2010), and p57^{Kip2} (Coley et al. 2012; Sui et al. 2002).

1.4 Strategies to overcome drug-resistance in EOC

As EOC accounts for the highest mortality of all gynaecological malignancies and its tendency to develop resistance after treatment, a strategy to overcome the drug-resistant phenotype is crucial.

Changing several aspects such as the treatment schedule, route of drug administration, drug dose, duration of drug administration and using combinations of agents give some scope in managing the EOC using current chemotherapy agents. Research to develop novel targeted therapeutic agents for the treatment of EOC is ongoing with three main objectives; reduction in toxicity, ease of drug administration and overcoming drug resistance. There are a number of areas that can be manipulated to tackle drug resistance in EOC. Currently, research in this area includes hormonal therapy, growth factor inhibitor, apoptosis induction, anti-angiogenic therapy, gene therapy and immunotherapy (reviewed in Agarwal et al. 2006).

Targeting the cell cycle has recently emerged as one of the new approaches in drug development for cancer treatment. Cell cycle components are often deregulated in cancer cells. Overexpression of cyclins and/or downregulation of CKIs often results in cancer progression (Dickson and Schwartz 2009). In ovarian cancer, CyclinE levels are often deregulated and expression of the low molecular weight form of CyclinE (less than 50KDa) is associated with oncogenesis. This along with histological findings may assist in clinical diagnosis of EOC and opens the possibility for CyclinE to become a target for cancer therapy because of its dual role in cell proliferation and apoptosis (Mazumder et al. 2004). Additionally, inhibition of cell cycle can induce apoptosis. This feature can be manipulated to cause apoptotic cell death to the cancer cell. Inhibition of RNA polymerase II, a key enzyme in transcription mechanism decreased the level of anti-apoptotic protein Mcl-1 thus promoting apoptosis. RNA polymerase II is regulated by several CDKs via phosphorylation. Inhibition of CDKs by a CDK inhibitor agents such as flavopiridol and seliciclib downregulates activation of RNA polymerase II and subsequently leads to apoptotic events in cancer cells (Aldoss et al. 2009; Dickson and Schwartz 2009). Since cell cycle alterations is significant in tumorigenesis and cancer progression, the study of cell cycle components is paramount in order to establish molecular features of cancer and provide further insight into its mechanism, in the hope that it assists with fighting cancers.

2. p57^{Kip2}

Figure 3-1: p57^{Kip2} protein structure.

2.1 The gene

Human p57^{Kip2} protein is encoded by the *CDKN1C* gene situated at the telomeric end of chromosome 11, at the locus 11p15.5. Several binding sites can be found in the promoter region of this gene, including Sp-1 (stimulatory protein-1), ETS (erythroblastosis virus E26 oncogene), egr1 (early growth response 1), a TATA box, OCT1 (octamer-binding transcription factor 1), NF1 (neurofibromin 1), CAT element, a GRE (glucocorticoid-response element), a binding site for p63 isoform and binding sites for Hes1 and Herp2 notch effectors (Pateras et al. 2009). p63 has been shown to regulate transcriptional activation and repression of p57^{Kip2} (Beretta et al. 2005) while treatment of HeLa cells with dexamethasone, a glucocorticoid, upregulates p57^{Kip2} expression (Kamura et al. 2003). Human p57^{Kip2} protein consists of 3 distinct domains. Domain I (N-terminal region) consists of KID (CDK inhibitory domain) that is conserved among family members. KID contains 3 regions: a cyclin binding sequence, a CDK-binding site and an additional binding motif 3₁₀ helix. Thus, KID is essential in p57^{Kip2} CDK inhibitory mechanism. Domain I also contains sequence important in interaction with certain transcription factor, for example MyoD. Domain II contains a distinct proline-alanine rich repeat region termed PAPA region, which is not available in mouse p57^{Kip2}. Domain III is a C-terminal region comprises of a conserved motif QT domain. QT domain interacts with PCNA (proliferating nuclear antigen) and block DNA synthesis. This domain is also important in nuclear localization of p57^{Kip2}. The amino- and carboxy-terminal domains are conserved between human and mouse. Features of this protein are lack of stable helical and β -sheet structure which make it a highly

unfolded molecules, degraded faster and have a higher rate of evolution in comparison with folded protein (Borriello et al. 2011; Pateras et al. 2009). Additionally, p57^{Kip2} only adopts tertiary protein structure after binding to target proteins, thus might explain its interaction with a broad range of proteins and its involvement in regulating numerous cellular activities (Borriello et al. 2011).

2.2 p57^{Kip2} mode of action

p57^{Kip2} is a member of Cip/Kip family of CKIs, other members include p21^{Cip1} and p27^{Kip1}. p57^{Kip2} exerts its inhibitory action on the following CDK-cyclin complexes; CDK2-CyclinE, CDK2-cyclinA, CDK3-CyclinE, CDK4-cyclinD1, CDK4-cyclinD2 and to a lesser extent CDK1(CDK2)-cyclinB and CDK6-cyclinD2; in a cyclin-dependent manner. Higher concentrations of p57^{Kip2} will inhibit kinase activity of CDK-cyclin complexes while at lower concentrations it forms an active complex with CDK2-cyclinA. Inhibition of CDK-cyclin kinase activity comes from the amino-terminal region of the protein, which enables it to bind inside the catalytic cleft in amino-terminal region of CDK2 mimicking adenosine triphosphate (ATP) action (Pateras et al. 2009). The central area of p57^{Kip2} may function in other non-CDK inhibitory roles (Pateras et al. 2009), and the PAPA region has been proposed to be involved in interaction between p57^{Kip2} and the p220^{NPAT}/HiNF-P complex which is a CDK2 substrates associated with nucleosome packaging during G₁-S phase transition (Mitra et al. 2009) (Figure 3-).

Figure 3-2: Inhibitory action of $p57^{Kip2}$ on Rbp/E2F and $p220^{NPAT}$ /HiNF-P pathways. CDK2-CyclinE phosphorylates $p220^{NPAT}$ and Rbp. $p57^{Kip2}$ binds and inhibit CDK2-CyclinE activity and able to bind to $p220^{NPAT}$ /HiNF-P complex and inactivate it.

2.3 Genomic imprinting of $p57^{Kip2}$

CDKN1C, the gene encoding $p57^{Kip2}$ is situated at 11p15.5, a locus well known for containing several imprinted genes that shows preferential parent-of-origin gene expression (Borriello et al. 2011).

Gene expression at this locus is controlled via two imprinting centre/imprinted control region (ICR):

ICR1 (H19) and ICR2 (KNCQ1). ICR1 controls expression of H19 and insulin-like growth factor 2

(IGF2), while ICR2 controls among several genes, *CDKN1C* expression. Both ICR sits near to each

other which pose different methylation status based on allele inheritance. As a result, $p57^{Kip2}$ is

expressed from maternally inherited allele but faint expression is also present from paternally

inherited alleles. However, the exact mechanism of ICR2 in regulating gene expression is unclear.

Translocation can disrupt physical distance between ICR2 and *CDKN1C*, which leads to

downregulation of $p57^{Kip2}$ expression via loss of imprinting (LOI). LOI leads to overexpression of one

gene with silencing of the other gene controlled by the same ICR (Borriello et al. 2011; Feinberg and

Tycko 2004; Schulz 2007).

2.4 p57^{Kip2} roles in normal tissues

As stated previously, CDK inhibitory function of p57^{Kip2} is essential in cell cycle regulation. p57^{Kip2} reaches its peak expression during G₀ and G₁ cell cycle phase, and declines subsequently. p57^{Kip2} also plays a role in endoreduplication, which is replication of the nuclear genome in the absence of cell division ultimately resulted in increased genome content and polyploidy (Borriello et al. 2011; Ullah et al. 2009). p57^{Kip2} controls organogenesis, embryogenesis and cell differentiation in a temporal, spatial and cellular specific manner. Its expression is most apparent during organogenesis. During this stage, the protein is widely localized in tissue and reaches peak at key stages of differentiation for each specific organ. It is present in all major organs during embryonic development. Precise regulation of p57^{Kip2} is important in embryonic growth. The p57^{Kip2} protein starts to decline afterwards and only organs that mature after birth, such as testis, exhibit strong p57^{Kip2} staining in certain tissue compartments (Borriello et al. 2011; Pateras et al. 2009). Differentiation of several types of tissues such as lens-fiber, podocytes, placental cells, keratinocytes, pancreatic cells, hepatocytes, T-lymphocytes, spermatozoa, Leydig cells, chondrocytes, adrenal cortex cells and the nervous system is regulated by p57^{Kip2}. It also takes part in myogenesis via interaction with MyoD, a specific transcription factor needed in myogenic differentiation (Borriello et al. 2011). In adult tissues, p57^{Kip2} expression is limited to chondrocytes and some differentiated epithelial cells such as gastrointestinal villi, smooth muscle cells of intestinal wall, and basal and parabasal cells of normal oesophageal epithelium (Rosenberg et al. 2001). Significant amounts of p57^{Kip2} mRNA can be detected in human adult tissue mainly in skeletal muscle, brain, heart, lung, kidney, pancreas, testis and placenta (Borriello et al. 2011; Pateras et al. 2009). This restricted pattern of expression is due to CpG methylation dependent imprinting of the gene (Mitra et al. 2009). Other roles of p57^{Kip2} includes promoting erythrocyte differentiation, regulating cytoskeletal dynamics and cell motility, inducing senescence phenotype in certain cell types, promoting apoptosis in cancer cells and it also plays a role in transcriptional regulation. Apoptosis-inducing role of p57^{Kip2} is through CDK-independent mechanism by translocation into mitochondria and activating the intrinsic apoptotic pathway. P57^{Kip2}

is able to inhibit transcription indirectly by CDK inhibition which prevents Rbp phosphorylation and directly via binding of p57^{Kip2} to E2F1 and reducing serine residue phosphorylation of RNA polymerase II (Borriello et al. 2011).

2.5 p57^{Kip2} expression in cancerous tissues

Because of its role in cell cycle, deregulation of p57^{Kip2} is one of the features of many human cancers (Pateras et al. 2009; Roeb et al. 2007). Its downregulation can be found, for example, in breast cancer, Wilms' tumour, ovarian carcinoma and Beckwith-Wiedemann Syndrome (BWS). p57^{Kip2} downregulation is associated with the level of tumour aggressiveness in some malignancies and inversely correlated with cell proliferation (Borriello et al. 2011; Lee and Yang 2001; Pateras et al. 2009; Yue et al. 2003). Reduced p57^{Kip2} expression predicts poor prognosis in cancers such as oral squamous cell carcinoma, hepatocellular carcinoma, breast cancer and in malignant ovarian tumours (Pateras et al. 2009). Reduced expression of p57^{Kip2} can be observed in patients with malignant trophoblastic neoplasm (Kim et al. 2005). In some of other malignancies, increased level of p57^{Kip2} together with the level of cyclinE can be observed which may suggest an activation of feedback mechanism to contain uncontrolled cell proliferation. To summarize, the level of p57^{Kip2} in early stages of cancer is elevated before steadily decreasing along with progression of the malignancy (Pateras et al. 2009). Decreased p57^{Kip2} gene expression is associated with the immortalization process and exogenous overexpression of p57^{Kip2} reverts malignant phenotypes (Borriello et al. 2011).

Downregulation of p57^{Kip2} can take place transcriptionally and translationally. However, mutation of the gene itself is not found in cancer samples analyzed, thus, the most probable mechanism of p57^{Kip2} downregulation in cancers comes from epigenetic silencing. This alteration leads to genome

instability with subsequent loss of heterozygosity (LOH), histone deacetylation, methylation at CpG islands in the promoter region and 5' regulatory sequences, and nucleosome remodelling. Epigenetic alterations in cancer cells lead to activation of oncogenes, silencing of tumour suppressor genes like $p57^{Kip2}$ and inactivation of the DNA repair system (Taylor 2006). Increase in promoter methylation and LOH accounts for a decrease in mRNA levels (Borriello et al. 2011; Pateras et al. 2009; Shin et al. 2000; Yue et al. 2003). Deacetylation of histones leads to the transcriptionally inactive state as it permits formation of normal nucleosome structure (Shin et al. 2000). Reactivation of genes by treatment with demethylating agents such 5'aza-cytidine or with histone deacetylase inhibitors offers an alternative potential strategy for the treatment of some cancers, as stated previously in Chapter 1 (Seidel et al. 2012).

In lung cancer, downregulation of $p57^{Kip2}$ may be as a result of several mechanisms such as decreased $p57^{Kip2}$ expression coupled with increased Skp2 level and decreased $p57^{Kip2}$ at the transcriptional level. Some subsets of cases investigated by Pateras et al. (2009) shows upregulation of $p57^{Kip2}$ mRNA together with a decreased protein level which might be an effect of increased Skp2 level. Since some miRNAs have been shown to regulate $p57^{Kip2}$ level (discussed in the next paragraph), increases in miRNA levels associated with decreased $p57^{Kip2}$ is seen in several cancers including gastric, ovarian, colorectal and hepatic carcinomas (Table 3-2). Finally, deregulation of some transcription factors such as p73 also affect $p57^{Kip2}$ activity (Borriello et al. 2011).

Tumour type	Specific sub-type	p57 ^{Kip2} status
Liver tumours	Hepatocellular carcinoma	<ul style="list-style-type: none"> - Downregulation of p57^{Kip2} serves as an independent prognostic factors associated with poor outcome - Comparison with nm23 (metastasis suppressor) expression - abnormal coexpression of nm23/p57^{Kip2} maybe a useful molecular marker for metastasis and unfavourable prognosis
Urothelial carcinomas	-	<ul style="list-style-type: none"> - Protein was decreased in approximately 50% of cases, correlated with the more aggressive forms of cancer
Colorectal cancers	-	<ul style="list-style-type: none"> - Significant correlation between low expression of p57^{Kip2} and large size of tumour - Significant decrease during the transition from adenoma to carcinoma
Adrenocortical cancers	-	<ul style="list-style-type: none"> - Absence of expression in malignant samples - Downregulation was associated with increased CDK2 activity
Thyroid cancers	-	<ul style="list-style-type: none"> - Overexpressed in follicular adenomas and minimally invasive follicular adenomas - Upregulation diminished in invasive follicular carcinomas - Reduced in poorly differentiated and undifferentiated carcinomas - Significant reduction of protein in > 50% of thyroid lymphoma cases
Ovarian cancers	-	<ul style="list-style-type: none"> - Frequent decrease of protein without any correlation with poor survival
Gastric cancers	-	<ul style="list-style-type: none"> - Lower level of protein compared to normal tissues, good correlation with lymph node involvement
Oral and laryngeal cancers	-	<ul style="list-style-type: none"> - Low protein expression. - Absence of p57^{Kip2} is correlated with worst 5-year survival rate (squamous cell carcinoma & leukoplakia) - Increased miR221 and miR222 expression in oral carcinoma cells
Lung cancers	Non-small cell lung carcinomas	<ul style="list-style-type: none"> - p57^{Kip2} expression is selectively downregulated - Reduction was due to epigenetic modification (methylation, allelic imprinting and increased degradation)
Breast cancers	-	<ul style="list-style-type: none"> - Hypermethylation & reduction of mRNA level
Haematological tumours	CML (chronic myelogenous leukaemia)	<ul style="list-style-type: none"> - Decreased protein expression
	ALL (acute lymphoblastic leukaemia)	<ul style="list-style-type: none"> - Increased promoter methylation - Epigenetic changes of the gene as an independent marker of ALL
	Diffuse large B-cell lymphoma	<ul style="list-style-type: none"> - Methylation of the gene in more than 50% cases - Methylation status as detection of minimal residual disease

Table 3-2: Summary of p57^{Kip2} status in several human malignancies (adapted from Borriello et al. 2011).

2.6 p57^{Kip2} regulation

p57^{Kip2} levels are regulated transcriptionally and post-translationally. Transcriptional regulation of p57^{Kip2} comes from an epigenetic control mechanism, as mutational inactivation of this gene and its family members is uncommon (Pateras et al. 2009; Shin et al. 2000). Epigenetic is defined as modified DNA other than in the DNA sequence itself. Genomic imprinting is an epigenetic event that leads to the silencing of gene's expression. DNA methylation and histone modification are closely related with the mechanism of genomic imprinting (Feinberg and Tycko 2004). Imprinting of p57^{Kip2} comes from CpG methylation and histone deacetylase activities. The p57^{Kip2} gene is rich in CpG islands both upstream and downstream of its transcriptional start site and binding of stimulatory proteins to the gene promoter is enhanced by histone hyperacetylation (Borriello et al. 2011; Pateras et al. 2009). In normal status, the p57^{Kip2} gene is expressed by the maternal allele while paternal allele is imprinted. However in most human tissues, low levels of p57^{Kip2} expression leakage from the paternal allele can be detected (Pateras et al. 2009; Yue et al. 2003). Imprinting control of p57^{Kip2} is also tissue-specific, as the gene is hypomethylated in normal human kidneys and methylated in normal human bronchial tissues. Treatment with a demethylating agent or a histone deacetylase inhibitor activates p57^{Kip2} expression by increasing accessibility of DNA to transcription factors (Pateras et al. 2009; Shin et al. 2000). Silencing of p57^{Kip2} expression can also come from various types of non-coding RNA transcripts, as recent findings show that a series of micro-RNAs (miR) miR221, miR-222 and miR-25 (to name a few) are able to downregulate p57^{Kip2} expression in various human cell lines and *in vivo* human hepatocellular and gastric carcinomas (Borriello et al. 2011; Pateras et al. 2009). The possibility of a post-transcriptional regulation mechanism is shown by a different expression pattern of p57^{Kip2} mRNA and protein in mouse placenta (Kim et al. 2005).

Various other pathways also contribute to controlling p57^{Kip2} expression by activation of epigenetic mechanisms, either in inducing or in downregulating its expression. Transforming growth factor β 1

(TGF β 1) and E47 transcription factor participating in human development, upregulates p57^{Kip2} expression (Ma and Cress 2006; Pateras et al. 2009). However in osteoblasts, TGF β causes p57^{Kip2} degradation (Lee and Yang 2001). Treatment with several agents such as histone deacetylase inhibitor, demethylating agent and several drugs such as glucocorticoids, vitamin D3 analog EB1089 and BMS-38703 also induces p57^{Kip2} expression (Ma and Cress 2006; Pateras et al. 2009). Other examples that induce upregulation of p57^{Kip2} are atrial natriuretic peptide and p73 β . Several molecules have been described as downregulators of p57^{Kip2} expression such as IGF2, Notch effectors Hes1 and Herp2, inhibitor of differentiation 2 (Id2) and Pax3-FOXO1. PAX3-FOXO1 is a fusion protein found in rhabdomyosarcoma which downregulates p57^{Kip2} through degradation of transcription factor egr1, thus interfering with p57^{Kip2} activation (Pateras et al. 2009; Roeb et al. 2007).

2.7 p57^{Kip2} relationship with Skp2

Regulation of p57^{Kip2} protein level post-translationally is provided by the ubiquitin-proteasome ligase system, specifically by interaction with Skp2. Skp2 is a subunit of SCF-type E3 ligase, which belongs to the F-box protein family. The SCF multisubunit complex consists of three constant subunits and one variable subunit. The constant subunits are Skp1, Cul1 and Rbx1/Roc1, while the variable subunit is an F-Box protein which performs as a receptor for target proteins. F-box protein thus determines substrate specificity for SCF ligase. Skp2 and another protein - Fbw7 - are among the F-Box proteins that are well characterized and have been shown to control protein levels in cancer cells. Identification of the target protein is in a phosphorylation-dependent manner, as E3 ligase often needs target modification to enable the recognition (Borriello et al. 2011; Kamura et al. 2003; Kitagawa et al. 2009; Pateras et al. 2009; Rodier et al. 2008). Target modification also serves as a way of controlling the activity of SCF E3 ligase (Rodier et al. 2008). Modification of Skp2 target, p57^{Kip2} comes from phosphorylation of p57^{Kip2} by CDK-cyclin complexes. CDK2-CyclinE phosphorylated

p57^{Kip2} at Thr310 residue which serves as a negative feedback loop (Kamura et al. 2003). Identification of the target protein is then followed by polyubiquitination and subsequent degradation by proteosome. Skp2 in turn is targeted by another E3 ligase APC^{Cdh1} for degradation and serves as another way to regulate the ligase activity (Rodier et al. 2008). Besides p57^{Kip2}, Skp2 also targets p27^{Kip1}, p21^{Cip1}, hyperphosphorylated Rbp, p130, E2F1, free CyclinE and c-Myc amongst others, for ubiquitination (Kamura et al. 2003; Kitagawa et al. 2009; Rodier et al. 2008; Sun et al. 2007). During the cell cycle, the Skp2 protein level is low in G₀/early G₁ before gradually increasing during S phase and continues until the cell reaches mitosis (Kamura et al. 2003; Rodier et al. 2008). As Skp2 promotes degradation of negative regulators of the cell cycle, it is considered as an oncogene. Furthermore, there is *Skp2* amplification in human gastric cancers and elevated expression of Skp2 can be found in various other human cancers (Kitagawa et al. 2009; Rodier et al. 2008).

Figure 3-3: SCF E3 ligase structure. Different F-box proteins have different tumour-associated substrates (adapted from Kitagawa et al. 2009).

3. CyclinE as a partner of p57^{Kip2}

CyclinE is the activator of CDK2. CDK2-CyclinE complex is essential in cell cycle progression from G₁ to S phase of the cell cycle and this complex is regulated by p57^{Kip2} among others. CyclinE protein is both synthesized and degraded in the cytoplasm. It is translocated into the nucleus and forms a complex with its partner CDK2. The CDK2-CyclinE complex is essential in cell cycle progression from G₁ to S phase of the cell cycle (Malumbres and Barbacid 2005; Mazumder et al. 2004). CDK2-CyclinE phosphorylates an array of substrates that promotes cell cycle progression. CyclinE also contributes

in the regulation of DNA replication and apoptosis. The cyclinE gene encodes for an approximately 50kDa protein. CyclinE has an isoform CyclinE2, approximately 47kDa molecular weight with 47% overall homology and sharing 75% homology to CyclinE's cyclin box. CDK2-CyclinE downstream targets include Rbp, Cdc6, NPAT and nucleophosmin (Mazumder et al. 2004).

3.1 CyclinE activation and functions

In addition to G₁-S cell cycle progression, CDK2-CyclinE complex interacts with Cdc6, a pre-replication protein that enables its binding to chromatin. Loading of Cdc6 onto chromatin initiates DNA replication. Continual CyclinE binding to chromatin during DNA replication progression might serve as a means of preventing DNA re-replication. Phosphorylation of CyclinE during mitosis prompted CyclinE dissociation from chromatin. This interaction is re-established again via CyclinE dephosphorylation after mitosis exit, thus promoting another round of DNA replication. CDK2-CyclinE promotes centriole disorientation with subsequent splitting by phosphorylating nucleophosmin, which is a part of the centrosome. Nucleophosmin phosphorylation promotes its dissociation from the centrosome thus allowing centriole disorientation. Apart from that, interaction of CDK2-CyclinE with p220^{NPAT} during the beginning of S phase induces histone gene transcription (Malumbres and Barbacid 2005; Mazumder et al. 2004). As stated earlier, p220^{NPAT}/HiNF-P complex is a CDK2 substrate associated with nucleosome packaging and is regulated by p57^{Kip2} (Figure 3-) (Mitra et al. 2009).

Apart from its role in DNA replication and cell cycle progression, CyclinE also plays a part in apoptosis. Following radiation treatment on multiple myeloma and lymphoma cells, CyclinE mRNA and protein level increased substantially followed by increased CDK2-CyclinE kinase activity. Activation of CDK2 is important in apoptosis induction, thus increase in CDK2-CyclinE in these cases

might point to the activation of apoptosis mechanism. In haematopoietic cells, CyclinE overexpression sensitizes cells to radiation-induced apoptosis, while its downregulation protect cells from death. During the progression of apoptosis after radiation and chemotherapeutic agent treatment, a smaller fragment of CyclinE called p18CyclinE is generated possibly by caspase-mediated cleavage of full-length CyclinE protein. P18CyclinE binds to Ku70, a component of non-homologous end-joining (NHEJ) DNA repair mechanism which is activated following DNA damage. Binding of p18CyclinE to Ku70 is proposed to act as regulatory mechanism, preventing DNA damage repair during the course of apoptosis. CyclinE is known to phosphorylates p27^{kip1} thus promoting p27^{kip1} degradation via the ubiquitin-proteasome pathway (Maddika et al. 2007; Mazumder et al. 2004).

3.2 CyclinE regulation and deregulation in cancers

CyclinE is regulated transcriptionally and post-transcriptionally. CyclinE falls under the control of the E2F transcription factor regulation since the *CyclinE* promoter contains several binding sites for E2F. The E2F binding site is also found in CyclinE repressor module that acts to repress CyclinE expression until late G1 phase. Within this site, E2F is bound with Rbp and pocket protein DP1. Rbp interacts with histone deacetylase (HDAC) and several other proteins to form a repressor complex. Phosphorylation of Rbp by CDK4-CyclinD releases Rbp from the repressor complex, enabling gene transcription (including *CyclinE*) by E2F. Post-translational regulation of CyclinE comes from its interaction with several proteins and its ability to autophosphorylate. CDC25A, an upstream regulator of CyclinE removes inhibitory phosphorylation on CDK2 thus indirectly influence CyclinE activation. Apart from activating CyclinE, CDK2 binding protects CyclinE from degradation since unbound CyclinE is degraded via ubiquitin-proteasome system. Skp2, an F-box protein involved in regulating p53^{kip2} has been found to play a role in CyclinE degradation. *Skp2* silencing leads to accumulation of CKIs with a subsequent negative effect on CDK2 activation and CyclinE

autophosphorylation, ultimately promoting CyclinE degradation. Another F-box protein, Fbw7 shows specific binding to CyclinE. Fbw7 phosphorylates Thr380 and Thr62, promoting CyclinE degradation via ubiquitin-proteasome pathway. Mutation of *Fbw7* is found in cancers with high CyclinE, suggesting its role in regulating CyclinE levels (Mazumder et al. 2004).

Deregulation of CyclinE had been reported in breast, colon, lung, ovary and prostate carcinomas (Maddika et al. 2007; Mazumder et al. 2004). In some tumours, CyclinE upregulation is an early event during the transformation however in others it is a late event. Several mechanisms contributing to high CyclinE level in tumours are increased protein synthesis, decreased degradation and impaired translocation into the nucleus. In ovarian cancer, loss of p27^{kip1} expression and CyclinE or CDK2 overexpression significantly correlates with malignancy. High CyclinE expression correlates with lower life expectancy and poor prognosis (Mazumder et al. 2004; Rosenberg et al. 2001). CyclinE deregulation might be associated with certain ovarian cancer sub-types, particularly clear cell, serous and poorly differentiated carcinomas of the ovary (Rosenberg et al. 2001; Stamatakis et al. 2010). Low p27^{kip1} expression coupled with high CyclinE level is also significantly correlated with poor prognosis in lymphoma. Malignant transformation of breast ductal carcinoma *in situ* to invasive carcinoma is associated with increased CyclinE expression. Another form of biochemically active low molecular weight CyclinE is found to be commonly expressed in majority of breast cancer cell lines and breast cancer patients. Its level is associated with increased CDK2 kinase activity, higher rate of cell proliferation and poor outcome (Mazumder et al. 2004). In ovarian cancer, CyclinE is associated with higher tumour grade, late stage disease, suboptimal cytoreduction and patients aged more than 60 years at the time of diagnosis (Stamatakis et al. 2010).

4. Exogenous modulation of the cell cycle by use of synthetic CDK inhibitors

4.1 Seliciclib: Mode of Action

Figure 3-4: Seliciclib (CYC202, R-roscovitine) chemical structure (Golsteyn 2005).

Seliciclib (CYC202, R-roscovitine) is the r-enantiomer of the cell cycle inhibitor agent roscovitine. It is a second generation of CDK inhibitory agent, with a higher selectivity for its CDK target compared to the first generation small molecule CDK inhibitors such as flavopiridol. In contrast to seliciclib, flavopiridol is a pan-CDK inhibitor with a global inhibition effects. Seliciclib on the other hand has a high specificity for CDK2-cyclinE, CDK9-cyclinT, CDK7-cyclinH and CDK1-cyclinB and it is a potent inhibitor of CDK2-cyclinE relatively (Aldoss et al. 2009; Coley et al. 2007; Whittaker et al. 2007). Seliciclib is able to block cell proliferation in almost all stages of the cell cycle by a number of mechanisms. The main mechanism involved in cell cycle blockage by seliciclib is through the ATP pocket of the kinase catalytic subunit. Seliciclib reversibly binds to and competes for the ATP-binding site which inhibits CDK-cyclin activities (Aldoss et al. 2009; Galimberti et al. 2010). Other mechanisms include cell cycle arrest as a consequence of CDK inhibition at G₁S and G₂M, and apoptosis promotion. Seliciclib promotes apoptosis by means of several ways. It inhibits CDK7 and CDK9 thus downregulating phosphorylation of RNA polymerase II carboxy terminal domain, which has a role in transcription machinery. This event leads to downregulation of anti-apoptotic proteins such as Mcl-1 and other proteins involved in transcription and translation, for example CyclinD1. Seliciclib also inhibits Rbp phosphorylation and downregulates expression of XIAP and survivin. All these events lead to cell cycle arrest at G₁S and G₂M, and initiation of caspase-dependent apoptosis (Aldoss et al. 2009; MacCallum et al. 2005; Whittaker et al. 2007). Inhibition of CDK9 by seliciclib also

affects the level of MDM2, a negative regulator of the tumour suppressor protein p53. Reduced level of MDM2 results in stabilization and accumulation of transcriptionally active p53 in nuclei which will lead to apoptosis. Cells with functional p53 are more susceptible to seliciclib effects (Paprskářová et al. 2009). However, seliciclib is also able to induce killing in cells with mutation of either *ATM* or *TP53* genes as in B-cell chronic lymphocytic leukaemia (B-CLL) through downregulation of transcription and cell survival without noticeable effect on normal lymphocytes (Alvi et al. 2005).

Figure 3-5: Summary of seliciclib mode of actions.

4.2 Seliciclib as single agent and in combination with other drugs

Seliciclib has shown its antitumour effect against a spectrum of human cancer cell lines, both haematological and solid tumours. Treatment of B-CLL cell lines with seliciclib promotes apoptosis irrespective of ATM/TP53 mutations while in combination with alemtuzumab (a monoclonal antibody drug) seliciclib promotes apoptosis of alemtuzumab-resistant cells (Aldoss et al. 2009; Alvi et al. 2005). Seliciclib also induces apoptosis in diffuse large B cell lymphoma (DLBCL) and mantle cell

lymphoma (MCL) by accumulation of lymphoma cells in G₁ or G₂M phases. A synergistic effect can also be seen in combination with doxorubicin or bortezomib (a proteasome inhibitor) against multiple myeloma (MM) cells. In lung cancer studies, seliciclib has shown induction of apoptotic activity either as a single agent or in combination with EGFR and HER-2 pathway inhibitors through formation of anaphase catastrophe during cell division (Aldoss et al. 2009; Galimberti et al. 2010). Treatment of breast cancer cell lines with seliciclib promotes apoptosis by sensitizing cells to tumour necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) followed by cell cycle arrest. Combination with doxorubicin or trastuzumab also produces a synergistic effect (Aldoss et al. 2009). In uterine sarcoma cell lines, seliciclib treatment shows synergism in combination with cisplatin. However, as a single agent seliciclib does not elicit a profound cell cycle effect as compared to other cytotoxic agents such as paclitaxel but a consistent G₂M accumulation has been reported. Again p53 status did not have an influence on seliciclib-cisplatin synergism (Aldoss et al. 2009; Coley et al. 2007b). Combination of seliciclib with paclitaxel also shows synergism, preferably by following the sequence of adding seliciclib first before paclitaxel treatment on SK-LMS-1 cell line (Coley et al., 2007b). Apoptotic activity caused by seliciclib treatment can also be observed in cervical cancer, malignant gliomas and undifferentiated nasopharyngeal carcinoma cell lines (Aldoss et al. 2009).

5. Platinum compounds (cisplatin and carboplatin)

Figure 3-6: Chemical structures of platinum-based compounds used in this study: a) Cisplatin (*cis*-diamminedichloroplatinum(II)) b) Carboplatin (*cis*-diammine-1,1-cyclobutane-dicarboxylatoplatinum(II)).

Cisplatin was introduced in the late 1970s for treatment of ovarian and testicular cancers, and since has become the most commonly used anticancer drug in the United States. Its less toxic analog, carboplatin was approved as chemotherapeutic agent used to treat ovarian cancer during the late 1980s. Both of these drugs together with another platinum compound oxaliplatin are widely used to treat solid tumours such as lung, colorectal, ovarian, breast, head and neck, bladder and testicular cancers. Both compounds differ in terms of chemical reactivity, pharmacokinetics and toxicity, among others. Carboplatin, apart from its lower toxicity compared to cisplatin is also more stable with comparable activity in some tumours (Burger et al. 2011; McWhinney et al. 2009).

5.1 Mode of action

Platinum compounds were classified under alkylating agents as they give rise to similar DNA damaging effects. They exert their effect by binding to DNA basepairs and creating DNA adducts, intra- and inter-strand crosslink and strand breaks. Cisplatin reacts with purine bases in DNA, causing DNA-protein and DNA-DNA inter-strand and intra-strand crosslinks. As a result, nuclear metabolism and chromatin organization is disrupted and DNA replication is inhibited (McWhinney et al. 2009; Siddik 2003). These events lead to activation of DNA damage response (DDR) mechanism via *Gadd45a* (growth arrest and DNA damage inducible) gene and nucleotide excision repair (NER). Apart from DDR, cisplatin-induced DNA damage also triggers several pathways including cell cycle checkpoints and apoptosis. To remove DNA adducts caused by cisplatin, NER complex is activated and cell cycle progression is halted to enable adducts removal. However, upon incomplete DNA repair caused by overwhelming damage, the apoptosis mechanism is activated. These three pathways (DDR, cell cycle arrest and apoptosis) are closely related with p53 and its downstream effector's activation. Experiments conducted on human col carcinoma cell line HCT-116 showed that cisplatin induced p53 activation via ATR (ATM and Rad3-related) (Damia et al. 2001; Siddik 2003). ATR phosphorylates Ser15 of p53 as well as Chk1 to facilitate additional p53 phosphorylation

and activation. Chk1 in turn phosphorylates p53 additionally at Ser20. Apart from Chk1, cisplatin is also found to activate Chk2, a downstream effector of ATM in an ATM-independent way (Damia et al. 2001; Siddik 2003). The CKI p21^{Cip1} is also activated (transactivation) via p53 after cisplatin treatment in experiments done on human ovarian cancer cell line 2008 (Delmastro et al. 1997; Siddik 2003). Observable cisplatin effects on cell cycle include transient S phase cell cycle blockage followed by G₂ or M cell cycle arrest via inhibition of CDK1-CyclinA/B. P16^{INK4a}, another CKI has been shown to facilitate inhibition of G₁ phase kinases (CDK4/CDK6) following cisplatin-induced DNA damage. However, accumulation of cells in G₁ is infrequent as cells mainly trap in G₂M phase (Shah and Schwartz 2001; Siddik 2003). Cisplatin increases S phase duration and cell cycle blockage in a dose-dependent manner. This event ultimately leads to aberrant mitosis (Shah and Schwartz 2001). *Bax*, a pro-apoptotic gene is also activated by cisplatin via p53. However, in cancer cells with defective apoptosis mechanism, p53 downregulation may not make cells resistant towards platinum. Instead, p53 disruption can sensitize cells to platinum drug treatment possibly through downregulation of p21^{Cip1} which results in premature mitosis and cell death (Shah and Schwartz 2001; Siddik 2003).

5.2 Platinum toxicity

The general toxicity profile for platinum compounds differs from each other. Cisplatin causes mild hematologic toxicity and cumulative ototoxicity. The most common toxicity profile caused by cisplatin is peripheral neurotoxicity, starting with painful paraesthesia (sensation of tingling or numbness of skin) followed by loss of vibration sense and ataxia (lack of voluntary coordination of muscle movement) in parallel with increasing treatment cycles. Cisplatin also induces severe nausea and vomiting. Severe renal tubular damage and decrease glomerular filtration are also side effects of cisplatin and manageable with saline hydration and mannitol diuresis to minimize kidney damage. Carboplatin on the other hand causes dose-dependent thrombocytopenia and higher rates of anaemia in comparison with cisplatin. However, neurotoxicity caused by carboplatin is less frequent

and less severe whilst no ototoxicity is observed in patients receiving carboplatin treatment. Patients also experience less nausea and/or vomiting and renal toxicity (McWhinney et al. 2009). Platinum-induced nephrotoxicity is thought to relate to transporter activity in renal tubular cells. Interaction between transporter SLC22A2 (solute carrier 22 family member A2/OCT2-organic cation transporter 2) and CTR1 (copper transporter 1) with cisplatin may induce its uptake and cause renal toxicity. Oral administration of imatinib together with platinum compounds is shown to reduce renal accumulation of that platinum compound; however the exact mechanism is unknown. SLC22A2 is also postulated as the primary mediator of ototoxicity by regulating cisplatin uptake in the cochlea hair cells (Burger et al. 2011).

5.3 Acquired resistance to platinum compounds

Tumours that are resistant to cisplatin are also resistant towards carboplatin treatment, suggesting these platinum compounds evoke similar cellular responses. Specific mechanisms for acquired resistance to cisplatin have been described. These include reduced intracellular drug accumulation by means of inhibition in drug uptake, increase in drug efflux or both (Burger et al. 2011; Siddik 2003). Binding of cisplatin to thiol-containing molecules such as glutathione (GSH) and cysteine-rich metallothionein inactivates cisplatin thus reducing its cytotoxic effect. Upregulation of GSH and metallothionein have been recorded in several cisplatin-resistant tumour models. Since cisplatin induces the DDR mechanism, alteration in components of DDR affect the cisplatin response in cells. Testicular tumours that are sensitive towards cisplatin treatment had been shown to express a very low level of XPA and ERCC1/XPF, proteins that are part of NER complex. Overexpression of XPA promotes cell repair and contributes towards cisplatin resistance. Loss of *hMLH1* or *hMSH2*, part of mismatch repair (MMR) complex is constantly seen in cisplatin resistance and are associated with microsatellite instability and resistance to cell death. Dysfunctional p53 has a close association with deficiency in MMR. Mutation in *p53* gene is observed in variety of cancers with correlation between

cisplatin sensitivity and p53 level. Lower response rate was seen in cancers with high p53 mutation level such as ovarian, head and neck and metastatic bladder cancers while seminomatous germ cell tumours with wild-type p53 showed greater cisplatin sensitivity. However, p53 status might not be the definitive characteristic of cisplatin resistance since resistance do occurs irrespective of *p53* status in some cases. Another mechanism by which cells acquire cisplatin resistance is inhibiting apoptosis pathway. Inhibitor of apoptosis, such as survivin and XIAP overexpression promotes cisplatin resistance. These proteins directly or indirectly affect caspases activities which are essential in apoptosis mechanism (Shah and Schwartz 2001; Siddik 2003). Other mechanisms of acquired cisplatin resistance include overexpression of *HER2*, increased activity of PI3K/Akt pathway and deregulation in ras/MAPK pathway, are not discussed here.

Limiting platinum-DNA adduct formation	- Impaired transport leading to reduced platinum accumulation: decreased drug uptake and increased drug efflux
	- Inactivation of platinum compounds: glutathione and metallothionein
Preventing cell death upon treatment	<div>- Increased repair of DNA adducts</div> <div>- Increased tolerance to DNA adducts</div> <div>- Failure to provoke apoptotic pathways</div>

Table 3-3: Major cellular mechanisms involved in platinum resistance (Burger et al. 2011).

Aim

This study focuses on p57^{kip2} and its association with platinum resistant ovarian cancer. From here we have gone on to look at the effects of the CDK inhibitor seliciclib in cells with p57^{kip2} deficient cells.

Hypotheses

- Downregulation of p57^{kip2} plays a functional role in mediating anticancer drug resistance
- In the absence of adequate p57^{kip2} function (due to low expression levels) the use of a synthetic CKI, such as seliciclib can compensate for this deficiency and possibly re-sensitise drug resistant cells to platinum

Methods

Cell lines used in this work are ovarian cancer cell lines PEO1 and its drug-resistant variant PEO1CarbR (carboplatin-resistant) and PEO1CisR (cisplatin-resistant); and SK-OV-3. Cells were cultured in a suitable media accordingly. Techniques used are viability assay using MTT, whole cell lysate preparation, SDS-PAGE, Western blotting, alcohol fixation and DNA analysis using flow cytometry, mRNA isolation, RT and qPCR analysis, siRNA transfection, AnnexinV-FITC assay and isobologram analysis. All techniques have been explained previously in Chapter 2. Graphs were constructed using Microsoft Excel and GraphPAD PRISM softwares. Student's paired T-Test and one-way ANOVA with Tukey's test for multiple comparisons were done where applicable, and the results were expressed as p values.

Results

1. Cross-resistance between platinum compounds in ovarian cancer

a) Dose-response curve of PEO1 panel treated with carboplatin

b) Dose response curve of PEO1 panel treated with cisplatin

Figure 3-7: Dose-response curve of PEO1, PEO1CisR and PEO1CarbR treated with carboplatin (a) and cisplatin (b). Both PEO1CisR and PEO1CarbR showed cross-resistance to cisplatin and carboplatin treatment, while PEO1 parental cell line remained relatively sensitive to both platinum compounds. This cross-resistance phenotype was widely documented in clinical cases (Siddik 2003). (n > 3)

Platinum treatment followed by viability test using MTT was performed on the PEO1 panel (drug-sensitive and drug-resistant). The dose-response curves were drawn to acquire IC₅₀ values of carboplatin and cisplatin for each cell lines in the respective panel. All cell lines used in this experiment and other subsequent experiments were tested negative for mycoplasma.

Table below was derived from the data obtained via viability assay results (Figure 3-):

	Carboplatin IC ₅₀ (μM)	RF	Cisplatin IC ₅₀ (μM)	RF
PEO1	20.1 (±9.2)	-	1.02 (±0.5)	-
PEO1CarbR	68.1 (±22.9)	3.39	5.17 (±1.8)	5.01
PEO1CisR	67.7 (±21.3)	3.37	5.64 (±1.0)	5.53

Table 3-4: Carboplatin and cisplatin IC₅₀ values in PEO1, PEO1CarbR and PEO1CisR. RF refers to resistance factor. To achieve similar effect as cisplatin, carboplatin needs to be used at higher concentration. Based on the IC₅₀ values, PEO1CarbR and PEO1CisR showed cross-resistance by having a very close IC₅₀ values to each other for both drugs. IC₅₀ values were derived from dose-response curve analysis (n >3).

Figure 3-7 and Table 3-4 shows the difference of drug dose needed to achieve similar effect between drug-sensitive and drug-resistant cancer cells. Both PEO1CarbR and PEO1CisR showed cross-resistance towards cisplatin and carboplatin with IC₅₀ values near to each other.

2. *p57^{Kip2}* and *Skp2* mRNA level in PEO1 panel

QPCR was performed in order to quantify *p57^{Kip2}* and *Skp2* mRNA level in PEO1 panel.

Figure 3-8: QPCR analysis of *p57^{Kip2}* and *Skp2* level in PEO1 parental and platinum-resistant PEO1CarbR and PEO1CisR. *Skp2* is the negative regulator of *p57^{Kip2}*. a) Result shown that *p57^{Kip2}* mRNA level in PEO1CisR was higher compared to PEO1. In PEO1CarbR, *p57^{Kip2}* mRNA level was significantly low. Thus the level of *p57^{Kip2}* expression was in the order of PEO1CisR > PEO1 > PEO1CarbR. b) *Skp2* mRNA expression was shown to be increased in cisplatin-resistant PEO1 in a higher magnitude compared to PEO1CarbR. The level of *Skp2* expression in this panel was PEO1CisR > PEO1CarbR > PEO1. Using One-way ANOVA with Tukey's test, the significant differences between expression levels of proteins in each line were calculated. The difference in *p57^{Kip2}* expression between PEO1, PEO1CisR and PEO1CarbR was significant. Similar results were also achieved for *Skp2* expression in all three cell lines. All results were normalized against GAPDH. (*p < 0.05, **p < 0.005) (n=7).

p57^{Kip2} and Skp2 gene expression in the PEO1 panel were quantified using qPCR analysis and results were presented as bar charts. Results obtained showed p57^{Kip2} and Skp2 upregulation in PEO1CisR compared to parental cell line PEO1. However, significant downregulation of p57^{Kip2} was seen in PEO1CarbR with higher Skp2 level in the same cell line compared to parental.

3. Level of target proteins in cell lines

Western blotting using several antibodies was performed to look at the protein levels in each cell lines. Antibodies used were against CKIs p57^{Kip2} and p27^{Kip1}, and G₁S cell cycle regulators CyclinE, E2F1 and Survivin. Results were scanned and labelled accordingly.

3.1 p57^{Kip2}

Figure 3-9: p57^{Kip2} level in PEO1, PEO1CisR and PEO1CarbR as assessed by western blotting. p57^{Kip2} protein band appeared in PEO1 and PEO1CisR. However, there was no p57^{Kip2} band in PEO1CarbR. Based on the results, expression of p57^{Kip2} in PEO1 and PEO1CisR were higher than in PEO1CarbR, which was almost undetected. This pattern reflected mRNA expression analysis of p57^{Kip2} using qPCR.

3.2 G₁S cell cycle regulators

Figure 3-10: Protein level of CyclinE (a), E2F1 (b), Survivin (c) and p27^{Kip1} (d) in PEO1, PEO1CarbR and PEO1CisR assessed by western blotting. Platinum-resistant cell lines have a higher expression of these proteins compared to the parental cell line (showed by higher density of the bands) except for p27^{Kip1}. p27^{Kip1} expression was diminished (as shown by the reduced band intensity) in PEO1CarbR compared to PEO1 and PEO1CisR, which also holds true for p57^{Kip2} expression. This might be caused by epigenetic modification presents in PEO1CarbR. Downregulation of these proteins might contribute to the higher sensitivity of PEO1CarbR towards seliciclib treatment compared to PEO1 and PEO1CisR. Expression of E2F1, a transcription factor is elevated in drug-resistant phenotype compared to PEO1 cell line, showed by the increased band intensity. This is an evidence of drug-resistant cell lines are transcriptionally more active than the parental cell line.

Based on the western blotting results, drug-resistant cell lines acquire changes in comparison with parental (drug-sensitive) cell line. These changes might play a role in the drug resistance development.

4. IC₅₀ values of cell lines treated with seliciclib

Seliciclib treatment followed by viability test using MTT was performed on PEO1 panel (drug-sensitive and drug-resistant). The dose-response curves were drawn to acquire Seliciclib IC₅₀ values for each cell lines in the panel.

Dose-response curve of PEO1 panel treated with seliciclib

Seliciclib IC ₅₀ (μM)	
PEO1	27.4 (±1.4)
PEO1CarbR	19.2 (±3.3)
PEO1CisR	33.0 (±2.7)

Figure 3-11: Comparison of IC₅₀ values of PEO1, PEO1CarbR and PEO1CisR treated with seliciclib. PEO1CisR shows highest IC₅₀ values and PEO1CarbR have the lowest IC₅₀ of seliciclib. Paired T-Test was done to determine the significance of IC₅₀ differences between PEO1CarbR and PEO1CisR. p value of less than 0.005 proved that the differences was statistically significant (n > 3).

Based on the results, PEO1CarbR with the lowest expression of p57^{Kip2} showed the highest response towards seliciclib treatment compared to PEO1 and PEO1CisR.

5. Level of target proteins in seliciclib-treated cell lines

Based on the IC₅₀ values obtained from the viability assay, PEO1 panel was treated with seliciclib to assess protein level of p57^{Kip2} and several G₁S cell cycle regulators (CyclinE, E2F1 and Survivin) before and after treatment. Control and treated cells were then harvested and used for western blotting.

5.1 p57^{Kip2} western blotting

Several attempts to do western blotting using p57^{Kip2} antibodies yield unsatisfactory results. This problem arises from the fact that the antibodies were not specific enough to give a strong signal in some of our cell lines. Most of the antibodies used produced non-specific bands despite several optimizations done to the procedures, such as changing the concentration, modification to the washing reagents and blocking reagents used and changing exposure times.

5.2 CyclinE

Figure 3-12: Western blotting results showing CyclinE level in PEO1 (a), PEO1CisR (b) and PEO1CarbR (c) cell lines treated with 15µM seliciclib and harvested on 0 and 24 hours post-treatment. Each cell line behaves differently in terms of cyclinE expression after seliciclib treatment. Seliciclib treatment in PEO1CisR results in an elevated CyclinE level showed by the size and the intensity of the protein band. In PEO1CarbR, cyclinE protein level is unchanged after 24 hour treatment with seliciclib, but overall expression of this cyclin is much higher in this cell line compared with the other two cell lines. (n=3)

5.3 Survivin

Figure 3-13: Western blotting results of Survivin level in PEO1 (a), PEO1CisR (b) and PEO1CarbR (c) cell lines treated with 15µM seliciclib and harvested on 0 and 24 hours post-treatment.Based on the results,the level of survivin expression in PEO1 is unchanged after the treatment with 15µM seliciclib for 24 hours. Result of the same seliciclib treatment to PEO1CisR yields higher level of survivin expression after 24 hours treatment compared with 0 hour control, indicated by the size and the intensity of the band. Survivin expression decreases in PEO1CarbR after seliciclib treatment. PEO1CarbR has the highest survivin expression compared with the other two cell lines while PEO1CisR has the lowest survivin expression among these three cell lines. (n=3)

5.4 E2F1

Figure 3-14: Western blotting results of E2F1 level in PEO1 (a), PEO1CisR (b) and PEO1CarbR (c) cell lines treated with 15µM seliciclib and harvested on 0 and 24 hours post-treatment. Based on the results, 15µM seliciclib treatment of PEO1 cells resulted in a slight decrease of E2F1 protein level on 24 hour post-treatment. Same seliciclib treatment on PEO1CisR yields higher protein expression after 24 hours, indicated by the increase of protein band size. PEO1CarbR shows the lowest E2F1 expression compared to the other two cell lines at 0 hour. Seliciclib treatment further reduces E2F1 protein level in PEO1CarbR. This downregulation was shown by the protein band that has almost disappeared 24 hours post-treatment. (n=3)

Based on the western blotting results of several G₁S cell cycle regulators, seliciclib treatment evokes different response in each cell line after 24 hours treatment.

6. Comparison of apoptotic response in seliciclib-treated cells

PEO1 panel was treated with seliciclib and subjected to Annexin V-FITC analysis to assess their apoptotic response towards seliciclib. Dose chosen was based on the IC₅₀ values obtained previously.

Figure 3-15: Annexin V-FITC analysis of PEO1, PEO1CarbR and PEO1CisR treated with seliciclib for 48 hours. Data shown are typical of repeat experiments (n = 3). Each quadrant in a cytogram represents different state of apoptosis and

percentage of cells. Lower left quadrant represents live cells, lower right represent cells in early apoptosis (Annexin V-FITC positive, PI negative), upper right represents cells in late apoptosis/necrotic (Annexin V-FITC and PI positive) and upper left represents debris. Control cells for all 3 cell lines were in a good condition (live cells > 90% of total cell percentage). Seliciclib treatment caused apoptosis in all 3 cell lines, with the highest rate in PEO1CarbR. PEO1 parental and PEO1CisR have approximately similar response towards seliciclib treatment.

7. Alteration in DNA content in cells treated with seliciclib

PEO1 panel was treated with seliciclib and subjected to flow cytometry analysis to assess DNA changes obtained after treatment. Seliciclib affects cell cycle components. Thus, treatment with seliciclib will influence DNA content of the cell as well. Dose chosen was based on the IC₅₀ values obtained previously.

Figure 3-16: Cell cycle phases percentage according to the DNA analysis of cell lines untreated and treated with 25µM seliciclib for 48 hours. Treatment induced apoptosis to all cell lines, represented by increased sub-G1 proportion after treatment. *p < 0.05 (n = 3)

Based on the DNA histogram analysis, treatment with 25 μ M seliciclib for 48 hours induced apoptosis in all cell lines treated. This was represented by increased sub-G₁ population in cells treated with seliciclib. Seliciclib produced more profound effects on PEO1CarbR compared to PEO1 and PEO1CisR. In treated PEO1CarbR cells, the higher cell cycle arrest level can be seen with an increase in S phase and decrease G₁ phase populations compared to untreated PEO1CarbR.

8. p57^{Kip2} gene silencing and its effect on seliciclib and cisplatin cellular response

PEO1 parental and cisplatin-resistant cell lines were transfected with p57^{Kip2} siRNA to look at the effect of p57^{Kip2} downregulation in these cell lines. Transfection was followed by treatments with seliciclib and cisplatin for 24 hours before being subjected to Annexin V-FITC and flow cytometry analysis. GAPDH siRNA was used as a control to assess transfection efficiency. mRNA from control and transfected cells were harvested and subjected to qPCR analysis. Based on the analysis, transfection of GAPDH siRNA reduced the expression of *GAPDH* mRNA in samples to approximately 32% (mean fold change 0.32 ± 0.09). Two variations of p57^{Kip2} siRNA (13 and 14) were used in this experiment. Transfection using siRNA p57^{Kip2} (13) failed to work as there is no downregulation of p57^{Kip2} mRNA after qPCR analysis. siRNA p57^{Kip2} (14) produced p57^{Kip2} silencing of only approximately 22% (mean fold change 0.78 ± 0.26). Several optimisations have been carried out such as changing of Dharmafect reagent concentration (from 4 μ L per 400 μ L reaction to 2 μ L per 400 μ L reaction) and changing seeding count of the cell (seeding count that have been used were 1×10^5 cells/mL, 2.5×10^5 cells/mL, 3×10^5 cells/mL and 4×10^5 cells/mL) but the silencing effect did not differ significantly from the previous values.

Because of difficulties working with PEO1 cell lines in terms of silencing efficiency, we have tried p57^{Kip2} silencing method on other cell line, SK-OV-3. SK-OV-3 is another type of human ovarian carcinoma cell line. siRNA p57^{Kip2} (13) produced approximately 50% silencing of p57^{Kip2} based on

qPCR analysis. Treatment of $p57^{Kip2}$ -silenced samples with seliciclib and cisplatin resulted in increased sensitivity to seliciclib and increased resistance to cisplatin (Figure 3-).

Figure 3-17: Annexin V-FITC results of SK-OV-3 control and $p57^{Kip2}$ -silenced samples. Control and silenced samples were treated with 50μM seliciclib and 20μM cisplatin for 48 hours before being subjected to the analysis using flow cytometry. Higher apoptotic response was seen in control cells treated with cisplatin compared to the silenced cells. However, the apoptotic pattern was reversed with seliciclib treatment. This represents the relationship between $p57^{Kip2}$ downregulation with platinum resistance and collateral sensitivity to seliciclib.

9. Isobologram analysis to assess drug synergism on PEO1 and PEO1CarbR

Isobologram analysis was performed in order to assess drug synergism for carboplatin and seliciclib combination treatment on PEO1 and PEO1CarbR. Cells were treated with several predetermined drug doses followed by viability assay. Values obtained were then used to calculate combination index (CI) values using CalcuSyn software.

9.1 Carboplatin-seliciclib combination treatment on PEO1

Figure 3-18: Median-effect plot (a) and combination index (CI) (b) of PEO1 treated with combination of seliciclib and carboplatin after data analysis in CalcuSyn software. Dotted line represents CI value of 1 (n = 7). This figure is a representative. The effects are additive but there is some antagonism at higher doses. Combination treatments of seliciclib and carboplatin on lower dose results in CI < 1 in PEO1 parental cell line. Subsequent higher doses produces CI = 1 and later CI > 1 at the highest dose used (40μM seliciclib and 320μM carboplatin).

9.2 Carboplatin-seliciclib combination treatment on PEO1CarbR

Figure 3-19: Median-effect plot (a) and combination index (CI) (b) of PEO1CarbR treated with combination of seliciclib and carboplatin after data analysis in CalcuSyn software. Dotted line represents CI value of 1 ($n = 7$). This figure is a representative. Seliciclib and carboplatin combination in PEO1CarbR results in $CI > 1$ at lower dosage before the index value decreasing to nearly $CI=1$ following increasing doses. The effects were mainly antagonism but with higher dosage the effect was nearly additive.

Discussion

In this first part of the PhD study, three cell lines were used. Two of them being drug-resistant - PEO1CarbR and PEO1CisR derived from the parental cell line PEO1. PEO1CarbR shows the highest sensitivity towards seliciclib treatment compared to the parental PEO1 and PEO1CisR cell lines based on the seliciclib IC₅₀ values. The analysis of apoptosis indicated an apoptotic level- that was appropriate and in line with the drug sensitivity results. Accumulation of cells at S phase, which is a feature of cell cycle blockade by seliciclib can be seen particularly in PEO1CarbR.

p57^{Kip2} is a cell regulatory proteins and its downregulation is a feature of several cancers including EOC. Downregulation of p57^{Kip2} in PEO1CarbR has been demonstrated before and treatment with demethylating agent reactivates the gene (Coley et al. 2012). The level of mRNA and protein expression of p57^{Kip2} in parental and drug-resistant cell lines used in this study was assessed. Another member of CIP/KIP family, p27^{Kip1} is also found to be downregulated in drug-resistant cell lines with PEO1CarbR has the lowest protein expression level. Other cell cycle regulatory proteins such as E2F1, CyclinE and survivin have also been found to be upregulated in drug-resistant cell lines compared to PEO1. The upregulation of CyclinE is associated with malignancy status of ovarian cancer together with the downregulation of p27^{Kip1} as reviewed by Mazumder et al. (2004) and D'Andrilli et al. (2008). Accumulation of CyclinE reflects either the increase in synthesis or decrease in degradation. Upregulation of CyclinE in these drug-resistant cell lines might be the end result of E2F1 upregulation and downregulation of p57^{Kip2}, as the Skp2 level which is a regulator of CyclinE does not change significantly with drug treatment although its level was significantly higher in both carboplatin- and cisplatin-resistant PEO1 compared to the parental cell line. Survivin is associated with cancer cell survival and has been shown to be upregulated in drug-resistant variant cell lines (Coley et al., 2007).

This study also looked at the effect of seliciclib treatment to the expression level of Skp2, CyclinE and Survivin in the cell lines. Based on the results, seliciclib treatment did not affect Skp2 protein levels in all experimented cell lines. The Survivin level after seliciclib treatment however presents quite complicated results. Taken into consideration, downregulation of Survivin after 24 hour seliciclib treatment is in agreement with work by Aldoss et al. (2009). Higher seliciclib dose usage is able to induce more downregulation of Survivin compared to the lower dose (30 μ M dose data not shown). However the increase in Survivin level after 48 hours treatment points to the effort of cells to evade apoptosis caused by seliciclib.

Collectively, seliciclib treatment caused steady upregulation of CyclinE in PEO1CisR towards 48 hours after treatment. With no noticeable changes in Skp2 level, upregulation of CyclinE in seliciclib-treated cells maybe a mechanism of compensation for lack of CDK2-CyclinE activity in these cells. The lack of activity comes from seliciclib binding to the CDK ATP pocket site thus renders them inactive. This event will lead to cell cycle blockade. More CyclinE is expressed in order to overcome the cell cycle blockade due to seliciclib which reflects in increased protein level. This result is in accordance with Mazumder et al. (2004) who reported that treatment with chemotherapeutic agents such as topoisomerase I inhibitor leads to increased CyclinE level in hematopoietic cells. On the other hand, CyclinE level in PEO1CarbR do not differ much 24 hour after seliciclib treatment.

E2F1 is an important transcription factor that promotes expression of various genes needed in G₁ to S phase transition. After seliciclib treatment E2F1 level become inconsistent in PEO1 cell line up until 48 hours post-treatment. Increasing levels of E2F1 can be taken as a sign of a transcriptionally active state. Thus, the increased CyclinE level in PEO1CisR and PEO1CarbR after seliciclib treatment is a result of increased E2F1 expression. This can be considered as an effect of the drug to the cells.

Upregulation of E2F1 after CKI treatment has been reported by Ma and Cress (2007). In their report, treatment of breast cancer cell lines with CKI BMS-387032 upregulated the E2F1 protein.

Additionally, BMS-387032 treatment caused more cell death in cells that lacked p57^{Kip2}, which is in line with our findings for seliciclib in PEO1CarbR. p57^{Kip2} is a direct target and inducible by E2F1 action thus upregulation of E2F1 induce expression of p57^{Kip2} which leads to apoptotic events.

Seliciclib has been shown to have a synergistic effect when combined with cisplatin and paclitaxel in uterine sarcoma cell lines (Coley et al., 2007). We investigated whether seliciclib could produce a similar effect in combination with carboplatin on PEO1 and PEO1CarbR. Based on the results, combinations of seliciclib and carboplatin produced an additive effect at lower dose and an antagonistic effect on higher dose on both cell lines. There was also no evidence of drug sequence dependence as changing the sequence of the drug produced similar results (data not shown).

To explain the reason behind PEO1CarbR sensitivity towards seliciclib treatment compared to PEO1 and PEO1CisR, the seliciclib mechanism of action and PEO1CarbR molecular biology have to be taken into consideration. In PEO1CarbR several genes have been found to be downregulated including p57^{Kip2}, a CDK inhibitor. Seliciclib which is also a CDK inhibitor drug works well in this cell line because it replaces p57^{Kip2} role as a cell cycle inhibitor through binding to the ATP site of the CDKs. Seliciclib also can inhibit other CDK-cyclin complexes but the most profound effect is on CDK2-CyclinE which is also a target of p57^{Kip2}. Inhibition of CDK-cyclin complexes results in cell cycle blockade and eventually apoptosis. The data presented herein are in agreement with the findings reported by Ma and Cress (2007) who showed that overexpression of p57^{Kip2} restrains E2F1-driven transcription thus reducing S phase. Seliciclib promotes expression of E2F1, which subsequently

leads to increased S phase fraction. However as seliciclib is able to inhibit phosphorylation of RNA polymerase II via inhibition of CDK7/CDK9, apoptosis is triggered.

Based on the present work, the cell lines presented a different level of sensitivity towards a cell cycle inhibitor seliciclib treatment. It is tempting to look more closely at the differences between platinum resistant cells at the level of gene methylation. Methylation-specific PCR and pyro-sequencing will be the methods used to address this and some of this work has already been carried out prior to this project. The hypotheses presented were partially correct, since re-sensitization is difficult to achieve based on isobologram analysis.

Conclusion

In conclusion, downregulation of p57^{Kip2} in cancer cell is a marker of seliciclib sensitivity and measurement of this could be considered as a biomarker in clinical trials of this agent. Seliciclib is also a potential drug to be used in advanced malignancies with drug-resistance phenotype.

Since the drug-resistant ovarian cancer cell lines have been shown to have multiple gene changes, the next step is to assess several regulatory protein such as *Stratifin* and *Wee1* in terms of their contribution to the drug-resistant phenotype. These regulatory genes have been shown to be downregulated in taxane-resistant cell lines, based on analysis using microarray screening.

Chapter 4

Key Players in Cell Cycle and their Deregulation in Cancers

Introduction

1. G₂M DNA damage checkpoint

DNA damage response (DDR) is important in recognizing and repairing damaged DNA before mitosis. Unrepaired DNA might lead to cell senescence or tumorigenesis. DDR comprises of proteins within networks that act immediately upon exogenous and endogenous causes of DNA damage. Within the signal transduction pathway, proteins involved in DDR can be divided into sensors, mediators, transducers and effectors. Ultimately, DDR will result in either DNA repair, cell cycle arrest or if the damage is overwhelming, apoptosis. DNA damage is detected by sensors which will recruit mediators, often proteins with BRCA1 C-terminus repeat (BRCT)-containing domain acting as protein-phosphoprotein interaction modules. Mediators will then recruit PIKK (Phosphoinositide-3-kinase-related protein kinase) family members, which are large proteins with the ability to phosphorylate a wide range of substrates that sometimes have overlapping functions with other family members, showing crosstalk between them. PIKK family members act as transducers of the signal and their activation depends on the type of DNA damage acquired. Subsequently the signal is passes over to the effectors by means of substrate phosphorylation. Activated effectors will phosphorylate downstream substrates and eventually cause cell cycle arrest and DNA repair or activation of apoptotic pathways if the damage is overwhelming (Giunta and Jackson 2011; Niida and Nakanishi 2006).

Figure 4-1: Flow of cell response following DNA damage. DNA damage can be either double strand break (DSB) or single strand break (SSB). Damage sustained is recognized by sensors, which recruit mediators that will pass the signal to transducers, which in turn will phosphorylate effectors. Further substrate phosphorylation by effectors will result in cell cycle arrest, DNA repair or apoptosis.

Function	Class	Gene
Sensors	Replication factor C (RFC1)-like	<i>RAD17</i> <i>RFC2-5</i>
	Proliferating cell nuclear antigen (PCNA)-like	<i>RAD9</i> <i>RAD1</i> <i>HUS1</i>
Mediators	BRCT-containing	<i>BRCA1</i> <i>53BP1</i> <i>TopBP1</i> <i>MDC1</i>
	DSB recognition/repair (MRN complex)	<i>Mre11</i> <i>RAD50</i> <i>NBS1</i>
Transducers	PI3 kinase-like protein	<i>ATM</i> <i>ATR</i>
	PIKK binding protein	<i>ATRIP</i>
	Protein kinase	<i>CHK1</i> <i>CHK2</i>
Effectors	Transcription factor	<i>p53</i>
	Phosphatase	<i>CDC25A,B,C</i>
	Protein kinase	<i>CDKs</i> <i>CDC7</i>

Table 4-1:Classification of genes involved in DDR (Adapted from Niida and Nakanishi 2006).

2. ATM

ATM (Ataxia-telangiectasia mutated) is a member of the PIKK family. ATM is a large serine/threonine protein kinase with the gene situated at chromosome 11q22-23. Mutation of ATM causes ataxia-telangiectasia, a rare autosomal recessive disorder characterized by cerebellar ataxia, neuro-degeneration, radiosensitivity, cell cycle checkpoint defects, genome instability and predisposition to cancer.

2.1 ATM activation and function

Activation and function of ATM is achieved via association with various proteins or protein complexes, a feature shared among PIKK family members. Inactive ATM exists as a multimer/homodimer. When activated, the ATM homodimer dissociates and binds to several

proteins, mainly those involved in cell cycle control. ATM plays a major role in maintaining DNA integrity through cell cycle control and DNA repair mechanisms. In cell cycle, ionizing radiation that produces DNA double strand break (DSB) induces ATM activation. The joined effort of mediator complex MRN (Mre11, Rad50 and Nbs1) and phosphorylation of a variant H2A histone, H2AX near the breakage recruits ATM to the site of the DSB. MRN recruitment to the damaged DNA was thought to be the earliest event following DSB occurrence since at least one of the protein (Nbs1) is recruited to the site independently of H2AX phosphorylation. Additionally, Nbs1 immobilizes ATM at the site of DSB. ATM then undergoes rapid phosphorylation at Ser1981 to dissociate into active monomers. However, acetylation of ATM by acetyl transferase Tip60 is thought to be the critical activating event in comparison to ATM phosphorylation. Apart from forming a complex with ATM, Tip60 also interacts with MRN complex via its cofactor Trrap. Downregulation of Tip60 leads to failed ATM activation and reduced cell survival following DNA damage. Active ATM then phosphorylates H2AX, producing γ -H2AX that is recognized by MDC1, another mediator protein. ATM also subsequently phosphorylates MDC1, which enables recruitment of E3 ubiquitin ligase RNF8. RNF8 promotes H2AX ubiquitination and recruitment of the DNA repair complex BRCA1 (BreastCancer1) and 53BP1 (p53 binding protein 1). BRCA1 mediates DNA repair via homologous recombination (HR) and 53BP1 via non-homologous end joining (NHEJ). Phosphorylation of RNF8 by CK2 promotes its binding to MRN resulting in the complex stabilization and enhancement of ATM auto-amplification loop (Derheimer and Kastan 2010; Niida and Nakanishi 2006; Reinhardt and Yaffe 2009) (Figure 4-2).

Following ATM activation, it phosphorylates p53 and proteins that interact with p53 including MDM2 and Chk2. Activation of p53 by ATM enables p53 to further activate downstream genes and triggers S phase and G₂M checkpoints. Activation of S phase arrest is achieved via phosphorylation of FANCD2 (Fanconi anaemia group D2) and SMC1 by ATM while G₂M checkpoint arrest via Chk2 phosphorylation and activation (Derheimer and Kastan 2010).

Figure 4-2: ATM activation sequence following DSB generated by ionizing radiation. (1) The MRN complex is recruited to the DSB site. DSB also promotes H2AX phosphorylation. ATM is recruited to the site following these events. Activated ATM then further phosphorylates the H2AX and the MDC1 (2). MDC1 mediates recruitment of the BRCA1 and the 53BP1 proteins which initiates the DNA repair mechanisms (HR or NHEJ, respectively) (3). ATM also phosphorylates p53 and Chk2, which activates the G₂M cell cycle arrest (4).

Apart from its critical role in cell cycle checkpoint, ATM is also involved in DNA repair via interaction with the DNA repair enzyme, Artemis in the G₂ phase. Optimal DNA damage repair might involve ATM activity. KAP1 (KRAB-associated protein 1) is a component of heterochromatin that is a direct target of ATM. ATM binding to KAP1 is believed to promote heterochromatin relaxation thus facilitating DNA repair. TRF2 protein, which is a part of shelterin is another protein that interacts with ATM. Shelterin is a complex of telomere cap functioning to prevent recognition of the free end of chromosome as the free end of a double strand break. By binding to ATM, TRF2 inhibits ATM activity locally at the telomeres (Derheimer and Kastan 2010).

3. ATR

ATR, a serine/threonine protein kinase is another family member of PIKK family sharing some similarities with ATM and Rad3, hence the name (ATM and Rad3-related). Other family members include DNA-PKcs (DNA dependent protein kinase catalytic subunits), mTOR (mammalian target of rapamycin) and hSMG1 (suppressor with morphological effect on genitalia). Generally, the PIKK family members share common structural features: N-terminal HEAT repeats, a FAT domain, a protein kinase domain and a C-Terminal FAT-C domain (Derheimer and Kastan 2010). ATR plays a role in DDR via recognition of single strand DNA (ssDNA) generated as a product of processed DSBs, replication stress caused by stalled replication forks or nucleotide excision repair. Treatment with drugs such as Aphidicolin, a DNA polymerase inhibitor or hydroxyurea, inhibitor of ribonucleotide reductase also results in ATR activation.

3.1 ATR activation and function

Activation of ATR in ATM-dependent manner takes place during late S or G₂ phase by the formation of RPA-coated ssDNA following endo- and/or exonuclease activity of Mre1/CtIP complexes. During S phase, ssDNA forms when MCM helicase continues unwinding the DNA template despite a stalled replication fork. ATR forms a complex with its regulator ATRIP (ATR-Interacting protein). ATR binds to ssDNA through ATRIP interaction with RPA (replication protein A). RPA is a ssDNA-binding protein that recognizes exposed ssDNA and can rapidly coat it. Following its recruitment to the site of exposed ssDNA, ATR is activated through binding of ATR-ATRIP to 9-1-1 (Rad9-Rad1-Hus1), a DNA-associated protein complex. 9-1-1 recruits BRCT domain-containing protein TopBP1 (Topoisomerase II binding protein 1) in a phospho-dependent manner which in turn activates ATR. ATR is also activated by Claspin and together with TopBP1, these proteins are co-mediators for ATR to activate downstream protein Chk1 (Chen et al. 2012; Lopez-Contreras and Fernandez-Capetillo 2010; Reinhardt and Yaffe 2009; Shechter et al. 2004). Claspin is thought to serve as an adaptor protein,

bringing the substrates together, as it has been shown that Claspin binds both Rad9 (part of 9-1-1 complex) and Chk1 (Shechter et al. 2004). Apart from Chk1 as the primary target and proteins that activate ATR/Chk1 pathway, ATR also phosphorylates several other proteins such as H2AX, WRN (Werner syndrome helicase), BLM (Blooms syndrome helicase), BRCA1 and FANCD2 showing crosstalk between ATR/Chk1 and ATM/Chk2 pathways (Chen et al. 2012) (Figure 4-3).

Figure 4-3: ATR activation sequence following generation of ssDNA. (1) The ssDNA generated is coated with the RPA. **(2)** The ATR-ATRIP complex is recruited by the RPA-coated ssDNA. **(3)** The RPA-ssDNA also recruited the Rad17. This followed by recruitment of 9-1-1 complex by rad17 to the damage site. **(4)** The 9-1-1 complex brings TopBP1, an ATR-ATRIP activator near to the complex, thus activating it. **(5)** Following the ATR activation, Chk1 is activated via phosphorylation.

Subsequently this event leads to the cell cycle arrest.

Besides the role in DDR, ATR is also indispensable in normal cells. At lower expression levels, ATR regulates the progression of DNA replications thus moderating the rate of S phase and prevents the accumulation of abnormal DNA structures (Shechter et al. 2004). In cells with low ATM protein, many of its substrates were eventually phosphorylated after IR exposure showing the possibility of ATR compensating for lack of ATM activity (Kastan and Bartek 2004).

4. Chk1

Figure 4- 4: Chk1 protein structure.

The Chk1 protein consists of a kinase domain in its N-terminal followed by the SQ/TQ cluster domain (SCD), a nuclear localization signal and a PCNA interacting protein (PIP) motif. The C-terminal domain of Chk1 is thought to function as its auto-regulator (Stracker et al. 2009). Chk1 serine/threonine checkpoint kinase is a downstream effector of ATR and is associated with chromatin. In proliferating cells, Chk1 expression peaks at S and G₂ phases while in quiescent and differentiated cells its expression is almost undetectable. Chk1 activation follows DNA damage signalling from ATR. ATR phosphorylates Chk1 at Ser345 and Ser317 and thus activates it. In turn, Chk1 phosphorylates CDC25 to initiate cell cycle arrest at S and G₂ phases (de Carcer et al. 2007; Niida and Nakanishi 2006). Activation of Chk1 by ATR phosphorylation requires Claspin as a mediator protein. Recruitment of Claspin to ssDNA is facilitated by ATR-mediated phosphorylation of Rad17, a ssDNA binding protein (Reinhardt and Yaffe 2009). Phosphorylation of Chk1 at Ser345 is essential for its activation while Ser317 serves as additional support. Phosphorylated Chk1 undergoes autophosphorylation at another Ser residue (Ser296) prompting its dissociation from the chromatin and then localizes to the cytoplasm and interphase centrosomes. Subsequently, activated Chk1 binds to and phosphorylates CDC25A to promote S phase cell cycle arrest and CDC25C to promote G₂ checkpoint via inhibitory phosphorylation mechanism. Apart from CDC25, Chk1 also binds and phosphorylates Wee1. Wee1 phosphorylation serves as a recognition site for SFN that in turn binds to and promotes Wee1 stability and kinase activity. Other Chk1 targets include Rad51, FANCD2 and FANCE (Fanconi anaemia group E), proliferating cell nuclear antigen (PCNA) and the TIMELESS complex of replication fork-associated proteins. By interacting with these proteins, Chk1 plays a role in regulation of DNA repair

and stability of stalled replication forks. Chk1 was shown to promote p53 activity by targeting and phosphorylating the ubiquitin ligase Mdm4/X together with Chk2 (Chen et al. 2012; Reinhardt and Yaffe 2009; Stracker et al. 2009). Inhibitory phosphorylation of TLK1 (Tousled like kinase 1) by Chk1 after DNA damage inhibits TLK1 activity at S phase. TLK1 targets Asf1, a protein required in maintaining chromatin dynamics and progression of replication fork during S phase (Stracker et al. 2009).

Chk1 and *Claspin* expression are under the control of the transcription factor E2F1 (Verlinden et al. 2007). Regulation of Chk1 comes from modulation of Claspin levels via proteosomal degradation. At the onset of mitosis, Plk1 (Polo like kinase 1) is activated and phosphorylates Claspin to promote its degradation via recognition by SCF δ Trcp ubiquitin ligase. Without Claspin, Chk1 activation is halted thus promoting mitosis. Following DNA damage signal, both Claspin and Plk1 are ubiquitinated by APC/C^{Cdh1}. While Plk1 is degraded, Claspin is protected from degradation by ubiquitin protease USP28 thus enabling activation of Chk1. Chk1 is also regulated via phosphatase action by stimulating PP2A (Protein phosphatase 2A) activity on itself. However, the exact mechanism still needs to be elucidated. p53-inducible type-2C phosphatase Wip1 also binds and regulates Chk1 activity (Stracker et al. 2009).

5. Chk2

Figure 4-5: Chk2 protein structure.

Albeit sharing conserved kinase domains with Chk1 and thus sharing some similar substrates, Chk2 is structurally and functionally different from Chk1. The Chk2 protein consists of an N-terminal SCD, a Forkhead associated (FHA) domain and kinase domain near the C-terminus. FHA is involved in phosphorylation-dependent protein-protein interactions. Atm carries out Chk2 activation following DSB formation via phosphorylation at Thr68 and several additional sites, creating a binding site recognized by FHA domain of the second Chk2 monomer. Multimerization of Chk2 is important for further autophosphorylation and activation of Chk2. Apart from direct phosphorylation of Chk2 by ATM, other PIKK family members (ATR, DNAPK) have been shown to phosphorylate Chk2 *in vitro* suggesting ATM-independent Chk2 activation is also possible. Following its activation, Chk2 phosphorylates CDC25A to promote G₁ and S phase cell cycle arrest in a similar manner as Chk1. It is thought that Chk2 plays accessory roles to Chk1 activity (de Carcer et al. 2007; Reinhardt and Yaffe 2009; Stracker et al. 2009). Additionally, Chk1 has been shown to compensate for lack of Chk2 activity (Bartek and Lukas 2003). Besides CDC25A, Chk2 also phosphorylates the transcription factor E2F1 that results in its stabilisation. Phosphorylation of Rbp by Chk2 also enhances its interaction with E2F1 following DNA damage. E2F1 requires ATM and Chk2 activity to initiate the transcription of proapoptotic genes (Apaf1 and p73) thus promoting apoptosis. Chk2 promotes IR-induced apoptosis partially through phosphorylation of the PML (promyelocytic leukaemia) protein in a p53-independent manner. FOXO1, a transcription factor responsible for apoptosis activation, is also regulated by Chk2. Phosphorylation of FOXO1 by Chk2 inhibits its activity. DNA damage results in checkpoint activation and inhibition of CDK2 activity, thus promoting FOXO1 translocation into the nucleus to initiate apoptosis. Another very important protein downstream of ATM is p53, which is a direct target of Chk2. Besides direct phosphorylation of p53, Chk2 regulates p53 degradation by inhibition of ubiquitin ligase MDM4/X. Phosphorylation of MDM4/X by Chk2 and Chk1 promotes its nuclear retention and degradation, and promotes dissociation of HAUSP ubiquitin protease. Phosphorylation of the Che1/AATF kinase by Chk2 and ATM further influences p53-dependent transcriptional responses by increasing Che1 stability and recruitment to the promoters of p53-

responsive genes after DNA damage (Castedo et al. 2004; Stracker et al. 2009). Other proteins that are phosphorylated by Chk2 include CDC25C and BRCA1 (Niida and Nakanishi 2006). Additionally, Chk2 is also an endogenous repressor of mitotic catastrophe. Inhibition of Chk2 or Chk2-activating partners sensitizes cells to mitotic catastrophe via prolonged activation of CDK1-CyclinB (Castedo et al. 2004).

Regulation of Chk2 by Plk1 is part of mechanism in checkpoint deactivation during mitosis and checkpoint recovery. This regulation comes via phosphorylation of the Chk2 FHA domain (van Vugt et al. 2010). Wip1 and PP2A which binds and regulate Chk1, also exert similar regulatory mechanisms to Chk2 (Stracker et al. 2009).

6. p53, guardian of the genome

p53 is a transcription factor central in regulating cell cycle processes. The central domain of the p53 protein acts as a DNA binding domain while N-terminal domain functions in transactivation. The C-terminal domain of p53 is required for oligomerization and regulates DNA binding. Upon activation, p53 binds directly to promoter or enhancer of a gene. The binding results in transcription rate alteration of the target genes (Liang et al. 2011; Schulz 2007). As a transcription factor, p53 is able to promote transcription of target genes and interacts with a wide range of genes. However, p53 interaction with the basal transcription factor TBP or with transcriptional repressors such as SIN3A results in transcriptional repression of several other genes as well (Schulz 2007). In the normal state, p53 activity is kept low due to its inactivity and short half-life of approximately 10-20 minutes which is insufficient for activity (Liang et al. 2011; Niida and Nakanishi 2006). The short half-life of p53 comes from its post-translational modification and degradation by MDM2/HDM2. MDM2 is responsible for p53 regulation by binding to the p53 N-terminal region, blocking its transcriptional

activity and initiating p53 nuclear export. MDM2 is also a specific E3 ubiquitin ligase for p53, promoting its ubiquitination and subsequent proteosomal degradation (Coschi and Dick 2012; Schulz 2007). Apart from phosphorylation, regulation of p53 activity occurs through other means of posttranslational modification such as acetylation, neddylation and sumoylation (Niida and Nakanishi 2006; Schulz 2007).

6.1 p53 activation and functions

p53 is activated through phosphorylation by several proteins. Upon activation, p53 forms a tetramer and binds to target DNA sequences (Liang et al. 2011; Schulz 2007). As stated before, ATM, ATR, Chk1 and Chk2 are shown to interact with and phosphorylates Ser residues of p53, promoting p53 stabilization and subsequently eliciting several pathway interactions after the cell has sustained genotoxic stress (Coschi and Dick 2012; Damia et al. 2001; Schulz 2007; Siddik 2003). Ser residue phosphorylation also increases p53 stability and half-life by preventing interaction with MDM2 (Coschi and Dick 2012). Downregulation of MDM2 also promotes p53 activity and MDM2 phosphorylation by ATM impairs its ability to promote nucleo-cytoplasmic shuttling of p53 (Niida and Nakanishi 2006). p53 phosphorylation at the N-terminal domain promotes p53 transcriptional activity while acetylation at Lys (Lysine) residues can modulate p53 DNA-binding activity, transcriptional activation and localization. Stabilization of p53 following DNA damage promotes transcriptional activation of target genes and determines the fate of the cell (Coschi and Dick 2012). Thus, p53 influences various cellular functions, including cell cycle arrest, apoptosis and DNA repair (Nam and Kim 2008; Schulz 2007). Activation of p21^{Cip1} and p27^{Kip1} by p53 can lead to induced cell cycle arrest via inhibition of CDK1-CyclinB and CDK2-CyclinE. p53 also activates GADD45, another CDK1-CyclinB inhibitor involved in DNA repair (Niida and Nakanishi 2006; Schulz 2007). p53 together with Rb serves as G₁ checkpoint moderators by supervising Ras, Myc and E2F oncogenic activity. E2F promotes transcription of genes responsible for cell cycle progression, including CKIs p16^{INK4a} and

p14^{ARF}. p14^{ARF} is responsible for inhibition of MDM2 thus promoting p53 stability and activity.

Hyperactivation of Ras or Myc promotes p14^{ARF} transcription and indirectly activates p53 (Massague 2004; Schulz 2007). Apoptosis mediated by p53 is through transcription of several pro-apoptotic genes and release of cytochrome C from the mitochondria. Cytochrome C subsequently binds to Apaf1 (apoptotic protease activating factor 1) which leads to activation of caspases (Coschi and Dick 2012; Maddika et al. 2007). Apart from activation, phosphorylation of Ser residues at different sites on p53 by Protein kinase C (PKC) and Chk2 modulates its activity. p53 also promotes MDM2 induction as a feedback mechanism controlling its activity (Schulz 2007). Other than roles stated previously, p53 also plays an additional role in maintaining genomic stability. p53 can recognize DNA damage-related structures and bind to ssDNA and dsDNA to promote strand exchange and repair. Additionally, p53 regulates DNA repair mechanisms such as homologous recombination and NHEJ (Coschi and Dick 2012). Due to its central role in controlling cell fate after DNA damage, p53 has acquired the title 'guardian of the genome' (Coschi and Dick 2012; Debernardis et al. 1997; Lane 1992).

Figure 4-6: ATM, ATR, Chk1, Chk2 and p53 in G₂M DDR control.

7. Deregulation in cancers

7.1 ATM/Chk2 deregulation

The majority of deleterious mutations in the *ATM* gene lead to the generation of the truncated version of the protein, while other mutations are missense substitutions and in-frame deletions (Derheimer and Kastan 2010; Kastan and Bartek 2004). This condition leads to radioresistant DNA synthesis and increased sensitivity towards radiotherapy (radiosensitivity). Radioresistant DNA synthesis is a term used to describe the condition of defect in DNA synthesis during S phase after exposure to ionizing radiation. These two characteristics are fundamental in identification of ataxia-telangiectasia, a disease caused by mutation of *ATM*. As described before, ataxia-telangiectasia is a rare, autosomal recessive disorder. Patients with this disorder showed progressive cerebral ataxia, neuro-degeneration, genome instability, predisposition to develop leukaemia and lymphoma together with radioresistant DNA synthesis and radiosensitivity. Ataxia-telangiectasia patients also exhibit resistance to insulin as ATM produces adverse effects on insulin-dependent signalling. Germline mutations of ATM-interacting proteins such as *Mre11* and *Nbs1* lead to disorders presented in a similar way to ataxia-telangiectasia phenotypes. Hypomorphic mutations in *Mre11* lead to an AT-like disorder (ATLD) while mutation in *Nbs1* leads to Nijmegen breakage syndrome (NBS) (reviewed by Curtin 2012; Derheimer and Kastan 2010; Kastan and Bartek 2004).

Mutations in *Chk2* have been reported in carcinomas of the breast, lung, vulva, urinary bladder, colon and ovary, osteosarcomas and lymphomas. These mutations are clustered in three domains of Chk2: N-terminal with the SCD regulatory domain, FHA protein-protein interaction domains and the catalytic C-terminal domain. In human prostate and breast cancer, *Chk2* mutations were considered as low penetrance alleles while in mice, mutations can promote genome instability. Mutations comprise of either missense or results in truncated protein sequences. Experiments in mice showed that *Chk2* deletion is insufficient to cause spontaneous cancers but causes increases in susceptibility

towards skin tumours induced by carcinogens. Additional mutation either in *BRCA1* or in *Mre11* in these cases promotes development of various other tumours. Conversely, in some human cancer cell lines particularly p53-deficient primary breast and colon carcinomas, upregulation of Chk2 activity via phosphorylation at Thr68 was found in the absence of chemotherapy or radiotherapy treatment. This shows the importance of Chk2 deregulation in carcinogenesis (Bartek and Lukas 2003; Stracker et al. 2009). Chk2 is dispensable for prenatal development, thus supporting the role of Chk2 as an ‘amplifier’ for Chk1 (Niida and Nakanishi 2006). A subset of patients with Li-Fraumeni syndrome was found to have heterozygous germline mutations in *Chk2* without any p53 mutations. Li-Fraumeni patients are also predisposed to develop sarcomas, leukaemias, brain tumours, adrenal tumours and other cancers (reviewed by Kastan and Bartek 2004).

7.2 ATR/Chk1 deregulation

As stated previously, ATR plays a significant role in normal cell development. Lack of ATR expression leads to a condition called Seckel syndrome. The syndrome’s characteristics include dwarfism and craniofacial abnormalities and are associated with severe hypomorphism of ATR. Downregulation of ATR in the mouse results in increased DNA damage during embryogenesis and replication. Loss of one allele of ATR or Chk1 was shown to modestly increase carcinogenesis and ATR activation leads to senescence. In adult mice, constitutive ATR hypomorphism or deletion leads to accelerated ageing (Lopez-Contreras and Fernandez-Capetillo 2010; Stracker et al. 2009).

Mutation of Chk1 is a rare event in cancer (Carrassa et al. 2012). Aberration of the gene is reported in endometrial, gastric and colorectal carcinomas. In colon and endometrial carcinomas presenting with microsatellite instability, frameshift mutations as a result of either insertion or deletion of single adenine in *Chk1* gene were found. Expression of a shorter isoform of Chk1 mRNA can be seen

in a subset of small cell lung carcinoma. This isoform translates into a Chk1 protein that is missing a part of its conserved catalytic domain. Since a total lack of Chk1 promotes early embryonic lethality in mice, it is understandable that Chk1 is essential in both normal and cancerous cells (reviewed by Bartek and Lukas 2003). Microcephalin (MCPH1/BRIT1), a gene mutated in hereditary microcephaly, was found to interact with Chk1. Microcephalin gene mutation shows impaired interaction with Chk1, thus influencing its transcriptional regulation and checkpoint induction. In mice, haploinsufficiency of Chk1 is associated with tumorigenesis phenotypes such as impaired cellular proliferation, genetic instability and elevated CDC25A following DNA damage. Additionally, Chk1 is indispensable for prenatal development (Niida and Nakanishi 2006; Stracker et al. 2009). Upregulation of Chk1 mRNA was found in *MYCN*-amplified neuroblastoma (Sorensen and Syljuasen 2012). Chk1 was found to be highly expressed in grade 3 breast carcinomas with triple negative phenotype (oestrogen receptor (ER)-/progesterone receptor (PR)-/HER2-) in comparison with other grade 3 tumours (Verlinden et al. 2007).

7.3 p53 deregulation

The p53 tumour suppressor protein is the most commonly deregulated gene in human cancers (Baird and Kaye 2003; Coschi and Dick 2012; Siddik 2003). Defects in *TP53* permit accumulation of genetic changes that favour malignant transformation over many cell generations. The effect of p53 mutation is evident in the Li-Fraumeni syndrome, first described by Li and Fraumeni (1969). Li-Fraumeni syndrome is based on observations of childhood rhabdomyosarcoma patients and identification of four families in which its children and young adults had an autosomal dominant pattern of soft tissue sarcoma, breast cancer and other neoplasms (Li et al. 1988; Malkin et al. 1990). Patients with Li-Fraumeni syndrome develop numerous cancers ranging from those of blood, lymphoid organs, soft tissues, the nervous system and epithelia. Malkin et al. first describes germline mutations of p53 via analysis of five families with Li-Fraumeni syndrome (Malkin et al. 1990). One

mutant copy of p53 gene is inherited in these patients, in combination with mutational inactivation of the other gene copy or loss of heterozygosity (LOH) at chromosome 17p (Coschi and Dick 2012; Schulz 2007). LOH is a term used to describe a process in which heterozygous sequences become homozygous through several mechanisms, such as loss of chromosomes with intact alleles leaving only the defective allele. Deletion of the intact allele - or recombination that results in two defective alleles - is also correlates with LOH. There are also other mechanisms of p53 inactivation in human cancers. Missense mutations of p53 in one allele combined with LOH of another allele is the most common mechanism of p53 inactivation in human cancers. Nonsense and splice mutations are also observed albeit less frequently (Schulz 2007). These alterations result in aneuploidy and aberration of chromosome structure. There is a significant correlation between *TP53* mutations and tumour aneuploidy. It is shown that cells with p53 mutations are tolerant of both tetraploidy and aneuploidy. Missense p53 mutation correlates with poor prognosis in comparison with p53-null patients. Loss of p53 altogether only results in the absence of p53 tumour suppressor action while dominant mutated p53 can also acquire gain-of-function of genes that are selected for malignancy apart from losing its' tumour suppressor action. This phenomenon is caused by the ability of mutated p53 to bind and transactivate genes encoding growth stimulators, such as Myc that provide survival and proliferative advantages (Coschi and Dick 2012). Overexpression of MDM2, which promotes accelerated p53 degradation, is another mechanism of p53 deregulation seen in cancers such as sarcomas and gliomas. Loss of the MDM2 negative regulator p14^{ARF} via gene deletion, mutation or promoter hypermethylation also affects the p53 level. Other mechanisms involved in p53 inactivation include downregulation of upstream activators such as ATM or Chk1 and inactivation by viral oncoproteins e.g. HPV E6 involved in cervical cancer (Kesis et al. 1993; Schulz 2007; Siddik 2003). Mutation of p53 can correlate with cisplatin response in certain types of cancers. Wild-type p53 cancers show a higher response rate towards cisplatin treatment compared to cancers with high frequency of p53 mutation such as ovarian, head and neck, and metastatic bladder

cancers (reviewed in Siddik 2003). This is due to increased cell ability to amplify drug resistance gene loci (Coschi and Dick 2012).

Aim

To screen for any deregulation of DNA damage response proteins and those relating to G₂M cell cycle regulation in several panels of ovarian, breast, head and neck and melanoma paired parental and drug-resistant cell lines.

Hypothesis

Changes in expression levels of DNA damage sensors and cell cycle checkpoint controllers underlie cell cycle changes in drug resistant cell lines – particularly at the G₂M phases – and these changes may be duplicated across many drug resistant cell line panels.

Method

Cell lines used were PEO1 parental and carboplatin-, cisplatin- and Taxol-resistant variants, SK-OV-3 parental and cisplatin- and Taxol-resistant variants, MCF7 parental and Taxol-resistant variant, T47D parental and Taxol-resistant variant, and HN5 parental and cisplatin-resistant variant. Cells were grown in suitable medium accordingly. Protein samples were harvested and Western blotting was carried out as described in Chapter 2. At least three repetitions of western blotting experiments were carried out.

Results

Western blotting using several antibodies against different G₂M cell cycle regulators was performed on selected panels of drug-sensitive and drug-resistant cancer cell lines: ovarian cancer (PEO1 and SK-OV-3), breast cancer (MCF7 and T47D) and head and neck cancer (HN5). The selected G₂M cell cycle regulators are ATM, ATR, Chk1, Chk2 and p53. The experiments were conducted on whole cell lysate of cells harvested at approximately 90% confluence to assess differences in protein levels between drug sensitive and drug resistant cancer cells.

1. Ovarian cancer: Western blotting results of PEO1 panel

Figure 4-7: Cell cycle regulators protein level in ovarian cancer cell line PEO1 and drug-resistant variants PEO1CarbR (carboplatin-resistant), PEO1CisR (cisplatin-resistant) and PEO1TaxR (Taxol-resistant) assessed by western blotting. ATM and Chk2 expression were unaltered in all cell lines. SFN, which is a downstream effector of ATM-Chk2 showed little differences between parental and drug-resistant variants. Wee1, a downstream effector of ATR-Chk1 pathway was expressed in parental cell line but the expression was less or diminished in drug-resistant cell lines. Expression level of p53 is unaltered in drug-resistant cell lines in comparison with parental cell line apart from PEO1CarbR, which shows p53 downregulation. For PEO1 the p53 status was reported as mutant (according to various literatures).

2. Ovarian cancer: Western blotting results of SK-OV-3 panel

Figure 4-8: Cell cycle regulators protein level in ovarian cancer cell line SK-OV-3 and its drug resistant variants SKOV3CR (carboplatin-resistant) and SKOV3TaxR (Taxol-resistant) assessed by western blotting. Across cell lines, ATM, Chk2 and SFN protein levels were unchanged. There was low ATR level in parental but higher level in drug-resistant. Chk1 level in cisplatin-resistant SK-OV-3 was higher compared to parental and Taxol-resistant SK-OV-3 and this was also true for Wee1 level, although the Wee1 band was hardly detected. The p53 expression for all cell lines was not detected using the p53 antibody as a consequence of the previously reported mutation.

3. Breast cancer: Western blotting results of MCF7 panel

Figure 4-9: Cell cycle regulators protein level in breast cancer cell line MCF7 and its Taxol-resistant variant MCF7TaxR assessed by western blotting. Apart from ATM and ATR level, other proteins were shown to be expressed differently in these two cell lines. There was higher Chk1 and Wee1 level in MCF7TaxR in comparison with parental cell line MCF7. SFN, while expressed in MCF7 was not expressed in MCF7TaxR. Chk2 appeared to be expressed in a similar level in both cell lines but in MCF7TaxR, the band was slightly lower than MCF7. In MCF7, p53 is expressed (in line with its wild-type status) but diminished in MCF7TaxR (shown to be deleted, Coley unpublished data).

4. Breast cancer: Western blotting results of T47D panel

Figure 4-10: Cell cycle regulators protein level in breast cancer cell line T47D and Taxol-resistant T47DTaxR assessed by western blotting. Apart from SFN, other proteins were expressed differently in both cell lines. There was higher expression of ATM, ATR and Chk2 in Taxol-resistant T47DTaxR however Wee1 level was slightly lower than parental cell lines. This was also the case for Chk1 expression. There was no significant difference of p53 expression between parental and Taxol-resistant T47D (reported as mutant).

5. Head and neck cancer: Western blotting results of HN5 panel

Figure 4-11: Cell cycle regulators protein level in head and neck cancer cell line HN5 and its cisplatin-resistant HN5CisR assessed by western blotting. Apart from slightly higher expression of Chk1 and SFN, other proteins of interest were expressed in a similar level in both cell lines. The p53 status has been reported to be mutated.

Based on the western blotting results, drug-resistant cancers showed differences in expression of almost all proteins screened compared to their parental cell lines, apart from cisplatin-resistant HN5 (head and neck cancer). It appears that the changes seen was cell type-specific and might be influenced by p53 status.

Western blotting results were scored accordingly and the results were presented in the following tables.

6. Western blotting scores for protein level screening in cell lines used

6.1 Summary of all cell lines

Differences in protein levels as determined by visual scoring of western blots are shown in Table 4-1. The results were grouped into ovarian, breast and head and neck cancers with sub-group of different cell lines. Scores were given based on differences seen in comparison with the respective parental cell lines.

	Ovarian cancers						Breast cancers				Head and neck	
	PEO1			SKOV3			A2780		MCF7		T47D	
	Parental	CarbR	CisR	TaxR	Parental	CR	TaxR	Parental	TaxR	Parental	TaxR	HNS5
ATM*	±	±	±	±	±	±	±	±	±	±	+	±
ATR	±	-	±	±	±	+	+	±	±	±	+	±
Chk1*	±	-	++	-	±	+	-	±	+	±	-	+
Chk2*	±	±	±	±	±	±	±	±	±	±	+	±
Wee1	±	-	-	-	±	±	±	±	++	±	-	±
SFN	±	±	±	-	±	±	±	±	0	±	±	±
p53	± (m)	-	±	±	0(n/m)	0	0	± (wt)	+	± (wt)	0	± (m)

Legends =

(±): Level in parental cell line. In comparison with parental cell line = (-): downregulated, (0): none expressed, (+): upregulated, (++): highly expressed
p53 status according to various literature: (wt) wild-type, (n/m) null/mutated

Table 4-2: Protein level of several key regulators of cell cycle checkpoints in selected ovarian, breast cancer and head and neck cancer cell lines, parental and its drug resistant counterparts. Shaded columns represent protein level in parental cell lines. In most parental cell lines, all proteins screened were expressed except p53 in SK-OV-3. Clear columns represent protein level in drug-resistant cell lines in comparison with parental cell lines respectively. Various alterations in protein level were detected in drug-resistant cell lines. This table includes A2780 and A2780TaxR cell lines, which are discussed in Chapter 5. *Present in all cell lines.

6.2 Protein level in parental and Taxol-resistant cell lines

Table 4-3 illustrates scoring results of cell lines based on Taxol as causative agent:

	Ovarian cancers						Breast cancers					
	PEO1		SKOV3		A2780		MCF7		T47D		Parental	TaxR
	Parental	TaxR	Parental	TaxR	Parental	TaxR	Parental	TaxR	Parental	TaxR		
ATM	±	±	±	±	±	±	±	±	±	±	±	+
ATR	±	±	±	+	±	0	±	±	±	±	±	+
Chk1*	±	-	±	-	±	-	±	+	±	+	±	-
Chk2	±	±	±	±	±	++	±	±	±	±	±	+
Wee1*	±	-	±	±	±	0	±	++	±	++	±	-
SFN	±	-	±	±	±	0	±	0	±	0	±	±
p53	± (m)	±	0(n/m)	0	± (wt)	+	± (wt)	0	± (m)	0	± (m)	±

Legends =
(±): Level in parental cell line.
In comparison with parental cell line = (-): downregulated, (0): none expressed, (+): upregulated, (++) : highly expressed
*Frequently deregulated
p53 status according to various literature: (wt) wild-type, (n/m) null/mutated

Table 4-3: Protein level of several key regulators of cell cycle checkpoints in selected ovarian and breast cancer cell lines, parental and its Taxol-resistant counterparts. Shaded columns represent protein level in parental cell lines. Based on the scores, Chk1 and Wee1 are proteins that were frequently deregulated in Taxol-resistant cell lines.

6.3 Protein level in parental and platinum-resistant cell lines

Table 4-4 illustrates scoring results of cell lines based on platinum (carboplatin and cisplatin) as causative agent:

	Ovarian cancers						Head and neck	
	PEO1			SKOV3			HN5	
	Parental	CarbR	CisR	Parental	CR		Parental	CisR
ATM	±	±	±	±	±		±	±
ATR	±	-	±	±	+		±	±
Chk1*	±	-	++	±	+		±	+
Chk2	±	±	±	±	±		±	±
Wee1*	±	-	-	±	±		±	±
SFN	±	±	±	±	±		±	±
p53	± (m)	-	±	O(n/m)	0		± (m)	±

Legends =
(±): Level in parental cell line.
In comparison with parental cell line = (-): downregulated, (0): none expressed, (+): upregulated, (++): highly expressed
p53 status according to various literature: (n/m) null/mutated

Table 4-4: Protein level of several key regulators of cell cycle checkpoints in selected ovarian and head and neck cancer cell lines, parental and its platinum-resistant counterparts. Shaded columns represent protein level in parental cell lines. Based on the scores, Chk1 and Wee1 are proteins that were frequently deregulated in Taxol-resistant cell lines.

Discussion

To describe cancers that may have arisen from multiple sites of origin is a challenge, since each type of cancer tends to have specific features, particularly in terms of genetic background. However, various tumour types do share some similarities in DDR component deregulation, and hence analysis of this can be done using a variety of different cancer cell line models. Cell lines used in this study were of epithelial origin. Drug-resistant cell lines were developed using agents commonly used to treat cancers; cisplatin, carboplatin and paclitaxel (Taxol™). Several key cell cycle regulators were found to have similar expression patterns across the panels of cell lines examined. Based on the data obtained, cell cycle regulators Chk1, Chk2, and ATM were present in the majority of cancer cell lines looked at, despite here being different expression patterns between parental and drug-resistant variants, and also between cell lines of different origin (refer Table 4.2). As previously stated, Chk1 is essential in DDR as well as in the normal cell cycle progression to ensure successful cell division. Downregulation of Chk1 leads to cell cycle arrest and loss of cell viability (refer 7.1 ATM/Chk2 deregulation in cancers). Thus, cancer cells are thought to maintain at least a minimum level of Chk1 expression to prevent cell death. Cancer cells might gain alterations that result in Chk1 downregulation and therefore this may result in changes in the Chk2 levels to compensate the lack of Chk1 activity. Acquired resistance towards anticancer drugs might alter expression levels as a part of the resistance mechanism but in doing so, ATR and Chk2 levels are maintained. Chk2, which is downstream of ATM, was found to be expressed at low levels in the majority of cell lines, with the exception of the T47D Taxol resistant line. ATM mutations which result in the absence of the functional protein is a feature of AT patients, showing that ATM does not drastically influence viability. The ATM protein was detected in all cell lines used in this study with different expression level between parental and drug-resistant variant in certain pair of cell lines. Interestingly, in the ovarian cancer cell line A2780TaxR, the ATR level was undetectable despite the fact that ATR downregulation might affect cell viability via induction of cell cycle arrest (refer to Introduction).

There is a possible explanation for the adaptations seen for this cell line, discussed further in Chapter 5.

Platinum compounds and Taxol are two chemotherapy agents used commonly in treating cancers. Various reports have been published about the ability of cancer cells to adapt and subsequently acquire resistance to these agents. Both agents have different mechanism of action. Platinum compounds (cisplatin and carboplatin) bind to DNA, causing DNA adducts and cross-links, which subsequently cause DNA strand breaks and inhibit DNA replication. Taxanes however do not directly bind to DNA strands but cause cell cycle arrest via interaction with microtubule dynamics and interfering with mitosis (Shah and Schwartz 2001). In the context of cell cycle, platinum compounds cause cell cycle arrest at G₁ and G₂ whilst taxanes induce G₂M cell cycle arrest. Thus it is more appropriate to discuss cell cycle deregulation in drug resistance according to the class of chemotherapy drug.

Based on western blotting data of PEO1, SK-OV-3 and HN5 parental and platinum-resistant cell lines, Chk1 was shown to be the most deregulated protein compared with other cell cycle regulators examined. Chk1 downregulation was seen in carboplatin-resistance while upregulation was seen in cisplatin-resistant cell lines, in comparison with parental cells (refer Table 4.3). Chk1 expression seems to follow the ATR expression pattern in the majority of platinum-resistant cancer cells. However, Wee1 is downregulated in both types of platinum resistance. Thus, in the context of this study, Wee1 downregulation is part of the survival mechanism adaptation by drug resistant cancer cells after platinum treatment (Figure 4-6). Apart from these deregulations seen in ATR-Chk1-Wee1 pathway, there were relatively little changes seen in the expression of the ATM-Chk2-SFN pathway seen in platinum-resistant cell lines compared with their parental cell lines respectively. Acquired resistance towards platinum compound had been associated with increased DNA repair and higher

tolerance towards DNA adducts formation. In these cases, alteration in ATR-Chk1-Wee1 pathway might serve as an adaptive response towards platinum treatment since platinum compounds induce cell cycle arrest at G₁ and G₂ in platinum-sensitive cells.

Figure 4-6: Alteration of G₂M cell cycle regulators in platinum resistance. a) Carboplatin resistance. b) Cisplatin resistance. p53 status is according to parental cell line. ATR, Chk1 and Wee1 are components mostly deregulated in platinum resistance. Downregulation of p53 can be seen in carboplatin-resistant cell line (PEO1CarbR) compared to parental but not in cisplatin-resistant cell lines. *ATR upregulation was seen in SKOV3CR.

Taxanes conversely, bind to microtubules and stabilized them, thus interfering with mitotic progression and ultimately causing G₂M cell cycle blockade and cell death. In Taxol-resistant cell lines, the ATR-Chk1-Wee1 pathway was again found to be mostly deregulated in comparison with ATM-Chk2-SFN pathway. The majority of Taxol-resistant cell lines showed upregulation of ATR compared to their respective parental cell lines. In Taxol-resistant breast cancer cell lines, Chk1 and Wee1 expression seemed matched in their relative expression (refer Table 4.4). This situation is conceivable since Wee1 has been shown to be downstream target of Chk1. Upregulation of Wee1 seen in the Taxol-resistant MCF-7 cell line might be due to the role of Wee1 in preventing mitotic catastrophe (discussed further in Chapter 5). Additionally, Wee1 expression does not reflect p53 status (wild-type or mutant) of cell lines, showing that p53 might not play a role in Wee1 regulation

but appears to influence SFN expression (being a downstream target), as seen for the MCF-7TaxR line. SFN is discussed further in Chapter 6.

Figure 4-7: Alteration of G₂M cell cycle regulators in Taxol-resistant breast cancer cell lines. p53 status is according to parental cell lines. a) Wild-type p53. b) Mutated p53.

In Taxol-resistant ovarian cancers, Chk1, Wee1 and SFN were found to be downregulated in the majority of cell lines. ATR deregulation was cell line-specific while Chk2 was shown to be highly expressed in A2780TaxR (refer Table 4.4) and all Taxol resistant lines possessed mutated or deleted p53. To summarize, the ATR-Chk1-Wee1 pathway is frequently deregulated in Taxol resistance of both breast and ovarian cancers (Figure 4-8).

Figure 4-8: Alteration of G₂M cell cycle regulators in Taxol-resistant ovarian cancers. Downregulation of Chk1, Wee1 and SFN were observed in majority of Taxol-resistant cell lines screened. *p53 status and its deregulation observed were different according to cell lines. **Massive upregulation was observed in A2780TaxR in comparison with parental cell line.

Analyses of deregulation of cell cycle components on the basis of agent specific resistance possess some exceptions which made a unifying explanatory model difficult to develop. This may have been influenced by the variety of p53 status seen in all cell lines studied but is also evidence of the multiplicity and complexity of cancers. Additionally, the ATM-Chk2-SFN pathway is known to carry out crosstalk activity with the ATR-Chk1-Wee1 pathway. The crosstalk is carried out by means of the pathway interactions via phosphorylation events either directly or indirectly, thus adding more complexity to the study of their deregulation in cancers. PEO1 has a mutated p53 while MCF7 has wild-type p53. Other cell lines (SK-OV-3, T47D and HN5) have either null or mutated p53 status. Apart from MCF7TaxR where the p53-deleted (confirmed via sequencing, unpublished data), p53 status of the other drug-resistant cell line variants are unknown. p53 known as the guardian of the genome, plays an important part in determining cell response towards various stress and the most frequently altered gene in cancers. Moreover, Taxol and platinum compounds have been shown to promote different patterns of p53 phosphorylation that affect its action on other cell cycle component. Taxol can induce Ser20 phosphorylation possibly via Chk2 which promotes its

stabilization. This also leads to induction of p21^{Cip1} activity. Cisplatin is also able to evoke such reaction with greater p21^{Cip1} induction, possibly via additional p53 phosphorylation at Ser15. P53 Ser15 phosphorylation event is known to promote p53-DNA binding stabilization (Damia et al. 2001). However, the p53 status of ovarian cancer cell line SK-OV-3 does not influence its sensitivity towards Taxol or cisplatin treatment (De Feudis et al. 1997; Debernardis et al. 1997).

Overall, the ATR-Chk1-Wee1 pathway seems to be the most deregulated in drug-resistant cancer cell lines based on comparisons of protein expression levels between parental and drug-resistant cell lines. Although Chk1 is frequently deregulated, its wide range of downstream targets may provide a challenge for drug development specifically aims at targeting Chk1. However, considering each cell line's genetic background, Wee1 emerges as a possible candidate for determination of drug-resistance and represents a viable druggable target, since it specifically targets CDK1-CyclinB.

The results presented fit the hypothesis. Expression levels of key components of G₂M cell cycle checkpoints were altered in drug-resistant cell lines and some duplication were seen in other cell lines with some similarities in term of genetic background and a specific causal agent.

Conclusion

Differential expression levels of key cell cycle regulation and DNA damage recognition factors such as Chk1 and ATR appear important in mediating anticancer drug resistance. Based on the data accrued from the cell lines studied, Wee1 and lesser so SFN emerge as interesting targets that appear to mediate cellular response towards taxanes and platinum compounds. Their dysregulation would appear to be important in the consideration of anticancer drug resistance mechanisms and it might depend on the individual cellular context.

The complexity of the cell cycle machinery and the biology of the cell itself make it difficult to determine precisely the mechanism(s) behind dysregulation of cell cycle and checkpoint control in Taxol- and platinum-resistance. However, this study that focuses on the cell cycle deregulation at the G₂M cell cycle checkpoint has attempted to delineate such mechanisms and work continues to define further the mechanism between Wee1 expression and drug resistance.

Chapter 5

Wee1 as a Candidate Biomarker in Taxol Resistance

Introduction and Background

1. Wee1

1.1 Wee1 gene structure

Figure 5-1: Primary structure of somatic Wee1 protein.

Wee1 tyrosine kinase derived its name from an after effect on the size of the cells resulting from *Wee1* silencing in *S. pombe*. Wee1 originally cloned in *S. pombe* and characterized by Russell and Nurse (1989) plays an important role in mitosis by allowing cells to attain an appropriate size before being allowed to go into mitosis. Silencing of *Wee1* promotes cell cycle progression into M phase prematurely and as a result, produced small or ‘wee’ cells (reviewed by Suryadinata and Sarcevic 2008). Wee1 is a part of the Wee1 kinases family, Myt1 being the only other family members (Perry and Kornbluth 2007). However in 2000, Nakanishi et al. characterized and proposed a new member of Wee1 family, naming it Wee1B. Wee1B possess a close sequence similarity with Wee1 by having a putative nuclear localization signal in its N-terminal domain and was shown to regulate mitosis (Nakanishi et al. 2000). Therefore, based on studies in mammalian and *Xenopus* systems, currently there are two isoforms of Wee1, maternal or embryonic isoform (Wee1A in *Xenopus*, Wee1B in human) and somatic isoform (Wee1B/Wee2 in *Xenopus* and Wee1A in human) (Perry and Kornbluth 2007).

1.2 Expression

Intracellularly, Wee1 is mainly found as a nuclear protein while Myt1 is membrane-bound (Lee and Yang 2001; Nakanishi et al. 2000). Analysis of Wee1 isoforms has revealed that Wee1A (somatic) isoform and Wee1B (embryonic) isoform plays a role in different stages of early embryogenesis. Using northern blot analysis, Wee1B was shown to be highly expressed in testis while Wee1A showed a broader spectrum of expression sites including brain, pancreas, uterus and lung apart from testis (Nakanishi et al. 2000).

	Maternal/embryonic Wee1	Somatic Wee1
Expression pattern	Expressed in oocytes and early embryos	Expressed later in development stage into adulthood
Function	Control meiotic cell cycle and early mitotic cell cycle	Control somatic cell cycle which commences after zygotic transcription begun

Table 5-1: Two isoforms of Wee1 in Xenopus and mammalian systems (Perry and Kornbluth 2007).

1.3 Mechanism of action of Wee1

Wee1 phosphorylates the tyrosine 15 (Tyr-15) residue on CDK1. Inhibitory phosphorylation by Wee1 ensures CDK1 inactivation until the completion of DNA replication - or until cells have reached a suitable size before progressing into mitosis (M phase). In comparison with Wee1, Myt1 can phosphorylate Thr-14 and Tyr-15 residues of cyclin-associated CDK1. Both residues lie within the ATP binding pocket of CDK1 (de Carcer et al. 2007). However, Myt1 has a restricted substrate since Wee1 can bind to other CDKs as well, including CDK2 via Thr residue phosphorylation (Lee and Yang 2001; Nakanishi et al. 2000; Reinhardt and Yaffe 2009; Sorensen and Syljuasen 2012; Suryadinata and Sarcevic 2008). As a predominantly nuclear protein, Wee1 acts by reducing the active CDK1-CyclinB level in the nucleus during interphase while Myt1 additionally binds to CDK1-CyclinB complex, thus sequestering it at the membrane. Wee1 and Myt1 are inactivated in the G₂ to M phase transition to allow mitotic progression (Perry and Kornbluth 2007). Inhibition of CDK1 phosphorylation activity is

also maintained in G₂ cell cycle arrest after DNA damage as a result of the G₂M checkpoint control (Lee and Yang 2001). This checkpoint is essential in order to avoid cell cycle progression with damaged DNA and results either in cell death or if faulty will result in carcinogenesis. DNA damage signals transmitted via Chk1/Chk2 (checkpoint kinases) subsequently activates Wee1 and concomitantly inactivates CDC25C (Cell Division Cycle 25-C). The function of CDC25C phosphatase is to activate CDK1 by removing its inhibitory phosphorylation; hence its inactivation halts CDK1 activation. Active CDK1-CyclinB has been shown to be phosphorylated by Wee1 and Myt1 or sequestered outside the nucleus by the action of another protein called Stratifin or 14-3-3 σ (described in detail in Chapter 6) and consequently, cell cycle progression will be halted (Indovina and Giordano 2010). Wee1 also might play other ancillary roles in mitosis via phosphorylation of Serine (Ser) 549 residue of CDK1 (Perry and Kornbluth 2007).

Wee1 interaction with CDK2-CyclinA is less well understood. CDK2 plays a role in promoting mitosis by reducing the phosphorylation of the CDK1-CyclinB complex, which normally gives rise to a delay in mitotic entry. Inhibition of CyclinA activity via siRNA or by using CDK1-CyclinA inhibitors leads to mitotic blockade and decreased CDC25 activity. Targeted deletion of CyclinA proved to be lethal in mouse embryo as well as in adult animals with evidence of G₂ cell accumulation (Enders 2010; Kalaszczyńska et al. 2009). However, the exact mechanism behind CyclinA inhibition and cell cycle effects remains elusive. Based on works in *Xenopus*, CDK2 has been shown to interact with Wee1 through its RxL1 binding motif. CDK2 binds and phosphorylates the Wee box and makes it inactive. Additionally, CDK2 might play a role in the cytoplasmic distribution of Wee1 by phosphorylating another residue that promotes Wee1 nuclear export via interaction with Crm1 (Enders 2010). This mechanism remains to be further clarified. As such, CDK2 is negative regulator of Wee1 (Figure 5-).

Figure 5-2: Relationship between CDK1-CyclinB, CDK2-CyclinA and Wee1. Wee1 negatively regulates CDK1-CyclinB by inhibitory phosphorylation (**). CDK2-CyclinA promotes mitotic entry by reducing inhibitory phosphorylation of CDK1-CyclinB through negative regulation of Wee1.

1.4 Regulation

Wee1 is expressed in S and G₂ phases and its activity corresponds with Wee1 protein concentration.

Wee1 activation comes from interaction with Chk1 and stimulation from CABLES (CDK5 and Abl enzyme substrate), a CDK-interacting protein (Sorensen and Syljuasen 2012). CABLES was first isolated by Zukerberg et al. (2000) via screening of a mouse cDNA library. CABLES links CDK5 to c-Abl tyrosine kinase, thereby promoting CDK5 Tyr residue phosphorylation by c-Abl (Zukerberg et al. 2000). Low levels of CDK activity in the G₂ phase can also contribute towards Wee1 activation in budding yeast (Sorensen and Syljuasen 2012). Wee1 is able to undergo autophosphorylation at Tyr residues, a process which acts as a positive regulator. Regulation of Wee1 also comes from interaction via phosphorylation and binding with 14-3-3 proteins during interphase. Human Wee1 interacts with 14-3-3 proteins by C-terminal consensus motif that enhances the stability and ability of Wee1 to maintain interphase. It has been shown that this binding occurs in interphase but not in mitosis. It requires phosphorylation of Ser residue in C-terminus of Wee1 to create a binding site for 14-3-3 proteins (Perry and Kornbluth 2007; Reinhardt and Yaffe 2009) (Figure 5-13). However, interaction between Wee1 and 14-3-3 proteins might also negatively regulate its activity. Akt promotes cell cycle progression by phosphorylating the Ser642 residue of Wee1, promoting its

cytoplasmic localization during late S and G₂ phases. Phosphorylation of Wee1 Ser642 creates binding site for 14-3-3 θ , an isoform of 14-3-3 proteins. Allegedly, binding with different isoforms of 14-3-3 determines Wee1 activity. Binding with β , γ and ζ isoforms will promote its activity while 14-3-3 θ promotes its nuclear sequestration (Perry and Kornbluth 2007).

Figure 5-13: Positive regulation of Wee1. Wee1 phosphorylates CDK1 thus making it inactive and results in G₂M cell cycle arrest. Low CDK activity and interaction with CABLES enhances Wee1 activity (1). Wee1 is also able to undergo auto-phosphorylation (2). Chk1 promotes Wee1 activity via phosphorylation that creates binding site for 14-3-3 protein which further promotes its activity and stability (3).

Inactivation of Wee1 is poorly understood. It is thought that inhibition of Wee1 activity at the beginning of mitosis is promoted by its phosphorylation and degradation. Wee1 phosphorylation is part of a negative feedback loop initiated by CDK1-CyclinB. CDK1-CyclinB phosphorylates the Ser123 residue of Wee1. Following phosphorylation, Wee1 becomes a target for ubiquitination by SCF ubiquitin ligase β -TrCP via interaction with its WD40 domain and later subjected to proteosomal degradation. Ser123 phosphorylation also acts as a docking site for polo-like kinase Plk1. Apart from Wee1, Plk1 is also able to inhibit Myt1. Another SCF-type ubiquitin ligase Tome-1 (Trigger of Mitotic Entry) might also play a role in enhancing β -TrCP activity. Tome-1, originally described by Ayad et al.

(2003), promotes Wee1 degradation and mitotic entry through Ser38 phosphorylation of *Xenopus* Wee1 as a recognition label (Perry and Kornbluth 2007; Sorensen and Syljuasen 2012). Thus, the level of active CDK increases thereby promoting cell cycle progression.

Nuclear Wee1 stability is controlled by DNA-responsive checkpoints. A study in *Xenopus* showed that upon inhibition of DNA replication or DNA damage, Wee1 protein was stabilized and degraded intranuclearly during mitotic entry. Wee1-interacting protein Hsl7 might play a role in intra-nuclear degradation of *Xenopus* Wee1 at G₂M transition. This was shown through overexpression and depletion experiments involving Hsl7. Hsl7 overexpression overrides checkpoint into mitosis by excess Wee1 degradation, whilst depletion of Hsl7 stabilized Wee1 could promote cell cycle arrest (Perry and Kornbluth 2007) (Figure 5-14).

Figure 5-14: Negative regulation of Wee1. CDK1-CyclinB phosphorylates Wee1 as a negative feedback loop. Wee1 phosphorylation creates binding site for Plk1 and β -TrCP ubiquitin ligase thus promoting its degradation. 14-3-3 isoform θ binds to phosphorylated Wee1 and promotes its nuclear export. Binding of Wee1 with Hsl7 directly without CDK phosphorylation also promotes Wee1 degradation internuclearly.

1.5 Expression of Wee1 in cancer

Deregulation of Wee1 has been reported in several cancers (Backert et al. 1999; Kiviharju-et al. 2007; Hallstrom et al. 2007; Magnussen et al. 2012; Masaki et al. 2003). Using cDNA array, Backert et al. (1999) reported significant downregulation of *Wee1* in colon carcinoma cells of both non-mucinous and mucinous phenotypes. These findings also correlated with *Wee1* expression levels in patients' tissue samples (Backert et al. 1999; Lee and Yang 2001). There is evidence for various microRNAs (miRs) that target *Wee1* and, therefore, play a role in *Wee1* transcriptional control, as described for some cancers. MiR-155, for example, has been shown to be overexpressed during the innate immune response, autoimmune disorders and various malignancies. Tili et al. examined the ability of microRNA-155 (miR-155) to induce mutation and found evidence for targeting *Wee1*, causing its downregulation (Sorensen and Syljuasen 2012; Tili et al. 2011). Additionally, low *Wee1* expression could increase susceptibility to carcinogenesis. Gamma-irradiation on human primary prostate epithelial cells and human prostate tissues revealed that prostate epithelium retained CDK2-associated kinase activity as a result of low inhibitory phosphorylation of CDK2. Ectopic *Wee1* expression however rescues the checkpoints. Thus it showed that prostate epithelium expressed very low levels of *Wee1* thus increasing its susceptibility to acquire DNA lesions and development into prostate cancer (Kiviharju-et al. 2007; Hallstrom et al. 2007; Sorensen and Syljuasen 2012). Downregulation of *Wee1* was also reported in non-small-cell lung cancer (Magnussen et al. 2012).

However, overexpression of *Wee1* has been reported in several cancers including in osteosarcoma, hepatocellular carcinoma (HCC), glioblastoma and breast cancer. A comparative study of HCC *versus* cirrhosis revealed that protein level and kinase activity of *Wee1* is higher in HCC compared with surrounding cirrhotic tissues (Masaki et al. 2003). There is higher *Wee1* expression in glioblastoma cell nuclei in comparison with normal brain regions and its expression was well correlated with patient survival (Mir et al. 2010). A high level of *Wee1* was associated with poor disease-free survival

in malignant melanoma and markers of increased tumour cell proliferation (thicker primary tumours and ulceration) based on analysis performed on paraffin-embedded patient derived tissues and cultured melanocytes (Magnussen et al. 2012).

1.6 Wee1 and DNA damage recognition (DDR)

DNA damage occurs naturally and also as a result of exposure to certain type of chemicals used in cancer treatment. DNA damaging agents as chemotherapeutic agents are classified according to their mode of action. There are radiomimetics, topoisomerase inhibitors (for example doxorubicin), anti-metabolites (5-fluorouracil) and alkylating agents (carboplatin and cisplatin) (Indovina and Giordano 2010). DNA damage evokes cellular responses which results in DNA repair, cell cycle arrest and/or apoptosis through DNA damage checkpoint control. These checkpoints consist of sensors, mediators, transducers and effectors (Niida and Nakanishi 2006). Depending on the type of DNA damage, different signalling pathways are activated. In a nutshell, cell cycle checkpoints at G₁S and G₂M are activated if the cell sustains DNA damage, stalled replication forks or stress (Bouwman and Jonkers 2012). ATM and ATR kinases are signal transducers that phosphorylate a large number of substrates including Chk1 and Chk2 kinases, another type of signal transducer. In turn, Chk1 activates effector kinases for p53 and Wee1 while at the same time blocking CDC25 phosphatase activity via phosphorylation which results in its direct inhibition, nuclear export or ubiquitination followed by its subsequent degradation (Niida and Nakanishi 2006). p53 activation and CDC25 inhibition are also triggered by Chk2 kinase which receives its activation signal from ATM. In conclusion, the activation of these DNA damage signalling pathways results in activation of cell cycle checkpoints via inhibition of CDK2-CyclinE activity at G₁S and CDK1-CyclinB activity at G₂M (Bouwman and Jonkers 2012).

Wee1's property as a negative regulator of the cell cycle has proven to be an interesting target for drug development. Hence MK1775, a Wee1 inhibitor has been in clinical trial as single and combination treatment. Wee1 inhibitors were developed to provide targeted therapy especially with cancers that are p53-deficient. In these cells, lack of p53 is hypothesized to make the cells more susceptible to G₂M checkpoint abrogation (Hirai et al. 2009) and in this context MK1775 can be described in this way.

2. MK1775: Synthetic inhibitor of Wee1

2.1 Mechanism of action

Figure 5-15: MK1775 chemical structure (Kreahling et al. 2012).

Cancer patients are typically treated with conventional DNA damaging agents such as ionizing radiation, alkylating agents and platinum compounds. However, these agents possess low specificity and are associated with considerable side effects. Combinations of these DNA damaging agents with more targeted treatment may enhance activity and narrow target choice. MK1775 (2-alkyl-6-anilino-1-aryl, 2-dihydro-3H-pyrazole [3,4-d]pyrimidin-3-one) originally described by Hirai et al. (2010) is a selective and potent small molecule inhibitor of Wee1 (Mizuarai et al. 2009). MK1775 inhibits Wee1 by competition with ATP for its Wee1 binding site, leading to decreased phosphorylation of CDK1. MK1775 has a >100 fold selectivity over human Myt1, another Wee1 family member that also inhibits CDK1 (Hirai et al. 2009).

2.2 MK1775 in combination and as a single agent

Combination treatment using DNA damaging agent gemcitabine, carboplatin and cisplatin in p53-deficient cells promotes apoptosis. *In vivo* combination treatment showed that MK1775 enhanced tumour growth inhibition and with no evidence of increased toxicity. MK1775 treatment *in vitro* with the microtubule targeting agent docetaxel and paclitaxel did not enhance their cytotoxic effects (Hirai et al. 2009; Indovina and Giordano 2010). Several other studies also demonstrated antitumorigenic effects of MK1775 in combination with cytotoxic agents in non-small-cell lung carcinoma, breast, prostate, pancreatic, ovarian and colon cancers (reviewed by Krehling et al. 2012). In mice, Wee1 knockout resulted in mice embryonic lethality, DNA damage and apoptosis. In rodents, Wee1 downregulation hinders tumour xenograft growth. Synergistic effects to inhibit cellular proliferation and promoting apoptosis were seen in combination treatments using the Chk1 inhibitor AR458323. It was also shown that cancer cell lines were more prone to this combination compared to non-cancerous cells (Davies et al. 2011). Another study carried out using the Chk1 inhibitor PF00477736 in combination with MK1775 showed that regardless of p53 status, this combination acts synergistically in breast, ovarian, colon and prostate cancer cell lines. *In vivo* combination treatment in mice results in higher tumour growth suppression in comparison with single agent treatment without any sign of toxicity (Carrassa et al. 2012). *In vivo* treatment using MK1775 alone in animal xenograft models produces limited antitumour efficacy in comparison with another Wee1 inhibitor PD0166285 (Hirai et al. 2009). However, MK1775 treatment in wild-type p53, mutant p53 and null p53 sarcoma cells produced similar degrees of cell death as a result of mitotic catastrophe caused by Wee1 inhibition alone (Krehling et al. 2012). These data suggest that MK1775 activity is based on cancer type and p53 status is not a definitive way of determining treatment response towards this agent.

3. Paclitaxel

3.1 Mechanism of action

Figure 5-16: Paclitaxel (TaxolTM) chemical structure (Orr et al. 2003).

Paclitaxel (TaxolTM) is an antimitotic agent derived from *Taxus brevifolia* and *Taxus yunnanensis*. There are two major groups of antimitotic agents, classified based on their action on the tubulin polymers/microtubule system. Vinca alkaloids are examples of agents that bind to α/β -tubulin heterodimers thus inhibit polymer assembly. In contrast, Taxol belongs to the group of antimitotic agents that binds to polymer and stabilized microtubules. Taxol only binds to polymerized tubulin, specifically the β -tubulin subunit in microtubules and not tubulin dimers themselves. Binding of Taxol to microtubule polymers is reversible and enhances polymerization of tubulin. In the presence of Taxol, microtubules have a higher degree of stability and resistance towards depolymerisation by Ca^{2+} , cold temperature and dilution. Taxol also alters microtubule assembly kinetics. Taxol can also interfere with mitotic progression by suppressing microtubule dynamics and consequently, produces an aneuploid population of cells by means of forced mitotic exit. Taxol induces several antimitotic effects that have been shown to be concentration-dependent (Kumar et al. 2010). Taxol was approved by FDA (US Food and Drug Administration) for treatment of several types of human cancers, including ovarian cancer (Kumar et al. 2010). There are several newer forms of paclitaxel developed by conjugation with other molecules for ease of administration, reducing toxicity and improving efficacy. These conjugates include albumin, docosahexaenoic acid, poly-L-glutamic acid and brain peptide vector angioprep-2 (Kumar et al. 2010; Yardley 2013).

3.2 Resistance mechanism to Taxanes

Acquired resistance to Taxol had been a “stumbling block” in cancer treatment. There are several known mechanisms of Taxol resistance. These include overexpression of the multidrug transporter p-glycoprotein and alteration in the drug’s metabolism. Orr et al. have listed several possible mechanisms of Taxol resistance concentrating on the microtubules. This includes changes to microtubule dynamics, altered expression of β -tubulin isotypes especially in class III and IV, point mutations in tubulin that altered microtubule dynamics, altered expression or post-translational modifications of tubulin or microtubule-regulatory proteins, altered binding of Taxol to the microtubule and alterations in signalling pathway (Orr et al. 2003).

3.3 Taxol and mitotic catastrophe

Taxol as a microtubular poison, directly affect mitosis via tubulin stabilization which in turn causes mitotic catastrophe. Mitotic catastrophe is a poorly defined event. Some researchers classify mitotic catastrophe as an event of cell destruction during or shortly after mitosis as an effect of abnormal chromosome segregation or DNA damage (Bouwman and Jonkers 2012; Galluzzi et al. 2011; Portugal et al. 2010). Galluzzi et al. (2011) further defined mitotic catastrophe as a signalling pathway triggered by abnormalities in mitotic apparatus sensed during mitosis that leads to mitotic arrest followed by cell death. This event can sometimes be accompanied by the release of pro-apoptotic proteins and caspase activation, pointing to cross-talk between mitosis and another form of cell death, apoptosis. Mitotic catastrophe had also been defined as the main cause of cell death by ionizing radiation (Portugal et al. 2010). Until recently, there have been no exact biochemical features that can define mitotic catastrophe. However, several biochemical events have been associated with mitotic catastrophe such as caspase-2 activation, prolonged activation of spindle-assembly checkpoint (SAC), activation of p53 and aberrant level of CyclinB (Galluzzi et al. 2011). Premature activation or overexpression of CDK1-CyclinB also leads to mitotic catastrophe by means

of early entry into mitosis before the completion of the G₂ phase. Prolonged activation of CDK1-CyclinB via Chk2 inhibition promotes progression towards mitosis followed by mitotic catastrophe (Castedo et al. 2004). Some morphological features have been associated with mitotic catastrophe, including micronucleation and multinucleation. Micronucleation is an aftermath of inconsistent chromosome and/or chromosome fragments division between two daughter cells. Multinucleation is a product of abnormal karyokinesis which generates two or more nuclei with similar or heterogeneous sizes (Galluzzi et al. 2011). The following table summarizes mitotic catastrophe.

Definitions and characteristic	Cell death occurring during or after a faulty mitosis Can lead to necrosis or apoptosis-like death (p53-independent)
Morphological features	Micronucleation Multinucleation Giant cells Partially condensed chromatin Apoptotic and/or necrotic features
Biochemical features/ associated genetic changes	Activation of caspase-2 Prolonged SAC signalling p53 activation Abnormal CDK1-CyclinB activation Stimulated by deficiencies in cell cycle checkpoint proteins and mitotic spindle assembly Can follow caspase-dependent or independent routes G ₂ M accumulation and polyploidy
Inflammatory/immune features	Poorly defined (might be dependent on the executioner mechanism)

Table 5-2: Summary of mitotic catastrophe (Galluzzi et al. 2011; Portugal et al. 2010).

Aim

Wee1 is one of the genes listed as downregulated in drug-resistant cell lines in comparison with parental cell lines in the original cDNA microarray analysis. This study aims to further confirm these earlier data and to try to establish if *Wee1* expression is pivotal in inducing anticancer drug resistance.

Hypotheses

- *Wee1*, identified as changing its expression levels in several drug resistant cancer cell lines, plays a role in mediating the change in drug sensitivity – particularly in the case of taxanes
- Downregulation of *Wee1* is due to epigenetic silencing and this plays a functional role in mediating cancer cell responsiveness to taxanes
- Conversely, drug resistant cell lines showing marked up regulation of *Wee1* may show collateral sensitivity to the *Wee1* kinase inhibitor MK-1775

Methods

Methods used in this study as described in Chapter 2 are protein harvest, western blotting, mRNA extraction, qPCR analysis, cytotoxicity assay using MTT, DNA harvest, PI staining, Annexin V-FITC, flow cytometric analysis, and siRNA transfection. Cell lines used were A2780 and A2780TaxR Taxol-resistant, and SK-OV-3 and SKOV3 Taxol-resistant variant cell lines. Dose-response curves were constructed using GraphPAD PRISM. Student's Paired T-Test was performed where applicable and result was expressed as p value. All methods have been described in Chapter 2.

Results

1. Taxol response in cell lines

Taxol treatment followed by viability test using MTT was performed on both A2780 and SK-OV-3 panel (drug-sensitive and drug-resistant). The dose-response curves were drawn to acquire IC₅₀ values of Taxol for each cell lines in the respective panel. All cell lines used in this experiments and other subsequent experiments were tested negative for mycoplasma.

1.1 Taxol IC₅₀ level in A2780 and SK-OV-3, parental and drug-resistant cell lines

Dose-Response Curve of Taxol in A2780 and A2780TaxR

Figure 5-7: Dose-response curve of Taxol treatment to A2780 and Taxol-resistant A2780TaxR. Cells were seeded at 3x10⁴ cells/mL in 96-well plate and treated with Taxol for 72 hours before termination using MTT reagent. Resistance factor (RF) was calculated using formulae (IC₅₀ parental/IC₅₀ drug-resistant). RF value for A2780TaxR was 3.189 (n=5).

Dose-Response Curve of Taxol in SK-OV-3 and SKOV3T6

Figure 5-17: Dose-response curve of Taxol treatment to SKOV3 and Taxol-resistant SKOV3T6. Cells were seeded at 3x10⁴ cells/mL in 96-well plate and treated with Taxol for 72 hours before termination using MTT reagent. RF value for SKOV3T6 was 20.33 (n=3).

1.2 DNA content analysis of A2780 panel without and with Taxol treatment

A2780 parental and A2780TaxR (Taxol-resistant) was treated with 10nM Taxol and harvested after 24 and 48 hours treatment. Table 5-3 summarized changes seen in DNA content of both cell lines after Taxol treatment, as analysed using flow cytometry. Experiment was conducted to assess differences of Taxol response (in terms of cell cycle changes) between Taxol-sensitive and Taxol-resistant ovarian cancer cells.

A2780		A2780TaxR
Control	Typical DNA content (G ₁ S and G ₂ M peaks)	Typical DNA content (G ₁ S and G ₂ M peaks), approximately similar as parental cell line
24 hour post-treatment	<div><div></div><div>- appearance of debris peaks and subG₁</div><div>- reduced G₁ peak size</div><div>- higher G₂M peak</div></div>	No significant changes to DNA profile
48 hour post-treatment	<div><div></div><div>- prominent debris peaks</div><div>- maintenance of G₂M peak</div></div>	No significant changes to DNA profile

Table 5-3: Summary of DNA cytogram of A2780 and A2780TaxR treated with Taxol. Taxol induced G₂M cell cycle arrest. This effect was clearly shown in Taxol-sensitive A2780 but not in Taxol-resistant A2780TaxR. The effect was more pronounced with higher Taxol dosage and longer incubation time.

1.3 DNA cytogram of A2780 panel with different Taxol dose at 48 hours post-treatment

Figure 5-9 shows cytograms of control (untreated) and Taxol-treated (10nM and 25nM) A2780 and A2780TaxR as analysed using flow cytometry. Experiment was conducted to assess DNA changes caused by different Taxol concentration on Taxol-sensitive and Taxol-resistant ovarian cancer cells.

Figure 5-9: DNA cytogram of A2780 and Taxol-resistant A2780TaxR without and with 48 hours of 10nM and 25nM Taxol treatment. Longer exposure to Taxol promotes cell death as seen by the appearance of undefined peaks (cell debris) right before the actual DNA peaks. However in Taxol-resistant A2780 there were hardly any changes seen in the DNA peaks compared to control. This figure is representative of cytograms obtained during repeated experiments (n=3).

2. Wee1 mRNA level in A2780 and SK-OV-3 panels

Wee1 expression in A2780 and SK-OV-3 panels were measured using qPCR analysis and results were presented in Figure 5-10.

Figure 5-18: qPCR analysis of Wee1 expression in 2 different parental and Taxol-resistant cell lines (A2780 and SK-OV-3). Based on qPCR analysis, *Wee1* expression was significantly downregulated in both Taxol-resistant cell lines compared to its respective parental cell lines. (* $p<0.05$, ** $p<0.005$) (A2780 pair $n=13$, SK-OV-3 pair $n=6$)

Following qPCR, western blotting using an antibody against Wee1 was performed in order to assess the protein level in the different cell lines.

3. Wee1 protein level in A2780 and SK-OV-3 panels

Figure 5-19: Western blotting analysis of Wee1 level in selected cell lines. a) Wee1 protein level in A2780 and Taxol-resistant A2780TaxR. In Taxol-resistant cell line, there was clear downregulation of Wee1 compared to its parental cell line, based on the decreasing size of Wee1 protein band in A2780TaxR compared to parental cell line A2780. **b)** Wee1 protein expression in SK-OV-3 and SKOV3TaxR Taxol-resistant. Wee1 level in Taxol-resistant was higher compared to parental cell lines as shown by the increased intensity of the band. ($n=3$).

The Wee1 level in A2780 panel was in agreement with qPCR analysis. However, the Wee1 protein level in SK-OV-3 panel did not reflect its qPCR analysis.

4. G₂M cell cycle control protein level in A2780 panel

Western blotting for several G₂M cell cycle proteins was done to assess and to compare their level in A2780 and A2780TaxR. Antibodies used in the experiment were against ATM, ATR, Chk1, Chk2 and p53. β-actin and GAPDH acted as positive controls. Experiments were performed on whole cell lysate samples.

Figure 5-12: Western blotting analysis of A2780 and its Taxol-resistant counterpart A2780TaxR. HeLa acted as positive control for most of the proteins detected. a) Level of G₂M cell cycle regulator proteins upstream of Wee1 in both cell lines. Apart from ATM, A2780TaxR Taxol-resistant showed different expression patterns compared to A2780 parental cell line. Upregulation was detected in p53 level **(b)** and massively seen in Chk2 expression. Downregulation was seen in ATR and Chk1 compared to parental cell line.

Downregulation of Wee1 in A2780TaxR might be due to epigenetic modification of the gene.

Demethylating treatment was done in order to assess this hypothesis.

5. Demethylating agent treatment in A2780 Taxol-resistant

Figure 5-13: Treatment with demethylating agents, 5'-aza-cytidine (5-Aza) and Trichostatin A (TSA) to taxol-resistant A2780. 5'-aza-cytidine treatment brings back Wee1 expression in this cell line, which is consistent with the cDNA microarray analysis.

6. Western blotting of Wee1 silenced whole cell lysates

Wee1 gene silencing was conducted on A2780 and SK-OV-3 to emulate Wee1 downregulation seen in their Taxol-resistant counterparts. Western blotting was performed on whole cell lysates harvested post silencing treatment to assess silencing efficiency.

6.1 A2780

Figure 5-14: Wee1 silencing experiment in the A2780 cell line. Cells were successfully silenced based on the western blotting result above. Wee1 protein band can still be seen in samples treated with “scramble” siRNA (acted as a negative control). Optimal condition for siRNA transfection in A2780 using 6-well plate was achieved at 5×10^5 cells/well with 50nM total siRNA concentration and 6 μ L transfection reagent per well.

6.2 SK-OV-3

Figure 5-15: Wee1 silencing experiment in the SK-OV-3 cell line. Cells were successfully silenced based on the western blotting result above. Wee1 protein band can still be seen in samples treated with scramble (negative control), showing that the silencing procedure without the Wee1 siRNA did not affect We1 expression significantly.

Based on the western blotting results of both A2780 (Figure 5-14) and SK-OV-3 (Figure 5-15), Wee1 silencing was successfully carried out in A2780 and SK-OV-3. Cells were treated with Taxol and subjected to flow cytometry analysis to compare changes of DNA content and apoptotic cell response post-treatment between control and *Wee1*-silenced cells.

7. *Wee1* silencing in A2780 followed by Taxol treatment

7.1 DNA content analysis before and after Taxol treatment using flow cytometry

Figure 5-16: *Wee1*-silenced A2780 cells were then subjected to Taxol treatment followed by flow cytometry analysis to compare the DNA content before and after treatment (n=3).

In drug-sensitive cells, Taxol induced G₂M cell cycle arrest that can be observed as increasing G₂M DNA content. *Wee1* silencing in Taxol cells did not alter cell response towards 10nM Taxol treatment after 48 hours. However sub-G₁ population in *Wee1*-silenced, Taxol-treated cells were higher compared to control and scramble cells treated similarly.

7.2 Apoptotic analysis before and after Taxol treatment

Figure 5-17: Annexin V-FITC analysis of A2780 control, scramble and Wee1-silenced before and after 48 hours of 10nM Taxol treatment (n=3). Wee1 silencing in A2780 did not affect live cell population (third cytogram from top left, bottom left quadrant) similarly as scramble siRNA transfection (second cytogram from top left). There were no significant differences between control, scramble and Wee1-silenced cells in terms of their response towards Taxol treatment (p value for differences of Taxol-treated, Wee1-silenced A2780 versus scramble with similar Taxol treatment is >0.02). Figure is representative of three repeated experiments.

Based on the flow cytometric analysis on DNA content and apoptotic analysis, Wee1 downregulation in A2780 do not give significant influence on Taxol response compared to control cells.

8. *Wee1* silencing in SK-OV-3 followed by Taxol treatment

8.1 DNA content analysis before and after Taxol treatment

Figure 5-18: *Wee1*-silenced cells were then subjected to DNA analysis using flow cytometry to compare between DNA content before and after treatment. In drug-sensitive cells, Taxol induced G₂M cell cycle arrest that can be observed as increasing G₂M DNA content. In SK-OV-3 scramble siRNA control cells, there was significant increase in G₂M peak percentage after 48 hour 500nM taxol treatment (**p value <0.005). Similar effect was seen in *Wee1*-silenced, Taxol-treated cells. However, this effect was much less compared to control. Higher percentage of sub-G₁ peak was also observable in *Wee1*-silenced sample compared to control (n=3).

8.2 Apoptotic analysis before and after Taxol treatment

Figure 5-19: Annexin V-FITC analysis of SK-OV-3 control, scramble and *Wee1*-silenced before and after 48 hours of 500nM Taxol treatment. Taxol treatment to control cells (first cytogram from bottom left) reduced live cell's population considerably as seen by the emergence of cell population in early (bottom right quadrant) and late-stage apoptosis (top right quadrant). Silencing of *Wee1* alone resulted in increased cell apoptosis (third cytogram from top left) but more viable cells seen after Taxol treatment (third cytogram from bottom left) in comparison with Taxol-treated scramble cells (second cytogram from bottom left). Live cell population in scramble and *Wee1* siRNA cytograms were artificially set to 100% for easier comparison and to correct for cell death induced by transfection and *Wee1* silencing (n=3). Figure is representative of three repeated experiments.

Based on the DNA content and the Annexin V-FITC analysis, *Wee1* silencing in SK-OV-3 cells influence Taxol response. *Wee1* silencing in SK-OV-3 appears to reduce sensitivity towards Taxol treatment,

showed by less G₂M cell cycle arrest and higher percentage of live cells in *Wee1*-silenced cells in comparison with controls (untreated and scramble siRNA).

9. MK1775 effect on viability of A2780 and SK-OV-3, parental and Taxol-resistant cell lines

MK1775 is a *Wee1* inhibitor and was used alternatively to downregulate *Wee1* expression in selected cancer cell lines. A2780 and MCF7 panels were chosen based on the *Wee1* expression level in A2780TaxR and MCF7. Both of these cell lines exhibit high *Wee1* expression level in comparison with their counterparts. Cells were treated with MK1775 followed by a viability assay. Dose-response curves were drawn accordingly to derive MK1775 IC₅₀ for all cell lines.

9.1 A2780 and A2780TaxR

Dose-Response Curve of MK1775 in A2780 and A2780TaxR

Figure 5-20: Dose-response curve of MK1775 treatment to A2780 and A2780 Taxol-resistant cell lines. A2780TaxR has a lower *Wee1* expression in comparison with parental cell line A2780 and responds less to MK1775 treatment (n=3).

9.2 MCF7 and MCF7TaxR

Dose-response curve of MK1775 in MCF7 and MCF7TaxR

Figure 5-21: Dose-response curve of MK1775 treatment to MCF7 and MCF7TaxR Taxol-resistant cell lines (n=3). MCF7TaxR was more sensitive towards MK1775 treatment compared to MCF7. Of note, MCF7TaxR expresses higher Wee1 protein level compared to its parental cell line MCF7 (see Chapter 4: Key players in cell cycle).

Based on the dose-response curves generated from the viability assay data for both A2780 panel (Figure 5-20) and MCF7 panel (Figure 5-21), cells with higher Wee1 expression level were more sensitive towards MK1775 treatment compared to their counterparts with lower Wee1 expression. These results shows that Wee1 plays a role in cell viability and Wee1 downregulation gives greater effect on cells with high Wee1 expression compared to cells with lower Wee1 expression.

Discussion

Deregulation of gene expression is a common event in cancer. By doing so, cancer cells acquire growth advantage compared to the surrounding tissues. *Wee1* had been reported as one of genes downregulated in several cancers (refer to Introduction). The fact that *Wee1* appears downregulated in drug-resistant cancers is also supported by our cDNA microarray analysis (Coley unpublished data 2008).

We examined *Wee1* deregulation in cancer cell cycle particularly in ovarian cancer cell lines A2780 and SK-OV-3. These cell lines confer different genetic backgrounds with A2780 having a wild-type p53 while SK-OV-3 is p53 mutant. Based on the results of the cytotoxicity assay, we established that drug-resistant cells have at least three-fold Taxol resistance in comparison with parental cell lines. This level of resistance is clinically relevant according to Hall et al. (2009). The minimum requirement of resistance to be considered significant is at least two-fold (Hall et al. 2009).

Deregulation of *Wee1* in cancer has been documented *in vitro* and *in vivo* as discussed earlier. cDNA microarray analysis that have been done previously also points out *Wee1* as one of the gene frequently downregulated in drug-resistant cancer cell lines with possibility of promoter hypermethylation as a reason for low expression level. Assessment of *Wee1* mRNA and protein level in A2780 Taxol-resistant counterpart *versus* the parental line shows clear downregulation of *Wee1*. Even though *Wee1* expression was low in SKOV3T6 this was not true for *Wee1* protein level, which was higher in Taxol-resistant SK-OV-3 compared to parental cell line. Discrepancies between mRNA and protein level might come from post-translational modification of *Wee1* protein in SK-OV-3 Taxol-resistant. Defects in *Wee1* regulation, for example in proteosomal degradation might also contribute to higher protein levels. Additionally, assessment of the *Wee1* protein level was performed in unsynchronized cells as constitutive expression to give the background indication of overall gene expression level. The use of

synchronized cells or time-point samples might give a clearer understanding of the state of Wee1 expression in SK-OV-3 parental and Taxol-resistant cells.

Since the A2780 cell line showed agreement of Wee1 downregulation on both mRNA and protein levels, this cell line was been chosen as a model to conduct a study of Wee1 in Taxol resistance. Screening of upstream cell cycle components showed that Taxol-resistant A2780TaxR acquired several changes that might give advantage to the cells to tolerate Taxol treatment. In drug-sensitive A2780, there was detectable expression of Chk1 and Wee1. Theoretically, downregulation of Wee1 will give growth advantages to cancer cells through unrestricted CDK activities. However, Chk1 and Wee1 are essential in avoiding replication stress in cells that have acquired other genetic changes that lead to increased replication stress via increased CDK activities. Replication stress will induce cell death if the burden of DNA damage is too high. Thus, Wee1 and Chk1 expression in A2780 is significant in protecting cells from death. It also explains A2780 sensitivity towards MK1775 treatment, since it depends on Wee1 to keep control of CDK activities. This explanation was consistent with the hypothesis described by Sorensen and Syljuasen (2012). Significant upregulation of p53 and Chk2 in Taxol-resistant A2780TaxR in comparison with A2780 might be the effect of Taxol treatment, since Taxol is able to activate Chk2 which in turn phosphorylates p53 at S20 residue, thus promoting its stabilization (Damia et al. 2001). Since p53 is downstream of Chk2, it might be that the upregulation of p53 was a response of elevated Chk2 level. High Chk2 expression might also serve as an additional benefit to these cells. Chk2 expression was shown to protect cells from mitotic catastrophe, an event initiated by Taxol (Castedo et al. 2004). ATM/ATR are DNA damage sensory proteins. Initiation of DNA damage will generate signals sensed by ATM/ATR which relays it to the downstream effector Chk1/Chk2. These proteins promote activation of cell cycle regulatory proteins including Wee1 that in turn halt cell cycle progression to allow for DNA damage repair. Downregulation of ATR and Wee1 might be as a result of increased Chk2 and p53 level.

Both pathways are involved in regulation of CDK1 activity and in this cell line, the ATR-Chk1-Wee1 level was preferentially downregulated in order to maintain the cell cycle progression even in the presence of Taxol. Since both pathways negatively regulate CDK1 activity, complete suppression of CDK1 activity will lead to cell cycle arrest and cell death and downregulation of ATR-Chk1-Wee1 seems to be the only way to keep the cells cycling whilst maintaining the CDK1 level in the presence of Taxol.

Previously in Chapter 3, it was shown that downregulation of p57^{KIP2} in platinum-resistant PEO1 was due to epigenetic modification, specifically hypermethylation at the gene's promoter site. This event leads to downregulation of gene expression and demethylating treatment has been able to bring the expression back. In this study, two types of chromatin-modifying agents (CMAs) had been utilised to assess Wee1 epigenetic changes. 5'-aza belongs to the class of nucleoside analogs of DNA methyltransferase (DNMT) inhibitors while TSA is a hydroxamate class of histone deacetylase (HDAC) inhibitor (HDACi) targeting HDAC class I and II. 5'-Aza acts by promoting DNA demethylation while TSA promotes histone acetylation (Seidel et al. 2012; Woo et al. 2007). Both have different mode of action but lead to transcriptionally active DNA. Expression of Wee1 has been re-established in Taxol-resistant A2780TaxR after 5'-Aza treatment, showing that hypermethylation of DNA does play a role in Wee1 downregulation. TSA treatment singly and in combination with 5'-Aza (combination result not shown) does not significantly restore Wee1 expression. It might be due to the toxic effect of TSA since TSA-treated cells were often died afterwards. TSA has been reported to induce growth inhibition and apoptotic cell death in U937 leukaemia cell line in a dose-dependent manner (Woo et al. 2007). Additionally, combination treatment using CMAs may result in adverse effects caused by genome-wide alterations apart from the lack of specific targets (Bouwman and Jonkers 2012). Investigation of hypermethylation sites on the Wee1 gene is currently ongoing. Taxol treatment leads to G₂ cell cycle arrest via stabilization of microtubules that prevent mitosis (Orr et al. 2003). This effect was clearly seen

in Taxol-sensitive A2780 by the appearance of debris (indicating cell death and subG₁ peaks), reduced G₁ peak and higher G₂M peak as early as post-24 hours Taxol treatment (results not shown). The effect was more pronounced after 48 hours treatment and with higher Taxol dose. In contrast, the cytotoxic effects of Taxol were not seen in Taxol-resistant cells in the same experiments. The DNA profile remains stable towards the end of the treatment, suggesting adaptation in coping with DNA damage stress induced by Taxol possibly through G₂M cell cycle checkpoint modification.

Silencing of *Wee1* in A2780 was carried out to try to emulate the downregulation seen in Taxol-resistant A2780TaxR. Unlike SK-OV-3, the A2780 cell line was not yet optimized for siRNA transfection using DharmaFECT transfection reagent, according to the manufacturer's protocols. Thus, method development to achieve optimal conditions was carried out using GAPDH siRNA in trial experiments. Optimization includes assessment of cell density, siRNA concentration and the amount of transfection reagent used. *Wee1* silencing was done based on the result of GAPDH silencing experiments (results not shown). Scramble siRNA used as a negative control produced approximately similar cell response as untreated control cells after Taxol treatment, proving that the transfection process itself did not initiate significant cytotoxic effects to cells and is less likely to produce high background levels of apoptosis. In *Wee1*-silenced cells, Taxol treatment produced significant cell death by appearance of sub-G₁ peak, and increased G₂M peak. A similar effect was seen in control and scramble cells. There were also no significant differences in apoptotic readout between non-drug treated control, scramble and *Wee1* siRNA-treated cells. Overall, silencing of *Wee1* in A2780 even though successful in downregulating *Wee1*, does not give any significant differences in response towards Taxol treatment. This result fits with the hypothesis that *Wee1* downregulation was a secondary effect of Chk2 upregulation in Taxol resistance developed in wild-type p53 expressing cells. However, it must be noted that the status of p53

is not yet verified in A2780TaxR and it might play a role in influencing Taxol response in this Taxol-resistant cell line.

Wee1 silencing was also carried out in SK-OV-3, an ovarian cancer cell line with a significantly different genetic background compared to A2780. SK-OV-3 has been reported to have mutated p53 and the western blotting data in Chapter 4 is in agreement with this. The optimal conditions used for *Wee1* silencing in A2780 were successfully adopted for silencing *Wee1* in SK-OV-3 cells. Taxol induced cell cycle arrest in scramble cells, as indicated by a decreased G₁ peak percentage showed concomitant higher G₂M content. Despite differences between control and Taxol-treated G₂M being significant in both pairs (scramble and *Wee1* siRNA-treated), data for *Wee1*-silenced SK-OV-3 showed a lower level of statistical significance (<0.05) compared to scramble (<0.005). We believe that this was down to the difficulties in determining the exact Taxol dose added with technical issues and financial restriction in performing gene silencing experiment itself. Alteration on the Taxol dose could provide a clearer picture but should result in the same conclusions. Based on the result obtained, *Wee1* silencing affects DNA content of SK-OV-3 cells following Taxol treatment. Instead of causing G₂M cell cycle arrest as observed in scramble (negative control) cells, Taxol treatment to *Wee1*-silenced SK-OV-3 did not elicit such response. Additionally, inhibition of *Wee1* expression alone had been reported to reduce cell viability (Garimella et al. 2012; Kreahling et al. 2012). This response was also observed following *Wee1* silencing in SK-OV-3 control cells. Overall, *Wee1* silencing plays a role in the cellular response to Taxol in p53-deficient SK-OV-3 cells. By silencing *Wee1*, the cytotoxic response towards Taxol was abrogated. Even though *Wee1* inhibition has been reported to promote cell death, surviving clones of cancer cells that have acquired *Wee1* downregulation may be predisposed to Taxol resistance.

Another way of downregulating Wee1 expression is through the use of an exogenous inhibitor. The Wee1 inhibitor MK1775 was used to assess the role of this checkpoint kinase in Taxol resistance. It is tempting to look at the effect of MK1775 and Taxol combination treatment and assess whether it will reflect the effects we saw with Wee1 silencing on Taxol induced apoptosis. Wee1 inhibition will promote early entry into mitosis and Taxol will induce cell death via mitotic catastrophe. Hypothetically, combinations of both agents will result in higher cell death than a single agent treatment. Simultaneous inhibition of Wee1 and CDC25 has been shown to promote mitotic collapse, an event described by failure to enter and progress through to the mitotic phase (Potapova et al. 2011).

Aside from ovarian cancer, MK1775 treatment was also carried out on MCF7 and its Taxol-resistant counterpart MCF7TaxR breast cancer cell lines. In contrast to the ovarian cancer drug resistant models, Wee1 has been found to be highly expressed in MCF7TaxR in comparison with MCF7 (explained further in Chapter 4). In line with this, MCF7TaxR was found to be sensitive to MK1775 treatment compared to MCF7, showing the importance of Wee1 in mediating the drug resistant phenotype in MCF7TaxR.

Overexpression of Wee1 had been shown to save cells from apoptosis and contributes to efficient DNA repair in G₂M (de Carcer et al. 2007; Mir et al. 2010). Being p53-deficient, MCF7TaxR cells rely more on G₂M checkpoint to be able to proceed successfully into mitosis without any kind of lethality. Wee1 overexpression appears to be a part of MCF7TaxR adaptation to allow sufficient time for DNA damage repair. The MCF7 cell line has been reported as being resistant towards Wee1 inhibitor but Wee1 inhibition sensitized cells to TRAIL-induced apoptosis (Garimella et al. 2012).

The results presented partially fit the hypotheses. Wee1 does play a role in Taxol resistance. Collateral sensitivity to Wee1 inhibitor MK1775 was seen in Taxol-resistant cell lines with high Wee1 expression. However, genetic background seems to have an influence towards Wee1 and Taxol response.

Conclusion

Wee1 deregulation has been reported in a large number of cancers and was thought to be caused by epigenetic modifications. In p53 wild-type cells, Wee1 downregulation does not appear to influence the cytotoxic response to Taxol. Conversely, in p53-deficient cells, Wee1 downregulation promotes cell death possibly through forced early entry into mitosis with subsequent mitotic catastrophe. However in p53-deficient cells that have acquired *Wee1* silencing, sensitivity towards Taxol treatment was decreased. Based on the data obtained, p53 status of the cell lines appears to play a role in influencing Wee1 in relative to cellular Taxol response. Since p53 appears to be closely related to Wee1 action, more studies are needed in order to establish this relationship that subsequently leads to a firm conclusion. Additionally, in a subset of ovarian cancer with Taxol resistance, low Wee1 was observed coupled with resistance towards MK1775 treatment. Therefore the possibility of Taxol resistance via Wee1 downregulation cannot be dismissed and warrant further analysis, but clearly the picture is complex and many factors are closely related and can influence the final outcome of the treatment approach.

To date, there are very few reports on Wee1 silencing effect and Taxol response. This study was conducted in hope to shed some light onto this matter.

Chapter 6

SFN as a Candidate Biomarker in Taxol Resistance

Introduction

1. 14-3-3 family

14-3-3 family members are highly conserved 30KDa acidic proteins originally discovered as abundantly expressed mammalian brain proteins (Ichimura et al. 1988). The characteristic migration pattern on two-dimensional DEAE-cellulose chromatography and starch gel electrophoresis plus their elution fraction gave rise to the name '14-3-3'. This family consist of 7 isoforms, named using the Greek letters of α , β , δ , ϵ , ζ , θ/τ and σ according to their respective positions on HPLC elution. α is phosphoform of β while δ is phosphoform of ζ . θ is widely expressed in tissues including brain and T cells while σ is the only isoform that is expressed exclusively in epithelial cells. 14-3-3 family members take part in a variety of cell events including cell signalling, cell cycle regulation, intracellular trafficking/targeting, cytoskeletal structure and transcription via binding to their phospho-Ser and phospho-Thr containing ligands (Aitken 2006; Gardino and Yaffe 2011; Lodygin and Hermeking 2006). They form a dimer and share structural similarity amongst family members with some differences in a few regions. 14-3-3 residues were shown to be conserved among all isoforms and are important in phosphopeptide binding (Aitken 2006).

2. SFN (14-3-3 σ , HME1)

2.1 Function

14-3-3 σ is also known as human mammary epithelial marker 1 (HME1) or stratifin (SFN). SFN was originally discovered via its expression in differentiating epithelial cells and was shown to be under direct regulation of p53 (Hermeking et al. 1997). SFN is the only 14-3-3 isoform that is expressed after DNA damage, showing its importance in DNA damage checkpoint. SFN was also shown to bind CDK1 via its nuclear exporting sequence (NES) and promoted CDK1 nuclear exclusion into cytoplasm. The NES is important in CDK sequestration since mutant NES of SFN was unable to sequester CDK out from nucleus

(Laronga et al. 2000). SFN also binds to G₁-specific CDK2 and CDK4. Overexpression of SFN inhibits CDK2- and CDK1-associated kinase activity (Laronga et al. 2000; Lee and Yang 2001; Lodygin and Hermeking 2006). Apart from that, SFN was reported to interact with Akt (Protein Kinase B-PKB) which in turn promotes Akt inhibition and leads to stabilization of CKI p27^{KIP1} via blocking of its Akt-mediated ubiquitination and degradation (Gardino and Yaffe 2011; Lodygin and Hermeking 2006). Based on its significant role in cell cycle checkpoints, SFN is being defined as a new class of CKI (Laronga et al. 2000; Lee and Yang 2001). SFN association with CDK1 is also important in preventing mitotic catastrophe during G₂ arrest. Downregulation of SFN after G₂ arrest permits CDK1-CyclinB entrance into nucleus thus promoting early entry into mitosis which results in mitotic catastrophe (Lee and Yang 2001). Apart from the interaction with CDKs, SFN also directly interacts with Wee1, an important cell cycle regulator that has been discussed in Chapter 5. SFN together with Chk1, act as Wee1 positive regulators by enhancing Wee1 kinase activity against CDK1 (Gardino and Yaffe 2011; Lee and Yang 2001). SFN is vital in maintaining genomic stability, since downregulation of SFN leads to genomic instability characterized by loss of telomeric repeat sequences, chromosome end-to-end fusions and non-reciprocal translocations (Lee and Yang 2001; Lodygin and Hermeking 2006). SFN also plays a role in the inhibition of cell transformation and inhibits the anchorage-independent growth of several breast cancer cell lines, reducing their transformation potency (Laronga et al. 2000; Lee and Yang 2001). Primary epithelial cells can grow indefinitely without stimulation from exogenous oncogenes or oncoviruses in the absence of SFN, showing the importance of SFN in cell transformation (Ren et al. 2010). Despite the fact that SFN regulates entry into mitosis, mitotic progression of cells that have already entered mitosis phase is promoted by SFN, particularly by enhancing cytokinesis completion via promoting the synthesis of mitotic-form CDK11. In normal epithelial cells, binding of SFN to a transcription factor eIF4B (eukaryotic initiation factor 4B) impairs its function. This event leads to the expression of the mitotic-form of CDK11 (p58-PITSLRE) and completion of cell division. In the absence of SFN, only the interphase form of CDK11

is synthesized that leads to incomplete cytokinesis which promotes the development of pre-cancerous tetraploid lesions (Gardino and Yaffe 2011; Hynes and Smirnova 2012; Wilker et al. 2007). In keratinocytes, SFN has been linked to expression of matrix metalloproteinase 1 (MMP1) via binding to aminopeptidase N (APN), an integral membrane protein that acts as a cell surface receptor (Ghaffari et al. 2010). MMPs are proteolytic enzymes with functions to degrade extracellular matrix (ECM) and involved in a variety of cellular processes including tumour metastasis. SFN has been shown to promote expression of fibroblast associated MMP1, MMP3, MMP8 and MMP24 via the p38 mitogen-activated protein kinase (MAPK) signalling pathway (Ghaffari et al. 2010). Another keratinocyte associated plasma membrane protein that has shown interaction with SFN is BP180 (bullous pemphigoid antigen-2) (Li et al. 2007).

2.2 Regulation of SFN

SFN is regulated mainly by the action of p53 and plays a role in G₂M phase by regulating cell cycle progression. This was shown by the occurrence of G₂M cell cycle arrest and increasing cell size after exogenous overexpression of SFN in cells. A similar event was also seen after DNA damage induced via γ irradiation or DNA-damaging agent treatment that caused activation of p53. Since mutant p53 does not induce SFN upregulation after DNA damage, it was concluded that SFN was regulated by p53 (Hermeking et al. 1997; Lodygin and Hermeking 2006). However in p53-negative cells, Tap53 induces SFN expression after DNA damage. This serves as evidence of SFN transactivation by other proteins. SFN expression was also shown to be induced by TAp73 in breast cancer cell lines while BRCA1 can also indirectly affect SFN expression via cooperation with p53 (Lodygin and Hermeking 2006). TNF-related apoptosis-inducing ligand (TRAIL) also causes SFN upregulation in prostate cancer cells. Efp, a target gene product of *ER α* (Oestrogen receptor α) was shown to regulate SFN via ubiquitin-mediated

degradation. Transfection of SFN and Efp together in COS7 cells showed cytoplasmic localization of both proteins and their ability to interact and form a complex. Interaction of Efp with SFN was shown to occur via the RING finger domain of Efp that acts as an E3 ubiquitin ligase and promotes ubiquitination with subsequent degradation of SFN which can then in turn dissociate it from CDK-Cyclin complex, therefore promoting cell cycle progression (Urano et al. 2002).

Figure 6-1: Function and regulation of SFN. SFN expression is under the control of p53. Apart from p53, other proteins such as TAp53, Tap73 and TRAIL can also induce SFN expression. SFN forms a dimer and interacts with various other proteins that result in expression of MMPs, regulation of cell cycle control and progression of mitosis. SFN is negatively regulated by Efp that promotes its degradation via ubiquitination followed by proteosomal degradation.

2.3 SFN Expression in normal and cancerous tissues

In contrast with other 14-3-3 isoforms, SFN was found to be deregulated in various human cancers (reviewed by Hynes and Smirnova 2012). In breast carcinoma, SFN was shown to be downregulated and cancer progression from atypical hyperplastic lesions to ductal carcinoma *in situ* was marked by loss of

expression. Other than breast cancer, downregulation of SFN was found to be related with loss of p16^{INK4a} expression in oral and vulval cancers specifically in HPV-negative lesions (Syrjanen et al. 2010). Downregulation of SFN was also seen in human hepatocellular carcinoma (Iwata et al. 2000). Other cancers that have downregulation of SFN include SV40-transformed human keratinocytes, head and neck squamous cell carcinoma lines, primary bladder tumours and colonic polyp specimens (Iwata et al. 2000; Laronga et al. 2000). A comparison study by Ren et al. (2010) between normal oesophageal epithelium and oesophageal squamous cell carcinoma (ESCC) showed that SFN mRNA and protein level were markedly downregulated. This observation was supported by immunohistochemistry staining of the samples. SFN downregulation was statistically correlated with tumour infiltration depth and metastasis. A shorter 5-year survival rate of ESCC patients was observed for those with lower SFN expression. Poor prognosis for patients with intrahepatic cholangiocarcinoma and prostate cancer was also associated with lower SFN expression (Ren et al. 2010). In the ErbB2KI transgenic model, loss of either one or both copies of *SFN* allele led to accelerated mammary growth (Hynes and Smirnova 2012). SFN downregulation was associated with advanced disease in many cancer of epithelial origin including stomach and lung. In ovarian cancer, loss of SFN mRNA significantly correlated with progression and invasiveness of tumour (Akahira et al. 2004). Downregulation of SFN was concomitant with the progression of glioma and SFN has been shown to be a significant and independent prognostic indicator for glioma patients (Deng et al. 2011).

In contrast, there are several reports of SFN upregulation in cancers. In comparison with normal cervical epithelium, SFN was upregulated in cervical carcinomas (CCs) and cervical intraepithelial neoplasia (CIN) lesions, higher grade of CC and CIN shows higher SFN expression in parallel. The mechanism of SFN upregulation in these cases was still unknown (Syrjanen et al. 2010). SFN upregulation is thought to play

a role in development and/or progression of pancreatic cancer, papillary or undifferentiated thyroid cancer and urinary bladder squamous cell cancer (Ren et al. 2010). A study in lung adenocarcinoma revealed high SFN levels associated with progression from *in situ* adenocarcinoma to invasive adenocarcinoma by enhancement of tumour cell proliferation. Stimulation of SFN expression in this case was found out to be potentially related to P13K/Akt signalling pathway in a p53-independent manner (Shiba-Ishii et al. 2011). In certain cancers, there is a difference of SFN expression and deregulation among cancer sub-types. In ovarian cancer, the SFN expression pattern differs based on histological type, whereas in small cell lung cancer (SCLC) SFN inactivation also followed in a histological type-specific manner (Akahira et al. 2004; Osada et al. 2002).

2.4 SFN gene hypermethylation

SFN downregulation was detected in several types of cancer as mentioned above. However, silencing of SFN was not due to genetic alteration in most cases but due to epigenetic modification specifically via CpG methylation. This was shown by the unmasking of the SFN expression after 5'-Aza treatment. CpG methylation of SFN was detected in cancer of gastric, liver, lung, skin, squamous cells, and endometrium with higher prevalence in breast, prostate and ovarian cancers. Gradual increase of SFN methylation was observed with progression of breast cancer and basal cell carcinoma of the skin. Frequent association seen between SFN methylation status and breast cancer prognosis served as a basis of SFN methylation status being proposed as a marker in diagnosing metaplastic breast carcinomas (reviewed by Lodygin and Hermeking 2006). In a study of gene promoter methylation in Indian breast cancer patients, the methylation status of *ERα* and *PR* (progesterone receptor) was significantly correlated with SFN methylation. Overall survival of patients with SFN expression was higher compared to patients with diminished SFN (Mirza et al. 2010). Even though SFN is a good candidate as a biomarker for breast

cancer metastasis, a careful approach needs to be taken since a small amount of SFN methylation was also detected in healthy subjects. These might have been associated with occult benign breast disease, age, leukocytes and/or benign breast disease (Zurita et al. 2010). Surprisingly, overexpression of SFN was found in pancreatic cancer despite *SFN* promoter hypermethylation and the mechanism is still unknown. *SFN* hypermethylation was also found in normal stromal and lymphoid cells during the development stage, showing that downregulation of SFN was achieved through epigenetic modification and was not restricted to cancer cells only (Lodygin and Hermeking 2006). Additionally, *SFN* was shown to be hypermethylated in both oral squamous cell carcinoma (OSCC) and cancer-free subjects. Hypermethylation in these cases was not associated with smoking, drinking habits or specific tumour characteristic (de Freitas Cordeiro-Silva et al. 2011).

2.5 SFN and drug resistance

In breast cancer cell lines, SFN expression is either positively or negatively associated with different drug resistant mechanisms (Deng et al. 2011; Hynes and Smirnova 2012). Functional proteomic analysis using two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry on MCF7 parental and multidrug-resistant MCF7/AdVp3000 revealed that SFN was one of the protein upregulated in MDR. *SFN* knock-down in MCF7/AdVp3000 decreased its resistance towards mitoxantrone and Adriamycin treatment while *SFN* overexpression in MCF7 showed increased resistance towards both agents. Comparable outcome was also observed in other cell line HEK293 (Liu et al. 2006). A similar approach was adopted in the investigation of multidrug resistant MCF7 induced by 5-fluorouracil treatment (MCF7/5-FU). In contrast, SFN was found to be downregulated in MCF7/5-FU along with other genes and overexpression of SFN via transfection induced sensitivity towards 5-FU and cisplatin treatment (Zheng et al. 2010).

Aim

SFN was shown to be downregulated in drug-resistant cancer cells along with other genes in a previous cDNA microarray analysis (Coley unpublished data). This study aims to confirm the changes seen *in vitro* and to attempt to ascribe a functional role for *SFN* expression levels in mediating drug responsiveness. This study aims to recreate *SFN* downregulation seen in drug-resistant cancer cells in parental, drug sensitive cancers and to provide an insight into *SFN* regulation in drug-resistant cancers specifically Taxol resistance.

Hypothesis

SFN plays a role in anticancer drug resistance and this is likely to be due to epigenetic silencing

Methods

Methods used in this study were, western blotting, mRNA extraction, qPCR analysis, viability assay using MTT, DNA harvest, PI staining, Annexin V-FITC, flow cytometric analysis, and siRNA transfection to silence *SFN* artificially in cancer cell lines. Cell lines used were A2780 and SK-OV-3. Graphs were prepared using Microsoft Excel software. Student's Paired T-Test was performed where applicable and result was expressed as p value. All methods have been explained in Chapter 2.

Results

SFN mRNA levels, which represents SFN gene expression in A2780 and SK-OV-3 panels were measured using qPCR analysis and results were presented as bar charts (Figure 6-2). Significant downregulation of SFN was seen in A2780TaxR compared to its parental. However, SFN expression in SKOV3TaxR was not statistically significant compared to SK-OV-3.

1. SFN mRNA level inA2780 and SK-OV-3 panels

Figure 6-2: Relative SFN mRNA level fold change of Taxol-resistant A2780 and SK-OV-3 in comparison with their parental cell line respectively. SFN mRNA level in A2780TaxR Taxol-resistant was undetected compared to A2780 (**p<0.005). Conversely, SFN mRNA level in Taxol-resistant SKOV3TaxR was shown to be higher than parental SK-OV-3. However, the difference was not statistically significant (p>0.2).

Following qPCR results, western blotting using antibody against SFN was performed in order to assess its protein level in the respective cell lines.

2. SFN protein level in A2780 and SK-OV-3 panels

Figure 6-3: Western blotting of SFN in A2780 and SK-OV-3 parental and Taxol-resistant cancer cell lines (n=3). a) Downregulation of SFN was observed in A2780TaxR Taxol-resistant compares to its parental cell line p53 wild-type A2780, shown by the disappearance of SFN band in A2780TaxR lane. b) Modest SFN downregulation was seen in Taxol-resistant SK-OV-3 compared to its parental, p53-deficient SKOV-3.

Western blotting result of SFN in A2780 panel reflects their qPCR analysis. However, this pattern of SFN downregulation was not reflected in SK-OV-3 panel. Higher SFN mRNA level with modest SFN protein downregulation was seen in SKOV3TaxR compared to the parental cell line. SFN downregulation was postulated as an effect of epigenetic modification. Thus, demethylating treatment was performed on A2780TaxR to assess this hypothesis.

3. Demethylating treatment in A2780TaxR Taxol-resistant

Figure 6-4: Treatment with demethylating agents, 5'-Aza and TSA singly and in combination to Taxol-resistant A2780TaxR. Treatment with demethylating agents restores SFN expression in A2780TaxR, which is in line with the previous cDNA microarray results.

Following previous results of qPCR analysis and western blotting, silencing of *SFN* in A2780 and SK-OV-3 was performed in order to recapitulate its downregulation seen in Taxol-resistant variants and to assess its effect on Taxol response of the cells.

4. Western blotting of *SFN* silencing samples

Western blotting was performed on whole cell lysates harvested by the end of the silencing treatment to assess silencing efficiency (Figure 6-5). The disappearance of *SFN* protein band in both *SFN*-silenced samples showed gene-silencing procedure was carried out successfully.

Figure 6-5: Western blotting of *SFN* gene silencing samples in A2780 and SK-OV-3 using siRNA transfection. **a)** *SFN* silencing in A2780 was carried out successfully based on the disappearance of *SFN* band in *SFN*-silenced sample compared to scramble siRNA (negative) and untreated controls. **b)** *SFN* silencing in SK-OV-3 was carried out successfully as judged from the appearance of the band in *SFN*-silenced sample compared to scramble siRNA and untreated controls.

5. *SFN* silencing in A2780 followed by Taxol treatment

5.1 DNA content analysis with and without Taxol treatment

Figure 6-6: *SFN*-silenced cells were subjected to 8nM Taxol treatment for 48 hours and analyzed using flow cytometry. Taxol treatment induces G₂M cell cycle arrest, observable by a higher G₂M DNA peak. The Taxol effect was seen in all Taxol-treated samples. There was no significant difference between untreated, scramble and *SFN*-silenced cells in Taxol response. (n=3)

SFN silencing in A2780 was then followed by 8nM Taxol treatment to assess changes of DNA content before and after treatment in comparison with controls (untreated and scramble cells). Samples were processed for DNA content analysis and Annexin V-FITC analysis using flow cytometry. Based on the DNA analysis, *SFN* silencing does not give significant influence to A2780 response towards Taxol treatment (Figure 6-6).

5.2 Apoptotic analysis with and without Taxol treatment

Figure 6-7: Annexin V-FITC analysis of A2780 control, scramble and *SFN*-silenced cells before and after 48 hours of 25nM Taxol treatment. *SFN* silencing did not affect cell viability significantly. <90% of cell population was still viable after transfection (first cytogram from left on second row, bottom left quadrant). Taxol treatment reduced live cell population to approximately 70% in control cells (first cytogram on top right). Similar effect was seen in scramble and *SFN*-silenced cells treated with Taxol. P value for differences of Taxol-treated, *SFN*-silenced A2780 versus scramble with similar Taxol treatment is >0.01. This result is a representative of repeated experiments (n=3).

6. *SFN* silencing in SK-OV-3 followed by Taxol treatment

6.1 Apoptotic analysis with and without Taxol treatment

Figure 6-8: Annexin V-FITC analysis of SK-OV-3 control, scramble and *SFN*-silenced cells before and after 48 hours of 25nM Taxol treatment. *SFN* silencing did not affect cell viability significantly. <90% of cell population was still viable after transfection (first cytogram from left on second row, bottom left quadrant). Taxol treatment reduced live cell population to approximately 90% in control cells (first cytogram on top right). Similar effect was seen in scramble and *SFN*-silenced cells treated with Taxol. P value for differences of Taxol-treated, *SFN*-silenced SK-OV-3 versus scramble with similar Taxol treatment is >0.1). This result is a representative of repeated experiments (n=3).

Annexin V-FITC analysis done on both *SFN*-silenced cells of A2780 and SK-OV-3 showed that *SFN* status does not give any significant influence on apoptotic response after Taxol treatment.

Discussion

SFN plays an important role in the regulation of G₂M checkpoint following DNA damage and in cytokinesis completion during mitosis. It had been shown that SFN downregulation in various cancers could promote progression. However, in certain types of cancer SFN was shown to be upregulated (refer Introduction). Based on the cDNA microarray analysis results (Coley unpublished data), SFN was among the genes downregulated in drug-resistant cancer cells. SFN downregulation was postulated as an effect of genetic modification, particularly gene hypermethylation.

Using qPCR and western blotting, downregulation of SFN in A2780TaxR Taxol-resistant in comparison with parental cells A2780 was confirmed. However in SK-OV-3 Taxol-resistant cell line, SFN mRNA level was not significantly different compared to parental cell line, even though there was downregulation of protein level albeit not as low as in A2780TaxR. Discrepancies between mRNA and protein level of SFN is not uncommon, since it had been shown that pancreatic cancer is characterized by SFN overexpression despite significant promoter hypermethylation that caused gene silencing (Lodygin and Hermeking 2006). Apart from promoter methylation, DNA hypoacetylation is another epigenetic modification that can influence the behaviour of gene expression. In this study, treatment with CMAs particularly TSA unmasked SFN expression in A2780TaxR cell line. However, 5'-Aza treatment on A2780TaxR, either singly or in combination with TSA, did not restore SFN expression to match parental cell line A2780. The result is in line with reports of restored SFN expression by means of CMA treatment (5'-Aza) on SFN-silenced breast cancer cells as reviewed by Lodygin and Hermeking (2006). Restored SFN expression in A2780TaxR also showed that epigenetic silencing plays a role in SFN downregulation in this cell line. Epigenetic silencing has been shown to be the main event that affects SFN expression in cancers. Significant downregulation of SFN was linked to hypermethylation and poor survival outcome in a

number of studies (see Introduction to this chapter). Thus, a number of studies were conducted to assess SFN methylation as a possible marker for tumour progression and cancer prognosis. Based on this result, we explored the possibility of SFN downregulation as a contributor towards Taxol resistance in ovarian cancer.

A role played by SFN in drug-resistance has been investigated before (Hynes and Smirnova 2012). In the present study, SFN silencing was introduced to cell lines via siRNA transfection in order to investigate its downregulation in Taxol resistance. Based on our results of Annexin V-FITC and DNA analysis, *SFN* silencing did not alter Taxol response significantly in ovarian cancer cell lines regardless of p53 status. The G₂M blockade seen after Taxol treatment in control non-silenced cells was also seen in *SFN*-silenced cells without any significant differences. In a study conducted by Chan et al (1999), mitotic catastrophe was seen in SFN-deficient cells with wild-type p53 after treatment with DNA damaging agent (Adriamycin) or ionizing radiation after failure in maintaining G₂M blockage. Similar treatment of p53 wild-type, SFN-expressed cells resulted in increased cell size, nuclear enlargement and DNA content akin to G₂M cell cycle arrest (Chan et al. 1999). Even though *SFN* was seen as a part of Taxol-resistant phenotype in our cell lines, it is possible that the cells acquired SFN downregulation together with other alterations that brings out the phenotype. Further, we were only looking at the consequences of *SFN* silencing following 48h of drug challenge and there is the possibility that a longer time frame might have revealed a different result. As shown in the previous chapter, A2780TaxR Taxol resistant cell line acquired deregulation of several other proteins of G₂M regulators including ATR and Wee1 apart from SFN. Even though *SFN* downregulation alone had been shown to be responsible for drug-resistance in some studies, we did not observe such an effect. Hence, for the cell line models used in our study there is a likelihood that it is the combination of several genes together that may bring about the drug

resistant phenotype and for some genes their influence on this process may be obvious when looked at in isolation.

Finally, the results presented partially fits the hypothesis. *SFN* downregulation in A2780TaxR appears because of epigenetic silencing. However, *SFN* appears to play no role in influencing Taxol response in the cell lines studied.

Conclusion

In this study, we have shown that *SFN* was downregulated in Taxol-resistant ovarian cancer cell lines. However, silencing of *SFN* does appear to play a role in inducing Taxol resistance in two cancer cell lines regardless of the p53 status. Expression of *SFN* was shown to be varied among cell lines and hence its role in mediating drug resistance may be context-dependent.

Chapter 7

Development of a Drug Resistant Cell Line

Introduction

1. INK4 family of CKIs

INK4 (Inhibitors of CDK4) is a family of CKIs which consists of four members. They are p16^{INK4a}, p15^{INK4b}, p18^{INK4c} and p19^{INK4d}. INK4 family members specifically inhibit CDK4 and CDK6 and thus play an important role in the regulation of G₁ progression into S phase of the cell cycle. These INK4 family are able to bind to both free and CyclinD-associated CDK4 and CDK6, consequently forming an inactive complex that leads to G₁ cell cycle arrest (Lee and Yang 2001; Maddika et al. 2007). Members of INK4 are highly conserved among species and shares approximately 40% homology among them (Roussel 1999).

1.1 p16^{INK4a} gene

Serrano et al. (1993) first isolated human p16 complementary DNA and showed inhibitory effect of p16^{INK4a} on CDK4-CyclinD. The p16^{INK4a} protein is encoded by p16 gene and resides in locus 9p21, or the *INK4a* locus (Serrano et al. 1996). Alternative reading frame of *INK4a* encodes p14^{ARF}, a structurally and functionally different protein that plays a role in p53 positive regulation via p53 protein stabilization (Maddika et al. 2007; Nam and Kim 2008). p16^{INK4a} shares similar ankyrin-like repeats with other INK4 members. The ankyrin-like repeats are thought to play a role in protein-protein interaction (Roussel 1999).

1.2 p16^{INK4a} role in cell cycle regulation

p16^{INK4a} is part of the p16-CDK4/6-CyclinD-Rbp pathway controlling cell cycle progression in G₁ phase. Rbp negatively regulates G₁ progression by sequestering the transcription factor E2F while CDK4/6-CyclinD complexes promote G₁ progression by inducing phosphorylation of Rbp and the subsequent release of E2F (Serrano et al. 1996). CDK4/6 kinase activity is inhibited by p16^{INK4a}, which in turn prevents phosphorylation of Rbp and consequently halts G₁ cell cycle progression (Nam and Kim 2008; Roussel 1999). ETS transcription factors are responsible for p16^{INK4a} expression and its accumulation in the cell is induced by the senescence process (Lee and Yang 2001; Massague 2004). Following p16^{INK4a} binding to CDK4/6, CyclinD is released. Destabilization of cyclinD couples with redistribution of Cip/Kip CKIs to CDK2 promotes G₁ arrest (Shapiro 2006; Sherr and Roberts 1999). Apart from senescence, p16^{INK4a} also plays a role in cellular response towards UV damage. Upregulation of p53 and p21^{Cip1} following UV exposure is mediated by p16^{INK4a} via redistribution of the RNA binding protein HuR (Hu antigen R) (Al-Mohanna et al. 2007). HuR is implicated in the regulation of mRNA stability by antagonizing the mRNA degradation pathway (reviewed by Brennan and Steitz 2001). Upon UV exposure, p16^{INK4a} promotes HuR cytoplasmic relocation from the nucleus which in turn binds to and stabilizes p21^{Cip1} and p53 mRNA (Al-Mohanna et al. 2007). Hyperactive Ras, Myc and E2F signalling also induces p16^{INK4a} expression which subsequently promotes either cell quiescence or senescence (Massague 2004).

Sharpless and Chin (2003). The *INK4a* locus coding for p16^{INK4a} and p15^{INK4b} is frequently mutated in human cancer cell lines and often altered in human malignancies (Lee and Yang 2001; Serrano et al. 1996). The observation of frequent inactivation of p16^{INK4a} in human cancers suggested its importance in cancer development. Indeed, Serrano et al. (1996) have shown that *INK4a* deficiency promotes tumorigenesis in mammals via generation of *INK4a*^{-/-} mice. Several mechanisms of p16^{INK4a} inactivation have been characterized and can be divided into three main mechanisms, which vary according to the cancer type. In cancers such as pancreatic adenocarcinomas, oesophageal carcinomas, biliary tract cancers and in families with hereditary predisposition to melanoma and pancreatic cancer, p16^{INK4a} inactivation is caused by point mutations and small deletions in the *INK4a* locus. These mutations can affect either p16^{INK4a} exclusively or together with p15^{INK4b}. The second mechanism of p16^{INK4a} inactivation is large homozygous deletions which eliminate the locus altogether and is commonly found in non-small cell lung carcinomas, malignant gliomas, renal cell carcinomas, head and neck tumours, prostate tumours, bladder carcinomas and acute lymphoblastic leukaemias. Finally, methylation of *INK4a* locus leads to gene silencing accounts for p16^{INK4a} loss in breast and colon cancers (Lee and Yang 2001; Nam and Kim 2008; Serrano et al. 1996). p16^{INK4a} inactivation is functionally equivalent to loss of Rbp function (Maddika et al. 2007) which allows for unsupervised cell cycle progression.

Apart from gene inactivation, a growth factor sensor p34^{SEI-1} encoded by *SEI-1* (Selected with *INK4A*) gene was found to antagonize p16^{INK4a} inhibitory action on CDK4-CyclinD complex thus rendering the complex resistant to inhibition (Figure 7-1) (Lee and Yang 2001; Sugimoto et al. 1999). Additionally, p34^{SEI-1} overexpression promotes fibroblast cell proliferation even under low serum conditions (Sugimoto et al. 1999) and the gene is mapped to 19q13.1-q13.2 which shows amplification in some cancers (Lee and Yang 2001). This suggests the importance of p34^{SEI-1} in tumorigenesis.

1.4 p16^{INK4a} and melanoma

Melanoma is clinically characterized by aggressive clinical behaviour, tendency of late metastasis and high resistance towards therapeutic treatment. Deletion of *INK4a* is one of several genetic events in melanoma. *INK4a* deletion is found in approximately 50% of primary tumours and in nearly all melanoma cell lines (Sharpless and Chin 2003; Walker et al. 1998). According to a review by Sharpless and Chin (2003), p16^{INK4a} inactivation via deletion and promoter methylation was found in 30-70% of primary melanoma tumours, while germline point mutations of p16^{INK4a} were associated with tumour susceptibility in familial melanoma subjects. Thus, p16^{INK4a} status serves as an interesting parameter in melanoma diagnosis and a rational target for treatment.

Loss or inactivation of CKIs, for example p16^{INK4a} in melanoma and p57^{Kip2} in ovarian cancers initiate research towards developing synthetic CKIs that would mimic action of endogenous CKIs and are able to influence cancer cell cycle progression. Drug development programs carried out to search for such compounds resulted in the development of potent small molecule CDK inhibitors originated from a variety of chemical classes. Examples are seliciclib, a purine analog and PD0332991, a pyridopyrimidines (Fry et al. 2004). Seliciclib has been discussed earlier in Chapter 3 and PD0332991 is discussed in the following paragraph.

2. PD0332991

Figure 7-2: PD0332991 chemical structure (Fry et al. 2004).

PD0332991 is a small, non-ATP competitive CDK inhibitor against CDK4 and CDK6 (Fry et al. 2004; Katsumi et al. 2011). PD0332991 treatment inhibits phosphorylation of Rbp as the downstream target of CDK4/6-CyclinD in a rapid and reversible manner (Collins and Garrett 2005). In Rbp-positive human cancers (breast, colon, lung carcinoma and leukaemias) PD0332991 works by interfering DNA formation via inhibition of thymidine incorporation. As a result, PD0332991 produces an exclusive cell cycle G_1 arrest. PD0332991 has been tested to a panel of 36 protein kinases and have been found to have negative activity. PD0332991 was also shown to have no activity against Rb-negative cells (Fry et al. 2004). Combination treatment with the proteasome inhibitor bortezomib to a myeloma cell line model 5T33 resulted in enhancement of cell killing (Menu et al. 2008). Synergistic action was observed when PD0332991 was coupled with tamoxifen and trastuzumab in ER+ and HER2-amplified human breast cancer cell lines respectively (Finn et al. 2009). PD0332991 has been tested in a Phase I study on Rbp-positive solid tumours and non-Hodgkin lymphoma and is currently in Phase II clinical trial for metastatic or advanced liposarcoma (Flaherty et al. 2012; Schwartz et al. 2011). The low level of p16^{INK4A} has been shown to be inversely correlated with PD0332991 sensitivity in malignant rhabdoid tumour (MRT), a rare and aggressive form of childhood malignancy. MRT arises from the silencing or mutation of *INI1* gene, responsible for transcriptional inhibition of CyclinD, the partner of CDK4 and CDK6. Since *INI1* also

promotes p16^{INK4A} expression, MRT also show a low level of p16^{INK4A} as a result of mutation or deletion of the gene (Katsumi et al. 2011).

PD0332991 therefore is a very promising drug clinically provided the correct patient cohort is selected. Nevertheless there is a window of opportunity for cancers to develop resistance to this drug since cancers are multifactorial and are able to adapt in order to survive. This forms the rationale for this last part of the thesis work by developing a novel PD0332991-resistant cell line, which is important to provide an insight into alterations that might hinder the drug's action on cancers.

3. Cell model consideration

Before developing a new drug-resistant cell line, several factors must be considered. Determination of tumour type and selecting agent is paramount since there should be a relevant explanation of choosing the said tumour type and agent. Specific molecular characteristics of the tumour might relate to the mode of action of the selecting agent. Development of the drug-resistant cell line should also aim at providing answers to questions like mechanisms behind the acquired resistance of a developmental drug to a specific type of tumour (Coley 2004).

3.1 A375

A375 originated from skin and a malignant melanoma cell line and was chosen as a model to develop PD0332991-resistance based on its specific genetic make-up. The A375 line is p16^{INK4A}-deleted thus lacking p16^{INK4A} expression, making it a desirable target for PD0332991 in line with recent report by Katsumi et al. (2011). Therefore, we predicted that A375 should show preferential sensitivity towards

PD0332991 treatment. However, this tumour might develop acquired resistance towards PD0332991 treatment over time. Thus, we are interested in investigating the resistance mechanism adopted by these PD0332991-resistant cells in order to avoid cell death (Figure 7-3).

Figure 7-3: a) Without p16^{INK4a} in A375 cell line, carcinogenic stimuli promote unrestricted cancer cell growth and proliferation. b) PD0332991 inhibits CDK4/6 activity thus promotes cell death via G₁ cell cycle arrest. However in time, some of these cells treated with PD0332991 may survive and develops PD0332991 resistance.

Aim

To develop a novel PD0332991-resistant cell line from melanoma cell line A375 and to investigate resistance mechanisms adopted by the PD0332991-resistant cells.

Hypothesis

The generation of a cell line with acquired drug resistance to the CDK4/6 inhibitor PD0332991 may help to identify biomarkers of response in cancer cells with a deficiency in p16^{INK4a}.

Materials

A375 cells were grown in RPMI supplemented with 10% FCS and 1x GlutaMAXTM. MTT reagent was prepared as previously mentioned. Antibodies used were ATM, ATR, CHK1, CHK2, Wee1 and GAPDH. PD0332991 (Selleck Chemicals, Houston, US) stock was prepared by diluting the drug in DMSO at a concentration of 10mM and 20mM. Stocks were stored at -20°C.

Methods

A375 (later designed as parental cell line A375) was seeded at 1:10 ratio. Cytotoxicity assay using MTT (spanning over two logs of concentration) was done on parental cell line to determine the initial dosage of PD0332991 (based on its IC₅₀ value) for resistant cell line development. Calculation of IC₅₀ was carried out using Microsoft excel and verified using graphPAD PRISM software. Cells were seeded at 4x10⁴ cells/mL and treated with PD0332991 for 72 hours before the addition of MTT reagent. Based on the

IC₅₀ value calculated from the results of MTT, an initial dose of PD0332991 was chosen, which is approximately 10% of the IC₅₀ value, as described in Coley (2004). The dose was increased to 10μM based on the overall cell growth characteristic after several passages. Cells were passaged 1:5 ratio and treated step-wise with increasing dose of PD0332991 for every other passage. This was to ensure that only a minority of cells tolerated the chemical insult. Surviving cells that escape cell death via drug treatment were then let to recover in the drug-free passage. Subsequent drug-resistant cells were designated as A375PDR and maintained with a 10μM dose of PD0332991 for every other passage. After several passages, A375 and A375PDR cells were subjected to viability assay using MTT to determine the level of resistance acquired. Cell count was kept identical for both cell lines (4x10⁴ cells/mL) and there was no obvious change in the growth rate of A375PDR relative to the A375 parent line. Cells were also harvested for protein as previously stated. Loading of 30μg/well protein lysates were then subjected to SDS-PAGE electrophoresis followed by western blotting to assess constitutive expression of several proteins of interest (ATM, ATR, CHK1, Chk2 and Wee1). 24- and 48-hour time-point protein harvest following treatment with PD0332991 was carried out to look at the expression level of survivin and p27^{Kip1} proteins in both cell lines. Cells were seeded in a T25 flask at a cell density of 4x10⁴ cells/mL in 10mL. Cells were then treated with 10μM PD0332991. After each time point, cells were harvested for protein as previously and lysates were run through SDS-PAGE and western blotting. Cells were also harvested for DNA content analysis, as previously described in other chapter and then subjected to flow cytometry to assess any cell cycle associated changes in DNA content before and after PD0332991 treatment.

Results

Viability assay testing on the A375 parental cell line indicated an IC_{50} of $9.1\mu M (\pm 0.07\mu M)$ ($n=3$) for PD0332991. A375 cells were then treated with increasing PD0332991 concentration for several alternative passages before the dose was increased to $8\mu M$ and subsequently to $10\mu M$ as a maintenance dose.

1. Assessment of resistance

Dose-response curve of PD0332991 in A375 and A375PDR

Figure 7-4: IC_{50} analysis of A375 melanoma parental cell line versus PD0332991-resistant derivative A375PDR. Log dose of PD0332991 (μM) was plotted against percentage viability of cells compared to untreated control cells. Each data point represents different drug dosage and its matching comparative cell viability. A375 parental cell line was represented by red dots while A375PDR was represented by blue square points ($n=3$).

The viability assay using MTT was carried out on both cell lines and showed the IC₅₀ value of 22.94μM (±0.06μM) for A375PDR. The resistance factor (ratio of IC₅₀ for resistant cell line relative to parental cell line) was shown to be a 2.5-fold for PD0332991.

2. Assessment of cell cycle regulators and DDR proteins

Figure 7-5: Western blotting result of A375 and PD0332991-resistant A375PDR showing the level of cell cycle regulators and DDR proteins. HeLa acted as a positive control while Actin as loading control (n=3).

Based on western blotting results, A375PDR showed a minor decrease in ATM and no significant difference of Chk2 expression compared to parental cell line. There was also no significant difference of ATR expression in both cell lines. However, there was a significant increase of Chk1 and a slight increase of Wee1 in A375PDR in comparison to A375. Of note, A375 expressed a wild-type p53 (Min et al. 2005) (the p53 western blotting result was not shown) while p53 status in the A375PDR is yet to be assessed.

3. Western blotting on time-point samples

3.1 Survivin

a) A375

b) A375PDR

Figure 7-6: Expression level of survivin in A375 and A375PDR at 0, 24 and 48 hours, without and with 10µM PD0332991.

The graph under each western blotting represents the densitometric analysis performed on western blotting results. **a)** Refers to the parental cell line A375. **b)** Refers to the PD0332991-resistant A375PDR. Actin acted as a loading control. Both sets of western blotting were run simultaneously with each other. (* $p < 0.05$) (n=3)

Based on western blotting results, survivin was expressed by both A375 and A375PDR cell lines.

However, the quantitative protein expression pattern varied between the cell lines. Survivin expression in untreated parental cells was unchanged throughout 48 hours. Treatment with PD0332991 brought about significant downregulation of protein 24 hours post-treatment. However by 48 hours, survivin was re-expressed albeit at a lower level compared to control untreated A375. In contrast, survivin expression in A375PDR increased over time and interestingly, PD0332991 treatment reduced survivin level as early as 24 hours post-treatment. The level was kept approximately similar as in untreated control at 0-hour time point without any significant differences.

3.2 p27^{Kip1}

a) A375

b) A375PDR

Figure 7-7: p27^{Kip1} protein expression in A375 and PD0332991-resistant A375PDR at 0, 24 and 48 hours, without and with 10μM PD0332991.

The graph under each figure represents densitometric analysis performed on western blotting results. **a)** Refers to the parental cell line A375. **b)** Refers to the PD0332991-resistant A375PDR. Actin was used as loading control (n=3). Both sets of western blotting were run simultaneously with each other.

Based on the western blotting results, p27^{Kip1} expression in PD0332991-resistant A375 was higher compared to the parental cell line A375 at 24 and 48 hour. p27^{Kip1} expression was induced by PD0332991 treatment in A375 as shown by the bands at 24 and 48 hour post-treatment in comparison with the control at the same time point. However, similar dose of PD0332991 treatment on the A375PDR does not give any significant changes to the level of p27^{Kip1} at 24 and 48 hours after treatment compared to the control.

4. DNA content before and after PD0332991 treatment

4.1 DNA histograms of A375 (parental cell line) before and after PD0332991 treatment

Figure 7-8: DNA histogram of parental cell line A375 before and after PD0332991 treatment. Analysis was done using flow cytometry. **a)** DNA content after 24 hours of PD0332991 treatment. Left panel was control cells while right panel was PD0332991-treated cells. Changes in peak sizes of both G_1S and G_2M peaks can be clearly seen. **b)** DNA content after 48 hours PD0332991 treatment. Left panel was control cells while right panel was PD0332991-treated cells. G_2M peak was evidently smaller. (n=2)

Based on the DNA histograms (Figure 7-8), treatment with PD0332991 considerably reduced the G_2M peak size, indicating a G_1 phase cell cycle arrest in A375 parental cell line compared to the untreated

control. The effect was evident after 24-hour PD0332991 treatment and prolonged until 48 hours post treatment without any significant differences seen between the two values (at 24 and 48 hours).

4.2 DNA histograms of A375PDR before and after PD0332991 treatment

Figure 7-9: DNA histogram of PD0332991-resistant cell line A375PDR before and after PD0332991 treatment. Analysis was done using flow cytometry. Similar pattern of response as A375 parental cell line was seen in A375PDR. **a)** DNA content after 24 hours of PD0332991 treatment. Left panel was control cells while right panel was PD0332991-treated cells. Changes in the peak sizes of both G₁S and G₂M peaks can be clearly seen. **b)** DNA content after 48 hours PD0332991 treatment. Left panel was control cells while right panel was PD0332991-treated cells. G₂M peak was evidently smaller. (n=2)

Based on the histograms above (Figure 7-9), similar DNA profile as the A375 parental cell line was observed in PD0332991-resistant A375PDR, without and with PD0332991 treatment for 24 and 48

hours. In control cells, a slightly bigger G₁ peak was observed in the untreated A375PDR compared to the untreated A375 parental cells. The G₁ cell cycle arrest was observed in 24 and 48-hour PD0332991-treated samples of A375PDR as shown by the evidently smaller G₂M peak relative to the respective controls.

Discussion

Cell cycle deregulation is a common event in cancer development. In normal cells, cell cycle regulation is paramount in order to make sure that only unaffected cells survive. This tight regulation comes from variety of proteins working together in a concert, involving a very complex web of mechanisms and pathways coming together to ensure the survival of normal cells. Treatment using chemotherapy (either single agent or combination of agents) or radiotherapy in most cases provides the means to stop cancerous growth. However, certain types of cancer can develop resistance towards agents used to treat them. An example is ovarian cancer, which can develop platinum refractory disease.

Development of a new class of more targeted therapy has been going on for quite some time. The main aim of this therapy is to provide treatment that is more effective with fewer side effects. There have been several success stories of targeted therapy. One of them is the development of Herceptin to treat breast cancer with higher success rate compared to conventional chemotherapy treatment. Several other compounds currently under development show promise as potential new therapeutic agents. This has in part been fuelled by the study of drug resistant mechanisms. Since cell cycle is the driving force of the cell itself, a lot of research has been conducted on the deregulation of its components in carcinogenesis since it provides many druggable targets. The work described in the present thesis emphasizes the close relationship between cell cycle deregulation and anticancer drug resistance.

In this study, we would like to look at the probability of cancer cells acquiring resistance towards targeted therapy designed to treat them effectively. The A375 cell line was chosen based on its specific genetic make-up i.e. p16^{INK4A}-deletion. p16^{INK4A} is a member of the CKI group INK4 which inhibits CDK4 and CDK6. Hypothetically, p16^{INK4A}-deleted A375 cells should have unchecked G₁S phase progression that comes from the downregulation of endogenous CKI and unrestrained CDK4/6 activity. Treatment with the CDK4/6 inhibitor PD0332991 will affect CDK4/6 activity, leading to the cell cycle arrest at G₁ phase.

Based on the IC₅₀ analysis, we successfully developed PD0332991-resistant A375 cell line to achieve clinically relevant level of resistance. In comparison with other established drug-resistant cell lines, resistance level acquired was adequate. There might be a possibility to produce a higher level of resistance in the future. Additionally, higher resistance level seen in other cell lines may reflect the selectivity of the agents themselves and the genetic make-up of the respective cell line.

Several changes were seen in cell cycle components collaterally with acquired drug resistant phenotype development. More focus was put on the deregulation of G₂M cell cycle components, which are ATM, ATR, Chk1, Chk2 and Wee1, mainly involved in DNA damage recognition. Wee1 negatively regulates CDK1 activity via inhibitory phosphorylation, reducing active CDK1 in the cell thus stalling cell cycle progression from G₂ to M phase. ATM and Chk2 were part of the double strand break (DSB) type of damage recognition sensors while ATR, Chk1 and Wee1 were part of sensors for a single strand break caused by UV or DNA intercalation agents such as platinum compounds. Higher expression of Chk1 and Wee1 seen in A375PDR cells might have been a part of the survival mechanism adopted by the cells upon PD0332991 treatment. Higher Wee1 and Chk1 expression in A375PDR might be due to the

upregulation of CDC25 (as yet not assessed) as a way of compensating for lack of CDK4/6 activity. In cell cycle, CDC25 promotes CDK4/6 activity by removing inhibitory phosphates (Qu et al. 2003). Hence, A375PDR cells might have elevated CDC25 levels, which may increase the possibility of activating CDK4/6 despite the inhibitory action of PD0332991. The level of activation might not be as high but adequate for Rb-phosphorylation, which enables cell cycle progression from G₁ to S phase. Elevated Chk1 and Wee1 might be a normal response to higher CDC25 levels, since Chk1 negatively regulates CDC25 and in turn promotes Wee1 expression. However further investigation was not conducted due to time constraint.

Survivin, as the name suggests is an inhibitor of apoptosis protein and a marker for cell survival (Yamamoto et al. 2008). Cancer cells have higher survivin expression compared to normal cells hence it is a good biomarker (Duffy et al. 2007; Jha et al. 2012). p27^{KIP1} conversely is a CKI within CIP/KIP family which involves in the regulation of CDK2-CyclinE activity at the restriction point between the G₁ and the S phase (Nam and Kim 2008). Based on western blotting results, changes were seen in Survivin and p27^{KIP1} protein expression level and pattern before and after PD0332991 treatment. A375PDR has a lower survivin expression compared to parental A375, however the level did return to match the parental cell line by 48 hours. Even though a similar effect as observed in the parental cell line was also seen in A375PDR after PD0332991 treatment, residual survivin expression still can be detected after 48 hours. This shown that the cells have gained some survival advantages that enables them to withstand chemical stress given. Treatment with PD0332991 promotes p27^{KIP1} expression in A375, which is evident after 48 hours treatment. This might be due to the p27^{KIP1} transactivation by p53 following chemical stress from PD0332991 treatment. According to literature, A375 express wild-type p53 (Min et al. 2005) and the status of p53 expression in A375 cells utilised in this study was assessed previously (data not

shown). The association of p27^{kip1} expression with p53 status in cancers have been documented in a number of studies (Casalini et al. 2007; Min et al. 2005). Based on the pattern of p27^{kip1} expression in both cell lines (parent and drug-resistant), p53 might play a role in the p27^{kip1} expression after PD0332991 treatment in A375, and possibly in A375PDR as well, as part of the stress response.

Flow cytometry results clearly showed the effect of PD0332991 treatment to cancer cells. However, there were no significant differences of DNA content between A375 and A375PDR control cells. It indicated that A375PDR somehow retained a similar DNA content despite changes acquired during resistance development. However, PD0332991 treatment caused a specific and prominent G₁ arrest on both cell lines seen as early as 24 hours post-treatment. There is similar observation of a specific G₁ cell cycle arrest reported by other researchers working with different cell lines (Collins and Garrett 2005; Finn et al. 2009). Despite drug treatment dose was a maintenance dose, PD0332991 still able to evoke such response in A375PDR. A possible explanation may be due to the phenotype instability of the resistant cell line.

Chapter 8

General Conclusion

Cell cycle perturbation is a common event in carcinogenesis. Changes associated with cell cycle are diverse and may reflect the tissue of origin. However, a general pattern of deregulation can be seen in several drug-sensitive and drug-resistant cancer types studied particularly concerning the components of the G₂M cell cycle checkpoint. To consider this further, in the context of anticancer drug resistance, cell cycle regulators such as Wee1 and other key cell cycle associated factors such as p53 and Chk1 were deregulated in several drug resistant cancer cell lines in comparison with their drug sensitive counterparts. This general trend also shows the importance of these proteins in regulating cell cycle thus makes the case for cell cycle control as being a key factor associated with cancer cells in order to obtain limitless growth advantage even in the presence of powerful chemotherapeutic drugs, such as Taxol.

Changes in cell cycle regulation in cancer cells are related to their dependence towards genetic alteration, a condition known as “oncogene addiction”. This is a term coined to explain the observation of persistent and similar genetic changes seen in cancer cells, including components of cell cycle. Examples of oncogene addiction are high expression of *myc* in osteogenic sarcoma and *cyclinD1* in pancreatic cancer cells (Jain et al. 2002; Nijman 2011; Weinstein 2002). Despite the fact that oncogene addiction gives growth advantage to cancer cells, it renders them vulnerable to sudden genetic changes introduced, such as inactivation of oncogenes or reactivation of tumour suppressors. Jain et al. (2002) working on transgenic mouse showed that *myc* overexpression preceded formation of osteogenic sarcoma but also brief loss of this oncogene overexpression prompted sarcoma cells to differentiate into normal osteocytes. However, reactivation of *myc* overexpression in these cells drives them into apoptosis, showing the vulnerability of cancer cells toward sudden genetic changes. In the present

study, oncogene addiction can be seen as a condition to describe in drug-resistant ovarian cancer cell lines. These cell lines showed overexpression of CyclinE, Survivin and E2F1 which are all implicated in cell cycle progression.

Molecular changes acquired by drug-resistant cancer cells distinguished them from their wild-type counterparts. Apart from gaining growth advantages, these molecular changes also mean genetic instability thus presenting a window of opportunity for possible new targeted therapies. In the study of p57^{Kip2} in platinum-resistant ovarian cancer, downregulation of p57^{Kip2} plays a role in platinum resistance. However, it is also confers collateral sensitivity towards seliciclib. This might be beneficial as a guide for treating ovarian cancer patients that relapsed after platinum treatment. According to Hall et al. (2009), the concept of collateral sensitivity was originally used to explain hypersensitivity of drug-resistant *E. coli* towards other unrelated agents. It is also used in drug study to explain a similar phenomenon observed in drug-resistant cells (Coley et al. 2012; Hall et al. 2009). An example of collateral sensitivity in drug-resistant cells was demonstrated by Nakajima et al. (2010) in human ovarian cancer cells PX2 and PX24 and also in MCF-7 cells with acquired resistance to paclitaxel (Ajabnoor et al. 2012). These cells acquired paclitaxel resistance with cross-resistance to docetaxel, another taxane agent. However, all drug resistant cell lines showed collateral sensitivity to platinum agent cisplatin (Coley et al. 2012; Nakajima et al. 2010). In this study, cross-resistance was seen in platinum-resistant ovarian cancer cell lines PEO1CarbR and PEO1CisR. Both cell lines have shown cross-resistance to cisplatin and carboplatin respectively even with a different genetic background such as the difference in the expression level of p57^{Kip2} and several other cell cycle proteins. Additionally, more cell death was seen in combination treatment compared to single drug treatment in drug-sensitive cancer and synergistic action of the two drugs was confirmed via isobologram analysis. This result might prove

to be useful in clinical settings in order to prevent carboplatin resistance in ovarian cancer patients as they have been shown to relapse after treatment with this agent. A subset of cancer patients that have gained drug resistance particularly towards Taxol might benefit from the study of Wee1 status. Even though the study of Wee1 presented here is still in its early phase, it shows the potential as a biomarker or as a treatment guide for a specific subset of cancer patients.

Drug resistance arises as a consequence of cellular alteration gained during the course of treatment. Ovarian cancer is notoriously known for its ability to relapse after chemotherapy after gaining drug resistance. Other types of cancer such as breast cancer are also known to acquire drug resistance. Alteration of cell cycle components is one of the molecular perturbations seen in drug-resistant cancer cells. The present study shows that alteration of cell cycle components as part of the drug resistance phenotype varies depending on the inducing agent and tissue of origin. p53 status is also believed to have an influence in the expression of other key cell cycle regulator in most cell lines and some of the data obtained in the present study support that. In addition, a general pattern was seen with alteration in ATR-Chk1-Wee1 pathway in the majority of cell lines studied. Epigenetic modification was believed to be involved in downregulation of Wee1 and SFN in selected drug-resistant cell lines studied. This cellular event was also shown to be the cause of p57^{Kip2} downregulation in drug-resistant ovarian cancer. Studies such as this, point to parameters associated with cell cycle perturbation such as a source of potential biomarker in cancer treatment, choosing the right agent to use and predicting the outcome of the treatment especially for a group of cancer patients with a specific genetic background. In this study, p57^{Kip2} and Wee1 was identified as potential candidate biomarkers for use as prognostic indicators. p57^{Kip2} in the form of its specific methylated DNA, is a possible candidate for predicting platinum resistance in ovarian cancer and determining seliciclib sensitivity. Wee1 also emerges as an interesting

parameter, particularly in the case of taxane sensitivity. However, this area still requires more investigation.

Finally, cell culture is an important *in vitro* instrument to study cellular mechanisms behind drug resistance and to study drug properties. It is accessible as the study can be conducted in the laboratory. Cell culture also provides an environment close enough to mimic *in vivo* milieu. It is particularly useful in the study of acquired drug resistance. Hence, the technique was utilized for the development of drug-resistant melanoma in order to study its mechanisms of PD0332991 resistance.

In conclusion, the study of cell cycle regulation has provided more insight into the problem of anticancer drug resistance.

Challenges and future work

Development of a novel drug-resistant cell lines require a set of trial and errors in order to determine the best culture conditions. It has to be noted that this project is in its early stage and there are possibilities of further analysis of the drug resistant phenotype. Development of higher resistance level coupled with further tests to confirm resistance and phenotype stability could be carried out. As explained previously, higher resistance level can be achieved via periodic exposure to increasing level of the drug for a certain period. Cancer cells with varying levels of resistance to each other might have acquired a different adaptation mechanism. Thus, analysis performed on these cell lines might give beneficial information to assist patients during different stages of cancer treatment using said agent. These cells may also possess different sensitivity towards other agents as well. Drug combination

treatment followed by isobologram analysis on these cells might yield additional information. However, it must be kept in mind that drug resistance achieved *in vitro* must correlates with the actual level of drug resistance acquired by patients in clinical settings. Nevertheless, cancer cell lines with higher drug resistance level might reveals other cellular mechanisms that can be an interesting subject to study.

Data presented in this study will benefit from more supporting data such as mRNA level for proteins of interest, and screening and assessment of other regulatory proteins involved in the pathway for instance CDC25, p53, Rbp and CDK4/6. Additionally, survivin as an inhibitor of apoptosis shows potential as an interesting target for a marker of PD0332991 resistance in melanoma thus requires further investigation on its exact role in acquired drug resistance mechanisms. Survivin might play a major role as part of an adaptation mechanism acquired by cancer cells to withstand chemical insults. However, another key player (p53) which is upstream of survivin might also influences cellular response towards drugs. Thus, establishing the role of p53 is vital in building the case of survivin role in PD0332991-resistant melanoma. This is also the case for Wee1 and SFN in Taxol resistance. Since they are downstream targets of p53 and p53 status might influence cellular response to Taxol as well. Consideration of the genetic background is also important in choosing the suitable model to study drug resistance.

Finally, knock-in experiment followed by drug treatment may be helpful in determining the role of protein of interest in a specific drug-resistance mechanism, for example Wee1 in Taxol resistance. It has been shown in this study that Wee1 downregulation was seen in a certain Taxol-resistant cancer cells and *Wee1* silencing influences cellular Taxol response. Additionally, works carried out by Mariam and Coley (unpublished data, 2013) on MCF7TaxR (higher Wee1 expression compared to parental cell line

MCF7) shown that Wee1 knock-out re-sensitized Taxol-resistant breast cancer to Taxol treatment. Thus, Wee1 knock-in experiment on the parental cell line MCF7 might give a clue on Wee1 role in Taxol resistance acquired by MCF7TaxR. Furthermore, this might also applicable to other cell lines with similar genetic background as well.

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