CHARACTERIZATION OF CHECKPOINT ADAPTATION IN HUMAN FIBROBLASTIC GLIOMA CELLS AND AN ANALYSIS OF PROTEIN PHOSPHATASE INHIBITORS

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Thesis Abstract

This thesis reports that checkpoint adaptation occurs in human brain cancer cells. M059K cells, after treatment with camptothecin (CPT), recruited γ-histone H2AX, phosphorylated Chk1 and arrested in the G2 phase. Strikingly, cells escaped the checkpoint, became rounded and entered mitosis as measured by phospho-histone H3 signals. Lamin A/C immunofluorescence microscopy revealed that 48% of the cells that survived checkpoint adaptation contained micronuclei. These data suggest that brain cancer cells undergo checkpoint adaptation and may have an altered genome. This thesis also explored if phosphatases participate in checkpoint adaptation. Human colon cancer cells were treated with CPT and the PP2A inhibitor cantharidin. Following treatment the cells became rounded and 65% were positive for phospho-histone H3 signals indicating that cantharidin caused cells to be in mitosis following CPT treatment. These data suggest that PP2A might have a role in checkpoint adaptation, or participate in a pathway that bypasses checkpoint adaptation.

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List of Abbreviations

AP – Alkaline phosphatase

ATM – Ataxia-telangiectasia mutated

ATP – Adenosine triphosphate

ATR – Ataxia-telangiectasia mutated Rad 3-related

ATRIP – ATR interacting protein

A-T – Ataxia telangiectasia

BBB – Blood brain barrier

BSA – Bovine serum albumin

CAK – Cyclin-dependent kinase-activating kinase

Cdk – Cyclin-dependent kinase

Chk – Checkpoint kinase

CHO – Chinese hamster ovary cells

CPT – Camptothecin

DAPI - 4',6-diamidino-2-phenylindole

DMSO – Dimethyl sulfoxide

DNA – Deoxyribose nucleic acid

DNA-PK – DNA dependent protein kinase

DOX – Doxorubicin

DSB – Double stranded break

DTT – Dithiothreitol

EGTA - Ethyleneglycol-bis (beta-aminoethylether)- *N*,*N*′-tetraacetic acid

GBM – Glioblastoma multiforme

Gwl – Greatwall kinase

G1 phase – Gap 1 phase

G2 phase – Gap 2 phase

HCC – Hepatocellular carcinoma

HT-29 - Human colon carcinoma cell line

HU – Hydroxyurea

IR – Ionizing radiation

LET – Linear energy transfer

MMS – Methyl methanesulfonate

M phase – Mitosis phase

Mre-11 – Meiotic recombination 11

MRN – Mre-11-Rad50-Nbs1

M059K – Human fibroblastic glioblastoma cell line

Myt1 – Myelin transcription factor 1

Nbs-1 – Nibrin

PBS – Phosphate-buffered saline

PIKK – Phosphoinositide 3-kinase related kinase

Plk1 – Polo-like kinase 1

PNUTS – Protein phosphatase 1 nuclear-targeting subunit

PP – Protein phosphatase

PPM – Protein phosphatase Mg^{2+} or Mn^{2+} dependent

PPP – Phosphoprotein phosphatase

PTP – Protein tyrosine phosphatase

RPA – Replication Protein A

S phase – Synthesis phase

ssDNA – single stranded DNA

TMZ – Temozolomide

Topo1 – Topoisomerase 1

UV – Ultraviolet radiation

U2OS – Human epithelial osteosarcoma cell line

4-NQO – 4- Nitroquinoline 1-oxide

Chapter 1

1. Literature Review

This thesis examines checkpoint adaptation in brain cancer cells. A comprehensive explanation of the eukaryotic cell cycle, DNA damage and checkpoint adaptation is provided, and how these topics relate to brain cancer will be illustrated.

1.1. The Cell Cycle

Progression through the cell cycle is a carefully regulated and coordinated process. Cell cycle regulation has been conserved from yeast to mammals and ensures the formation of genetically identical progeny cells (George, 2003). Not surprisingly, defects in cell cycle regulation are a common cause of abnormal proliferation and the genomic instability of cancer cells (Cooper and Hausman, 2009). Studies of the cell cycle and cancer have become closely interconnected.

In eukaryotic cells, the cell cycle is made up of two main phases: interphase and mitotic phase (M phase). Interphase, taking up nearly 95% of the cell cycle is the phase during which both cell growth and DNA replication occur in sequential stages in preparation for cell division (Cooper and Hausman, 2009). Interphase is composed of three stages: Gap 1 phase (G1 phase), Synthesis phase (S phase), and Gap 2 phase (G2 phase) (Norbury and Nurse, 1992). G1 phase corresponds to the interval between M phase and the initiation of DNA replication. During G1 phase, the cell is metabolically active and continuously

growing. G1 phase is followed by S phase during which DNA replication takes place. The purpose of S phase is to replicate DNA into identical, semi-conserved chromosomes, which will be passed down to daughter cells. The completion of DNA synthesis is followed by G2 phase, during which cell growth continues and proteins are synthesized in preparation for mitosis.

M phase of the cell cycle corresponds to mitosis ending in cell division. Mitosis is characterized by the following processes: chromatin condensation and nuclear envelope breakdown, chromosome alignment, sister chromatid separation and migration to opposite poles of the cell, reformation of the nuclear membrane forming two nuclei, followed by chromosome decondensation (Rieder and Khodjakov, 2003). Chromosome decondensation occurs simultaneously with or is immediately followed by cytokinesis. Cytokinesis separates the cytoplasm, organelles and nuclei into two identical daughter cells, signifying completion of M phase. Mitosis is also characterized by morphological changes such as cell rounding, making them easily distinguishable from flattened interphase cells, as well as phosphorylation of histones, including histone H3 on serine 10 (Harris, 1973; Hendzel *et al*., 1997). These characteristics make it possible to follow cells as they exit from G2 phase, enter into mitosis and subsequently exit from mitosis (Juan *et al*., 1998).

1.1.1. Cell Cycle Regulators

Progression through the cell cycle is governed by a number of control points and regulators to ensure efficient and accurate cell growth, DNA replication and cell division. Some of these regulators are extracellular signals, such as nutrients and growth factors; whereas, a large number of them are intracellular proteins (Hartwell and Kastan, 1994). A subset of these intracellular protein regulators are the cyclin-dependent kinases (Cdks) and their protein regulatory partners the cyclins (Kastan and Bartek, 2004). Cyclins have no enzymatic activity, but are required to facilitate the catalytic function of the Cdks. Within the cell there are at least eight catalytic Cdks and regulatory cyclins working together to ensure proper regulation throughout the cell cycle (Nigg, 1995). For example, the Cdk4/cyclin D complex functions early in G1 phase in response to growth factors, whereas, the Cdk2/cyclin E complex regulates transition to S phase.

Cdk1/cyclin B is a protein complex that is important in the regulation of mitosis. This complex acts by controlling entry into and exit from the M phase of the cell cycle and is composed of two protein subunits: Cdk1 and cyclin B (Pines and Hunter, 1990). These protein subunits have two mechanisms by which they control entry and exit of the M phase: 1) Oscillations in cyclin B levels during the cell cycle control Cdk1 activity, thereby controlling mitotic entry and exit (Evans *et al*., 1983). Cyclin B levels rise during G2 until a peak is reached during mitosis. Levels then fall at the middle of mitosis, due to cyclin B degradation,

resulting in conclusion and exit from mitosis. 2) Cdk1/cyclin B complex activity is also regulated by the phosphorylation status of Cdk1, which is controlled by protein phosphatases and kinases (Forsburg and Nurse, 1991). Once the Cdk1 and cyclin B complex forms, it undergoes a conformational change that increases the accessibility of the Cdk1 residue threonine 161 (Thr161). Cdk1 is then phosphorylated at Thr161 by cyclin-dependent kinase (Cdk)-activating kinase (CAK) (Norbury *et al*., 1991). This phosphorylation results in a change in protein structure that allows ATP access to the catalytic site of Cdk1. However, this activating Thr161 phosphorylation does not activate the complex due to inhibitory phosphorylations on threonine 14 (Thr14) and tyrosine 15 (Tyr15) of Cdk1. These sites are phosphorylated by the protein kinases Myt1 and Wee1, respectively (Parker and Piwnica-Worms, 1992; Mueller *et al*., 1995). For entry into mitosis, the inhibitory phosphorylations are removed by the Cdc25 phosphatases, thereby exposing the ATP binding site of Cdk1 (Sebastian *et al*., 1993). The dephosphorylation by Cdc25 phosphatases results in Cdk1/cyclin B complex activation, thereby triggering entry into mitosis. This dual control mechanism is one example of the many cell cycle regulators, and mechanisms within each regulator, working to ensure precise and properly coordinated cell cycle progression.

1.1.2. Cell Cycle Checkpoints

In addition to cell cycle regulators, the cell cycle is also controlled by a series of control points known as cell cycle checkpoints (Hartwell and Weinert, 1989). Cell cycle checkpoints ensure the fidelity of cell division by preventing entry into the next phase of the cell cycle until the events of the preceding phase have been completed. Checkpoints also prevent progression through the cell cycle if the genome is damaged or improperly structured (Kastan and Bartek, 2004; Bartek and Lukas, 2007). This is accomplished through four main cell cycle checkpoints, one is called the spindle assembly checkpoint and the other three are called DNA damage checkpoints (Abraham, 2001).

The spindle assembly checkpoint occurs toward the end of mitosis and monitors spindle structure as well as the alignment of chromosomes on the mitotic spindle, thus ensuring that a complete set of chromosomes is distributed accurately to the daughter cells (Rudner and Murray, 1996). Failure of one or more chromosomes to align properly, or the occurrence of inaccurate spindle structure, will be detected at the spindle assembly checkpoint resulting in a cell cycle arrest in metaphase of mitosis (Abraham, 2001). This M phase arrest is released after proper chromosome alignment or when correct spindle structure has been restored, at which point the cell progresses from metaphase to anaphase.

1.2. DNA Damage Checkpoints

When dividing cells contain damaged DNA they initiate a response named the DNA damage checkpoint (Hartwell and Weinert, 1989; Carr, 2002). This checkpoint ensures genome fidelity by arresting cell cycle progression to provide time for repair, thereby preventing proliferation of cells with damaged DNA (Toczyski *et al*., 1997; van Vugt *et al*., 2005). A DNA damage checkpoint functions in each of the three stages of interphase (Cooper and Hausman, 2009). The G1 checkpoint will halt cell cycle progression in the presence of damaged DNA to allow time for DNA repair before the cell enters S phase (Abraham, 2001). This G1 checkpoint prevents damaged DNA from being replicated in the synthesis phase. The S phase checkpoint not only provides continual monitoring of the integrity of DNA to ensure damaged DNA is repaired before replication, but it also promotes the repair of errors that occur during DNA replication.

1.2.1. G2 DNA Damage Checkpoint

The G2 DNA damage checkpoint is one of the main processes under investigation in this thesis and therefore, a comprehensive understanding of how it functions is essential to this discussion. The G2 DNA damage checkpoint is present in the G2 phase of the cell cycle and prevents the initiation of mitosis in cells that contain damaged DNA (Melo and Toczyski, 2002).

1.2.1.1. Activation of the G2 DNA Damage Checkpoint

The G2 DNA damage checkpoint is a molecular system composed of sensors, transducers and effectors. Collectively, these create a phosphorylation cascade that ultimately results in a G2 phase arrest of the cell cycle (Melo and Toczyski, 2002). The type of damaged DNA, either DNA double stranded break (DSB) or DNA single stranded break, will result in the activation of either the ataxia telangiectasia mutated (ATM) or ataxia telangiectasia mutated Rad 3-related (ATR) kinases, respectively (Smith *et al*., 2010). Both ATM and ATR are protein kinases belonging to the phosphoinositide 3-kinase related kinases (PIKKs) family, whose activation initiates the checkpoint pathway. DSB damage is recognized by the Mre-11-Rad50-Nbs1 (MRN) complex, which ultimately results in ATM pathway activation. Whereas, DNA single stranded breaks result in the rapid accumulation of the single stranded binding protein, Replication Protein A (RPA), which results in the activation of the ATR pathway (Harper and Elledge, 2007).

In response to damaged DNA, ATM and ATR phosphorylate Checkpoint kinase 2 (Chk2) and Checkpoint kinase 1 (Chk1) substrates, respectively (Jazayeri *et al*., 2006). ATM and ATR also phosphorylate histone H2AX at Ser139, denoted γ-H2AX following phosphorylation (Rogakou *et al*., 1998; Burma *et al*., 2001; Ward and Chen, 2001). This phosphorylation occurs within minutes of DNA damage and thus H2AX phosphorylation on Ser139 has become a standard marker for the presence of damaged DNA (Rogakou *et al*., 1998; Bonner *et al*.,

2008). This phosphorylation is required for DNA repair, and is removed by the phosphatase PP2A once repair has been completed (Chowdhury *et al*., 2005). Upon detection of DSB damage, the MRN complex recruits ATM to the site of the DSB resulting in ATM activation. Once ATM has been activated, there is the initiation of a sequence of events, including DSB resection, which results in single stranded DNA (ssDNA) formation (Jazayeri *et al*., 2006). This ssDNA in turn facilitates the recruitment of RPA. The prolonged presence of RPA at a site causes the recruitment of the heterodimer ATR interacting protein (ATRIP) (Burrows and Elledge, 2008). ATR complexes with its protein partner ATRIP enabling the complex to bind to ssDNA (Liu *et al*., 2000; Zhao and Piwnica-Worms, 2001). The now active ATR, with assistance from the adaptor protein claspin, phosphorylates the signal transducer, Chk1 on serines 317 and 345 thereby activating it (Figure 1). Checkpoint kinases inhibit entry into mitosis through two mechanisms: 1) upregulation of Wee1 kinase and 2) downregulation of the Cdc25 phosphatases (Sancar *et al*., 2004). These two mechanisms work in unison to control Cdk1/cyclin B complex activity through Cdk1 inhibitory phosphorylations, and inhibition of the dephosphorylation mechanisms, respectively. Upon ATR phosphorylation, the now active Chk1 phosphorylates Cdc25C, a protein phosphatase, on serine 216 (Ser216), rendering Cdc25C inactive. In mammalian cells, three isoforms of Cdc25 have been identified: Cdc25A, Cdc25B and Cdc25C (Boutros *et al*., 2006). By knockout studies in mice, Cdc25C has been identified as the main effector of the G2/M checkpoint

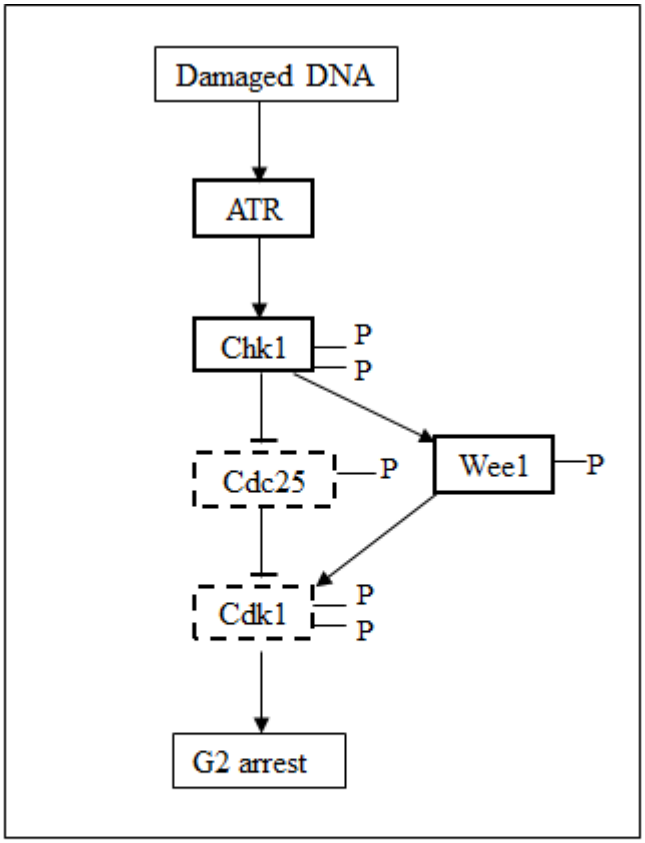


Figure 1. A simplified model for the activation of the G2 DNA damage checkpoint. A bold outline indicates an active protein and a dashed outline indicates a protein that is not active.

pathway. Upon Ser216 phosphorylation, the Cdc25C phosphatase binds to the protein unit 14-3-3, and becomes sequestered in the cytoplasm where it is degraded by the ubiquitin-proteasome pathway (Margolis *et al*., 2003; Sancar *et al*., 2004). With Cdc25C sequestered and degraded, there is an accumulation of inactive phosphorylated Cdk1, resulting in a G2 phase cell cycle arrest (Toczyski *et al*., 1997; Bartek and Lukas, 2007).

1.3. DNA Damaging Agents

Genotoxic agents damage the DNA of a cell, which if not repaired, may result in cell death (Bartek and Lukas, 2007). Current cancer treatments, such as chemotherapy or radiation, exploit the action of genotoxic agents in an attempt to kill cancer cells. In fact, the majority of cancer patients will be exposed to genotoxic agents over the course of their treatment (Siegel *et al*., 2011). An example of a genotoxic agent that is used in anti-cancer treatment is camptothecin (CPT), which is an active ingredient used in chemotherapy. The pharmacokinetics of CPT and its derivatives have been investigated extensively in humans, providing valuable information to model the genotoxic response in cancer cells (Pizzolato and Saltz, 2003). The mechanism of action for CPT is topoisomerase 1 (Topo1) inhibition. Topo1 is an enzyme that removes DNA supercoils that occur during transcription and DNA replication due to the intertwined nature of the double helix (Roca, 1995). Normally Topo1 induces nicks or single stranded DNA cleavages in the DNA backbone to release torsional

strain. However if CPT is present, CPT binds to Topo1 and DNA, resulting in the formation of a ternary complex (Hsiang *et al*., 1989). This ternary complex stabilizes the covalent binding of Topo1 to the 3' end of the broken DNA preventing the religation of the nicked strand and the release of the enzyme. When this stabilized complex encounters a replication fork, the single stranded breaks are converted into irreversible double stranded breaks (Beretta and Zunino, 2007).

1.4. Outcomes of Initiating the G2 DNA Damage Checkpoint

Until recently, there were thought to be two main outcomes of the DNA damage checkpoint, depending on the severity of the damage (Zhou and Elledge, 2000). One outcome is cell cycle arrest, DNA repair and re-entry into mitosis, a process known as checkpoint recovery (Bartek and Lukas, 2007). The second outcome being the inability for DNA to be repaired, resulting in the initiation of the apoptosis pathway that ends with programmed cell death. However in 1997, Toczyski *et al.* introduced a third outcome following initiation of the DNA damage checkpoint in which the cells, after a G2 phase cell cycle arrest, enter into mitosis regardless of the presence of damaged DNA (Toczyski *et al*., 1997). This process is called checkpoint adaptation.

1.4.1. Checkpoint Recovery

The DNA damage checkpoint allows time for DNA damage repair, after which cells can resume cell cycle progression and undergo cell division. Checkpoint recovery is the process by which repaired cells inactivate the DNA damage checkpoint and enter into mitosis (Zhou and Elledge, 2000). Upon repair of damaged DNA, the DNA damage checkpoint sensors become inactivated. With these sensors inactive, the activation of downstream substrates is suppressed. This cascade of substrate inactivation results in the cells ability to exit the G2 block and enter into mitosis (Clemenson and Marsolier-Kergoat, 2009).

1.4.2. Apoptosis

Programmed cell death, or apoptosis, is a regulated form of cell death, which is essential for various biological events such as morphogenesis. However, it is also important for the elimination of potentially harmful cells. If a cell becomes damaged and cannot be repaired, the cell may undergo apoptosis (Cheng *et al*., 2011). Regulated destruction of a cell is a complicated process, in which proper execution requires the coordinated activation and operation of multiple mechanisms. The terminal apoptotic pathway of mammalian cells depends on the activation of caspases, which is a family of proteases, and their modification of protein substrates within the nucleus and cytoplasm (Sancar *et al*., 2004). Activation of the apoptotic pathway results in a set of morphological and

biochemical features, making this a distinguishable biological process. These characteristics include: cell blebbing (irregular bulging), cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation (Kerr *et al*., 1972). The end result of apoptosis is controlled death and removal of a damaged cell to prevent further damage to the multicellular organism.

1.5. Checkpoint Adaptation

Checkpoint adaptation is a process defined by three features in cells with damaged DNA: 1) cell cycle arrest, 2) entry into mitosis, 3) damaged DNA in mitotic cells (Toczyski *et al*., 1997). This process is not well understood and is the central theme for my thesis work.

1.5.1. History of Checkpoint Adaptation

Checkpoint adaptation was originally described in *Saccharomyces cerevisiae* in 1993 as the ability of yeast cells to divide following a checkpoint arrest, despite the presence of irreparable chromosome damage (Sandell and Zakian, 1993). However it was not until 1997 that this surprising result was credited to be checkpoint adaptation (Toczyski *et al*., 1997). In the 1993 study, yeast strains were constructed to enable a single telomere to be eliminated from the end of a dispensable chromosome. The elimination of a telomere caused a cell cycle arrest. However, many cells recovered from the arrest, without repairing the damaged chromosome, and continued replicating and segregating for as many as

ten cell divisions. Further studies led to the conclusion that checkpoint adaptation in yeast is a process that is accompanied by loss of Rad53p checkpoint kinase activity and Chk1 phosphorylation (Pellicioli *et al*., 2001). It was hypothesized that checkpoint adaptation could potentially be a beneficial process in single cell organisms, which may repair the damage in subsequent cell cycles (Lee *et al*., 2000; Galgoczy and Toczyski, 2001).

In 2004, checkpoint adaptation was reported to occur in *Xenopus* egg extracts in response to the DNA polymerase alpha inhibitor aphidicolin, which inhibits DNA synthesis (Yoo *et al*., 2004). Yoo *et al.* reported that following a prolonged interphase arrest, aphidicolin-treated egg extracts underwent checkpoint adaptation and entered into mitosis despite the presence of incompletely replicated DNA. This adaptation was accompanied by phosphorylation of the checkpoint mediator protein claspin, by Plx1, which resulted in inactivation of the Chk1 kinase. The demonstration of checkpoint adaptation in multicellular eukaryotes raised interest regarding whether checkpoint adaptation also exists in human cells.

In 2006, Syljuåsen *et al.* confirmed that checkpoint adaptation did occur in human cells exposed to ionizing radiation. The paper reported two important conclusions: 1) Upon exposure to ionizing radiation, human U2OS osteosarcoma cells underwent an extended G2 phase cell cycle arrest; however, entered mitosis with damaged DNA and 2) Chk1 inhibition was necessary for the exit from the G2 checkpoint arrest. The first of these conclusions was based on two separate

experiments. Firstly, by flow cytometry it was demonstrated that the irradiated cells had been arrested in the G2/M phase then abrogated the checkpoint and progressed into G1 phase. Secondly, they were able to show by immunofluorescence microscopy and phospho-ser10 histone H3 and histone γ-H2AX staining that the irradiated cells were in mitosis with damaged DNA. However, they did not report what fraction of the population entered into mitosis with damaged DNA, therefore it was not known if checkpoint adaptation was a major event. By flow cytometry and Western blotting, Syljuåsen *et al.* demonstrated that during an extended G2 phase arrest, Chk1 was active and phosphorylated on three distinct serine residues. However, cells that had undergone checkpoint adaptation, Chk1 was dephosphorylated at Ser317/345. This result suggested that the exit from the prolonged IR-induced G2/M checkpoint was based upon the activity of Chk1 kinase. Syljuåsen and colleagues speculated that most of the cells carrying damaged DNA would eventually die; however, some cells might survive with DNA lesions that would make them prone to the development of further genomic instability. They also speculated that genomic instability would have implications in human cancer and could potentially contribute to cancer development.

1.5.2. Significance of Checkpoint Adaptation

Both the precise mechanism and biological significance of checkpoint adaptation are still unknown. With the demonstration of checkpoint adaptation in

human cells, a number of questions and concerns have arisen regarding the potential effects this process may have on multicellular organisms including: 1) does this happen in all human cancer cells or is this specific to certain cell types? 2) what becomes of the cells that survive checkpoint adaptation?

It may be speculated that the biological function of checkpoint adaptation in vertebrates is similar to the function that has been proposed for mitotic catastrophe, where defective cells with irreparable damage are eliminated through a mitosis-linked death, distinct from apoptosis (Vitale *et al*., 2011). It may also be speculated that checkpoint adaptation is a testimony to the resilience of life, in a last ditch attempt by the cell to increase its chance for survival. Nonetheless, current research has shown that 2% of cells that undergo checkpoint adaptation survive and complete at least one round of cellular division (Kubara *et al*., 2012). It has been speculated that the cells that survive checkpoint adaptation may contain a highly rearranged genome and may continue to divide, potentially creating a progeny with a modified genome (Syljuåsen *et al*., 2006; Kubara *et al*., 2012). What subsequently happens to these surviving cells and their progeny remains largely unknown.

Following the report of checkpoint adaptation in human cancer cells in 2006, a number of concerns have been identified regarding the issue of cellular division with damaged DNA. Firstly, mutated DNA is the molecular foundation for many diseases including human-cancer-susceptibility syndromes and can result in further genomic instability, a hallmark of cancer (Stratton *et al*., 2009).

Secondly, the most common form of cancer treatments currently used is treatment with DNA damaging agents (Siegel *et al*., 2011). Furthermore, cancer cells often have defects or impaired function of their DNA damage checkpoints (Wang *et al*., 2008; On *et al*., 2011). Therefore, we are currently treating cancer cells with agents that may result in checkpoint adaptation. Checkpoint adaptation has also been suggested to be a cause of genomic instability and further carcinogenesis (Kastan and Bartek, 2004; Kubara *et al*., 2012). Checkpoint adaptation and cells that survive it may then provide insight into additional cancer developments, such as secondary tumors that contain major genomic rearrangements (Kastan and Bartek, 2004; Kubara *et al*., 2012). Checkpoint adaptation may also provide a potential explanation for the resistance of human cancer cells to genotoxic therapy (chemoresistance) or cancer relapse (Syljuåsen *et al*., 2006; Wang *et al*., 2008; Kuntz and O'Connell, 2009; Kubara *et al*., 2012).

1.5.3. Checkpoint Adaptation in Human Cells

Studies have demonstrated aberrant mitosis in human cells following exposure to radiation or treatment with genotoxic compounds (Demarcq *et al*., 1994; Clifford *et al*., 2003; Hall and Giaccia, 2006; Vakifahmetoglu *et al*., 2008; Cahuzac *et al*., 2010). These studies report cells undergoing mitotic catastrophe. Mitotic catastrophe is a process that lacks a distinct and uniformly accepted definition; however, it is characterized as a form of cell death that includes aberrant mitosis (Vakifahmetoglu *et al*., 2008). It has been suggested that

checkpoint adaptation may be an inducer of, or happen prior to, mitotic catastrophe.

Since 2006, there have been subsequent reports of checkpoint adaptation in two other human cell lines, lymphocytic leukemia cells and colon carcinoma cells, which have demonstrated the three features outlined by Toczyski *et al*. (1997). There have also been reports suggesting checkpoint adaptation has occurred in other cell lines; however, the focus of these studies was not to demonstrate checkpoint adaptation. In 2008, a study was done in metastatic hepatocellular carcinoma (HCC) cells treated with the genotoxic alkylating agent cisplatin, in which an accumulation of DNA damage lesions was believed to be the result of checkpoint adaptation (Wang *et al*., 2008). This study was done to understand how cell cycle pathways (particularly Plk1 and Cdc25A pathway) critically affect the fate of metastatic cells. The result of the study determined that the metastatic HCC cells showed a defective cell checkpoint arrest, whereby cells continued to enter into mitosis following cisplatin treatment. This precocious entry into mitosis following cisplatin treatment resulted in an accumulation of DNA lesions suggested to be the result of HCC cells undergoing checkpoint adaptation. It was also noted in the study that although checkpoint adaptation often results in the elimination of excessively damaged DNA by cell death (such as through mitotic catastrophe), the metastatic cells used in this study predominantly avoided this fate, and were able to survive checkpoint adaptation with accumulated DNA lesions.

In 2011, a study was done to determine how fractionated radiation (smaller more frequent doses of radiation as opposed to one larger dose of radiation), affects the ability of lymphocytic leukemia cells (MOLT4) and normal human fibroblastic cells to repair induced DNA DSB (Rezacova *et al*., 2011). They reported that both lymphocytic leukemia and human fibroblastic cells were unable to repair all DSBs before the next radiation dose, which produced further DNA damage. Interestingly, they observed that following fractionated radiation doses, the MOLT4 cell line experienced a time of delayed cell proliferation that was due to a DNA damage checkpoint arrest. Depending on the treatment doses and frequencies, this delay in cell proliferation lasted anywhere from 24 h to 72 h as determined by cell counting and flow cytometry. However the MOLT4 cells were able to abrogate the DNA damage induced arrest and enter into mitosis with unrepaired DSB DNA damage. Damaged DNA was detected by γ-H2AX immunofluorescence and entry into mitosis was determined by an increase and subsequent decrease in cyclin B levels at 24 h and 48 h after the first and second irradiation as determined by Western blotting. It was reported that the normal human fibroblastic cell line had undergone a similar process in response to radiation, however they did not demonstrate a delay of cell proliferation or a checkpoint arrest in these cells and the level of DNA damage present following radiation was insufficient. Therefore further studies on normal human fibroblastic cells would need to be done to determine if they also undergo checkpoint adaptation.

Recent studies have demonstrated the human colon carcinoma cell line HT-29, which are well characterized epithelial cells, undergo checkpoint adaptation when treated with pharmacologically relevant concentrations of CPT (Kubara *et al*., 2012). The results of this study demonstrated that HT-29 cells, following treatment with 25 nM CPT, recruit multiple γ-H2AX foci (indicating damaged DNA), become phosphorylated at Ser345- of Chk1 (indicating activation of the DNA damage checkpoint), and enter into a G2 phase arrest. Then starting at 40 hours following the CPT treatment nearly all the cells escape the G2 phase arrest and become rounded up, a morphological characteristic of cells in mitosis. These rounded cells had high levels of cyclin B1, lacked Tyr15-Cdk1 phosphorylation and were positive for phospho-ser10 histone H3, all markers for cells in mitosis. It was also reported that the cells that entered into mitosis with damaged DNA were dephosphorylated at Ser345-Chk1, indicating a relationship between Chk1 dephosphorylation and checkpoint adaptation. Video microscopy demonstrated that nearly 90% of CPT treated cells entered into mitosis before cell death, indicating that this was a major cell cycle event that links cell cycle arrest to cell death. However, it was also reported that although nearly all of the cells that entered into mitosis with damaged DNA died, 2% of these cells survived. Figure 2 is a model proposed by Kubara *et al.* on the steps required for checkpoint adaptation after treatment with genotoxic agents.

Using ataxia telangiectasia (A-T) cells, a cell line that has defects in both checkpoint arrest and DSB repair, a 2007 study proposed a threshold hypothesis

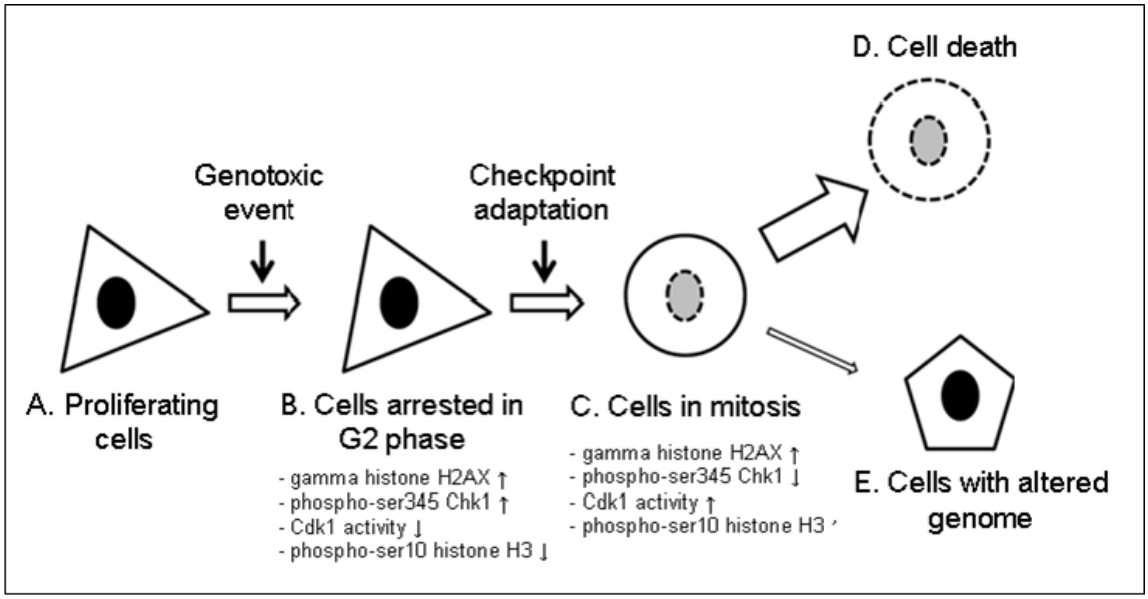


Figure 2. A model of the steps required for checkpoint adaptation after treatment with cytotoxic concentrations of genotoxic agents (Kubara *et al.*, 2012).

for the activation and release of the G2 checkpoint (Deckbar *et al*., 2007). This hypothesis suggested that a threshold level of radiation induced DSB is needed to activate and maintain the G2 checkpoint. In 2011, a study explored the potential of a DNA damage threshold in human cells as a determinant for DNA damage checkpoint activation and checkpoint adaptation (Tkacz-Stachowska *et al*., 2011). To do this, individual cells from human fibroblasts and U2OS osteosarcoma cells were irradiated and examined for checkpoint activation and release based on γ-H2AX foci and phospho-histone H3 staining. The results of the study indicated that the number of γ-H2AX foci varied between individual cells for all radiation doses and times. This result suggests that the amount of damaged DNA needed to activate and maintain the G2 checkpoint varies between single cells within a cell population, thus the checkpoint, and checkpoint adaptation are not dependent on a threshold level of damaged DNA.

The work that has been done since 2006, has established that checkpoint adaptation occurs in osteosarcoma, lymphocytic leukemia and colon carcinoma cell types (Syljuåsen *et al*., 2006; Rezacova *et al*., 2011; Kubara *et al*., 2012). These cell lines underwent the three criteria for checkpoint adaptation: 1) a cell cycle arrest, 2) entry into mitosis, and 3) damaged DNA in mitotic cells, following exposure to radiation and CPT (Toczyski *et al*., 1997). Checkpoint adaptation has also been suggested to occur in at least two other cell types including: hepatocellular carcinoma cells and normal human fibroblastic cells (Wang *et al*., 2008; Rezacova *et al*., 2011). As well as prior to cell death by

mitotic catastrophe, checkpoint adaptation has also been suggested to occur in fibrosarcoma and adenocarcinoma cells (Clifford *et al*., 2003; Cahuzac *et al*., 2010). Although checkpoint adaptation may be occurring in these cell types following treatment with a DNA damaging agent, all three of the criteria for checkpoint adaptation were either not explored or insufficiently supported in these reports. Therefore further work would need to be done to confirm that these cell lines undergo checkpoint adaptation. Checkpoint adaptation also appears to be independent of the type of DNA damage including damage due to radiation, CPT, cisplatin, bleomycin, S23906 and etoposide (Syljuåsen *et al*., 2006; Wang *et al*., 2008; Cahuzac *et al*., 2010; Rezacova *et al*., 2011; Kubara *et al*., 2012). Checkpoint adaptation also appears to be insensitive to a threshold of damaged DNA (Deckbar *et al*., 2007; Tkacz-Stachowska *et al*., 2011). Prior to this thesis work, checkpoint adaptation had not been reported in brain cancer cells.

1.6. Phosphorylation Status of Chk1 following Checkpoint Adaptation

The precise mechanism by which cells undergo checkpoint adaptation is still unknown. However, one component that has been proposed to be involved in establishing checkpoint adaptation is Chk1. It is a component in the initiation of the DNA damage checkpoint and in establishing checkpoint recovery, and hence is a candidate for initiation of checkpoint adaptation (Zhang *et al*., 2005). Recent studies have explored the possibility of Chk1 as a mediator for checkpoint adaptation. One such study was done by characterizing Chk1 inhibitors and

tracking the effect of Chk1 inhibition on cells that had been treated with a genotoxic agent (Ferry *et al*., 2011). In this study, HT-29 cells were treated with CPT and followed by treatment with Chk1 inhibitors that were found to be active in checkpoint bypass. The results of this study determined that a novel Chk1 inhibitor (S27888) was effective in inducing human cancer cells to enter mitosis with damaged DNA and an activated Chk1. This precocious cellular entry into mitosis with damaged DNA, following Chk1 inhibition, provides evidence that Chk1 inactivation is a component in checkpoint adaptation.

There are two proposed mechanisms for the inactivation of Chk1 which therefore initiates checkpoint adaptation: 1) Chk1 degradation or 2) Chk1 dephosphorylation (Syljuåsen *et al*., 2006; Zhang *et al*., 2009). In either case, both mechanisms would disable the DNA damage checkpoint by removing the activity of Chk1, thereby resulting in activation of Cdk1 and resumption of the cell cycle.

1.6.1. Chk1 Degradation Hypothesis

Studies have demonstrated that at CPT concentrations higher than 500 nM, human cancer cells will degrade Chk1 resulting in checkpoint termination (Zhang *et al*., 2005; Zhang *et al*., 2009). Nevertheless, the plasma concentration of CPT analogues in patients is limited to 75 nM because of toxicity (Raymond *et al*., 2002b). With this in consideration, the previous results indicating Chk1 degradation as the cause of checkpoint abrogation in cells with damaged DNA,

are at concentrations over 6 times higher than what is found in a clinical setting. Further studies regarding the Chk1 degradation hypothesis were conducted and demonstrated Chk1 protein presence after treatment with clinically significant concentrations of genotoxic compounds (Heffernan *et al*., 2002; Kurz *et al*., 2004; Cahuzac *et al*., 2010). In 2012, a study was done that determined the role of proteolysis in checkpoint adaptation (Kubara *et al*., 2012). This study demonstrated that in HT-29 cells, following 25 nM CPT treatment in conjunction with 300 nM MG132, a proteasome inhibitor, the process of checkpoint adaptation was not inhibited. This indicated that Chk1 degradation was not the mechanism by which cells undergo checkpoint adaptation.

1.6.2. Chk1 Dephosphorylation Hypothesis

It has been shown that in yeast and *Xenopus* egg extracts, checkpoint adaptation is related to the loss of Chk1 phosphorylation, the release of claspin and therefore inactivation of Chk1 kinase (Pellicioli *et al*., 2001; Yoo *et al*., 2004). In humans, dephosphorylation at Ser317/345 of Chk1 was reported in U2OS cells that had undergone checkpoint adaptation (Syljuåsen *et al*., 2006). Together these results provided evidence that Chk1 dephosphorylation, not degradation, is a mechanism in checkpoint adaptation. This hypothesis has since been confirmed in 2012 by Kubara *et al.* in HT-29 cells. In this study, Chk1 was dephosphorylated at Ser345 following checkpoint adaptation, in comparison to interphase cells of the same population that remained phosphorylated at Ser345.

However these results could not distinguish whether dephosphorylation of Chk1 occurred as the initiating event that drives cells into mitosis during the adaptation process, or alternatively, if dephosphorylation of Chk1 is a consequence of checkpoint adaptation. If Chk1 dephosphorylation initiates checkpoint adaptation, then blocking Chk1 dephosphorylation should block checkpoint adaptation. This could be tested by preventing Chk1 dephosphorylation, using phosphatase inhibitors. The phosphatase that dephosphorylates Ser317/345 of Chk1 in cells has not yet been identified; however, there have been a number of phosphatases reported to dephosphorylate Chk1 at these amino acids.

1.6.3. Protein Phosphatases

Reversible protein phosphorylation is recognized to be an essential and widespread regulatory mechanism of a myriad of fundamental cellular processes, including the DNA damage response. Reversible protein phosphorylations occur on about one-third of all proteins in human cells, and each protein phosphorylation (and dephosphorylation) affects the proteins activity and biological properties (Cohen, 2001). For example, protein phosphorylation allows the regulation of enzymatic activities by mediating conformational changes, or by directly blocking access to enzyme catalytic sites, such as with Cdk1. The level of protein phosphorylation is a reflection of a delicate balance between the phosphorylating activity of kinases and the dephosphorylating activity of phosphatases. The specificity and reversible nature of these phosphorylations

would suggest there is a similar ratio of protein kinases to protein phosphatases in order to counteract their actions. Surprisingly, the number of protein phosphatases encoded by the human genome is an order of magnitude lower than that of the number of kinases (Lander *et al*., 2001; Venter *et al*., 2001; Shi, 2009). This ratio is, in part, a result of distinct phosphatase isoforms that result in varied distribution and subcellular localizations. However it is predominantly due to the ability of the phosphatase catalytic subunit to form heterodimeric or heterotrimeric holoenzymatic complexes with many protein regulatory subunits and targeting domains in a mutually exclusive manner, resulting in considerable structural diversity (Liu and Xu, 2011). These changes in phosphatase confirmation result in distinct substrate specifications, restricted subcellular functions and diverse regulation. This permits numerous cellular functions to be controlled independently by relatively few phosphatases (Barford *et al*., 1998). While the protein kinases responsible for cellular phosphorylations involved in the DNA damage response have been well documented, the role of protein phosphatases is less well known (Freeman and Monteiro, 2010). Only recently has a better understanding of these phosphatases been developed.

Phosphatases can be grouped into three distinct families based on substrate specificity, structure and catalytic function. The largest class of protein phosphatases is the phosphoprotein phosphatase (PPP) family. Next is the protein phosphatase Mg^{2+} or Mn^{2+} dependent (PPM) family, followed by the protein tyrosine phosphatase (PTP) family. The PPP and PPM families dephosphorylate

phosphoserine and phosphothreonine residues (Ser/Thr) in a single reaction step using a metal-activated nucleophilic water molecule. This makes them distinct from the PTP family, which dephosphorylates phosphotyrosine residues by use of a cysteinyl-phosphate enzyme intermediate (Barford *et al*., 1998). For the purposes of this paper, only the phosphatases involved in the DNA damage response, the Ser/Thr phosphatases, will be discussed.

Representative members of the PPP family include protein phosphatase 1 (PP1), PP2A, PP2B (commonly known as calcineurin), PP4, PP5, PP6 and PP7 (Barton *et al*., 1994; Cohen, 1997). The PPM family includes protein phosphatases dependent on magnesium/manganese ions, such as PP2C, as well as the PP2C delta isoform Wip1 phosphatase (Shi, 2009).

1.6.4. Candidate Phosphatases for the Dephosphorylation of Chk1 at Ser317/345

In 2004, a study was done in *Schizosaccharomyces pombe* to identify proteins required for checkpoint release (den Elzen and O'Connell, 2004). In this study, wild type, Dis2 overexpressing and Dis2 null cells were exposed to a variety of DNA damaging agents and then cell survival, repair rate and percent of cells passing through mitosis was monitored. The DNA damaging agents used in this study included ionizing radiation (IR), ultraviolet radiation (UV), methyl methanesulfonate (MMS) and 4-nitroquinoline-N-oxide (4-NQO). It was reported that dephosphorylation of Chk1 at Ser345 by Dis2, a PP1 homolog, allowed for mitotic entry upon completion of DNA repair in G2. It was also demonstrated

that Dis2 null cells have a prolonged Chk1-dependent arrest and a compromised ability to downregulate Chk1 activity for checkpoint release. Importantly, it was also reported that the overexpression of Dis2 caused precocious entry into mitosis in the presence of un- or partially repaired damaged DNA, resulting in an unequal division of DNA between daughter cells. These results indicated that in *S. pombe*, overexpression of the PP1 homolog Dis2, and therefore enhanced Chk1 dephosphorylation, caused aberrant mitosis. Furthermore, they were able to show that Chk1 dephosphorylation by Dis2 could be abolished by inhibitor-2, which specifically inhibits PP1.

In contrast, Petersen *et al*. (2006) studied the effects of induced DSB and PP2A inhibition on Chk1 phosphorylation at Ser344 (homologous to Ser345 of human Chk1) in *Xenopus* egg extracts. As a source of DSBs, they used DNA digested with HaeIII, which generates blunt-ended double-stranded fragments that mimic DSBs in genomic DNA. They analyzed the presence of Ser344-Chk1 phosphorylation by SDS-PAGE and Western blotting. It was reported that DSB-induced Chk1 phosphorylation on Ser344 was strongly reversed by the addition of PP2A, suggesting that Chk1 is a substrate of PP2A. They also found that treatment with the PP2A phosphatase inhibitor okadaic acid enhanced the DSB-induced Chk1 phosphorylation at Ser344. This finding suggested that PP2A was an antagonist of the Chk1 phosphorylation on Ser344.

In human U20S cells, a 2005 study was done by Lu *et al*. to determine the effects of PPM1D (or Wip1, a PP2C delta isoform) on the dephosphorylation of

Ser345 of Chk1 and the effect it has on cell cycle checkpoints (Lu *et al*., 2005). By Western blotting and co-immunoprecipitation it was established that the phosphatase PPM1D, binds to Chk1. By an *in vitro* phosphatase assay it was also reported that PPM1D dephosphorylates Ser345 of Chk1 and that this dephosphorylation lead to decreased Chk1 kinase activity. It was also reported that PPM1D dephosphorylation correlated with reduced G2/M checkpoint activity in response to radiation induced damaged DNA. At varying times following irradiation, U2OS cells were fixed and analyzed for DNA content and mitosis by flow cytometry and phospho-histone H3 content. They reported that the reduction of PPM1D by PPM1D siRNA produced an 85% reduction in M-phase cells by 8 h which remained reduced up to 48 h. Based on these results, Lu and colleagues suggested that the function of PPM1D may be to reverse the Chk1 induced DNA damage checkpoint following completion of DNA repair. There was no investigation into the level of DNA damage present in the cells that entered into mitosis following irradiation. And in the absence of PPM1D, the level of cells in mitosis was 85% reduced for up to 48 h only, therefore PPM1D inhibition did not completely prevent cells from entering into mitosis following irradiation.

Conversely, in 2006 Leung-Pineda *et al.* had several observations regarding Chk1 dephosphorylation in human cells. Human HeLa cells were treated with the genotoxic agent, hydroxyurea (HU), and then incubated with okadaic acid or fostriecin, both PP2A inhibitors. Using SDS-PAGE and Western blotting they demonstrated that treatment of cells with HU, followed by exposure

to either fostriecin or okadaic acid, blocked Chk1 dephosphorylation at Ser317/345. On the contrary, when cells were incubated with I-2, a PP1 inhibitor, Chk1 dephosphorylation was not blocked in cell extracts. Secondly Ser317/345-phosphorylated Chk1 accumulated in cells with PP2A levels reduced by siRNA treatment, and PP2A was able to directly dephosphorylate Chk1 on Ser317/345 *in vitro*. Collectively, these results argued that PP2A regulates Chk1 *in vivo*. However, Leung-Pineda *et al.* also speculated that PP2A might not be the sole phosphatase that dephosphorylates Chk1 and that PP4, PP6 and Wip1 may also have roles in the dephosphorylation of Chk1 at Ser317/345. This study provided supporting evidence that PP2A may be the protein phosphatase that dephosphorylates Ser317/345 of Chk1. However they did not investigate 1) any changes in cell cycle arrest or abrogation, 2) the number of cells that entered into mitosis following HU treatment or 3) the level of DNA damage present in cells. Therefore it was not clear as to whether PP2A inhibition was able to prevent cells from entering into mitosis with damaged DNA.

In 2010 Landsverk *et al.* demonstrated PNUTS (protein phosphatase 1 nuclear-targeting subunit), one of the most abundant nuclear-targeting subunits of PP1, played a distinct role in the DNA damage response by facilitating the dephosphorylation of Chk1 following DNA damage. In this study, the human cervical cancer cell line HeLa was exposed to radiation at a dose rate of 4.3 Gy/min and the phosphorylation of Ser317 and Ser345 of Chk1 was determined by Western blotting. They reported Chk1 remained phosphorylated at Ser317/345

for up to 24 h, following PNUTS depletion. They were also able to demonstrate that PNUTS overexpression inhibited a G2 arrest. These results indicated that the inhibition of PP1 activity was able to prevent the dephosphorylation of Ser317/345 of Chk1 following exposure to ionizing radiation for up to 24 h. However this study did not discuss if inhibition of PNUTS was able to prevent cells from entering into mitosis with damaged DNA.

Taken together, three protein phosphatases, PP1, PP2A, and PP2C (PPM1D or Wip1), have been proposed as candidates for Chk1 dephosphorylation at Ser317/345 in response to checkpoint activation. In order to determine if Chk1 dephosphorylation is responsible for priming checkpoint adaptation, inhibition of these candidate phosphatases, to prevent the dephosphorylation of Ser345-Chk1, will be essential in order to determine the effect of this inhibition on checkpoint adaptation.

1.6.5. Protein Phosphatase Inhibitors

The activity of protein phosphatases can be impaired by a number of inhibitors that have been reported to produce a wide range of physiological effects, such as tumor suppression or promotion (Cohen *et al*., 1990). Several of these phosphatase inhibitors were first identified as naturally occurring human toxins, and proved useful in identifying the physiological responses and processes regulated by serine/threonine protein phosphatases. The degree of the effects these inhibitors have is often determined by 1) the exposure duration and

concentration of the inhibitor; 2) the cell type exposed to the inhibitor and 3) the cell permeability of the inhibitor (Mackintosh and Mackintosh, 1994). One inhibitor can differentially inhibit several different protein phosphatases depending on inhibitor concentration (Pereira *et al*., 2011). In determining phosphatase inhibitors for the three candidate phosphatases for dephosphorylating Ser345 of Chk1 (PP1, PP2A, PP2C) the inhibitors for PP1 and PP2A are separated into three categories: 1) those inhibiting PP1 and PP2A with equal selectivity including microcystin-LR and calyculin A; 2) those inhibiting PP1 more potently than PP2A, including tautomycin and 3) those inhibiting PP2A more potently than PP1, including okadaic acid, cantharidin, nodularins and fostriecin. Phosphatase PP2C has one primary inhibitor called sanguinarine (Aburai *et al*., 2010). Phosphatase inhibitor specificity and efficiency, including each phosphatase IC50 as well as intracellular phosphatase inhibition concentrations is summarized in Table 1. Intracellular phosphatase inhibition concentrations have been included for an accurate determination of *in vivo* phosphatase inhibition efficiency.

The cell cycle is regulated through an intricate network of reversible phosphorylations by protein kinases and phosphatases. As such, inhibition of key processes in this regulation system, including both phosphorylation and dephosphorylation, can result in abnormalities in cell cycle functioning, such as checkpoint abrogation. Such abnormalities have been reported following cellular treatment with various phosphatase inhibitors. In particular, cantharidin, okadaic

Table 1 Serine/Threonine specific protein phosphatase inhibitors and their respective IC50 values and intracellular phosphatase inhibition concentrations					
Phosphatase Inhibitor	**PP1 IC50 (nM)**	**PP2A IC50 (nM)**	**PP2C IC50 (μM)**	**Intracellular Inhibition and Cell line**	
Okadaic Acid	1.24-60 (Pereira e*t al.*, 2011)	0.1-1 (Pereira e*t al.*, 2011)	-	PP2A- 0.25 μM E18 rat primary cortical neuron (Rudrabhatla *et* al., 2009)	PP2A- 500 nM Human lung fibroblasts MRC-5 cell line (Cairns *et* al., 1994)
Cantharidin	500-1700 (Pereira e*t al.*, 2011)	40-160 (Pereira e*t al.*, 2011)	-	PP2A- 20 μM Human lung fibroblasts MRC-5 cell line (Cairns *et* al., 1994)	PP2A- 0.2 μM Human endothelial cells (Loktionova & Kabakov, 1998)
Tautomycin	0.19-0.67 (Pereira e*t al.*, 2011)	0.94-10 (Pereira e*t al.*, 2011)	-	PP1- 10 μM Human breast cancer cell MCF7 cell line (Favre *et* al., 1997)	
Microcystin-LR	0.1-6 (Toivola and Eriksson, 1999)	0.04-2 (Toivola and Eriksson, 1999)	-	PP2A- 15 μM E18 Rat primary cortical neuron (Rudrabhatla *et al*., 2009)	
Calyculin A	0.3-2.5 (Pereira e*t al.*, 2011)	0.5-7.3 (Pereira e*t al.*, 2011)	-	Both PP1 and PP2A- 100 nM Human kidney cells HEK293 cell line (Spurney, 2001)	Both PP1 and PP2A- 10 nM Human breast cancer cell MCF7 cell line (Favre *et al*., 1997)
Nodularins	0.3-1.8 (Pereira e*t al.*, 2011)	0.03-0.2 (Pereira e*t al.*, 2011)	-	Phosphatase not specified- 2 μM Rat hepatocytes (Eriksson *et al*., 1990)	
Fostriecin	4000-131,000 (Pereira e*t al.*, 2011)	1.5-40 (Pereira e*t al.*, 2011)	-	PP2A- 1 μM E18 Rat primary cortical neuron	
Sodium Fluoride	2.1 mM (Stubbs *et al*., 2001)	40 mM (Fathi *et al*., 2002)	-	PP2A- 10 mM Human breast cancer PMC42 cell line (cell extract) (Li *et al*., 1997)	
Sanguinarine	-	-	2.38-2.62 μM (Aburai *et al*., 2010)	PP2C- 0.37 μM Promyelocytic Human leukemia HL60 cell line (Aburai *et al*., 2010)	
Note. IC50 determined by enzyme assay (*in vitro*)					

acid and fostriecin have been shown to cause cells to enter prematurely into mitosis, inducing cell death (Cheng *et al*., 1998; Sakoff *et al*., 2004; Bonness *et al*., 2006). In 1998 Cheng *et al.* treated Chinese hamster ovary cells (CHO) with the protein phosphatase inhibitors fostriecin, okadaic acid and cantharidin, all which resulted in a G2/M phase "mitotic block". CHO cell homogenates were treated with either: 10 µM fostriecin, 30 µM okadaic acid or 5 µM cantharidin, concentrations at which the protein phosphatase PP2A was inhibited, as determined by phosphatase activity assay. Following treatment, flow cytometry results indicated a dose dependent increase in 4N DNA content, suggesting that cell cycle arrest was occurring after DNA replication but prior to completion of anaphase. Light microscopy revealed that following phosphatase inhibitor treatment, the cells became rounded in appearance and were easily detached from the flask, a morphologic characteristic of cells in mitosis. Trypan blue exclusion revealed rounded cells were viable, and cells treated 18 h or less could recover when plated in fresh media without phosphatase inhibitors. However when treated for more than 24 h, cells did not recover and died by apoptosis. They were also able to determine that the "mitotic block" was associated with aberrant spindle apparatus formation and abnormal chromosome replication. This study was not in the scope of checkpoint adaptation as they did not treat cells with a DNA damaging agent.

1.7. Checkpoint Adaptation in Brain Cancer

Prior to this thesis work there were no published demonstrations of checkpoint adaptation in brain cancer cells. Therefore the demonstration of checkpoint adaptation in brain cancer cells would be a valuable contribution to the still limited knowledge we have on checkpoint adaptation.

1.7.1. Brain Cancer

Brain cancer is typically classified by cell origin and frequently on a grading system, based on aggressiveness and rate of growth. Clinically, the grade system ranges from Grade I (least malignant) to Grade IV (most malignant). A glioma is a type of tumor that starts in the brain or spine arising from glial cells (Kleihues and Sobin, 2000). An astrocytoma is a glioma that develops from star-shaped glial cells, called astrocytes, which support nerve cells. Astrocytomas are classified based on the grading system, with the glioblastoma also referred to as glioblastoma multiforme (GBM) being the most common grade IV astrocytoma in humans.

GBM is the most common primary malignant glioma in adults comprising about one-fifth of all adult primary brain tumours and is considered to be one of the deadliest human cancers (Alves *et al*., 2011). GBMs are characterized by a high proliferative rate, aggressive invasiveness and insensitivity to radio or chemotherapeutic treatments. They are also characterized with a high mortality rate and a short patient survival period of less than one year.

1.7.2. Standard Treatment for Glioblastoma

Treatment of GBM is difficult and has a poor success rate due to several complicating factors including tumor cell resistance to conventional therapies and limited chemical accessibility to the brain by the blood brain barrier (BBB) (Lawson *et al*., 2007). The brain is also highly susceptible to damage and has limited repair capabilities. Standard treatment for GBM generally consists of surgical resection for the majority of the tumor mass (when possible) followed by radiation therapy, chemotherapy or a combination of both (Kanu *et al*., 2009; Minniti *et al*., 2009). The goal of surgical resection is to remove as much of the tumour as possible. Whereas surgical removal is a mainstay of therapy, radiation and chemotherapy are often needed to address the microscopic tumour cells that may remain from the surgery or may have spread through nearby tissues.

A variety of chemotherapeutic drugs have been tested and used in chemotherapy treatment for GBM. Among the list of chemicals used in the treatment of primary brain tumours is CPT, as well as a number of CPT analogs including topotecan and irinotecan (Friedman *et al*., 1999; Raymond *et al*., 2002a; Raymond *et al*., 2002b; Feun and Savaraj, 2008). Although there have been several advances in primary brain tumour treatment, the efficacy of chemotherapeutic treatment is limited, given that few therapeutic agents have proven to be effective in treating brain tumours. This lack of effective treatment remains a significant challenge for clinicians and necessitates the development of new strategies for brain cancer therapy.

1.7.3. Significance for Investigating Checkpoint Adaptation in Brain Cancer

Although there has been a vast amount of research done exploring glioblastomas and even glioblastoma cells treated with DNA damaging agents, there were no published reports of checkpoint adaptation in cells from a primary brain tumor. Much of the work done with glioblastomas and DNA damaging agents has been in the context of exploring the initiation and control of the DNA damage checkpoint following exposure to radiation or chemotherapeutic agents (Fernet *et al*., 2008; Sorensen *et al*., 2010; Cheng *et al*., 2011).

There has been a report on the possibility of mitotic catastrophe occurring in glioblastoma cells exposed to a genotoxic agent. In 2001, a study was done exploring the anti-tumour potential of the DNA methylating agent temozolomide (TMZ) (Hirose *et al*., 2001). During this study a p53-deficient glioblastoma cell line, U87MG-E6, was treated with 100 µM TMZ, resulting in a G2/M phase arrest that was thereafter bypassed, ending in cell death. This result was reported to be mitotic catastrophe. However there was no investigation into the level of DNA damage present in the cells that entered into aberrant mitosis, therefore it cannot be confirmed whether these cells had undergone checkpoint adaptation.

M059K is one glioblastoma cell line commonly used to study the effects of genotoxic agents and the DNA damage checkpoint. The M059K cell line is a p53-deficient human fibroblastic glial cell line cultured from the glioblastoma of 33 year old male (Allalunis-Turner *et al*., 1993; Lees-Miller *et al*., 1995). This cell line is unique and a prominent candidate for research in DNA damaging

agents and the DNA damage checkpoint because M059K has a sister cell line called the M059J cell line. The M059J cell line was isolated from the same tumour; however, it is the only human cell line known to lack DNA-PK catalytic subunit (DNA-PKcs). Therefore it fails to produce the DNA-PK protein, a protein important in the DNA damage response. The inability of the M059J cell line to produce the DNA-PK protein, results in a decreased ability of this cell line to repair DNA DSBs and increased sensitivity to DNA damaging agents. This factor has resulted in the M059K and M059J cell lines becoming frequently used in cancer cell research studying the cellular response to genotoxic agents, such as radiation or chemotherapeutic agents. However, the general focus of research on the M059K cell line, when treated with DNA damaging agents, has often been resolved around progression through the cell cycle, as well as initiation and control of the G2/M checkpoint and DNA damage repair (Virsik-Kopp *et al*., 2003; Block *et al*., 2004; Liu *et al*., 2008; Dejmek *et al*., 2009). Prior to this thesis, there had been no demonstration of checkpoint adaptation in the M059K cell line or in any brain cancer cell line.

In 2003 it was reported that following exposure to low and high LET (linear energy transfer) radiation there was an increase in radiation induced-giant cells as well as cells displaying 'mitotic catastrophe', in the M059J cell line at 144 h post-irradiation (Holgersson *et al*., 2003). The detection of giant-cells and 'mitotic catastrophe' was based on morphological changes and the number of cells that displayed these two categories was scored out of 500 cells total. Taken

together the number of cells displaying either or both of these two categories increased from 0.7% at a background level to 4% for low (4 Gy) and 10% for high (1 and 4 Gy) LET radiation exposure. However, radiation-induced giant cells and 'mitotic catastrophe' was not detected in the M059K cell line. In 2005 Holgersson *et al.* reported that following exposure to high LET there was a substantial increase in the number of M059J and M059K cells in the G2/M phase arrest at 48 h post exposure. They also reported that following treatment with 10 or 40 μg/mL of the radiomimetic drug bleomycin, a large portion of both M059J and M059K cells remained in a G2/M phase arrest as detected by flow cytometry. They further reported that this distinct cell cycle block was released in both cell types but was dependent on the amount of LET exposure and the presence of DNA-PK. However, neither of these reports investigated the level of DNA damage present in cells and 'mitotic catastrophe' was determined based on morphological changes alone. Therefore it cannot be confirmed whether these cells had undergone checkpoint adaptation.

Investigation of checkpoint adaptation in cells from a glioblastoma would be a valuable contribution to the current body of knowledge regarding checkpoint adaptation for a number of reasons. First, the glioblastoma is one of the most common types of primary brain cancer, and the current standard of care includes genotoxic therapies with chemicals including CPT and analogs. Also, there is a need for improved brain cancer treatment. This is a result of the difficulty in successful treatment of brain tumors due to the relatively poor response to

standard genotoxic treatments, as well as the poor prognosis for survival following diagnosis. The demonstration of checkpoint adaptation in a glioblastoma would further broaden the base of our still limited core knowledge on this poorly understood process and strengthen the argument that checkpoint adaptation occurs universally in cancer cells following genotoxic treatment.

1.8. Overview of Thesis

Checkpoint adaptation is a poorly understood process defined by a cell entering into mitosis, after an extended G2/M checkpoint arrest, despite the presence of damaged DNA. Checkpoint adaptation was first demonstrated in human cancer cells in 2006, following treatment with radiation, a commonly used form of cancer treatment (Syljuåsen *et al*., 2006). Checkpoint adaptation occurs in three cancer cell lines including osteosarcoma, lymphocytic leukemia and colon carcinoma, following genotoxic treatment with radiation or CPT (Syljuåsen *et al*., 2006; Rezacova *et al*., 2011; Kubara *et al*., 2012). Checkpoint adaptation has also been suggested to occur in other cancer cell lines (Wang *et al*., 2008; Cahuzac *et al*., 2010; Rezacova *et al*., 2011). It was shown recently that the majority of cells that enter into mitosis with damaged DNA die, however approximately 2% of them survive and continue to divide and may have a highly rearranged genome (Kubara *et al*., 2012). The outcome for the cells that survive checkpoint adaptation is unknown, however further genome instability has been implicated, which may provide insight into cancer developments such as secondary tumours

and cancer cell resistance to genotoxic treatments (Nakada *et al*., 2006; Nitiss, 2009).

Prior to my thesis work, a demonstration of checkpoint adaptation in a glioblastoma cell line had not yet been done. Investigation into whether checkpoint adaptation occurs in cells from a glioblastoma would provide further evidence that this is a universal process in cells exposed to genotoxic agents. There have also been implications regarding Chk1 inactivation by dephosphorylation, as the potential mechanism or inciting event that allows for checkpoint adaptation to occur. Exploring the potential effects of inhibiting Chk1 dephosphorylation using phosphatase inhibitors, and what effect this has on checkpoint adaptation may provide a deeper insight into the molecular mechanism of checkpoint adaptation. There remains much to be discovered and understood regarding the biological process and significance of checkpoint adaptation. In the future we may find that either inhibiting or exploiting checkpoint adaptation may improve and/or change our current approaches to cancer treatments.

1.8.1. Research Objectives

The purpose of this research project was to determine if checkpoint adaptation occurs in the brain cancer cell line M059K when treated with the genotoxic anticancer agent CPT. It was also focused on determining how inhibition of Chk1 dephosphorylation at Ser345, using phosphatase inhibitors,

would affect the process of checkpoint adaptation. Several smaller objectives were addressed to achieve this purpose:

1. Introduce and characterize the brain cancer cell line M059K in the laboratory.

2. Determine a cytotoxic concentration of CPT for the brain cancer cell line M059K (Chapter 2).

3. Assess if M059K cells enter into an extended DNA damage induced G2 phase cell cycle arrest following treatment with CPT (Chapter 2).

4. Determine if cells overcome this G2 phase arrest and enter into mitosis with damaged DNA (Chapter 2).

5. Find the cytotoxicity of several candidate phosphatase inhibitors for the HT-29 cell line to determine treatment concentration (Chapter 3).

6. Determine if there is an effect on the number of cells that undergo checkpoint adaptation following treatment with candidate phosphatase inhibitors (Chapter 3).

Chapter 2

2. Checkpoint Adaptation in the M059K Cell Line

2.1. Introduction

In 2006, checkpoint adaptation was demonstrated in human osteosarcoma cells following treatment with radiation (Syljuåsen *et al*., 2006). This was the first time checkpoint adaptation was observed in human cells. A study published by the Golsteyn laboratory reported that checkpoint adaptation also occurs in human colon cancer cells treated with CPT or in cells treated with novel DNA alkyating agents (Kubara *et al*., 2012). For this research project, human brain cancer cells were tested to determine if they undergo checkpoint adaptation following treatment with CPT. Knowledge of this is important because gliomas are difficult to treat, and typically receive forms of treatment that have been shown to cause checkpoint adaptation in other cell lines, including CPT and radiation. To identify checkpoint adaptation in brain cancer cells, I followed the criteria established by Toczyski and colleagues (1997). The process of checkpoint adaptation is defined by three features in cells with damaged DNA: 1) cell cycle arrest, 2) entry into mitosis, and 3) damaged DNA in mitotic cells.

Damaged DNA in cells results in the activation of the DNA damage checkpoint (Kastan and Bartek, 2004; Bartek and Lukas, 2007). This checkpoint activation results in phosphorylation of the protein histone H2AX, which is then known as γ-H2AX (Rogakou *et al*., 1998). Gamma-H2AX is a standard marker

for the presence of damaged DNA (Bonner *et al*., 2008). Checkpoint activation also results in the phosphorylation of protein substrates, including Chk1 at Ser317/345, causing a G2 phase cell cycle arrest. In cells that undergo checkpoint adaptation, the G2 cell cycle arrest is abrogated and cells enter into mitosis despite having damaged DNA (Toczyski *et al*., 1997; Syljuåsen *et al*., 2006; Kubara *et al*., 2012). Entry into mitosis is characterized by morphological changes at cellular and nuclear levels, such as cell rounding and chromatin condensation (Harris, 1973). It is also characterized by biochemical changes such as phosphorylation of histone H3, which is a common marker of cells in mitosis (Hendzel *et al*., 1997).

Most of the cells that undergo checkpoint adaptation die; however, a small number of them survive (Kubara *et al*., 2012). It is not known whether the cells that survive checkpoint adaptation have a changed genome as one might expect if cells are dividing with damaged DNA. These experiments provided the opportunity to examine the outcome of cells that survived checkpoint adaptation for signs of genomic instability.

To investigate whether checkpoint adaptation occurs in brain cancer cells, and to determine if there is evidence of genomic change in surviving cells, the human brain cancer cell line M059K was studied. This cell line is from a human fibroblastic glioma and was chosen because it is frequently used in research on the DNA damage response, has a mutant p53 tumour suppressor status and is sensitive to CPT treatment (Virsik-Kopp *et al*., 2003; Holgersson *et al*., 2005; Li

and Stern, 2005; Mi *et al*., 2009). This thesis reports that following treatment with cytotoxic and pharmacologically relevant concentrations of CPT, that M059K cells enter a G2 checkpoint arrest. The arrested cells abrogate the checkpoint and enter into mitosis with damaged DNA. This thesis also reports that after treatment with CPT, M059K cells acquire micronuclei, a marker consistent with cells that have genomic change (Hovhannisyan *et al*., 2009). These findings suggest M059K cells undergo checkpoint adaptation and surviving cells have a rearranged genome.

2.2. Materials and Methods

2.2.1. Cell Culture

Human M059K cells (American Type Culture Collection) were maintained in Dulbecco's Modified Eagle medium- DMEM/F-12 (Invitrogen) supplemented with 10% decomplemented fetal calf serum (PAA Laboratories, Etobicoke, Ontario), 2 mM non-essential amino acids (Invitrogen) and 15 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.4. Cells were grown at 37°C in 5 % CO_2 and media were changed every second or third day. M059K cells were plated at 150,000 cells/25 cm^2 flask cultivated for 48 h prior to treatment. The compound CPT (Sigma) was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10 mM and stored at -20°C until further use.

2.2.2. Cytotoxicity Assay

Cytotoxicity of CPT on M059K cells was measured by the microculture tetrazolium assay (MTT; (3-(4,5)- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Mosmann, 1983). M059K cells were plated at 4,000 cells/31 mm^2 well and cultivated for 48 h prior to treatment. Results were expressed as IC50, the compound concentrations that reduced by 50% the absorbance at 590 nm, compared to DMSO treated cells. All measurements were done in triplicate at 48, 72 and 96 h.

2.2.3. Flow Cytometry

Cells were plated at 150,000 cells/25 cm^2 flask cultivated for 48 h prior to treatment. At desired times after treatment, total cell cultures were trypsinized, washed in phosphatase-buffered saline (PBS) and fixed in 90% ethanol (-20°C) for at least 24 h. Fixed cell suspensions were blocked for 1 h with labelling buffer (PBS, 5% serum, 1% bovine serum albumin (BSA), 0.1% sodium azide). Cells were incubated with anti-serine 10 phosphorylated histone H3 antibody for 2 h, then incubated with wash buffer twice for 20 min (No. 06 -570, Millipore, 1:100 dilution) and 30 min incubation with FITC-conjugated secondary antibody (No. SC-2012, Santa Cruz, 1:100 dilution) in labelling buffer. Cells were washed with wash buffer (PBS, 1% BSA, 0.1% sodium azide) and separated by centrifugation (Kubara *et al*., 2012). For analysis, samples were incubated for 20 min in wash buffer with 0.02 mg/mL propidium iodide (Invitrogen) and 0.2 mg/mL RNAse A

(Sigma), and analysed with a FACS Canto II™ flow cytometer (BD Biosciences) using BD FACSDiva™ software. Gating was set using control samples without primary antibody. Experiments were repeated at least twice.

2.2.4. Extract Preparation

Cells were suspended in extraction buffer (50 mM HEPES, 50 mM NaF, 10 mM EGTA (ethyleneglycol-bis (beta-aminoethylether)- *N*,*N'*-tetraacetic acid), 50 mM β-glycerophosphate, 1 mM ATP, 1 mM DTT (dithiothreitol), 1% Triton X-100, 10 μg/mL RNAse A, 0.4 U/mL DNAse I, with Roche protease inhibitors cocktail) at a concentration of 20,000 cells per uL, on ice for 30 min. After five passages through a 26 g needle at 4°C, the suspension was centrifuged at 12,000 x g for 10 min at 4°C (Kubara *et al*., 2012). Extracts were further used for electrophoresis after being boiled for 3 min in presence of 2x SDS sample buffer (20% Glycerol, 10% DTT, 6% SDS, 500mM Tris, pH 6.84).

2.2.5. Electrophoresis and Western Blotting

Cell extract samples were run on polyacrylamide gels. Protein loading was confirmed by Coomassie blue staining of gels run in parallel or by anti-actin Western blotting. Molecular weight markers (Precision Plus) were from BioRad. Proteins were wet transferred to nitrocellulose for 16 hours at 25 volts. Subsequently, the membrane was blocked with either 5% low fat milk in Tris buffered saline TBS-0.1% Tween-20 (TBST) or 5% BSA in TBS-0.1% Tween-20

(TBS-T), and incubated overnight with the primary antibody anti-Chk1 (SC-8408, Santa Cruz, 1:200), anti-phospho ser345 Chk1 (2341S, Cell Signalling, 1:1000), or anti-actin (SC-58673, Santa Cruz, 1:200). Following washes, the membrane was incubated with alkaline phosphatase-coupled anti-mouse IgG or anti-rabbit IgG for alkaline phosphatase (AP) detection (BioRad) (Kubara *et al*., 2012).

2.2.6. Light Microscopy

Cell images were taken with an Infinity 1.5 camera powered by Infinity Capture (Lumenera Corporation) software. Live cells were detected by vital dye exclusion method using Amresco® Trypan Blue 0.4% solution (VWR, Canada).

2.2.7. Immunofluorescence Microscopy

Cells were plated on glass coverslips at 25,000 cells/9.6cm^2 well and cultivated for 48 h prior to treatment, then fixed in 3% formaldehyde for 20 min at room temperature. Cells were permeabilized for 5 min in 0.2% Triton X-100 then incubated with either anti-histone gamma H2AX (No. 05-636, Millipore), or anti-serine 10 phosphorylated histone H3 (No. 06-570, Millipore), or anti-Lamin A/C (No. SC-6215, Santa Cruz Biotechnology) for 2 h at room temperature (Cahuzac *et al*., 2010). Secondary antibodies: Texas Red anti-rabbit (Jackson/Beckman Boulter) for anti-histone H3, Alexa488 anti-mouse (Molecular Probes/Invitrogen) for anti-histone gamma H2AX, and Alexa488 anti-goat (Molecular Probes/Invitrogen) for anti-Lamin A/C were added for 2 h. Nuclei were stained

with 300 nM 4',6-diamidino-2-phenylindole **(**DAPI) in PBS for 15 min prior to mounting (Kubara *et al*., 2012). Cells were observed on a Zeiss microscope operated by Axiovision 3.1 software. Images were collected by Zeiss MR camera within the linear dynamic range. Images were prepared with Adobe Photoshop CS3 10.0 software. Cells positive for phospho-histone H3, gamma-H2AX or Lamin A/C were counted. Experiments were repeated at least twice.

2.2.8. Statistics

Graphing was done using Microsoft Excel 2007. Data was collected and plotted as means ± standard deviations of the mean. GraphPad Prism 5 software was used to generate dose response curves and estimate IC50.

2.3. Results

2.3.1. Cytotoxic Concentrations of CPT in the M059K Cell Line

To assess whether M059K cells undergo checkpoint adaptation, a concentration of CPT with which to treat the cells was determined. M059K cells were treated with increasing concentrations of CPT and their viability was measured at 48, 72 and 96 h by the MTT assay (Mosmann, 1983). The IC50 for CPT treated cells was 19.95 nM at 48 h, 5.67 nM at 72 h and 2.78 nM at 96 h post treatment (Figure 3). These values were within the range of CPT and CPT derivatives found in human patient serum following cancer treatment (Rivory *et*

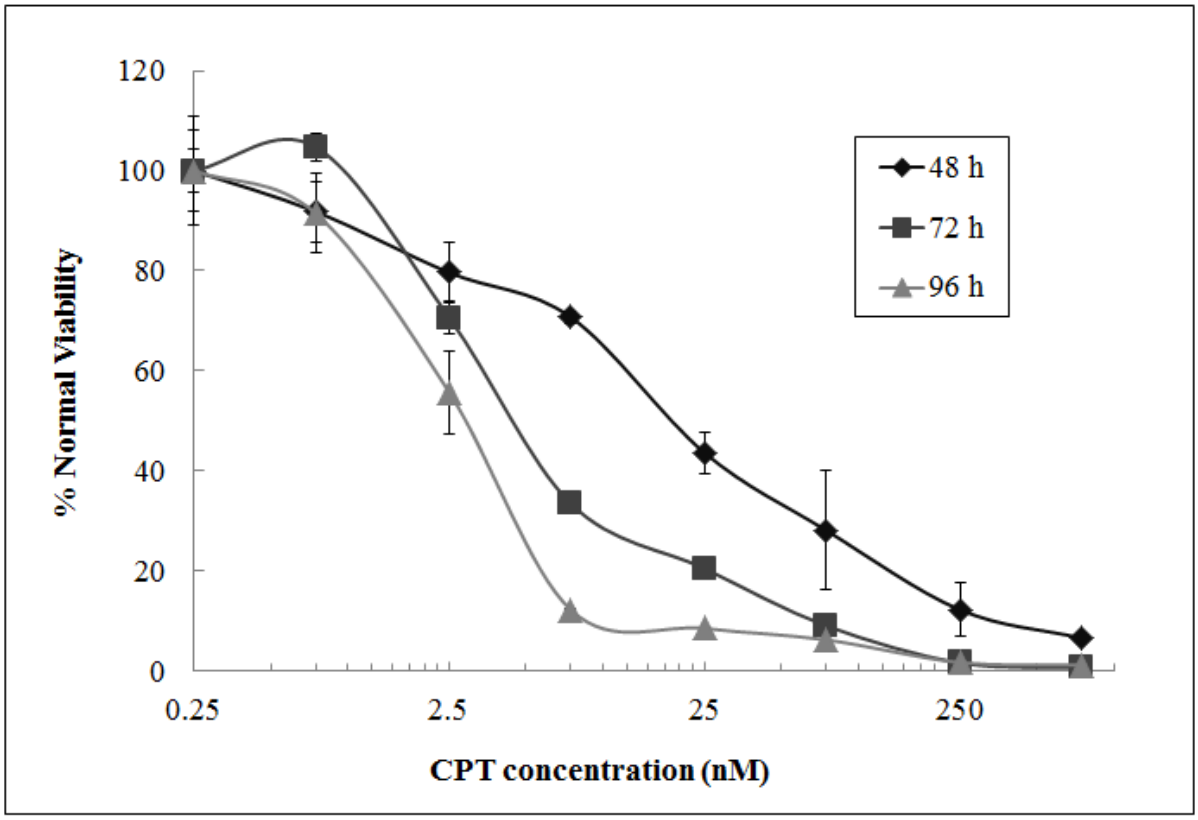


Figure 3. Cells treated with a cytotoxic concentration of CPT undergo a time dependent effect between 48 h and 96 h. M059K cells were treated with increasing concentrations of camptothecin (CPT) for 48 h (diamonds), 72 h (squares) and 96 h (triangles). Cell viability was measured by the MTT assay. The experiment was performed twice. Error bars represent standard deviation with each data point collected in triplicate.

al., 1997; Raymond *et al*., 2002b). Therefore a concentration of 25 nM was chosen for subsequent experiments because it is similar to human patient values, validating our pharmacological approach. Data from the MTT assay also revealed that at 48 h cells treated with 7.5 nM, and to a lesser extent 25 nM, were alive but would die by 96 h. The concentration of 7.5 nM provided a larger window at which the cells were viable prior to cell death. For this reason, a concentration of 7.5 nM CPT was also used in subsequent experiments. These data indicate that 25 nM and 7.5 nM are cytotoxic and pharmacologically relevant concentrations of CPT for the M059K cell line.

2.3.2. Cells Treated with CPT Become Arrested in the G2 Phase of the Cell Cycle

Having established that M059K cells are sensitive to pharmacologically relevant concentrations of CPT, next it was addressed whether they undergo checkpoint adaptation. First cells were examined by flow cytometry to measure DNA content, in order to detect a cell cycle arrest. Cells have one copy of DNA prior to DNA replication in S phase, denoted 2N DNA. Whereas cells that have undergone DNA replication contain 2 copies of DNA, denoted 4N DNA, until the two copies are separated in anaphase of mitosis at which point they have 2N DNA again. M059K cells were treated with 25 nM CPT for 24 h or 48 h then analyzed for DNA content by flow cytometry. At 24 h following treatment, cells had accumulated predominantly in S phase, as indicated by the accumulation of 2N DNA. By 48 h the cells accumulated in the G2 phase of the cell cycle, as

indicated by the accumulation of 4N DNA (Figure 4). The flow cytometry data indicated M059K cells were alive and arrested in the G2 phase of the cell cycle following treatment with a pharmacologically relevant and cytotoxic concentration of CPT.

2.3.3. Cell Treated with CPT Activate the DNA Damage Checkpoint

Typically cells that are arrested in the G2 phase of the cell cycle have a phosphorylated Chk1 at Ser317/345. Phosphorylation of Chk1 at Ser317/345 is a result of the activation of the DNA damage checkpoint. Therefore untreated and 7.5 nM CPT treated M059K cells were examined for the presence of Chk1 and phosphorylated Ser345-Chk1. These results were compared to untreated and treated HT-29 cells as a control. All cell extracts, both untreated and CPT treated, contained Chk1, and as predicted, CPT treated cells contained phosphorylated Ser345-Chk1 (Figure 5). These findings confirmed that M059K cells are sensitive to CPT at a pharmacologically relevant concentration. They also indicated that following treatment with CPT, M059K cells arrest in the cell cycle and contain activated Chk1.

2.3.4. Cells Treated with CPT Acquire a Rounded Shape

The next step was to determine if the cells that had become arrested in the G2 phase of the cell cycle would abrogate the cell cycle checkpoint and enter into

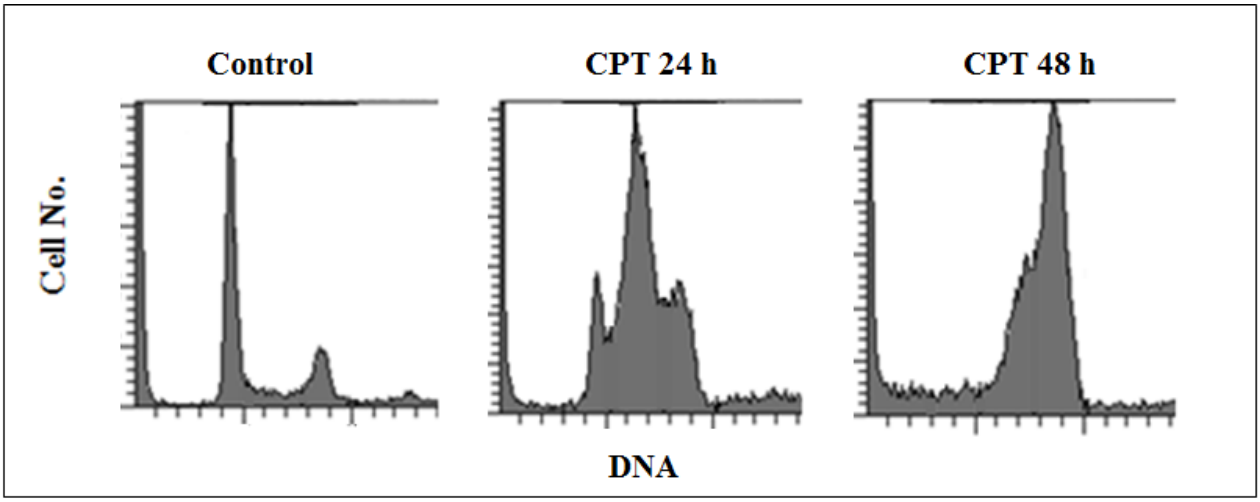


Figure 4. Cells treated with CPT are slowed through S phase and arrest in G2 phase of the cell cycle. M059K cells were either untreated or treated with 25 nM CPT for 24 h and 48 h. Samples were analyzed by flow cytometry for DNA content as determined by propidium iodide staining. The experiment was performed twice.

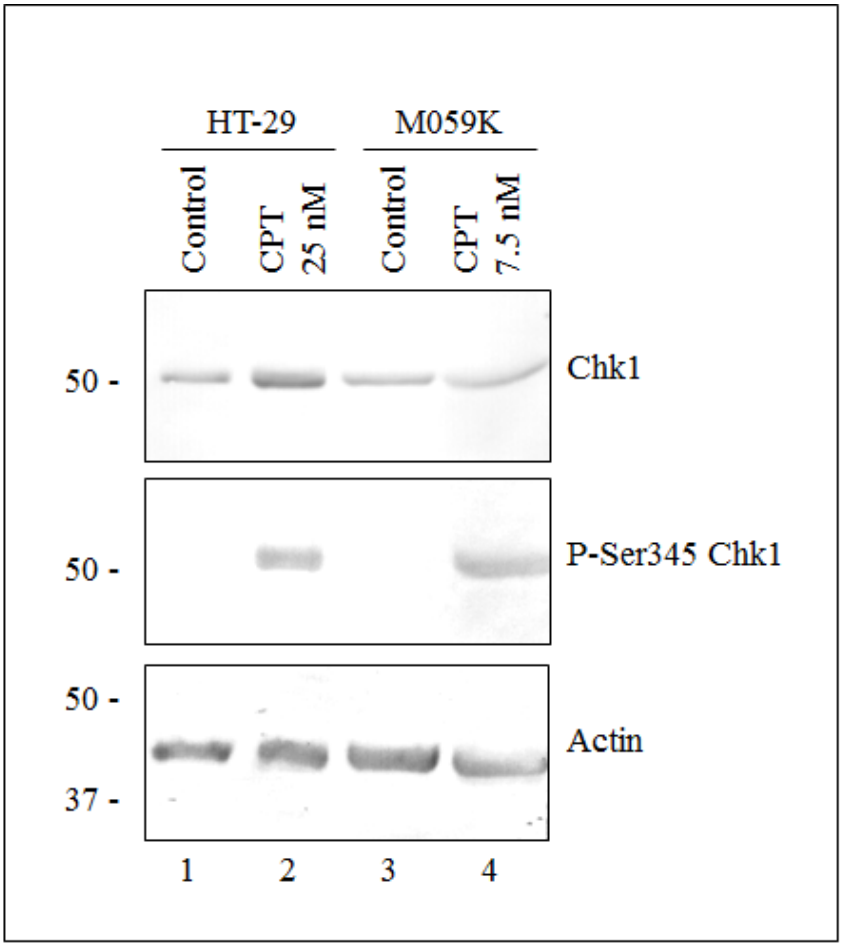


Figure 5. Cells treated with CPT contain phosphorylated-Ser345 Chk1. HT-29 cells were either untreated or treated with 25 nM CPT for 48 h. M059K cells were either untreated or treated with 7.5 nM CPT for 48 h. Extracts were prepared and analyzed by Western blotting with either Chk1, phospho-Ser345 Chk1 or actin antibodies. Positions of molecular weight markers (kD) are shown on left. The experiment was performed twice.

mitosis, the second defining feature of cells that have undergone checkpoint adaptation. M059K cells were treated with 25 nM CPT and observed by light microscopy for morphological changes, such as cell rounding. Following 24 h treatment, the majority of the cells were flat and strongly adherent to the flask; however at 48 h following treatment, cells became rounded (Figure 6). These rounded cells were weakly adherent and could be collected by a mechanical shake-off, leaving behind only flattened adherent cells. The remaining adherent culture was recultivated and observed. After 2 h more rounded cells appeared indicating that the flattened cells were the source of rounded cells and that this was a continuous process.

2.3.5. Cells Enter into Mitosis following the G2 Phase Checkpoint Arrest

The next step was to confirm that the rounded cells were in mitosis. Chromosomes in mitotic cells contain phospho-ser10 histone H3 therefore cells were examined for phospho-ser10 histone H3 by flow cytometry. Cells were either untreated, treated with 25 nM CPT for various times or treated with nocodazole for 24 h then stained for phospho-ser10 histone H3 and analyzed by flow cytometry. Nocodazole was used as a positive control as it interferes with microtubule polymerization resulting in cells that are blocked in mitosis. The number of cells positive for phospho-ser10 histone H3 in the untreated fraction was 1.5% and 7.0% in the nocodazole treated fraction. At 24 h CPT treatment the

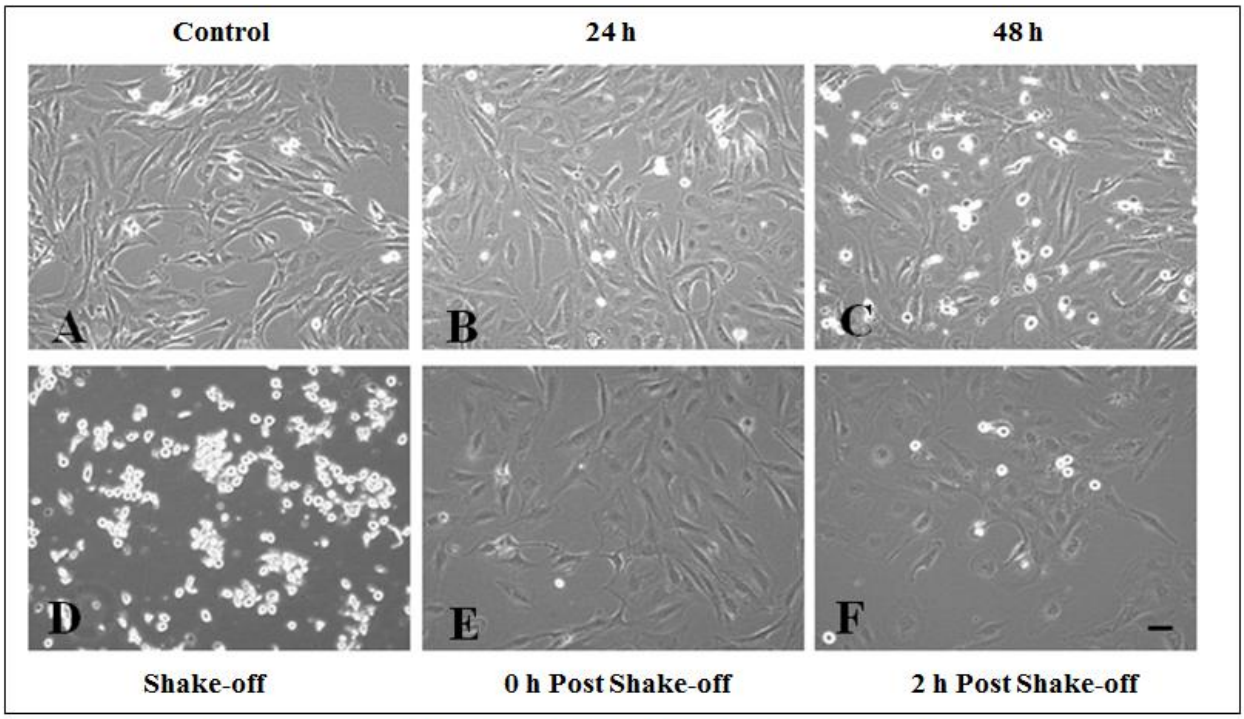


Figure 6. Rounded cells accumulate after 48 h treatment with CPT. M059K cells were either **A**) untreated or treated with 25 nM CPT for **B**) 24 h or **C**) 48 h and observed by phase contrast light microscopy. **D**) Rounded cells were collected by mechanical shake-off, leaving behind **E**) flattened interphase cells. **F**) Remaining interphase cells were observed at 2 h post shake-off at which time new rounded cells were present. The experiment was performed three times. Scale bar = 100 µm.

number of cells positive for phospho-ser10 histone H3 was 0.8%, which increased to 3.4% at 48 h, 4.9% at 72 h and 4.6% at 96 h (Figure 7). The results of this experiment demonstrated that in comparison to untreated cells or cells in the DNA damage checkpoint (24 h), there is an increase in the number of cells entering into mitosis following 48 -72 h treatment with 25 nM CPT.

2.3.6. Cells are in Mitosis with Damaged DNA

The third feature of checkpoint adaptation is entry into mitosis with damaged DNA. It was demonstrated that CPT treated M059K cells activated the DNA damage checkpoint, arrested in the G2 phase of the cell cycle, and subsequently entered into mitosis. Next was to test if the cells that entered into mitosis had damaged DNA. Cells were examined by immunofluorescence microscopy for both phospho-ser10 histone H3 as well as γ-histone H2AX. If cells were positive for phospho-ser10 histone H3 and positive for γ-histone H2AX following treatment with CPT for 48 h, then M059K cells were entering into mitosis with damaged DNA.

Cells were either untreated or treated with 7.5 nM CPT for 48 h then fixed and stained for either γ-histone H2AX or phospho-ser10 histone H3. Immunofluorescence showed 3% of the untreated population were positive for γ-histone H2AX, in comparison to 95% of the CPT treated population (Figure 8). In parallel, the number of cells positive for phospho-ser10 histone H3 was 5% in the untreated and 13% in the CPT treated population. With 95% of cells

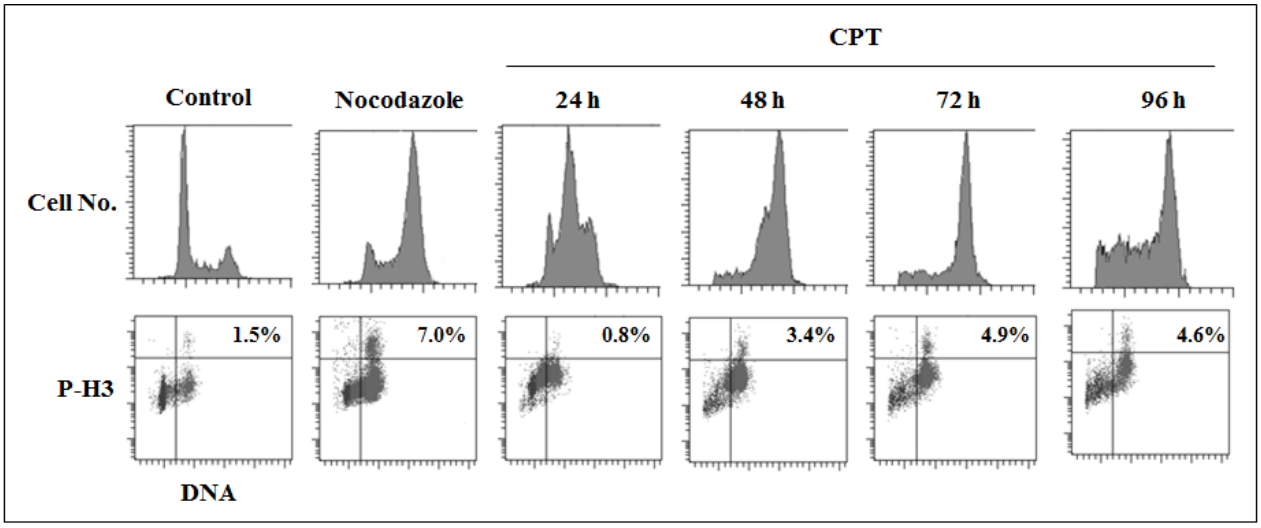


Figure 7. Mitotic cells accumulate after 48 h treatment with CPT. M059K cells were either untreated or treated with nocodazole or 25 nM CPT for 24, 48, 72, 96 h. Samples were analyzed by flow cytometry for DNA content and phospho-Ser10 histone H3 signals. The percentage of cells positive for phospho-Ser10 histone H3 is listed in the upper right quadrant. DNA content was determined by propidium iodide staining. Each experiment was performed twice.

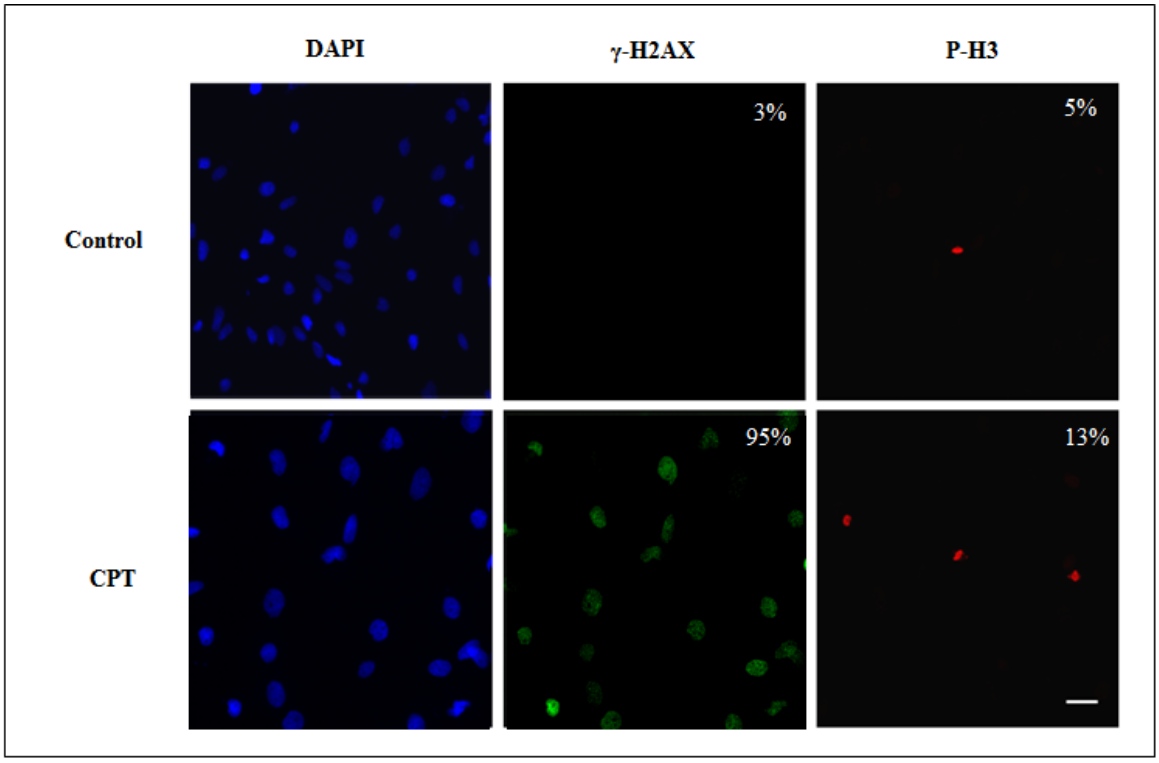


Figure 8. Cells that are in mitosis after 48 h treatment with CPT contain damaged DNA. Cells were either untreated (top panels) or treated with 7.5 nM CPT for 48 h (bottom panels) and stained with DAPI (left panels), γ-histone H2AX (center panels) or phospho-Ser10 histone H3 (right panels). Cells were observed by immunofluorescence microscopy. Percent of cells positive for staining condition was determined and is shown in the top right corner of panel. Scale bar = 100 μm. The experiment was performed twice.

containing damaged DNA following CPT treatment and 13% of these cells in mitosis, these data indicate that a minimum of 8% of the treated cells that are in mitosis have damaged DNA. Another feature of mitotic cells is chromosome condensation, which can be visualized by immunofluorescence microscopy. It was observed that of the cells with a condensed 4',6-diamidino-2-phenylindole (DAPI) nuclear stain, 94% of them were also positive for γ-histone H2AX (Figure 9).

Together, this data suggest that M059K cells treated with pharmacologically relevant concentrations of CPT: 1) activate the DNA damage checkpoint and become arrested in the G2 phase of the cell cycle, 2) enter into mitosis, and 3) contain damaged DNA; suggesting that M059K cells undergo checkpoint adaptation when treated with CPT.

2.3.7. Cells that Survive Mitosis following Treatment with CPT Acquire Micronuclei

This thesis demonstrated that M059K cells fulfilled the three features that define checkpoint adaptation when exposed to CPT. The next step was to address if there was evidence of genomic change in the cells that survive checkpoint adaptation by looking for micronuclei. A micronucleus appears as a small membrane-bound nucleus that forms due to improper incorporation of acentric fragments or chromosomes into daughter nuclei during cell division (Fenech *et al*., 2011). Micronuclei are commonly used as evidence of chromosome and/or genome

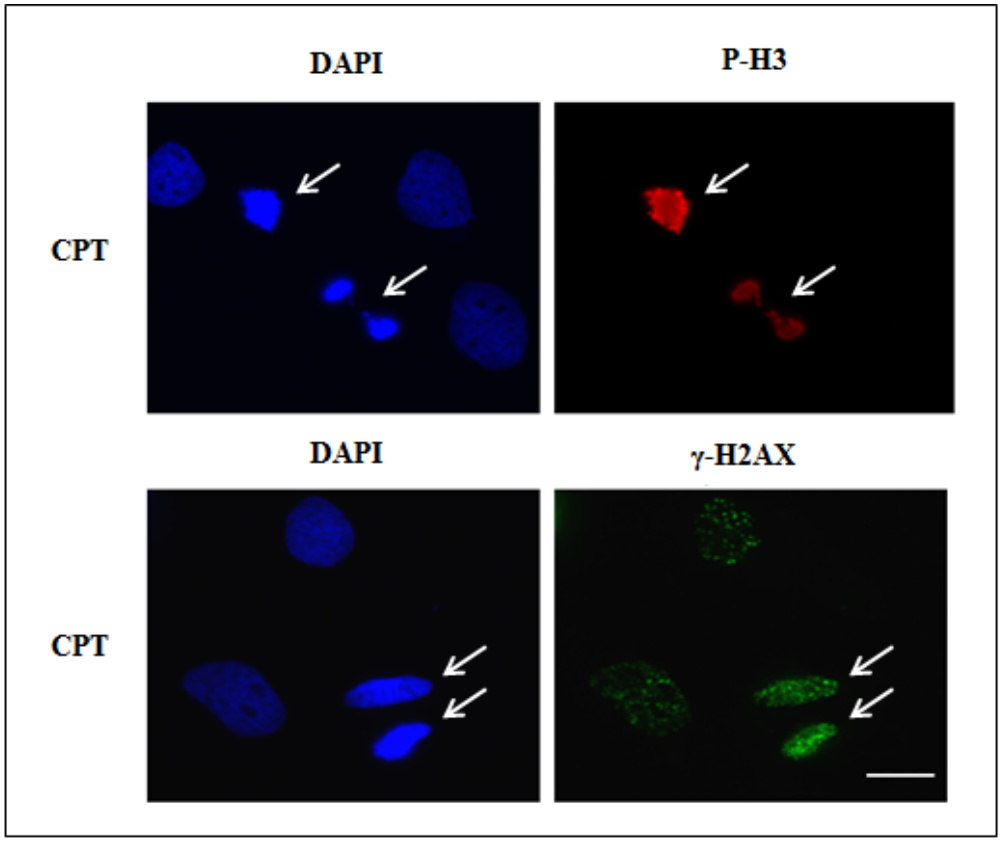


Figure 9. Chromosomes of cells that are in mitosis after CPT treatment are positive for gamma-histone H2AX staining. Cells were treated with 7.5 nM CPT for 48 h and stained with DAPI (left panels), γ-histone H2AX (bottom right) or phospho-Ser10 histone H3 (top right). Cells were observed by immunofluorescence microscopy. Arrows indicate cells in mitosis. Scale bar = 100 μm. The experiment was performed twice.

mutations caused by damaged DNA (Hovhannisyan *et al*., 2009). An experiment was designed to look for M059K cells that survive checkpoint adaptation then examined these survivors for evidence of micronuclei. Cells were either untreated or treated with 25 nM CPT over a period of 120 h. The cells that survived CPT treatment were observed through phase contrast light microscopy. I found that M059K surviving cells do contain micronuclei (Figure 10A). In the untreated population, 1% of cells contained micronuclei. Cells in the CPT treated population acquired one or more micronuclei increasingly over time with a total of 48% of the population containing micronuclei at 120 h (Figure 10B). These data provide evidence that the cells that undergo checkpoint adaptation do have a rearranged genome.

To confirm the observation of micronuclei by phase contrast light microscopy, cells were examined for a component of the nuclear membrane, lamin A/C. Lamin A/C is a major protein of the nuclear lamina, therefore nuclei can be visualized by detecting this protein using immunofluorescence microscopy (Tanaka and Shimizu, 2000). Cells were either untreated or treated with 25 nM CPT for 120 h then fixed and stained for lamin A/C, and observed by immunofluorescence microscopy. Cells that were lamin A/C positive for micronuclei were counted. In the untreated population 5% of cells were positive for micronuclei, whereas 47% were positive for micronuclei in the treated cells (Figure 11A and 11B). These data provided evidence that some cells survive

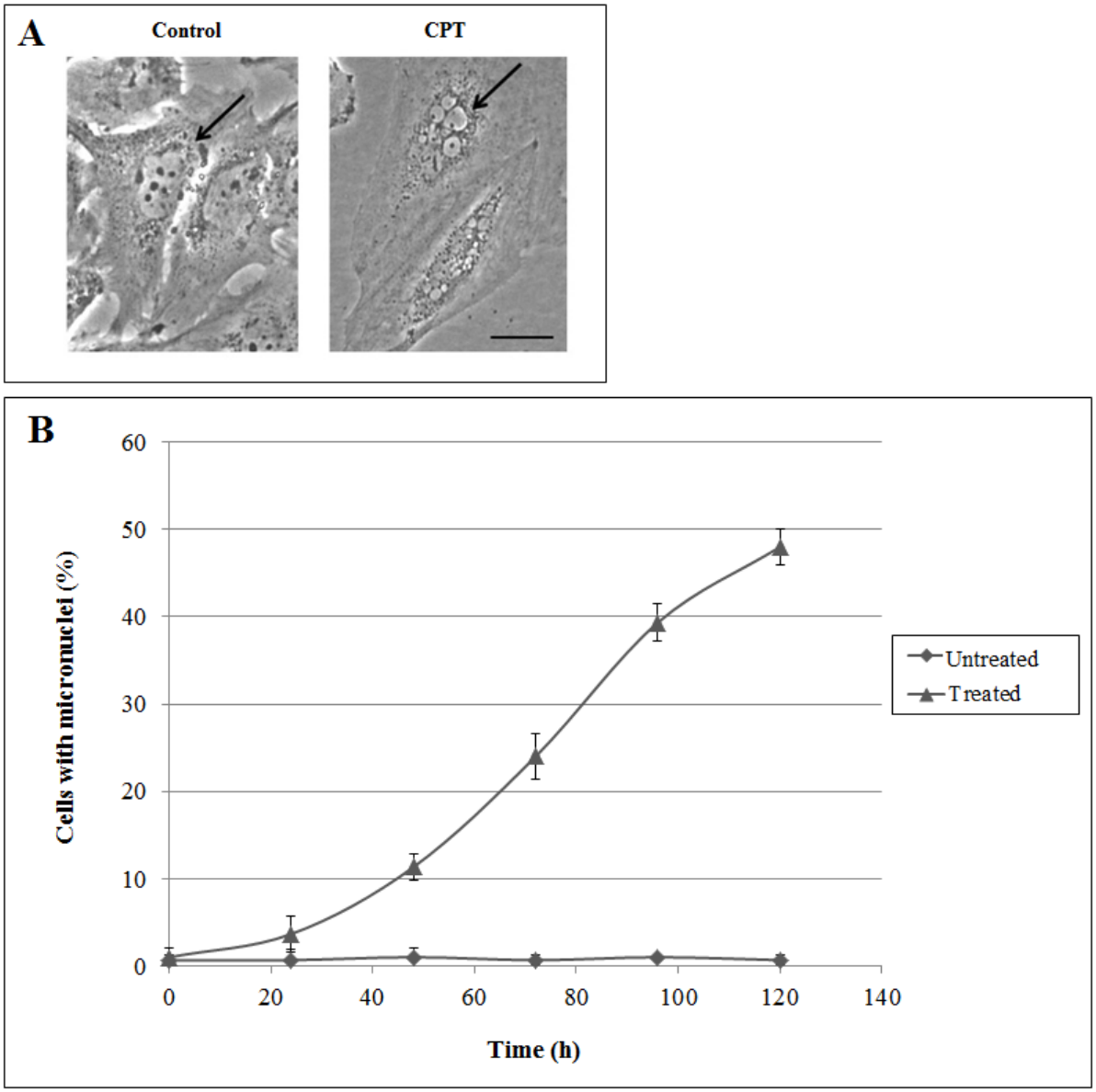
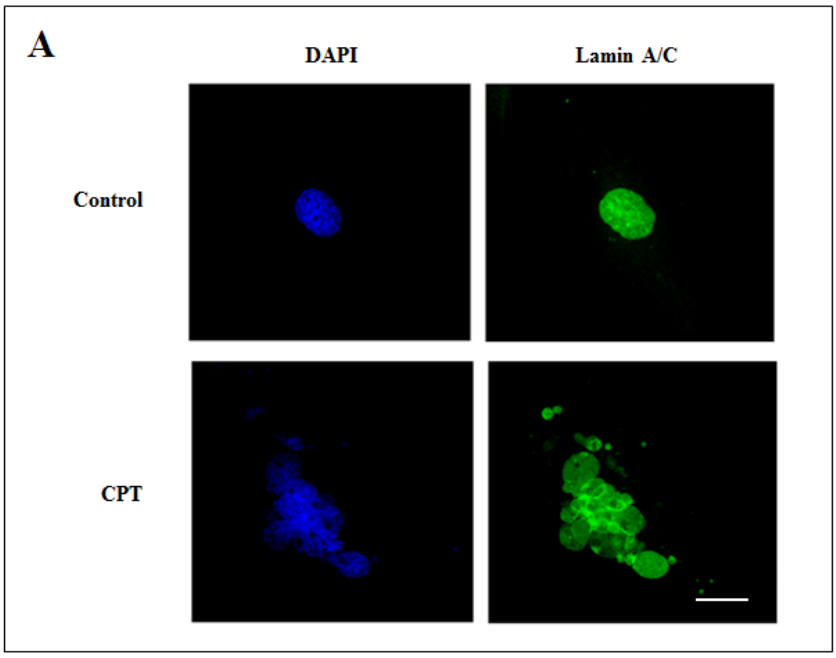


Figure 10A and 10B. Cells that survive mitosis following treatment with CPT acquire micronuclei. M059K cells were either untreated (left) or treated with 25 nM CPT (right) and observed by phase contrast light microscopy. **A**) Arrows indicate nuclei. Scale bar = 100 µM. **B**) The average percent of micronucleated cells of the total population was determined every 24 h for 120 h. The experiment was performed three times. Error bars represent standard deviation with each data point collected in triplicate.



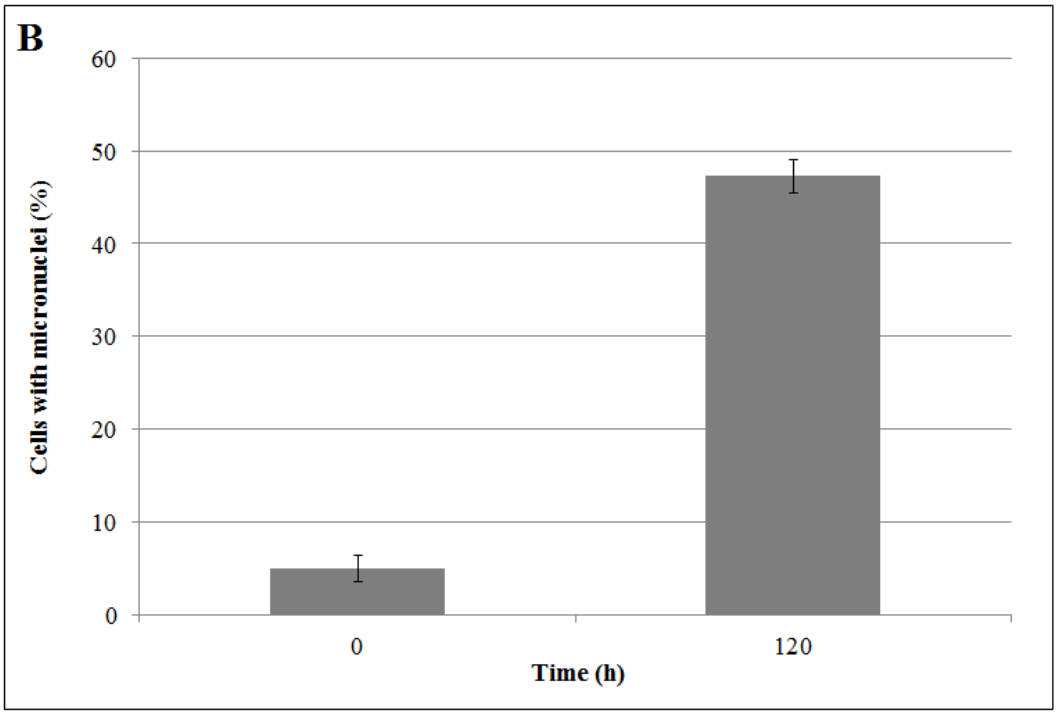


Figure 11A and 11B. Cells that survive mitosis following CPT treatment contain micronuclei that are positive for lamin A/C staining. M059K cells were either untreated or treated with 25 nM CPT for 120 h. **A**) Cells were stained with DAPI (left) and for lamin A/C (right). Images were collected by immunofluorescence microscopy. Scale bar = 100 µm. **B**) The average number of cells positive for lamin A/C micronuclei was counted. The experiment was performed three times. Error bars represent standard deviation with each data point collected in triplicate.

checkpoint adaptation and the surviving cells often contain micronuclei, which is a characteristic of genomic rearrangement.

2.4. Discussion

Checkpoint adaptation is characterized by an arrest in the cell cycle, overcoming this arrest and then entering into mitosis despite the presence of damaged DNA (Toczyski *et al*., 1997). Checkpoint adaptation has been demonstrated in a small number of human cancer cell lines including human bone, lymph and colon cancer following treatment with radiation or CPT (Syljuåsen *et al*., 2006; Rezacova *et al*., 2011; Kubara *et al*., 2012). This thesis addressed whether checkpoint adaptation occurs in brain cancer cells, specifically the brain cancer cell line M059K.

Brain cancer cells were chosen because checkpoint adaptation had not yet been reported in brain cancer cells. There have however been reports on the possibility of mitotic catastrophe and mitotic slippage in cells from gliomas treated with genotoxic agents (Hirose *et al*., 2001; Holgersson *et al*., 2003; Riffell *et al*., 2009). The glioblastoma also represents one of the deadliest forms of cancer and is difficult to treat. And it has been reported to be sensitive to, and is treated using, the genotoxic agent CPT (Friedman *et al*., 1999; Raymond *et al*., 2002b; Feun and Savaraj, 2008). This research would also broaden the limited base of cell origins for which checkpoint adaptation has been reported.

The brain cancer cell line M059K was chosen because this is a well characterized cell line from a glioblastoma. It is p53 deficient and has a sister cell line M059J, which lacks the DNA-PK catalytic subunit, both factors have made this a commonly researched cell line when exploring the DNA damage response (Virsik-Kopp *et al*., 2003; Block *et al*., 2004; Liu *et al*., 2008; Dejmek *et al*., 2009). Prior to this thesis work, there had been no reports of checkpoint adaptation in the M059K cell line. There have however been demonstrations of M059K cells accumulating in a G2/M phase arrest following exposure to radiation or the radiomimetic drug bleomycin (Holgersson *et al*., 2003; Holgersson *et al*., 2005). This G2/M phase arrest was reported to be followed by a release from the G2/M block at 48 h, as determined by flow cytometry. There was no exploration into the level of damaged DNA in cells that were released from this block. However it was suggested that the M059J cell line may have undergone mitotic catastrophe, which was not detected in the M059K cell line. Another reason the M059K cell line was chosen is because it is a fibroblastic cancer cell, which could bridge the link between reports of checkpoint adaptation in cancer cells with the report suggesting checkpoint adaptation occurred in a non-cancerous fibroblast (Rezacova *et al*., 2011). This would provide the opportunity to compare the effects of checkpoint adaptation in cancerous and non-cancerous cells when exposed to DNA damaging agents.

To examine whether checkpoint adaptation occurs in brain cancer, the M059K brain cancer cell line was first introduced and characterized in our

laboratory. Next, a concentration of the anticancer agent CPT was determined that M059K cells were sensitive to and that was also pharmacologically relevant. The effects of CPT treatment on the M059K cells were tested next. The findings of this thesis research suggest that M059K cells do undergo checkpoint adaptation when treated with CPT, as they fulfil the three features that define checkpoint adaptation. These data support that checkpoint adaptation is a universal characteristic in human cancer cells when treated with genotoxic agents. One limitation of this research was the inability to look at a single cell for both markers of damaged DNA and mitosis. In the future to address this, co-staining of cells with γ-histone H2AX and phospho-histone H3 may be done then observed by confocal microscopy that would allow for observation of signals from both antibodies in a single cell (Suzuki *et al*., 1997). This thesis also reported the cells that survive checkpoint adaptation acquire micronuclei. The presence of micronuclei in M059K cells following treatment, confirms these cells are entering mitosis with damaged DNA and the cells that survive mitosis, have a rearranged genome.

In 2012, Crasta *et al*. suggested errors in mitosis can generate DNA breaks or chromosome pulverization followed by chromosome rearrangement, a phenomenon called chromothripsis (Crasta *et al*., 2012). In this study both processes, DNA breakage and chromothripsis, resulted in the formation of cells containing micronuclei. They also noted the DNA rearrangements and mutations acquired in micronuclei could be incorporated into the cellular genome, which

may result in cancer development. This paper did not address checkpoint adaptation as a means for cellular entry into mitosis with damaged DNA. But it could be speculated that if additional DNA breaks and whole genome rearrangements can occur from entry into mitosis with damaged DNA, then checkpoint adaptation may be the cause of this and may have grave implications on the genome of daughter cells and further cancer developments.

Studies have established that nearly all the cells that undergo checkpoint adaptation eventually die (Syljuåsen *et al*., 2006; Kubara *et al*., 2012). One study suggested that perhaps checkpoint adaptation is a biochemical pathway for cell death, distinct from apoptosis, whose biological significance is to eradicate cells that have irreparable damaged DNA (Vitale *et al*., 2011). This argument must be considered. However a study in 2012 reported that up to 2% of the cells that enter into mitosis with damaged DNA survive (Kubara *et al*., 2012). The results of this thesis work suggest that these surviving cells contain micronuclei, indicating genomic rearrangement has occurred. What becomes of the cells that survive checkpoint adaptation with rearranged genomes remains unknown. However, the demonstration of micronuclei in surviving cells supports the prediction that cells surviving genotoxic treatments may be the source of tumour cells with major genomic rearrangements (Nakada *et al*., 2006; Nitiss, 2009).

Overall, these data suggest M059K cells do undergo checkpoint adaptation when exposed to CPT, and that the cells that survive checkpoint adaptation have a rearranged genome. We propose that checkpoint adaptation is a universal process

in cancer cells when treated with genotoxic agents, and the cells that survive checkpoint adaptation may be the source of secondary tumours. This may also provide a potential explanation for chemoresistance of cancer and/or cancer relapse following treatment. In the future it would be advantageous to explore the structure, life cycle and genome of surviving cells, to better understand what becomes of these cells. It would be advantageous to look deeper into the level of DNA damage in surviving cells, which could potentially be done through assays such as the comet assay. We could also look at the level of genome rearrangement in the surviving cells with techniques such as with karyotyping. It would also be advantageous to explore whether we can exploit or prevent checkpoint adaptation from occurring. Preliminary results indicate there may be chemical compounds that kill cells undergoing checkpoint adaptation more efficiently while they are in mitosis. This would diminish the number of cells that survive checkpoint adaptation with rearranged genomes. It may also be advantageous to see if we can prevent checkpoint adaptation from occurring by either inhibiting or eliminating biochemical pathways that allow or initiate checkpoint adaptation. A broader understanding of checkpoint adaptation may have long term significance in improving or rerouting the current course of cancer treatment.

Chapter 3

3. The Effect of Phosphatase Inhibition upon Cells Treated with Genotoxic Agents

3.1. Introduction

The process of checkpoint adaptation is defined by three features in cells with damaged DNA: 1) cell cycle arrest, 2) entry into mitosis, 3) damaged DNA in mitotic cells (Toczyski *et al*., 1997). In 2006, checkpoint adaptation was demonstrated in human osteosarcoma cells following treatment with radiation (Syljuåsen *et al*., 2006). Checkpoint adaptation has since been demonstrated in the human colon carcinoma cell line HT-29 and lymphocytic leukemia cell line MOLT4, when treated with CPT and radiation (Rezacova *et al*., 2011; Kubara *et al*., 2012). These data along with data in the brain cancer cell model suggest that checkpoint adaptation might be a common biological event in cancer cells. The precise mechanism by which checkpoint adaptation occurs is unknown (Clemenson and Marsolier-Kergoat, 2009).

Studies have shown that Chk1 is dephosphorylated at Ser317/345 in cells that have undergone checkpoint adaptation (Syljuåsen *et al*., 2006; Kubara *et al*., 2012). The phosphatase that dephosphorylates Ser317/345 of Chk1 is not known; however PP2A, PP1 and PP2C have been implicated as candidates for this dephosphorylation (den Elzen and O'Connell, 2004; Lu *et al*., 2005; Leung-Pineda *et al*., 2006; Petersen *et al*., 2006; Landsverk *et al*., 2010). I hypothesize that dephosphorylation of Chk1 at Ser345 is the mechanism that allows

checkpoint adaptation to occur. To test this hypothesis the HT-29 model, described in Kubara *et al*., 2012, was used. Briefly, cells were treated with CPT for 48 h to induce damaged DNA, a time that cells would be undergoing checkpoint adaptation. Cells were then treated with phosphatase inhibitors, to inhibit candidate phosphatases. Cells were then analyzed for markers of checkpoint adaptation. If Chk1 dephosphorylation at Ser345 is essential for cells to undergo checkpoint adaptation, then inhibiting this dephosphorylation should prevent checkpoint adaptation.

Contrary to my hypothesis, this thesis reports that following treatment with CPT and a cytotoxic concentration of the potent PP2A inhibitor cantharidin, HT-29 cells became rounded and entered into mitosis, in a greater proportion than cells treated with CPT alone.

3.2. Materials and Methods

3.2.1. Cell Culture

Human HT-29 cells (American Type Culture Collection) were maintained in RPMI 1640 (Invitrogen) medium supplemented with 10% decomplemented fetal calf serum (PAA Laboratories, Etobicoke, Ontario), 2 mM L-glutamine (Invitrogen), and 10mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.4. Cells were grown at 37°C in 5 % CO_2 and media were changed every second or third day. HT-29 cells were plated at 500,000 cells/25 cm^2 flask

and cultivated for 48 h prior to treatment. The compound camptothecin (Sigma) was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10 mM and stored at -20°C until further use. The phosphatase inhibitors cantharidin, calyculin A, cyclosporin and okadaic acid were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 1 mM, 0.5 mM or 0.1 mM, respectively and stored at -20°C until use.

3.2.2. Cytotoxicity Assay

Cytotoxicity of each phosphatase inhibitor on HT-29 cells was measured by the microculture tetrazolium assay (MTT; (3-(4,5)- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Mosmann, 1983). HT-29 cells were plated at 7,000 cells/31 mm^2 well and cultivated for 48 h prior to treatment. Results were expressed as IC50, the compound concentrations that reduced by 50% the absorbance at 590 nm, compared to DMSO treated cells. All measurements were done in triplicate at 24 h.

3.2.3. Light Microscopy

Cell images were taken with an Infinity 1.5 camera powered by Infinity Capture (Lumenera Corporation) software. Live cells were detected by vital dye exclusion method using Amresco® Trypan Blue 0.4% solution (VWR, Canada).

3.2.4. Immunofluorescence Microscopy

Cells were plated on glass coverslips at 50,000 cells/9.6cm^2 well and cultivated for 48 h prior to treatment, then fixed in 3% formaldehyde for 20 min at room temperature. Cells were permeabilized for 5 min in 0.2% Triton X-100 then incubated with either anti-histone gamma H2AX (No. 05-636, Millipore), or anti-serine 10 phosphorylated histone H3 (No. 06-570, Millipore). The secondary antibody Texas Red anti-rabbit (Jackson/Beckman Boulter) for anti-histone H3 was added for 2 h. Nuclei were stained with 300 nM 4',6-diamidino-2-phenylindole **(**DAPI) in PBS for 15 min prior to mounting (Kubara *et al*., 2012). Cells were observed on a Zeiss microscope operated by Axiovision 3.1 software. Images were collected by Zeiss MR camera within the linear dynamic range. Images were prepared with Adobe Photoshop CS3 10.0 software. Cells positive for phospho-histone H3 were counted. Experiments were repeated at least twice.

3.2.5. Statistics

Graphing was done using Microsoft Excel 2007. Data was collected and plotted as means ± standard deviations of the mean. GraphPad Prism 5 software was used to generate dose response curves and estimate IC50.

3.3. Results

3.3.1. Cytotoxicity of Candidate Phosphatase Inhibitors in HT-29 Cells

To assess if the inhibition of phosphatases could affect checkpoint adaptation, first a cytotoxic concentrations of candidate phosphatase inhibitors was determined in HT-29 cells by the MTT assay. This information provided a concentration with which to treat cells without killing them during the course of the experiment. The MTT cytotoxicity assay was performed on HT-29 cells using the following phosphatase inhibitors: cantharidin, sodium fluoride and okadaic acid to inhibit PP2A; calyculin A to inhibit PP1 and PP2A; cyclosporin to inhibit PP2B. Based on the MTT assay results, IC50 values were determined for each phosphatase inhibitor: cantharidin 1.9 μM, calyculin A 3.5 nM, sodium fluoride 3.6 mM, okadaic acid 37 nM, cyclosporin 0.4 μM (Figure 12). Based on these data, concentrations of 2.5 μM cantharidin and 5 nM calyculin A were used for subsequent experiments. Due to time constraints, no further experiments were conducted on the effects of sodium fluoride, okadaic acid or cyclosporin treatment on checkpoint adaptation.

3.3.2. HT-29 Cells Co-treated with CPT and Cantharidin or CPT and Calyculin A Acquire a Rounded Shape

To assess if the inhibition of phosphatases could affect checkpoint adaptation, HT-29 cells were co-treated with CPT and phosphatase inhibitors. HT-29 cells were either untreated or treated with 25 nM CPT for 48 h to induce checkpoint adaptation. The rounded cells were collected and removed by a

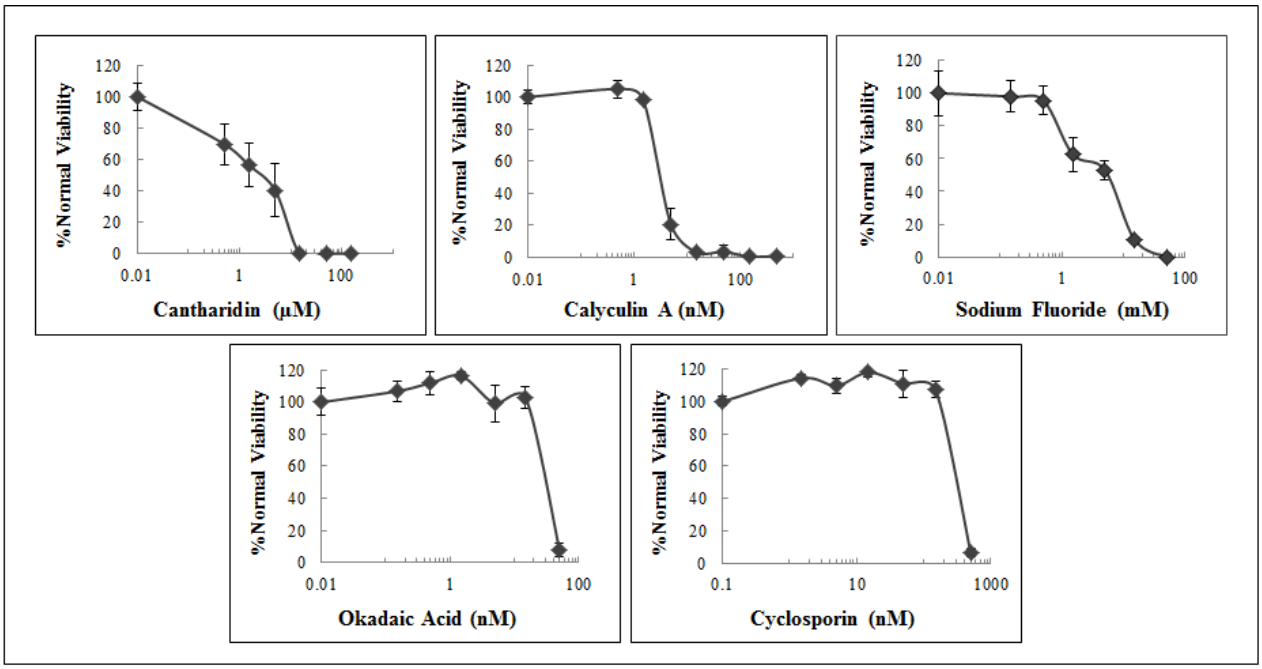


Figure 12. Cytotoxic concentrations for phosphatase inhibitors. HT-29 cells were incubated with increasing concentrations of cantharidin, calyculin A, sodium fluoride, cyclosporin, okadaic acid. Cell viability was measured by the MTT assay and an IC50 was calculated for each compound: cantharidin 1.9 µM, calyculin A 3.5 nM, sodium fluoride 3.7 mM, okadiac acid 37 nM, cyclosporin 0.4 µM. Each experiment was performed at least twice. Error bars represent standard deviation with each data point collected in triplicate.

mechanical shake-off, leaving behind only interphase cells. The interphase cells were the test group because they would undergo checkpoint adaptation during the course of the experiment. The interphase cells were either treated with DMSO (as a control) or 2.5 μM cantharidin. After 2 h, a time sufficient for new rounded cells to appear, the culture was observed by light microscopy. Interestingly, in the group co-treated with CPT and cantharidin, 95% of the cells became uniformly rounded. In parallel, the untreated population had 2.6% rounded cells whereas the CPT alone treated cells had 21%. The cells treated with cantharidin alone were not uniformly rounded, but appeared to be irregular in shape (Figure 13).

The next step was to test the effect of treatment with calyculin A on checkpoint adaptation. HT-29 cells were either untreated or treated with 25 nM CPT for 48 h to induce checkpoint adaptation. The rounded cells were collected and removed by a mechanical shake-off, leaving behind only interphase cells. The interphase cells were either treated with DMSO or 5 nM calyculin A. After 3 h, the culture was observed by light microscopy. In the group co-treated with CPT and calyculin A, 100% of the cells were uniformly rounded. In parallel, only 1.2% in the untreated population and 22% in the CPT alone treated population were rounded (Figure 14). In contrast to my predicted result of blocking checkpoint adaptation, two phosphatase inhibitors were identified that increased the number of rounded cells in a culture that was first treated with CPT. Further

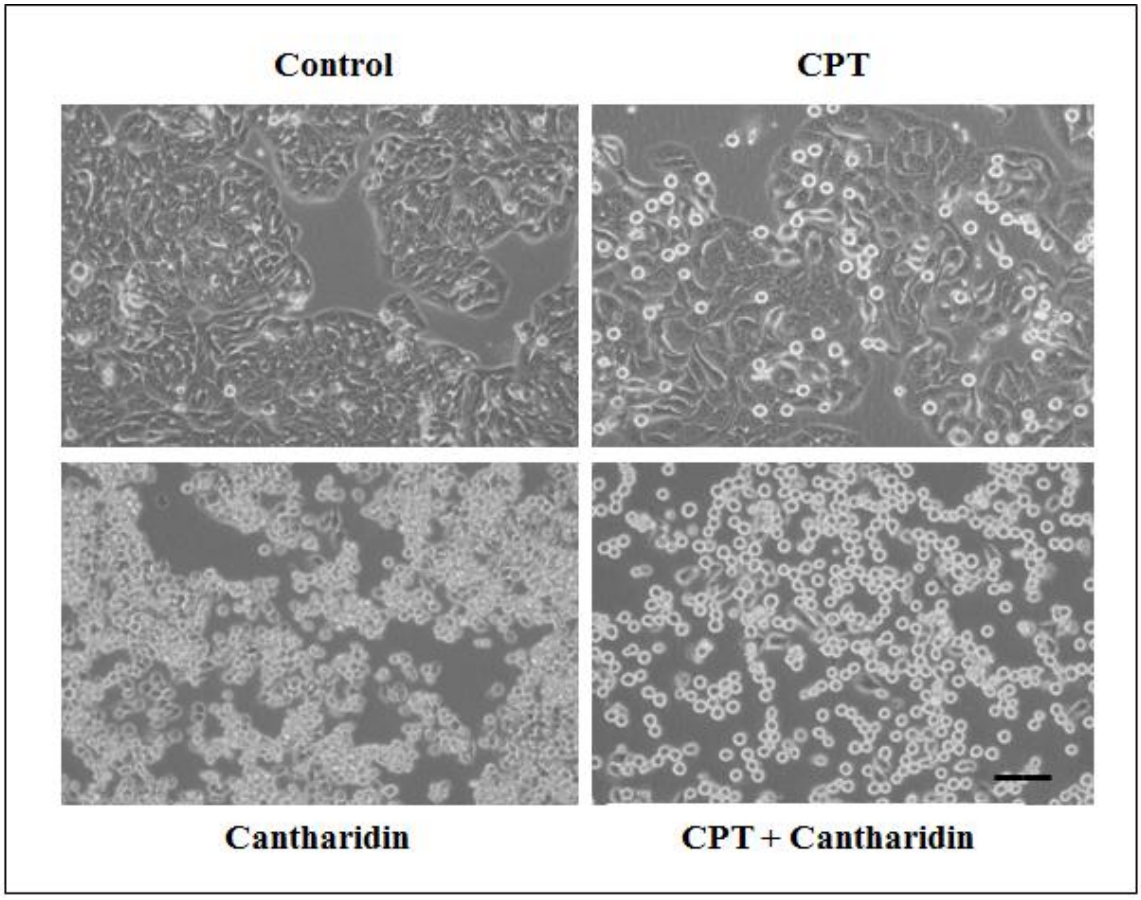


Figure 13. Rounded cells accumulate after co-treatment with CPT and cantharidin. HT-29 cells were either untreated or treated with 25 nM CPT for 48 h, 2.5 µM cantharidin for 2 h, or co-treated with 25 nM CPT for 48 h then 2.5 µM cantharidin was added for an additional 2 h, and observed by phase contrast light microscopy. Scale bar = 200 µm. The experiment was performed three times.

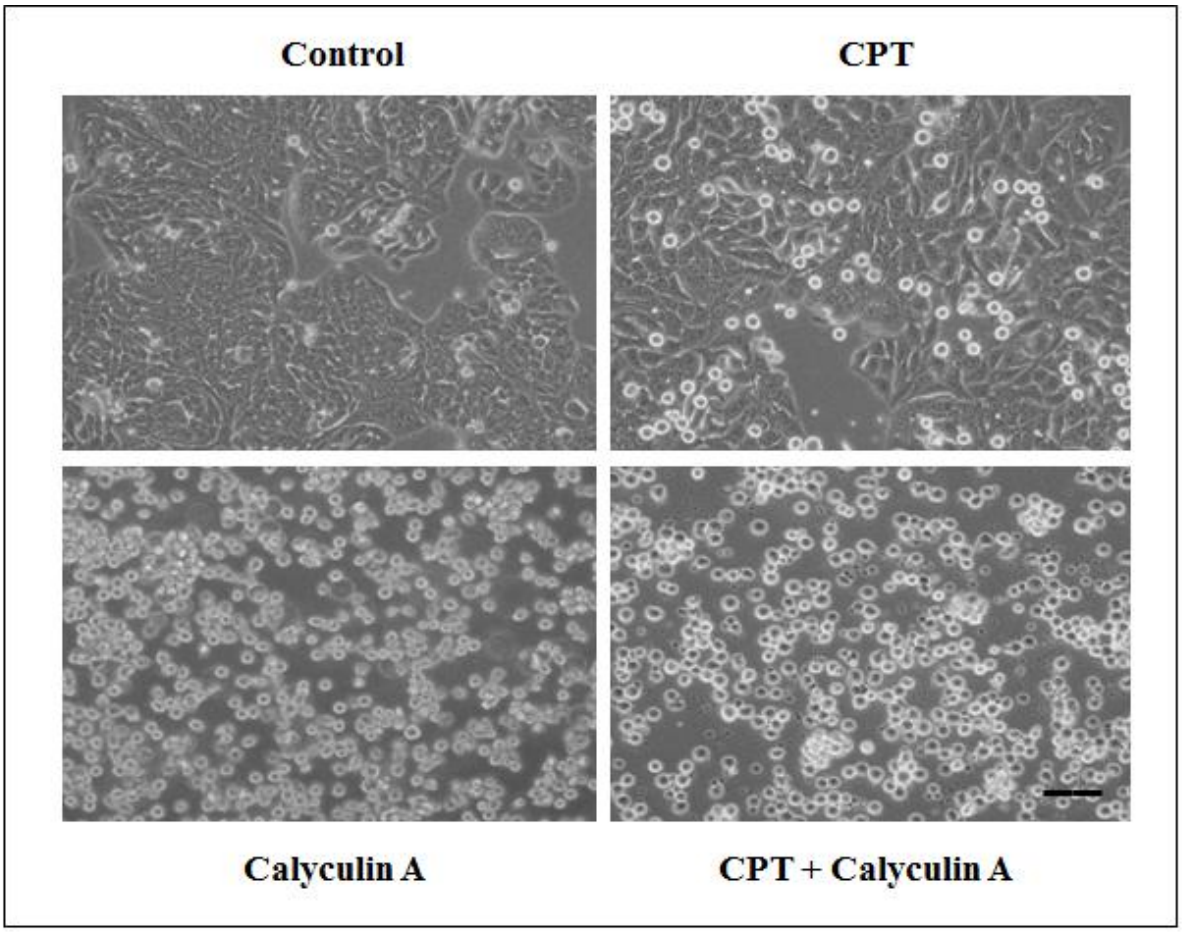


Figure 14. Rounded cells accumulate after co-treatment with CPT and calyculin A. HT-29 cells were either untreated or treated with 25 nM CPT, 5 nM calyculin A, or co-treated with 25 nM CPT for 48 h then 5 nM calyculin A was added for an additional 3 h and observed by phase contrast light microscopy. Scale bar = 200 μm. The experiment was performed two times.

experiments were conducted to confirm that these rounded cells were in mitosis following co-treatment with CPT and cantharidin. Due to time constraints, no further experiments were conducted on the effect of co-treating cells with CPT and calyculin A.

3.3.3. HT-29 Cells Treated with CPT and Cantharidin are in Mitosis

To determine if the rounded cells co-treated with CPT and cantharidin were in mitosis, cells were examined for phospho-ser10 histone H3, a marker of cells in mitosis (Hendzel *et al*., 1997). Cells were either untreated or treated with 25 nM CPT for 48 h then either DMSO or 2.5 µM cantharidin was added to the sample for an additional 2 h. Cells were fixed and stained for phospho-ser10 histone H3 and analyzed by immunofluorescence microscopy (Figure 15A). In the CPT-cantharidin sample, 65% of the cells were positive for phospho-ser10 histone H3, whereas 18% were positive in the CPT only, 9% in cantharidin only and 2% in the untreated populations (Figure 15B). These data confirmed the observation that co-treatment of cells with CPT and cantharidin causes HT-29 cells to enter into mitosis at a much higher number than cells treated with CPT or cantharidin alone.

3.4. Discussion

Exposure to DNA damaging agents results in activation of the DNA damage checkpoint (Carr, 2002). The DNA damage checkpoint initiates a

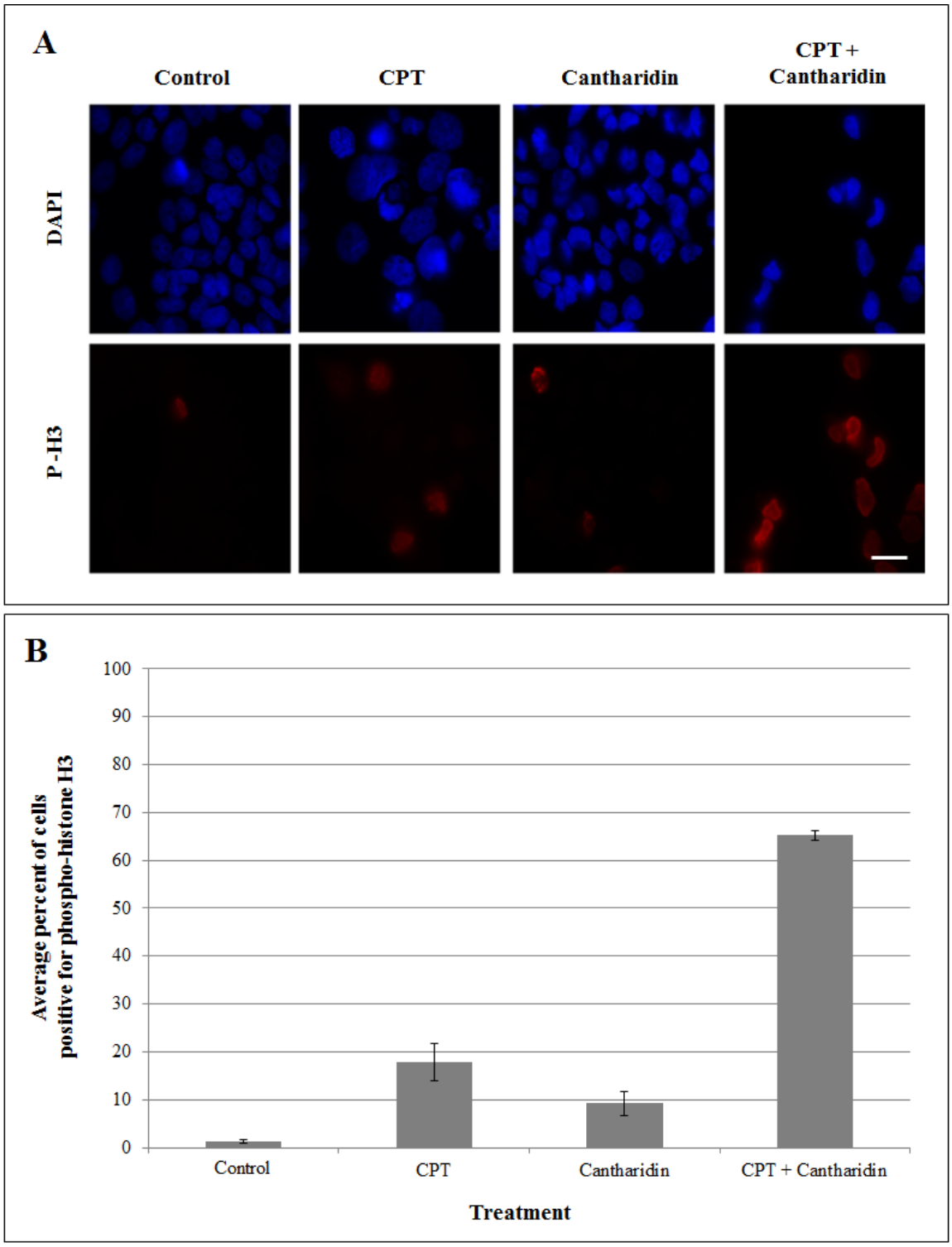


Figure 15A and 15B. Mitotic cells accumulate after co-treatment with CPT and cantharidin. HT-29 cells were either untreated or treated with 25 nM CPT, 2.5 µM cantharidin, or co-treated with 25 nM CPT for 48 h then 2.5 µM cantharidin was added for an additional 2 h. **A**) Cells were stained for DAPI (top panel), and phospho-Ser10 histone H3 (bottom panel). Images were collected by immunofluorescence microscopy. Scale bar = 100 µm. **B**) Cells positive for phospho-Ser10 histone H3 were counted. Error bars represent standard deviation with each data point collected in triplicate.

phosphorylation cascade of substrates including: Chk1 at Ser317/345, Cdc25 at Ser216, as well as phosphorylation of Cdk1 at Thr14 and Tyr15 (Liu *et al*., 2000; Zhao and Piwnica-Worms, 2001; Boutros *et al*., 2006). With inhibitory phosphorylations present on Cdk1, the Cdk1/cyclin B complex is inactive, resulting in a G2-phase arrest of the cell cycle (Melo and Toczyski, 2002). The precise biochemical process that enables cells to abrogate this DNA damage checkpoint and enter into mitosis with damaged DNA is unknown. Previous studies have demonstrated that the cells are in mitosis, indicated by high Cdk1 activity and phospho-histone H3 signals. And strikingly, Chk1 is dephosphorylated at Ser345 in cells that have undergone checkpoint adaptation (Kubara *et al*., 2012). Chk1 dephosphorylation has been identified as a candidate for the initiation of checkpoint adaptation (Syljuåsen *et al*., 2006; Kubara *et al*., 2012). However, it is not known if Chk1 dephosphorylation is causal for checkpoint adaptation.

Previous studies have linked phosphatase inhibitors to the inhibition or delayed dephosphorylation of Chk1 at Ser317/345. A study in 2006 demonstrated Chk1 dephosphorylation at Ser317/345 was inhibited using either the PP2A inhibitor okadaic acid or fostriecin, following exposure to the DNA damaging agent hydroxyurea (Leung-Pineda *et al*., 2006). In addition, this study showed that PP2A depletion, through siRNA, also resulted in inhibition of Chk1 dephosphorylation at Ser317/345. This paper did not however explore what

effects phosphatase inhibitor treatment or Chk1 dephosphorylation had on the cell cycle or mitosis in cells that have been treated with a DNA damaging agent.

Studies have been done on phosphatase inhibitor treatments resulting in abnormalities in the cell cycle however these studies were not done in the scope of checkpoint adaptation. For example, cantharidin, okadaic acid and fostriecin have been shown to push cells prematurely into mitosis, often causing cell death (Cheng *et al*., 1998; Sakoff *et al*., 2004; Bonness *et al*., 2006). Cheng and colleagues (1998) demonstrated that treating Chinese hamster ovary cells (CHO) with the PP2A inhibitors cantharidin, okadaic acid and fostriecin resulted in a mitotic block, and rounded cell morphology. They also found that treatment over 24 h with phosphatase inhibitors resulted in cell death via apoptosis. Other studies have demonstrated that the PP1 and PP2A inhibitors okadaic acid and cantharidin inhibit cancer cell growth and cause cell death (Hart *et al*., 2004; Bonness *et al*., 2006; Lu *et al*., 2008). As a result of this, phosphatase inhibition has been explored for its potential anticancer effects. In 2009, Lu *et al*. explored the effects of treating U87MG cells with a synthesized PP2A inhibitor, LB-1.2, alone or in tandem with the common chemotherapeutic agents temolozolomide (TMZ) or doxorubicin (DOX). They found that PP2A inhibition, through LB-1.2, enhanced the effects of cancer chemotherapy when cells were co-treated with TMZ or DOX. When mice were co-treated with LB-1.2 and either TMZ or DOX, Lu *et al*. reported delayed tumour growth or tumour regression, as well as delayed or absent recurrence of cancer cells. In cell culture, they found that treatment of

U87MG cells with LB-1.2 in combination with either TMZ or DOX resulted in inappropriate entry of cells into mitosis, accelerating the timing of cell death. This study was not performed in the context of checkpoint adaptation; however, the increase in cell death was suggested to be due to mitotic catastrophe.

To explore whether Chk1 dephosphorylation initiates checkpoint adaptation, it was addressed whether the inhibition of Chk1 dephosphorylation at Ser345 would inhibit checkpoint adaptation. With the knowledge that PP2A inhibition has been shown to inhibit the dephosphorylation of Chk1 at Ser345 and cantharidin is an inhibitor of PP2A, HT-29 cells were treated with CPT (to induce checkpoint adaptation) and then cells were treated with cantharidin. The concentration of cantharidin used in these assays was selected based on the results of the MTT cytotoxicity assay, this was also a concentration demonstrated to inhibit PP2A in human cells (Cairns *et al*., 1994). I hypothesized that if Chk1 dephosphorylation is an essential step in checkpoint adaptation, then treating cells with cantharidin should inhibit checkpoint adaptation. Our research data indicated that co-treatment of cells with CPT and cantharidin or CPT and calyculin A, resulted in rounded cells. Of the cells co-treated with CPT and cantharidin, 65% were positive for phospho-ser10 histone H3, confirming they were in mitosis. Whereas only 18% of the cells treated with CPT alone were positive for phospho-ser10 histone H3. Although 35% of the rounded cells were not positive for phopho-ser10 histone H3, as would have been expected from the rounded cell morphology, phospho-ser10 histone H3 is the more precise marker

for determining cells in mitosis. The remainder of the cells may have been undergoing a different cellular process, such as apoptosis; however, further investigation would need to be to done. These results indicated that PP2A inhibition caused more cells to be in mitosis, following treatment with CPT. This increase in the number of cells undergoing checkpoint adaptation upon PP2A inhibition was an unanticipated result and lead to the conclusion that other pathways might be involved in the regulation of checkpoint adaptation.

Recent studies have indicated that Greatwall kinase (Gwl) is a factor in the regulation of mitosis through its inhibition of PP2A (Vigneron *et al*., 2009; Gharbi-Ayachi *et al*., 2010; Mochida *et al*., 2010; Boke and Hagan, 2011). These studies have reported that PP2A is believed to antagonize the activity of the mitotic protein kinase Cdk1; in mitosis Cdk1 is active and PP2A is inactive. One study reported that Gwl inhibits PP2A (Figure 16), causing at least four major MPF substrates (Cdc2, Cdc27, MAPK, and Erp1/Emi2) to remain phosphorylated and thereby maintaining the mitotic state of cells (Vigneron *et al*., 2009). It was also reported that PP1/PP2A inhibitors, such as okadaic acid, were able to rescue the phenotype induced by Gwl inactivation. Subsequent studies reported that Gwl phosphorylates and activates the two proteins Arpp19 and Ensa, which are potent inhibitors of PP2A (Gharbi-Ayachi *et al*., 2010; Mochida *et al*., 2010). These studies suggest that the results obtained in this study, a massive number of cells entering into mitosis even though they contain damaged DNA, is due to PP2A inhibition through cantharidin. This result indicates that the inhibition of PP2A is

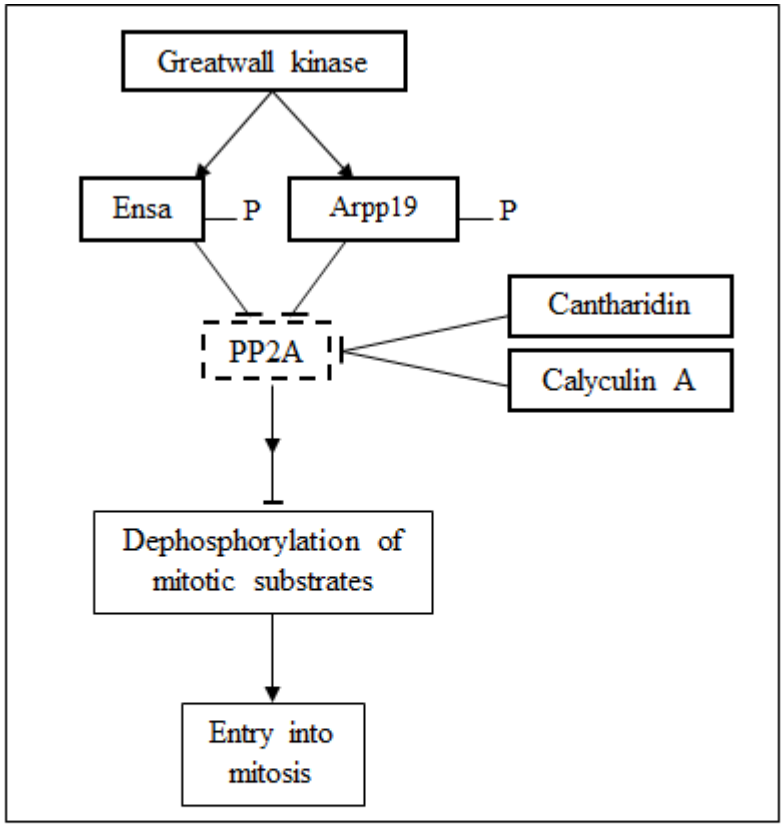


Figure 16. A simplified model of the Gwl pathway and the effect of PP2A inhibition on entry into mitosis. Active greatwall kinase phosphorylates substrates that will bind to and inhibit PP2A. Without PP2A activity, mitotic substrates become dephosphorylated resulting in entry into mitosis. Cantharidin and Calyculin A mimic this process through direct PP2A inhibition. A bold outline indicates an activate protein, a dashed outline indicates a protein that is not active.

perhaps a prevailing biochemical process over the precise process that results in checkpoint adaptation or accelerates the process that results in checkpoint adaptation.

To confirm that PP2A inhibition resulted in a larger proportion of cells undergoing checkpoint adaptation, it would need to be confirmed that the cells that were in mitosis following CPT and cantharidin treatment still contain damaged DNA. However, the results of this experiment suggest the cells that entered into mitosis do have damaged DNA based on two facts: 1) Prior to cantharidin treatment, cells were treated with CPT for 48 h, conditions that have been shown to result in entry into mitosis with damaged DNA, and 2) PP2A inhibition results in inefficient DNA repair and causes cells to be hypersensitive to DNA damage (Chowdhury *et al*., 2005). In the future, a different marker for the detection of DNA damage would be needed. The marker used in previous studies of checkpoint adaptation (including my own) γ-H2AX, is dephosphorylated by PP2A upon DNA repair, and as such, inhibition of PP2A could potentially give a false positive result for the presence of DNA damage. An example of an alternative method for the detection of damaged DNA is the comet assay (Collins, 2004).

To confirm that PP2A inhibition resulted in a larger proportion of cells undergoing checkpoint adaptation, it would also be necessary to confirm that the cantharidin treatment only inhibited PP2A in cells. Phosphatase inhibitors are often responsible for the inhibition of more than one phosphatase (Leung-Pineda

et al., 2006; Pereira *et al*., 2011). For example, cantharidin can also inhibit PP4 and PP6 at concentrations that inhibit PP2A, and inhibit PP1 at concentrations above those which inhibit PP2A. It would therefore need to be confirmed that the cantharidin treatment inhibited PP2A alone. The effects of PP2A inhibition could be confirmed by using RNAi. PP2A knockdown would result in the depletion of PP2A, at which point the effects on checkpoint adaptation could be observed.

The results of this research indicate that PP2A inhibition, through cantharidin treatment, pushes cells into mitosis when co-treated with CPT. Treatment of cells with CPT for 48 h primes cells for entry into mitosis as evidenced by the accumulation of 4N DNA, high cyclin B levels and low Cdk1 activity (Kubara *et al*., 2012). Treatment of cells with the PP2A inhibitor cantharidin possibly pushes these primed cells into mitosis through the Gwl pathway. A remaining question is whether PP2A inhibition was successful in preventing the dephosphorylation of Chk1 at Ser345. If Chk1 dephosphorylation was not inhibited with PP2A inhibition, then Chk1 is dephosphorylated by another protein phosphatase. The other candidate phosphatase proposed to be involved in Chk1 dephosphorylation at Ser345 is PP1. If Chk1 is dephosphorylated following cantharidin treatment, then PP1 may be the phosphatase responsible for Chk1 dephosphorylation. In the future, treatment with the PP1 phosphatase inhibitor tautomycin could be explored, to determine if PP1 inhibition has an effect on checkpoint adaptation. However, if PP2A inhibition was successful in preventing the dephosphorylation of Chk1 at

Ser317/345, then it can be assumed that the effect of PP2A inhibition on the initiation of mitosis through the Gwl pathway is independent of or bypasses the precise biochemical process that results in checkpoint adaptation. Cells were also co-treated with calyculin A, an inhibitor of PP1 and PP2A with equal potency, which also resulted in rounded cells following CPT treatment. This result does not exclude PP1 as a potential candidate for the dephosphorylation of Chk1, as entry into mitosis through PP2A inhibition and the Gwl pathway, may be a prevailing biochemical process.

To address whether Chk1 dephosphorylation at Ser345 is important in the initiation or maintenance of checkpoint adaptation, exploration into pathways that prevent Chk1 dephosphorylation, aside from PP2A inhibition would be necessary. One way to do this may be to produce Chk1 constructs with altered Ser317/345 regulatory sites. This may be done through the use of a previously published, C-terminal domain truncated, permanently active Chk1 variant, containing only the amino acids 1-289 (Ferry *et al*., 2011). Another option for altering the Chk1 Ser317/345 regulatory sites may be to replace the serine317/345 residues of Chk1 with negatively charged glutamate residues, which might result in a permanently active Chk1. This could then permit us to explore what effects a permanently active Chk1 has on checkpoint adaptation.

Chapter 4

4. Review of Thesis

Progression through the cell cycle is a carefully regulated and coordinated process. Cell cycle regulation has been conserved from yeast to mammals and ensures the formation of genetically identical progeny cells (George, 2003). Cells are equipped with a molecular system composed of sensors, transducers and effectors called the DNA damage checkpoint. The DNA damage checkpoint prevents the proliferation of cells with damaged DNA in order to maintain genomic integrity (Melo and Toczyski, 2002). Until recently, the two main outcomes of the DNA damage checkpoint in human cells were believed to be: 1) checkpoint recovery, in which cell cycle arrest is followed by DNA repair and re-entry into mitosis, 2) apoptosis, in which the DNA damage could not be repaired resulting in programmed cell death (Sancar *et al*., 2004).

Checkpoint adaptation has recently been proposed as a third possible outcome following the initiation of the DNA damage checkpoint. In 1993 checkpoint adaptation was originally reported in *Saccharomyces cerevisiae*, although not called checkpoint adaptation at that time. It was reported as the ability of yeast cells to divide following a checkpoint arrest, despite the presence of irreparable chromosome damage (Sandell and Zakian, 1993). Checkpoint adaptation is now defined by three features in cells with damaged DNA: 1) cell cycle arrest, 2) entry into mitosis, 3) damaged DNA in mitotic cells (Toczyski *et al*., 1997). In 2006, Syljuåsen *et al*. reported that checkpoint adaptation also

occurred in human cancer cells. In this study, human bone cancer cells were irradiated to damage the DNA (Syljuåsen *et al*., 2006). Following exposure to radiation, the cells entered into a DNA damage checkpoint arrest. The treated cells were able to overcome this cell cycle arrest and enter into mitosis despite the presence of damaged DNA. Since 2006, checkpoint adaptation has been clearly demonstrated in both human colon carcinoma and lymphocytic leukemia cells (Rezacova *et al*., 2011; Kubara *et al*., 2012). Checkpoint adaptation has also been suggested to occur in at least two other cancer cell lines including a non-cancerous cell line (Wang *et al*., 2008; Rezacova *et al*., 2011). Checkpoint adaptation has since been hypothesized to be a universal event in cancer cells when exposed to DNA damaging agents.

Prior to this thesis work, checkpoint adaptation had not yet been demonstrated in a brain cancer cell line. Within the literature, however, several laboratories have described cell phenotypes that suggest checkpoint adaptation might occur in human brain cancer cells. For example, M059K cells, the cell line that was used for this thesis work, have been reported to undergo mitotic catastrophe or form giant cells after genotoxic treatments (Hirose *et al*., 2001; Holgersson *et al*., 2003). There have also been reports on M059K or M059J cells entering into a G2/M phase block that was later released, following genotoxic treatments (Holgersson *et al*., 2003; Holgersson *et al*., 2005). However, none of these reports explored the criteria for checkpoint adaptation as described by Toczyski *et al.* in 1997. A portion of this thesis was dedicated to determine if

checkpoint adaptation occurs in human brain cancer cells. This information is important, because if we understand the biochemical basis of checkpoint adaptation in major cancers, such as brain cancer, we will likely be able to propose methods to improve treatments.

This thesis reports that brain cancer cells, treated with the anticancer agent CPT, undergo checkpoint adaptation. Following 48 h treatment with CPT, M059K cells exhibit the three criteria of checkpoint adaptation (Toczyski *et al*., 1997). Using flow cytometry and Western blotting, it was demonstrated that CPT treated cells enter into a G2 phase arrest of the cell cycle. Thereafter the cells enter into mitosis with damaged DNA, as demonstrated with phospho-histone H3 and γ-H2AX immunofluorescence microscopy. An important finding from this study was that the M059K cells that survive checkpoint adaptation acquire micronuclei, a marker for chromosome or genome damage (Hovhannisyan *et al*., 2009). At 120 h CPT treatment, approximately half the cell population contained one or more micronuclei. The presence of micronuclei in cells that have undergone checkpoint adaptation provides evidence that the survivors of mitosis with damaged DNA have a rearranged genome.

Glioblastomas are one of the deadliest forms of cancer due to their high proliferative rate, aggressive invasiveness and insensitivity to radio or chemotherapeutic treatments (Alves *et al*., 2011). These factors have resulted in an urgent need to improve brain cancer treatments. The demonstration of checkpoint adaptation in cells from a glioblastoma provides us with a better

understanding of how this cancer reacts to clinically relevant amounts of the anti-cancer agent CPT. With the knowledge that brain cancer cells undergo checkpoint adaptation, we may now focus our attention on altering the course of checkpoint adaptation. One way to inhibit checkpoint adaptation may be through the use of Cdk1 inhibitors (Figure 17). This will enable us to continue to utilize the cytotoxicity of genotoxic treatments however Cdk1 inhibition may enable us to prevent entry into mitosis with damaged DNA. Another method may be to exploit checkpoint adaptation through the use of Chk1 inhibitors. Treating DNA damaged cells with a Chk1 inhibitor, would eliminate the DNA damage response (Cahuzac *et al*., 2010). Eliminating the DNA damage response would then accelerate entry into mitosis with damaged DNA as well as accelerate a mitosis-linked cell death. The demonstration of checkpoint adaptation in a cancerous fibroblastic cell line also provides us with an opportunity to explore if checkpoint adaptation also occurs in human non-cancerous cells exposed to genotoxic agents. Non-cancerous epithelial cells are difficult to cultivate in a laboratory, therefore demonstrating that checkpoint adaptation occurs in cancerous fibroblastic cells provides us with a setting in which we can test if non-cancerous fibroblastic cells also undergo checkpoint adaptation. Investigating whether non-cancerous fibroblastic cells undergo checkpoint adaptation may provide insight on how every cell, cancerous or not, reacts when exposed to DNA damaging agents.

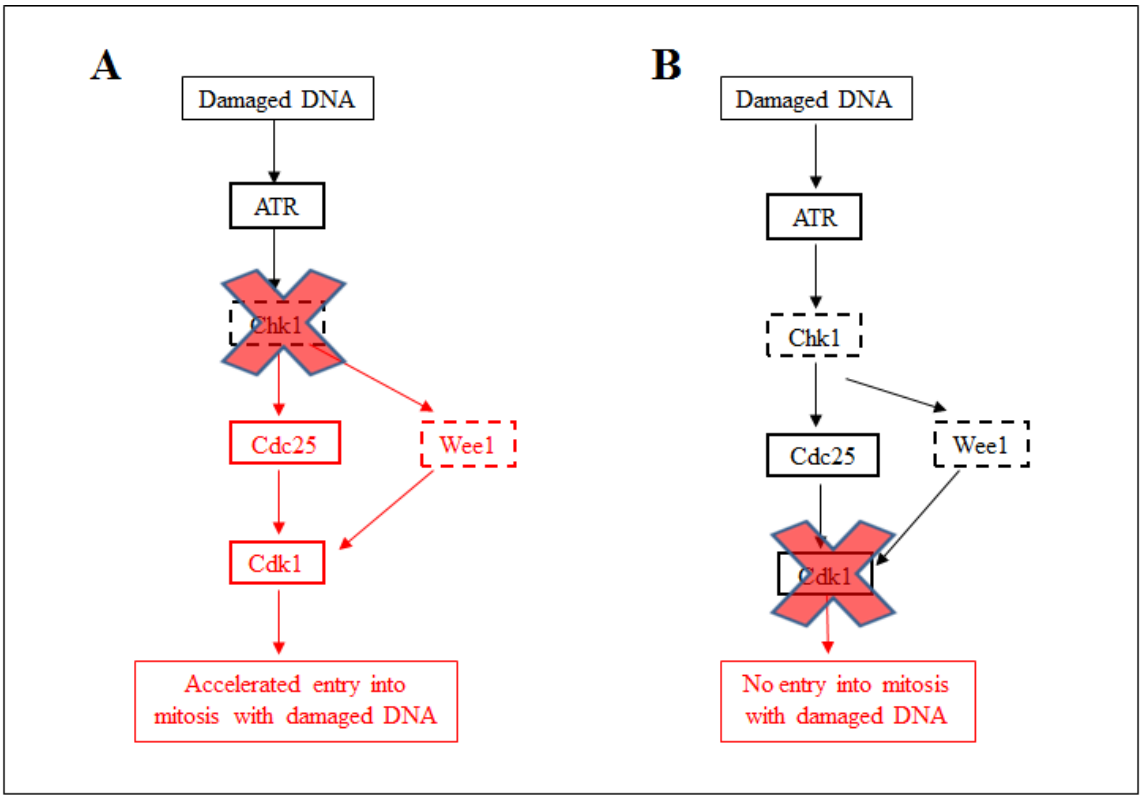


Figure 17. Identifying the pathway of checkpoint adaptation provides mechanisms to control its outcome. A) If Chk1 is inhibited when cells have damaged DNA, then Cdc25 remains active, which activates Cdk1, which permits cells to enter mitosis. B) If Cdk1 is inhibited when cells have damaged DNA, then cells cannot enter mitosis.

The report of checkpoint adaptation in brain cancer cells not only provides us with knowledge on how brain cancer cells react to anti-cancer treatments it also expands our still limited knowledge of checkpoint adaptation. The demonstration of checkpoint adaptation in the M059K cell line, adds a new origin and tumour type (brain and glioma), to the short list of origins and tumour types in which checkpoint adaptation has been demonstrated. The demonstration of checkpoint adaptation provides support that checkpoint adaptation is a universal event in cancer cells following treatment with genotoxic agents.

The presence of micronuclei in brain cancer cells that have undergone checkpoint adaptation suggests that checkpoint adaptation results in cells with genomic rearrangement. In 2012, Crasta *et al*. suggested errors in mitosis can generate DNA breaks or chromosome pulverization followed by chromosome rearrangement, a phenomenon called chromothripsis. In this study both processes, DNA breakage and chromothripsis, resulted in the formation of cells containing micronuclei (Crasta *et al*., 2012). They also noted that the DNA rearrangements and mutations acquired in micronuclei could be incorporated into the genome, which may result in further cancer development. The presence of micronuclei in brain cancer cells that have undergone checkpoint adaptation may provide a potential explanation for the insensitivity or resistance of brain cancer to genotoxic treatments. It may also provide an explanation for cancer relapse or the formation of secondary tumours (Nakada *et al*., 2006; Wang *et al*., 2008; Kuntz and O'Connell, 2009; Nitiss, 2009). One explanation for cancer cell resistance,

following genotoxic treatment, is the production of genomic mutations or rearrangements that enable cancer cells to survive therapy. The cells that survive therapy then proliferate resulting in a population of cells resistant to therapy (Kerbel, 1997; Gottesman *et al*., 2002). The presence of micronuclei in cells is a marker for genomic mutations or genomic rearrangements (Hovhannisyan *et al*., 2009). The demonstration of micronuclei in cells that have undergone checkpoint adaptation, suggests that checkpoint adaptation may be the link between genomic mutations that lead to cancer cell resistance. Future research directions may include further exploration of the cells that survive checkpoint adaptation; such as the proliferation rates, cell viability, and chromosome or genome stability of these surviving cells, to understand better what becomes of these surviving cells.

Mitotic catastrophe, or aberrant mitosis ending in cell death, is a process that has been suggested in many cell types, including brain cancers. Checkpoint adaptation, entry into mitosis with damaged DNA, has also been demonstrated in a few cell types, including brain cancer cells. It has been suggested that mitotic catastrophe and checkpoint adaptation may be connected (Bartek and Lukas, 2007; Vakifahmetoglu *et al*., 2008). The demonstration of checkpoint adaptation in brain cancer cells, along with the previous demonstrations of mitotic catastrophe in brain cancer cells supports the hypothesis that these two processes may be related. In the future it may be advantageous to explore the potential link between mitotic catastrophe and checkpoint adaptation.

The precise biochemical process that allows cells to abrogate the DNA damage checkpoint, even though they still contain damaged DNA remains to be understood. However, there have been reports that the cells that have undergone checkpoint adaptation have dephosphorylated Ser317/345 residues of Chk1 (Syljuåsen *et al*., 2006; Kubara *et al*., 2012). Chk1 dephosphorylation at Ser317/345 has been hypothesized to be the biochemical event that regulates checkpoint adaptation. This event would require the activity of protein phosphatases. The remaining portion of this thesis was dedicated to testing the relationship between checkpoint adaptation and protein phosphatase activity.

HT-29 cells were treated with CPT for 48 h, conditions that result in checkpoint adaptation. Then these cells, which were primed to undergo checkpoint adaptation, were treated with cytotoxic concentrations of the phosphatase inhibitor cantharidin and calyculin A. Cantharidin inhibits protein phosphatase PP2A, and calyculin A inhibits both PP2A and PP1. Both PP2A and PP1 have been suggested to be involved in Chk1 dephosphorylation at Ser345 (den Elzen and O'Connell, 2004; Leung-Pineda *et al*., 2006; Petersen *et al*., 2006; Landsverk *et al*., 2010). If Chk1 dephosphorylation at Ser345 is an essential step in initiating checkpoint adaptation, then preventing this step, would prevent checkpoint adaptation. In contrast to my prediction, that cantharidin or calyculin A treatment would block checkpoint adaptation, it resulted in a massive and rapid entry of cells into mitosis.

Although this result was contrary to my predication, it suggested that another major pathway may participate in checkpoint adaptation. Recent studies suggest that PP2A inhibition and entry into mitosis is linked to the Greatwall (Gwl) pathway. Gwl is a protein kinase that regulates mitosis through its inhibition of PP2A (Vigneron *et al*., 2009; Gharbi-Ayachi *et al*., 2010; Mochida *et al*., 2010; Boke and Hagan, 2011). By inhibiting PP2A, through treatment with cantharidin and calyculin A, the cells were pushed into mitosis presumably with damaged DNA (see Figure 16). I hypothesize then that the inhibition of PP2A is a prevailing biochemical process over the biochemical process that results in checkpoint adaptation, or bypasses it.

This finding demonstrated that in the future we may be able to alter the course of checkpoint adaptation; in this case it was accelerated and enhanced. This knowledge may eventually lead to improvements in cancer treatments. Future directions include exploring if PP2A inhibition was successful in preventing the dephosphorylation of Ser317/345 of Chk1, and looking further into a potential mechanism that may be able to inhibit cells from entering into mitosis with damaged DNA, such as Cdk1 inhibition.

4.1. Thesis Conclusions

The purpose of this thesis was two-fold 1) to determine if checkpoint adaptation occurs in the brain cancer cell line M059K following treatment with the genotoxic agent CPT and 2) to test the relationship between checkpoint

adaptation and protein phosphatase activity. This was done to provide evidence that checkpoint adaptation is a universal feature in cancer cells exposed to genotoxic agents and to better understand the biochemical basis of checkpoint adaptation. A better understanding of the biological significance and biochemical process of checkpoint adaptation may eventually provide us with an opportunity to alter the fate of checkpoint adaptation. Altering the course of checkpoint adaptation may eventually have long term significance for improving current cancer treatment. Based on the work presented in this thesis, the following conclusions were reached:

1. Following treatment with cytotoxic concentrations of CPT, M059K cells enter into a DNA damage induced G2 phase arrest of the cell cycle, and contain the active form of Chk1 (P-Ser345).

2. Following CPT treatment, M059K cells become rounded and enter into mitosis with damaged DNA, therefore M059K cells do undergo checkpoint adaptation.

3. Following treatment with CPT, M059K cells acquire micronuclei, an indicator of genome damage. This provides evidence that the cells that survive checkpoint adaptation contain a rearranged genome.

4. HT-29 cells treated with CPT, at conditions that result in checkpoint adaptation, followed by treatment with cytotoxic concentration of the phosphatase inhibitor cantharidin, rapidly enter into mitosis.

5. The entry of cells into mitosis following treatment with CPT and cantharidin is likely due to inhibition of protein phosphatase PP2A.

References

Abraham, R. 2001. Cell cycle checkpoint signaling through the ATM and ATR kinases. *Genes and Development*. 15:2177-2196.

Aburai, N., M. Yoshida, M. Ohnishi, and K. Kimura. 2010. Sanguinarine as a potent and specific inhibitor of protein phosphatase 2C *in vitro* and induces apoptosis via phosphorylation of p38 in HL60 cells. *Bioscience Biotechnology and Biochemistry*. 74:548-552.

Allalunis-Turner, M., G. Barron, R. Day, K. Dobler, and R. Mirzayans. 1993. Isolation of two cell lines from a human malignant glioma specimen differing in sensitivity to radiation and chemotherapeutic drugs. *Radiation Research*. 134:349-354.

Alves, T., F. Lima, S. Kahn, D. Lobo, L. Dubois, R. Soletti, H. Borges, and V. Neto. 2011. Glioblastoma cells: A heterogeneous and fatal tumor interacting with the parenchyma. *Life Sciences*. 89:532-539.

Barford, D., A. Das, and M. Egloff. 1998. The structure and mechanism of protein phosphatases: Insights into catalysis and regulation. *Annual Review of Biophysics and Biomolecular Structure*. 27:133-164.

Bartek, J., and J. Lukas. 2007. DNA damage checkpoints: from initiation to recovery or adaptation. *Current Opinion in Cell Biology*. 19:238-245.

Barton, G., P. Cohen, and D. Barford. 1994. Conservation analysis and structure prediction of the protein serine/threonine phosphatases - Sequence similarity with diadenosine tetraphosphatase from *Escherichia coli* suggests homology to the protein phosphatases. *European Journal of Biochemistry*. 220:225-237.

Beretta, G., and F. Zunino. 2007. Relevance of extracellular and intracellular interactions of camptothecins as determinants of antitumor activity. *Biochemical Pharmacology*. 74:1437-1444.

Block, W., Y. Yu, and S. Lees-Miller. 2004. Phosphatidyl inositol 3-kinase-like serine/threonine protein kinases (PIKKs) are required for DNA damage-induced phosphorylation of the 32 kDa subunit of replication protein A at threonine 21. *Nucleic Acids Research*. 32:997-1005.

Boke, E., and I. Hagan. 2011. Polo, Greatwall, and protein phosphatase PP2A jostle for pole position. *PLoS Genetics*. 7: e1002213.

Bonner, W., C. Redon, J. Dickey, A. Nakamura, O. Sedelnikova, S. Solier, and Y. Pommier. 2008. GammaH2AX and cancer. *Nature Reviews Cancer*. 8:957-967.

Bonness, K., I. Aragon, B. Rutland, S. Ofori-Acquah, N. Dean, and R. Honkanen. 2006. Cantharidin-induced mitotic arrest is associated with the formation of aberrant mitotic spindles and lagging chromosomes resulting, in part, from the suppression of PP2A alpha. *Molecular Cancer Therapeutics*. 5:2727-2736.

Boutros, R., C. Dozier, and B. Ducommun. 2006. The when and wheres of CDC25 phosphatases. *Current Opinions in Cell Biology*. 18:185-191.

Burma, S., B. Chen, M. Murphy, A. Kurimasa, and D. Chen. 2001. ATM phosphorylates histone H2AX in response to DNA double-strand breaks. *Journal of Biological Chemistry*. 276:42462-42467.

Burrows, A., and S. Elledge. 2008. How ATR turns on: TopBP1 goes on ATRIP with ATR. *Genes & Development*. 22:1416-1421.

Cahuzac, N., A. Studeny, K. Marshall, I. Versteege, K. Wetenhall, B. Pfeiffer, S. Leonce, J.A. Hickman, A. Pierre, and R.M. Golsteyn. 2010. An unusual DNA binding compound, S23906, induces mitotic catastrophe in cultured human cells. *Cancer Letters*. 289:178-187.

Cairns, J., S. Qin, R. Philp, Y. Tan, and G. Guy. 1994. Dephosphorylation of the small heat-shock protein Hsp27 *in-vivo* by protein phosphatase 2A. *Journal of Biological Chemistry*. 269:9176-9183.

Carr, A. 2002. DNA structure dependent checkpoints as regulators of DNA repair. *DNA Repair*. 1:983-994.

Cheng, A., R. Balczon, Z. Zuo, J. Koons, A. Walsh, and R. Honkanen. 1998. Fostriecin-mediated G(2)-M-phase growth arrest correlates with abnormal centrosome replication, the formation of aberrant mitotic spindles, and the inhibition of serine/threonine protein phosphatase activity. *Cancer Research*. 58:3611-3619.

Cheng, L., Q. Wu, Z. Huang, O. Guryanova, Q. Huang, W. Shou, J. Rich, and S. Bao. 2011. L1CAM regulates DNA damage checkpoint response of glioblastoma stem cells through NBS1. *EMBO Journal*. 30:800-813.

Chowdhury, D., M. Keogh, H. Ishii, C. Peterson, S. Buratowski, and J. Lieberman. 2005. gamma-H2AX dephosphorylation by protein phosphatase 2A facilitates DNA double-strand break repair. *Molecular Cell*. 20:801-809.

Clemenson, C., and M. Marsolier-Kergoat. 2009. DNA damage checkpoint inactivation: Adaptation and recovery. *DNA Repair*. 8:1101-1109.

Clifford, B., M. Beljin, G. Stark, and W. Taylor. 2003. G2 arrest in response to topoisomerase II inhibitors: The role of p53. *Cancer Research*. 63:4074-4081.

Cohen, P. 2001. The role of protein phosphorylation in human health and disease. *European Journal of Biochemistry*. 268:5001-5010.

Cohen, P. 1997. Novel protein serine/threonine phosphatases: Variety is the spice of life. *Trends in Biochemical Sciences*. 22:245-251.

Cohen, P., N. Brewis, V. Hughes, and D. Mann. 1990. Protein serine threonine phosphatases - an expanding family. *FEBS Letters*. 268:355-359.

Collins, A. 2004. The comet assay for DNA damage and repair principles, applications, and limitations. *Molecular Biotechnology*. 26:249-261.

Cooper, G., and R. Hausman. 2009. The cell: A molecular approach. ASM Press; Sinauer Associates, Washington, D.C. Sunderland, Mass. 820.

Crasta, K., N. Ganem, R. Dagher, A. Lantermann, E. Ivanova, Y. Pan, L. Nezi, A. Protopopov, D. Chowdhury, and D. Pellman. 2012. DNA breaks and chromosome pulverization from errors in mitosis. *Nature*. 482:53.

Deckbar, D., J. Birraux, A. Krempler, L. Tchouandong, A. Beucher, S. Walker, T. Stiff, P. Jeggo, and M. Lobrich. 2007. Chromosome breakage after G2 checkpoint release. *Journal of Cell Biology*. 176:749-755.

Dejmek, J., J. Iglehart, and J. Lazaro. 2009. DNA-dependent protein kinase (DNA-PK)-dependent cisplatin-induced loss of nucleolar facilitator of chromatin transcription (FACT) and regulation of cisplatin sensitivity by DNA-PK and FACT. *Molecular Cancer Research*. 7:581-591.

Demarcq, C., R. Bunch, D. Creswell, and A. Eastman. 1994. The role of cell cycle progression in cisplatin-induced apoptosis in Chinese Hamster ovary cells. *Cell Growth and Differentiation*. 5:983-993.

den Elzen, N., and M. O'Connell. 2004. Recovery from DNA damage checkpoint arrest by PP1-mediated inhibition of Chk1. *EMBO Journal*. 23:908-918.

Eriksson, J., D. Toivola, J. Meriluoto, H. Karaki, Y. Han, and D. Hartshorne. 1990. Hepatocyte deformation induced by cyanobacterial toxins reflects inhibition of protein phosphatases. *Biochemical and Biophysical Research Communications*. 173:1347-1353.

Evans, T., E. Rosenthal, J. Youngblom, D. Distel, and T. Hunt. 1983. Cyclin a protein specified by maternal messenger RNA in sea-urchin eggs that is destroyed at each cleavage division. *Cell*. 33:389-396.

Fathi, A., A. Krautheim, S. Lucke, K. Becker, and H. Steinfelder. 2002. Nonradioactive technique to measure protein phosphatase 2A-like activity and its inhibition by drugs in cell extracts. *Analytical Biochemistry*. 310:208-214.

Favre, B., P. Turowski, and B. Hemmings. 1997. Differential inhibition and posttranslational modification of protein phosphatase 1 and 2A in MCF7 cells treated with calyculin-A, okadaic acid, and tautomycine. *Journal of Biological Chemistry*. 272:13856-13863.

Fenech, M., M. Kirsch-Volders, A. Natarajan, J. Surralles, J. Crott, J. Parry, H. Norppa, D. Eastmond, J. Tucker, and P. Thomas. 2011. Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. *Mutagenesis*. 26:125-132.

Fernet, M., F. Megnin-Chanet, V. Favaudon, and J. Hall. 2008. Control of the G2/M checkpoint after exposure to low dose of ionising radiation - implications for hyper-radiosensitivity. *European Journal of Cancer Supplements*. 6:92-92.

Ferry, G., A. Studeny, C. Bossard, P. Kubara, D. Zeyer, J. Renaud, P. Casara, G. de Nanteuil, M. Wierzbicki, B. Pfeiffer, M. Prudhomme, S. Leonce, A. Pierre, J. Boutin, and R. Golsteyn. 2011. Characterization of novel Checkpoint kinase 1 inhibitors by *in vitro* assays and in human cancer cells treated with topoisomerase inhibitors. *Life Sciences*. 89:259-268.

Feun, L., and N. Savaraj. 2008. Topoisomerase I inhibitors for the treatment of brain tumors. *Experimental Review of Anticancer Therapy*. 8:707-716.

Forsburg, S., and P. Nurse. 1991. Cell cycle regulation in the yeasts *Saccharomyces-cerevisiae* and *Schizosaccharomyces-pombe*. *Annual Review of Cell Biology*. 7:227-256.

Freeman, A., and A. Monteiro. 2010. Phosphatases in the cellular response to DNA damage. *Cell Communication and Signaling*. 8:27.

Friedman, H., W. Petros, A. Friedman, L. Schaaf, T. Kerby, J. Lawyer, M. Parry, P. Houghton, S. Lovell, K. Rasheed, T. Cloughsey, E. Stewart, O. Colvin, J. Provenzale, R. McLendon, D. Bigner, I. Cokgor, M. Haglund, J. Rich, D. Ashley, J. Malczyn, G. Elfring, and L. Miller. 1999. Irinotecan therapy in adults with recurrent or progressive malignant glioma. *Journal of Clinical Oncology*. 17:1516-1525.

Galgoczy, D., and D. Toczyski. 2001. Checkpoint adaptation precedes spontaneous and damage-induced genomic instability in yeast. *Molecular and Cellular Biology*. 21:1710-1718.

George, M. 2003. Cells: building blocks of life. Creative Education, Mankato, Minn. 25.

Gharbi-Ayachi, A., J. Labbe, A. Burgess, S. Vigneron, J. Strub, E. Brioudes, A. Van-Dorsselaer, A. Castro, and T. Lorca. 2010. The substrate of greatwall kinase, Arpp19, controls mitosis by inhibiting protein phosphatase 2A. *Science*. 330:1673-1677.

Gottesman, M., T. Fojo, and S. Bates. 2002. Multidrug resistance in cancer: Role of ATP-dependent transporters. *Nature Reviews Cancer*. 2:48-58.

Hall, E., and A. Giaccia. 2006. Radiobiology for the radiologist. Lippincott Williams & Wilkins, Philadelphia ; London. 546.

Harper, J., and S. Elledge. 2007. The DNA damage response: ten years after. *Molecular Cell*. 28:739-745.

Harris, A. 1973. Location of cellular adhesions to solid substrata. *Developmental Biology*. 35:97-114.

Hart, M., A. Chamberlin, C. Walkom, J. Sakoff, and A. McCluskey. 2004. Modified norcantharidins: Synthesis, protein phosphatases 1 and 2A inhibition, and anticancer activity. *Bioorganic & Medicinal Chemistry Letters*. 14:1969-1973.

Hartwell, L., and M. Kastan. 1994. Cell cycle control and cancer. *Science*. 266:1821-1828.

Hartwell, L., and T. Weinert. 1989. Checkpoints - Controls that ensure the order of cell-cycle events. *Science*. 246:629-634.

Heffernan, T., D. Simpson, A. Frank, A. Heinloth, R. Paules, M. Cordeiro-Stone, and W. Kaufmann. 2002. An ATR- and Chk1-dependent S checkpoint inhibits replicon initiation following UVC-induced DNA damage. *Molecular and Cellular Biology*. 22:8552-8561.

Hendzel, M., Y. Wei, M. Mancini, A. Van Hooser, T. Ranalli, B. Brinkley, D. Bazett-Jones, and C. Allis. 1997. Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma*. 106:348-360.

Hirose, Y., M. Berger, and R. Pieper. 2001. p53 effects both the duration of G(2)/M arrest and the fate of temozolomide-treated human glioblastoma cells. *Cancer Research*. 61:1957-1963.

Holgersson, A., T. Heiden, J. Castro, M. Edgren, R. Lewensohn, and A. Meijer. 2005. Different G2/M accumulation in M059J and M059K cells after exposure to DNA double-strand break-inducing agents. *International Journal of Radiation Oncology Biology Physics*. 61:915-921.

Holgersson, A., A. Jernberg, L. Persson, M. Edgren, R. Lewensohn, A. Nilsson, A. Brahme, and A. Meijer. 2003. Low and high LET radiation-induced apoptosis in M059J and M059K cells. *International Journal of Radiation Biology*. 79:611-621.

Hovhannisyan, G., R. Aroutiounian, and M. Glei. 2009. Butyrate reduces the frequency of micronuclei in human colon carcinoma cells *in vitro*. *Toxicology In Vitro*. 23:1028-1033.

Hsiang, Y., M. Lihou, and L. Liu. 1989. Arrest of replication forks by drug-stabilized topoisomerase I-DNA cleavable complexes as a mechanism of cell killing by camptothecin. *Cancer Research*. 49:5077-5082.

Jazayeri, A., J. Falck, C. Lukas, J. Bartek, G. Smith, J. Lukas, and S. Jackson. 2006. ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. *Nature Cell Biology*. 8:37.

Juan, G., F. Traganos, W. James, J. Ray, M. Roberge, D. Sauve, H. Anderson, and Z. Darzynkiewicz. 1998. Histone H3 phosphorylation and expression of cyclins A and B1 measured in individual cells during their progression through G2 and mitosis. *Cytometry*. 32:71-77.

Kanu, O., A. Mehta, C. Di, N. Lin, K. Bortoff, D. Bigner, H. Yan, and D. Adamson. 2009. Glioblastoma multiforme: A review of therapeutic targets. *Experimental Opinion on Therapeutic Targets*. 13:701-718.

Kastan, M., and J. Bartek. 2004. Cell-cycle checkpoints and cancer. *Nature*. 432:316-323.

Kerbel, R. 1997. A cancer therapy resistant to resistance. *Nature*. 390:335-336.

Kerr, J., A. Wyllie, and A. Currie. 1972. Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer*. 26:239-257.

Kleihues, P., and L. Sobin. 2000. World Health Organization classification of tumors. *Cancer*. 88:2887-2887.

Kubara, P., S. Kerneis-Golsteyn, A. Studeny, B. Lanser, L. Meijer, and R. Golsteyn. 2012. Human cells enter mitosis with damaged DNA after treatment with pharmacological concentrations of genotoxic agents. *Biochemical Journal*. 446:373-381.

Kuntz, K., and M. O'Connell. 2009. The G(2) DNA damage checkpoint. Could this ancient regulator be the achilles heel of cancer? *Cancer Biology & Therapy*. 8:1433-1439.

Kurz, E., P. Douglas, and S. Lees-Miller. 2004. Doxorubicin activates ATM-dependent phosphorylation of multiple downstream targets in part through the generation of reactive oxygen species. *Journal of Biological Chemistry*. 279:53272-53281.

Lander, E., L. Linton, B. Birren, C. Nusbaum, M. Zody, J. Baldwin, K. Devon, K. Dewar, M. Doyle, W. FitzHugh, R. Funke, D. Gage, K. Harris, A. Heaford, J. Howland, L. Kann, J. Lehoczky, R. LeVine, P. McEwan, K. McKernan, J. Meldrim, J. Mesirov, C. Miranda, W. Morris, J. Naylor, C. Raymond, M. Rosetti, R. Santos, A. Sheridan, C. Sougnez, N. Stange-Thomann, N. Stojanovic, A. Subramanian, D. Wyman, J. Rogers, J. Sulston, R. Ainscough, S. Beck, D. Bentley, J. Burton, C. Clee, N. Carter, A. Coulson, R. Deadman, P. Deloukas, A. Dunham, I. Dunham, R. Durbin, L. French, D. Grafham, S. Gregory, T. Hubbard, S. Humphray, A. Hunt, M. Jones, C. Lloyd, A. McMurray, L. Matthews, S. Mercer, S. Milne, J. Mullikin, A. Mungall, R. Plumb, M. Ross, R. Shownkeen, S. Sims, R. Waterston, R. Wilson, L. Hillier, J. McPherson, M. Marra, E. Mardis, L. Fulton, A. Chinwalla, K. Pepin, W. Gish, S. Chissoe, M. Wendl, K. Delehaunty, T. Miner, A. Delehaunty, J. Kramer, L. Cook, R. Fulton, D. Johnson, P. Minx, S. Clifton, T. Hawkins, E. Branscomb, P. Predki, P. Richardson, S. Wenning, T. Slezak, N. Doggett, J. Cheng, A. Olsen, S. Lucas, C. Elkin, E. Uberbacher, M. Frazier, *et al*. 2001. Initial sequencing and analysis of the human genome. *Nature*. 409:860-921.

Landsverk, H., F. Mora-Bermudez, O. Landsverk, G. Hasvold, S. Naderi, O. Bakke, J. Ellenberg, P. Collas, R. Syljuåsen, and T. Kuntziger. 2010. The protein phosphatase 1 regulator PNUTS is a new component of the DNA damage response. *EMBO Reports*. 11:868-875.

Lawson, H., P. Sampath, E. Bohan, M. Park, N. Hussain, A. Olivi, J. Weingart, L. Kleinberg, and H. Brem. 2007. Interstitial chemotherapy for malignant gliomas: the Johns Hopkins experience. *Journal of Neurooncology*. 83:61-70.

Lee, S., A. Pellicioli, J. Demeter, M. Vaze, A. Gasch, A. Malkova, P. Brown, D. Botstein, T. Stearns, M. Foiani, and J. Haber. 2000. Arrest, adaptation, and recovery

following a chromosome double-strand break in Saccharomyces cerevisiae. *Cold Spring Harbour Symposium of Quantitative Biology*. 65:303-314.

Lees-Miller, S., R. Godbout, D. Chan, M. Weinfeld, R. Day, 3rd, G. Barron, and J. Allalunis-Turner. 1995. Absence of p350 subunit of DNA-activated protein kinase from a radiosensitive human cell line. *Science*. 267:1183-1185.

Leung-Pineda, V., C. Ryan, and H. Piwnica-Worms. 2006. Phosphorylation of Chk1 by ATR is antagonized by a Chk1-regulated protein phosphatase 2A circuit. *Molecular and Cellular Biology*. 26:7529-7538.

Li, H., L. Zhao, J. Funder, and J. Liu. 1997. Protein phosphatase 2A inhibits nuclear telomerase activity in human breast cancer cells. *Journal of Biological Chemistry*. 272:16729-16732.

Li, J., and D. Stern. 2005. Regulation of CHK2 by DNA-dependent protein kinase. *Journal of Biological Chemistry*. 280:12041-12050.

Liu, B., and X. Xu. 2011. Serine/threonine protein phosphatases in DNA damage response. *Chinese Science Bulletin*. 56:3122-3131.

Liu, Q., S. Guntuku, X. Cui, S. Matsuoka, D. Cortez, K. Tamai, G. Luo, S. Carattini-Rivera, F. DeMayo, A. Bradley, L. Donehower, and S. Elledge. 2000. Chk1 is an essential kinase that is regulated by ATR and required for the G2/M DNA damage checkpoint. *Genes and Development*. 14:1448-1459.

Liu, X., A. Matsuda, and W. Plunkett. 2008. Ataxia-telangiectasia and Rad3-related and DNA-dependent protein kinase cooperate in G2 checkpoint activation by the DNA strand-breaking nucleoside analogue 2'-C-cyano-2'-deoxy-1-beta-D-arabino-pentofuranosylcytosine. *Molecular Cancer Therapeutics*. 7:133-142.

Loktionova, S., and A. Kabakov. 1998. Protein phosphatase inhibitors and heat preconditioning prevent Hsp27 dephosphorylation, F-actin disruption and deterioration of morphology in ATP-depleted endothelial cells. *FEBS Letters*. 433:294-300.

Lu, J., B. Ikejiri, N. Golpayegani, S. Kim, H. Mushlin, F. Johnson, J. Kovach, R. Lonser, and Z. Zhuang. 2008. LB-1, an inhibitor of serine-threonine protein phosphatase PP2A, suppresses the growth of glioblastoma cells *in vitro* and *in vivo*. *Proceedings of the American Association for Cancer Research Annual Meeting*. 49:1353.

Lu, J., J. Kovach, F. Johnson, J. Chiang, R. Hodes, R. Lonser, and Z. Zhuang. 2009. Inhibition of serine/threonine phosphatase PP2A enhances cancer chemotherapy

by blocking DNA damage induced defense mechanisms. *Proceedings of the National Academy of Sciences U S A*. 106:11697-11702.

Lu, X., B. Nannenga, and L. Donehower. 2005. PPM1D dephosphorylates Chk1 and p53 and abrogates cell cycle checkpoints. *Genes & Development*. 19:1162-1174.

Mackintosh, C., and R. Mackintosh. 1994. Inhibitors of protein-kinases and phosphatases. *Trends in Biochemical Sciences*. 19:444-448.

Margolis, S., S. Walsh, D. Weiser, M. Yoshida, S. Shenolikar, and S. Kornbluth. 2003. PP1 control of M phase entry exerted through 14-3-3-regulated Cdc25 dephosphorylation. *EMBO Journal*. 22:5734-5745.

Melo, J., and D. Toczyski. 2002. A unified view of the DNA-damage checkpoint. *Current Opinion in Cell Biology*. 14:237-245.

Mi, J., J. Dziegielewski, E. Bolesta, D.L. Brautigan, and J. Larner. 2009. Activation of DNA-PK by ionizing radiation is mediated by protein phosphatase 6. *PLoS One*. 4: e4395.

Minniti, G., R. Muni, G. Lanzetta, P. Marchetti, and R. Enrici. 2009. Chemotherapy for glioblastoma: current treatment and future perspectives for cytotoxic and targeted agents. *Anticancer Research*. 29:5171-5184.

Mochida, S., S. Maslen, M. Skehel, and T. Hunt. 2010. Greatwall phosphorylates an inhibitor of protein phosphatase 2A that is essential for mitosis. *Science*. 330:1670-1673.

Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*. 65:55-63.

Mueller, P., T. Coleman, A. Kumagai, and W. Dunphy. 1995. Myt1: A membrane-associated inhibitory kinase that phosphorylates Cdc2 on both threonine-14 and tyrosine-15. *Science*. 270:86-90.

Nakada, S., Y. Katsuki, I. Imoto, T. Yokoyama, M. Nagasawa, J. Inazawa, and S. Mizutani. 2006. Early G2/M checkpoint failure as a molecular mechanism underlying etoposide-induced chromosomal aberrations. *Journal of Clinical Investigation*. 116:80-89.

Nigg, E.A. 1995. Cyclin-dependent protein kinases: Key regulators of the eukaryotic cell cycle. *Bioessays*. 17:471-480.

Nitiss, J. 2009. Targeting DNA topoisomerase II in cancer chemotherapy. *Nature Reviews Cancer*. 9:338-350.

Norbury, C., J. Blow, and P. Nurse. 1991. Regulatory phosphorylation of the P34c-D-C-2 protein kinase in vertebrates. *EMBO Journal*. 10:3321-3330.

Norbury, C., and P. Nurse. 1992. Animal Cell Cycles and Their Control.61:441-470.

On, K., Y. Chen, H. Ma, J. Chow, and R. Poon. 2011. Determinants of mitotic catastrophe on abrogation of the G(2) DNA damage checkpoint by UCN-01. *Molecular Cancer Therapeutics*. 10:784-794.

Parker, L., and H. Piwnica-Worms. 1992. Inactivation of the p34-cdc2-cyclin B complex by the human WEE 1 tyrosine kinase. *Science*. 257:1955-1957.

Pellicioli, A., S. Lee, C. Lucca, M. Foiani, and J. Haber. 2001. Regulation of Saccharomyces Rad53 checkpoint kinase during adaptation from DNA damage-induced G2/M arrest. *Molecular Cell*. 7:293-300.

Pereira, S., V. Vasconcelos, and A. Antunes. 2011. The phosphoprotein phosphatase family of Ser/Thr phosphatases as principal targets of naturally occurring toxins. *Critical Reviews in Toxicology*. 41:83-110.

Petersen, P., D. Chou, Z. You, T. Hunter, J. Walter, and G. Walter. 2006. Protein phosphatase 2A antagonizes ATM and ATR in a Cdk2- and Cdc7-independent DNA damage checkpoint. *Molecular and Cellular Biology*. 26:1997-2011.

Pines, J., and T. Hunter. 1990. Human cyclin A is adenovirus e1a-associated protein P60 and behaves differently from cyclin B. *Nature*. 346:760-763.

Pizzolato, J., and L. Saltz. 2003. The camptothecins. *Lancet*. 361:2235-2242.

Raymond, E., V. Boige, S. Faivre, G.J. Sanderink, O. Rixe, L. Vernillet, C. Jacques, M. Gatineau, M. Ducreux, and J. Armand. 2002. Dosage adjustment and pharmacokinetic profile of irinotecan in cancer patients with hepatic dysfunction. *Journal of Clinical Oncology*. 20:4303-4312.

Raymond, E., M. Campone, R. Stupp, J. Menten, P. Chollet, T. Lesimple, R. Fety-Deporte, D. Lacombe, X. Paoletti, and P. Fumoleau. Multicentre phase II and pharmacokinetic study of RFS2000 (9-nitro-camptothecin) administered orally 5 days a week in patients with glioblastoma multiforme. *European Journal of Cancer*. 38:1348-1350.

Rezacova, M., G. Rudolfova, A. Tichy, A. Bacikova, D. Mutna, R. Havelek, J. Vavrova, K. Odrazka, E. Lukasova, and S. Kozubek. 2011. Accumulation of DNA Damage and Cell Death after Fractionated Irradiation. *Radiation Research*. 175:708-718.

Rieder, C., and A. Khodjakov. 2003. Mitosis through the microscope: Advances in seeing inside live dividing cells. *Science*. 300:91-96.

Riffell, J., C. Zimmerman, A. Khong, L. McHardy, and M. Roberge. 2009. Effects of chemical manipulation of mitotic arrest and slippage on cancer cell survival and proliferation. *Cell Cycle*. 8:3025-3038.

Rivory, L., M. Haaz, P. Canal, F. Lokiec, J.P. Armand, and J. Robert. 1997. Pharmacokinetic interrelationships of irinotecan (CPT-11) and its three major plasma metabolites in patients enrolled in phase I/II trials. *Clinical Cancer Research*. 3:1261-1266.

Roca, J. 1995. The mechanisms of DNA topoisomerases. *Trends in Biochemical Sciences*. 20:156-160.

Rogakou, E., D. Pilch, A. Orr, V. Ivanova, and W. Bonner. 1998. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *Journal of Biological Chemistry*. 273:5858-5868.

Rudner, A., and A. Murray. 1996. The spindle assembly checkpoint. *Current Opinion in Cell Biology*. 8:773-780.

Rudrabhatla, P., W. Albers, and H. Pant. 2009. Peptidyl-prolyl isomerase 1 regulates protein phosphatase 2A-mediated topographic phosphorylation of neurofilament proteins. *Journal of Neuroscience*. 29:14869-14880.

Sakoff, J., I. Howitt, S. Ackland, and A. McCluskey. 2004. Serine/threonine protein phosphatase inhibition enhances the effect of thymidylate synthase inhibition. *Cancer Chemotherapy and Pharmacology*. 53:225-232.

Sancar, A., L. Lindsey-Boltz, K. Unsal-Kacmaz, and S. Linn. 2004. Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. *Annual Review of Biochemistry*. 73:39-85.

Sandell, L., and V. Zakian. 1993. Loss of a yeast telomere: Arrest, recovery, and chromosome loss. *Cell*. 75:729-739.

Scholey, J., I. Brust-Mascher, and A. Mogilner. 2003. Cell division. *Nature*. 422:746-752.

Sebastian, B., A. Kakizuka, and T. Hunter. 1993. Cdc25M2 activation of cyclin-dependent kinases by dephosphorylation of threonine-14 and tyrosine-15. *Proceedings of the National Academy of Sciences of the United States of America*. 90:3521-3524.

Shi, Y.G. 2009. Serine/threonine phosphatases: Mechanism through structure. *Cell*. 139:468-484.

Siegel, R., E. Ward, O. Brawley, and A. Jemal. 2011. Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths. *CA A Cancer Journal for Clinicians*. 61:212-236.

Smith, J., L. Tho, N. Xu, and D. Gillespie. 2010. The ATM-Chk2 and ATR-Chk1 pathways in DNA damage signaling and cancer. *Advances in Cancer Research*. 108:73-112.

Sorensen, C., M. Melixetian, D. Klein, and K. Helin. 2010. NEK11 linking CHK1 and CDC25A in DNA damage checkpoint signaling. *Cell Cycle*. 9:450-455.

Spurney, R. 2001. Regulation of thromboxane receptor (TP) phosphorylation by protein phosphatase 1 (PP1) and PP2A. *Journal of Pharmacology and Experimental Therapeutics*. 296:592-599.

Stratton, M., P. Campbell, and P. Futreal. 2009. The cancer genome. *Nature*. 458:719-724.

Stubbs, M., H. Tran, A. Atwell, C. Smith, D. Olson, and G. Moorhead. 2001. Purification and properties of *Arabidopsis thaliana* type 1 protein phosphatase (PP1). *Biochimica et Biophysica Acta-Protein Structure and Molecular Enzymology*. 1550:52-63.

Suzuki, T., K. Fujikura, T. Higashiyama, and K. Takata. 1997. DNA staining for fluorescence and laser confocal microscopy. *Journal of Histochemistry and Cytochemistry*. 45:49-53.

Syljuåsen, R., S. Jensen, J. Bartek, and J. Lukas. 2006. Adaptation to the ionizing radiation-induced G(2) checkpoint occurs in human cells and depends on checkpoint kinase 1 and Polo-like kinase 1 kinases. *Cancer Research*. 66:10253-10257.

Tanaka, T., and N. Shimizu. 2000. Induced detachment of acentric chromatin from mitotic chromosomes leads to their cytoplasmic localization at G(1) and the micronucleation by lamin reorganization at S phase. *Journal of Cell Science*. 113:697-707.

Tkacz-Stachowska, K., C. Lund-Andersen, A. Velissarou, J.H. Myklebust, T. Stokke, and R. Syljuåsen. 2011. The amount of DNA damage needed to activate the radiation-induced G2 checkpoint varies between single cells. *Radiotherapy and Oncology*. 101:24-27.

Toczyski, D., D. Galgoczy, and L. Hartwell. 1997. CDC5 and CKII control adaptation to the yeast DNA damage checkpoint. *Cell*. 90:1097-1106.

Toivola, D., and J. Eriksson. 1999. Toxins affecting cell signalling and alteration of cytoskeletal structure. *Toxicology in Vitro*. 13:521-530.

Vakifahmetoglu, H., M. Olsson, and B. Zhivotovsky. 2008. Death through a tragedy: mitotic catastrophe. *Cell Death and Differentiation*. 15:1153-1162.

van Vugt, M., A. Bras, and R. Medema. 2005. Restarting the cell cycle when the checkpoint comes to a halt. *Cancer Research*. 65:7037-7040.

Venter, J., M. Adams, E. Myers, P. Li, R. Mural, G. Sutton, H. Smith, M. Yandell, C. Evans, R. Holt, J. Gocayne, P. Amanatides, R. Ballew, D. Huson, J. Wortman, Q. Zhang, C. Kodira, X. Zheng, L. Chen, M. Skupski, G. Subramanian, P. Thomas, J. Zhang, G. Miklos, C. Nelson, S. Broder, A. Clark, J. Nadeau, V. McKusick, N. Zinder, A. Levine, R. Roberts, M. Simon, C. Slayman, M. Hunkapiller, R. Bolanos, A. Delcher, I. Dew, D. Fasulo, M. Flanigan, L. Florea, A. Halpern, S. Hannenhalli, S. Kravitz, S. Levy, C. Mobarry, K. Reinert, K. Remington, J. Abu-Threideh, E. Beasley, K. Biddick, V. Bonazzi, R. Brandon, M. Cargill, I. Chandramouliswaran, R. Charlab, K. Chaturvedi, Z. Deng, V. Di Francesco, P. Dunn, K. Eilbeck, C. Evangelista, A. Gabrielian, W. Gan, W. Ge, F. Gong, Z. Gu, P. Guan, T. Heiman, M. Higgins, R. Ji, Z. Ke, K. Ketchum, Z. Lai, Y. Lei, Z. Li, J. Li, Y. Liang, X. Lin, F. Lu, G. Merkulov, N. Milshina, H. Moore, A. Naik, V. Narayan, B. Neelam, D. Nusskern, D. Rusch, S. Salzberg, W. Shao, B. Shue, J. Sun, Z. Wang, A. Wang, X. Wang, J. Wang, M. Wei, R. Wides, C. Xiao, and C. Yan. 2001. The sequence of the human genome. *Science*. 291:1304-1351.

Vigneron, S., E. Brioudes, A. Burgess, J. Labbe, T. Lorca, and A. Castro. 2009. Greatwall maintains mitosis through regulation of PP2A. *EMBO Journal*. 28:2786-2793.

Virsik-Kopp, P., M. Rave-Frank, H. Hofman-Huther, and H. Schmidberger. 2003. Role of DNA-PK in the process of aberration formation as studied in irradiated human glioblastoma cell lines M059K and M059J. *International Journal of Radiation Biology*. 79:61-68.

Vitale, I., L. Galluzzi, M. Castedo, and G. Kroemer. 2011. Mitotic catastrophe: a mechanism for avoiding genomic instability. *Nature Reviews Molecular Cell Biology*. 12:384-391.

Wang, X., Y. Zhu, K. Lui, Q. Cai, P. Lu, and R. Poon. 2008. Aberrant polo-like kinase 1-Cdc25A pathway in metastatic hepatocellular carcinoma. *Clinical Cancer Research*. 14:6813-6820.

Ward, I., and J. Chen. 2001. Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress. *Journal of Biological Chemistry*. 276:47759-47762.

Yoo, H., A. Kumagai, A. Shevchenko, A. Shevchenko, and W. Dunphy. 2004. Adaptation of a DNA replication checkpoint response depends upon inactivation of Claspin by the Polo-like kinase. *Cell*. 117:575-588.

Zhang, Y., J. Brognard, C. Coughlin, Z. You, M. Dolled-Filhart, A. Aslanian, G. Manning, R. Abraham, and T. Hunter. 2009. The F box protein Fbx6 regulates Chk1 stability and cellular sensitivity to replication stress. *Molecular Cell*. 35:442-453.

Zhang, Y., D. Otterness, G. Chiang, W. Xie, Y. Liu, F. Mercurio, and R. Abraham. 2005. Genotoxic stress targets human Chk1 for degradation by the ubiquitin-proteasome pathway. *Molecular Cell*. 19:607-618.

Zhao, H., and H. Piwnica-Worms. 2001. ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1. *Molecular and Cellular Biology*. 21:4129-4139.

Zhou, B., and S. Elledge. 2000. The DNA damage response: Putting checkpoints in perspective. *Nature*. 408:433-439.