The role of Mcl-1 in the response of human colorectal cancer cells to treatment with dichloroacetate

by

Leanne Delaney

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ABSTRACT

The role of Mcl-1 in the response of human colorectal cancer cells to treatment with dichloroacetate

Leanne Delaney
University of Guelph, 2013

Advisor: Dr. B. L. Coomber

Dichloroacetate (DCA) is a metabolic reprogramming agent that is used to target the unique metabolism of cancer cells, but is not always effective in colorectal cancer cells. In HCT116 cells, DCA was unable to induce apoptosis, but did decrease proliferation when compared to untreated cells. A decrease in full length Mcl-1 protein expression 7 hours following DCA treatment did not correspond with changes in mRNA production or changes in expression of inhibitory binding partners, but may be due to altered proteasomal degradation. Similar reduction in levels of a lower molecular weight Mcl-1 band occurred, which did not result from alternative splicing or from caspase-mediated cleavage. Mcl-1 showed primarily nuclear localization within the cell, and expression changes in full-length Mcl-1 were seen in nuclear lysate but not cytoplasmic lysate after 7 hours of DCA treatment. Changes in nuclear Mcl-1 expression did not correspond with cell cycle arrest or progression. These results suggest that proteasomal degradation of Mcl-1 may be altered following treatment with DCA, and this change may be associated with decreased proliferation, independent of cell cycle arrest. This may indicate a novel role of nuclear Mcl-1 in response of colorectal cancer to DCA exposure.
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DECLARATION OF WORK PERFORMED

I declare that all work reported in this thesis was performed by me.
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>2DG</td>
<td>2-deoxy-D-glucose</td>
</tr>
<tr>
<td>3PO</td>
<td>3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Bad</td>
<td>B-cell lymphoma-associated agonist of cell death</td>
</tr>
<tr>
<td>Bag-1</td>
<td>B-cell lymphoma-associated athanogene</td>
</tr>
<tr>
<td>Bak</td>
<td>B-cell lymphoma-antagonist/killer</td>
</tr>
<tr>
<td>Bax</td>
<td>B-cell lymphoma-associated X protein</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell CLL/lymphoma 2</td>
</tr>
<tr>
<td>Bcl-x</td>
<td>B-cell lymphoma-extra</td>
</tr>
<tr>
<td>Bid</td>
<td>BH3 interacting domain death agonist</td>
</tr>
<tr>
<td>Bim</td>
<td>B-cell lymphoma-like protein 11</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CAPS</td>
<td>3-(Cyclohexylamino)-1-propanesulfonic acid</td>
</tr>
<tr>
<td>CDK1</td>
<td>Cyclin-dependent kinase 1</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-Diamidino-2-Phenylindole</td>
</tr>
<tr>
<td>DCA</td>
<td>Dichloroacetate</td>
</tr>
<tr>
<td>DIPA</td>
<td>Diisopropylammonium dichloroacetate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Media</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxyribonucleotide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-signal-regulated kinase</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FH</td>
<td>Fumarate hydratase</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyeraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase</td>
</tr>
<tr>
<td>HIF1α</td>
<td>Hypoxia inducible factor 1 α</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxide</td>
</tr>
<tr>
<td>IDH1</td>
<td>Isocitrate Dehydrogenase</td>
</tr>
<tr>
<td>IEX-1</td>
<td>Radiation-inducible immediate-early gene</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>Mcl-1</td>
<td>Myeloid cell leukemia sequence 1</td>
</tr>
<tr>
<td>MMP</td>
<td>Mitochondrial membrane potential</td>
</tr>
<tr>
<td>MOMP</td>
<td>Mitochondrial outer membrane permeabilization</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td>MULE</td>
<td>Mcl-1 ubiquitin ligase E3</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>NBS1</td>
<td>Nijmegen Breakage Syndrome 1</td>
</tr>
<tr>
<td>p53</td>
<td>Protein 53</td>
</tr>
<tr>
<td>PAH</td>
<td>Pulmonary arterial hypertension</td>
</tr>
<tr>
<td>PASMC</td>
<td>Pulmonary artery smooth muscle cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDH</td>
<td>Pyruvate dehydrogenase</td>
</tr>
<tr>
<td>PDK</td>
<td>Pyruvate dehydrogenase kinase</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PLK1</td>
<td>Polo-like kinase 1</td>
</tr>
<tr>
<td>PKM2</td>
<td>Pyruvate kinase muscle isoenzyme 2</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulfonylfluoride</td>
</tr>
<tr>
<td>PPP</td>
<td>Pentose phosphate pathway</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>Puma</td>
<td>p53 upregulated modulator of apoptosis</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>R5P</td>
<td>Ribose 5-phosphate</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase PCR</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Real time quantitative PCR</td>
</tr>
<tr>
<td>SDH</td>
<td>Succinate dehydrogenase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris buffered saline - tween 20</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N,N-Tetramethylethylene diamine</td>
</tr>
<tr>
<td>TNF-α</td>
<td>4’,6-Diamidino-2-Phenylindole</td>
</tr>
<tr>
<td>USP9X</td>
<td>Probable ubiquitin carboxyl-terminal hydrolase FAF-X</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>γ-H2AX</td>
<td>H2A histone family, member X</td>
</tr>
</tbody>
</table>
INTRODUCTION

The development and progression of cancer is a complex process in which molecular changes occur to enable phenotypes typically associated with the development of malignancy: rapid and uncontrolled proliferation, resistance to apoptosis, evasion of growth suppressors, development of replicative immortality, induction of angiogenesis, and the ability to invade and metastasize to surrounding tissues (1). More recently, the altered metabolism of cancer cells emerged as another enabling characteristic of cancer development, and provided a unique opportunity in terms of cancer therapy (1). Although traditional cancer treatment involves cytotoxic chemotherapy that targets populations of proliferating cells, this event is not exclusive to cancer cells and thus chemotherapy has detrimental side effects to the human body through its interference with normal rapidly proliferating cells (2). Cancer cells preferentially utilize aerobic glycolysis to metabolize glucose, rather than using glucose oxidation in the mitochondria (3). Targeting this altered metabolism of cancer cells, referred to as the Warburg effect, is a more exclusive therapy that has been shown to leave normal cells largely unaffected (4-6).

Development of a specific cancer therapy has the potential to not only inhibit the progression of tumours alone, but also to overcome resistance of cancer to current therapeutic interventions (7-11). One proposed mechanism by which cancer therapy can target the Warburg effect is through reversing the altered metabolism of cancer cells back to that of normal cells, thereby inhibiting the advantages sustained by this phenotype. The Warburg effect allows cells to increase generation of biomolecules required for proliferation through the accumulation of metabolic intermediates (12). It also contributes to apoptosis resistance by creating hyperpolarized mitochondria and generating reducing
power, allowing control over reactive oxygen species (ROS) which, if uncontrolled, leads to oxidative stress and apoptosis (13).

One mechanism by which cancer cells maintain their metabolic phenotype is through the overproduction of pyruvate dehydrogenase kinase (PDK), which inhibits pyruvate dehydrogenase (PDH) (14). This inhibition prevents pyruvate, the end product of glycolysis, from entering mitochondria and undergoing TCA cycle and oxidative phosphorylation (14). Instead, pyruvate is converted into lactate and shuttled out of the cell, leading to an acidic microenvironment that also contributes to malignancy (15). Inhibition of PDK, therefore, could reverse the Warburg effect and therefore decrease cancer malignancy by slowing cell proliferation and increasing apoptosis.

Dichloroacetate (DCA) is a PDK inhibitor that has shown promise as a cancer treatment that does not harm somatic cells that are normally affected by chemotherapy (4-6). Currently, it has been experimentally shown to induce apoptosis in certain types of cancer, and abolishing therapeutic resistance to other anti-cancer compounds in combination treatments (7-11). In colorectal cancer, however, the effects of DCA are not as expected, and many reports indicate that DCA is ineffective or effective only at doses well above physiological relevance (16-18). As colorectal cancer is the second leading cause of cancer-related deaths in North America, and its high mortality rate at late stages is due to a high incidence of acquired resistance to therapy, it is of interest to determine the molecular mechanisms by which these cells are resisting DCA-induced apoptosis (19).

The Bcl-2 family is comprised of pro- and anti-apoptotic proteins that interact with each other to determine whether the mitochondrial outer membrane will become
permeabilized, leading to cytochrome c release, caspase activation, and apoptosis (20). This family of proteins is commonly dysregulated in cancer, sometimes as a byproduct of aerobic glycolysis, and contributes to their resistance to apoptosis. Mcl-1, an anti-apoptotic protein is very important in determining whether or not apoptosis will occur; its expression alone has been shown to confer resistance to several cancer therapies (21-23). These same therapies have been shown to interact with DCA to enhance or overcome resistance to apoptosis, indicating a potential role of Mcl-1 in cellular responses to DCA treatment (7,8,10,11,24). Mcl-1 also plays an additional role in the cell; nuclear localization of Mcl-1 has been shown to correspond to cell cycle regulation, and Mcl-1 has been shown to interact with checkpoint regulators as well as localize to sites of DNA damage during DNA damage responses (25-29). This dual role of Mcl-1 within the cells creates several possibilities for its activity in response to DCA. Mcl-1 levels could relate to mitochondrial-induced apoptosis, or proliferation and advancement through the cell cycle. The role of Mcl-1 in response to DCA treatment has not been evaluated, and may provide critical insight into a mechanism by which cancer cells evade DCA-induced apoptosis.

This thesis will provide evidence to support the use of metabolic remodeling agents in cancer therapy, and discuss the use of one particular agent, DCA, in reducing proliferation and increasing cell death. It will then establish a potential link between DCA and Mcl-1, and investigate the regulation of Mcl-1 following DCA treatment in colorectal cancer cells.
LITERATURE REVIEW

Colorectal Cancer

Although the incidence and mortality of colorectal cancer has decreased in recent years as a result of improved screening standards, colorectal cancer remains one of the deadliest cancers in North America (19). While surgery can be curative if the cancer is detected early, in late stages of this cancer, treatment with cytotoxic chemotherapy is used in attempts to eradicate the disease. However, this type of non-specific treatment has considerable side effects (19). Current chemotherapeutics include replication inhibitors such as 5-fluorouracil, which inhibits any rapidly proliferating cells by interfering with DNA replication (30). Although cancer cells are indeed rapidly dividing and therefore susceptible to this drug, side effects occur due to interference with normal populations of rapidly proliferating cells resulting in problems with the digestive tract (31), fatigue, hair loss, weight loss, and mouth ulcers (2). Heterogeneity in the genome within colorectal cancer tumours results in a large incidence of chemoresistance in patients, contributing to the poor prognosis of late-stage disease (32). More effective therapy could be developed that targets phenotypes not only essential for the maintenance of malignancy, but also that are unique to cancer cells. This would not only decrease devastating side effects seen with traditional chemotherapy, but may also help to overcome acquired resistance to current treatments.

Cancer cell metabolism

The Warburg effect

Decades ago, Otto Warburg provided the first evidence of altered glucose metabolism in cancer cells by comparing rates of oxidative phosphorylation and lactic
acid fermentation between malignant and normal tissues (3). Since then, it has been consistently noted that a relatively high glycolytic rate is maintained in cancer cells regardless of oxygen availability. The metabolism of glucose via glycolysis followed by lactic acid fermentation in normoxia is referred to as aerobic glycolysis, and in hypoxia it is referred to as anaerobic glycolysis. The preferential use of aerobic glycolysis in cancer cells is in contrast to most normal tissue, where oxidative phosphorylation is preferred due to the higher energy output per glucose molecule. One consequence of this altered metabolism is a drastic increase in glucose uptake by cancer cells, which has been exploited in tumour imaging using fluorescently labeled glucose and positron emission tomography (PET) (33). Exploitation of the Warburg effect also holds potential in the development of cancer specific therapies; the Warburg effect is unique and common to the majority of cancer cells, and is associated with the development of a malignant phenotypes (34).

**Mitochondrial dysfunction in initiation of the Warburg effect**

The first popular theory explaining the initiation of the Warburg effect was that cancer cells have dysfunctional mitochondria, which creates a need for alternative metabolic processing of glucose. Defects in enzymes involved in the mitochondrial tricarboxylic acid (TCA) cycle lead to changes in metabolic intermediate production, which can enhance the Warburg effect through the activation and stabilization of the transcription factor hypoxia-inducible factor 1α (HIF1α). Somatic mutations in isocitrate-dehydrogenase 1 (IDH1) in gliomas cause reduced levels of α-ketogluterate, leading to increased levels of HIF1α (35). Additionally, germline mutations in fumarate hydratase (FH) and succinate dehydrogenase (SDH) correspond with a high risk of developing
leiomyomas or renal cell cancer (36), and paragangliomas or phaeochromocytomas (37), respectively. Mutations in these two mitochondrial enzymes cause a buildup of fumarate and succinate, which correlates to the overexpression and stabilization of HIF1α in vitro and in vivo (38,39).

Mutations in mitochondrial DNA (mtDNA), which encodes enzymes in the electron transport chain (ETC), have also been shown to contribute to the Warburg effect. mtDNA mutations resulting in dysfunctional or decreased NADH dehydrogenase, cytochrome oxidase, and ATP synthase subunits have been associated with a rise in reactive oxygen species (ROS) in human cells in vitro and in primary tumours (40-42). Increased ROS production is seen in human leukemia, ovarian cancer, colorectal cancer, and breast cancer in vitro (43,44). Elevated ROS has been shown to lead to transcriptional upregulation of HIF1α and its activation (45,46).

Although mitochondrial dysfunction can contribute to the Warburg effect, it has been shown that human cancer cells still possess the ability to metabolize glucose in the mitochondria when glucose levels are low in vitro (47). Increased ATP production via oxidative phosphorylation can be achieved in cancer cells by altering their energy substrate from glucose to glutamine in human cells in vitro (48,49). Cancer cell mitochondria remain active through generation of TCA cycle intermediates in glutamine metabolism (50) and β-oxidation of fatty acids (51). Therefore, mitochondrial defects might not always be the reason for the Warburg phenotype, and instead this altered metabolism may be attributed to genetic and molecular changes associated with tumourigenesis.
Molecular regulation of the Warburg effect

Molecular changes associated with the Warburg effect can be largely attributed to the effect of signaling pathways, tumour suppressors, and oncogenes on important enzymes in glucose metabolism. Particularly, upregulation of glucose transporters facilitates the drastic increase in glucose uptake seen in human cancer cells, whereas upregulation of glycolytic machinery, combined with suppression of pyruvate oxidation, can explain the cancer cell’s preference for aerobic glycolysis (52-54).

The serine/threonine kinase AKT is commonly dysregulated in cancers by constitutive activation, and its overexpression has been reported as an early event during sporadic colon carcinogenesis (55). AKT increases glucose intake by increasing glucose transporter synthesis and translocation to the plasma membrane (53). Its involvement in aerobic glycolysis is shown by its ability to increase glucose metabolism independently of an increase in oxygen consumption in vitro (56).

The tumour suppressor p53 is one of the most commonly mutated genes in human cancers including colorectal cancer; its impaired function has shown a strong correlation with malignancy (57,58). Loss of p53 function contributes to the Warburg effect as p53 downregulates glucose transporters (59), and upregulates proteins responsible for inhibiting glycolysis (60) and promoting mitochondrial oxygen consumption (52).

The oncogenic transcription factor HIF1α is usually rapidly degraded in normoxia, however its expression and activation is upregulated in many human cancers including colorectal cancer (61). It acts as a driving force for the Warburg effect through its transcriptional control over many proteins responsible for facilitating aerobic glycolysis. It is involved in upregulation of glucose transporters (62), glycolytic enzymes
and lactate dehydrogenase A (54), proteins that transport lactate out of the cell (63), and pyruvate dehydrogenase kinase (PDK) (14). PDK inhibits the enzyme pyruvate dehydrogenase (PDH), which is responsible for linking glycolysis to mitochondrial metabolism through the conversion of pyruvate to acetyl-CoA, which then enters the TCA cycle (14). PDK may play a critical role in the reprogramming of cancer metabolism by inhibiting mitochondrial processing of glucose following glycolysis.

The molecules contributing to the initiation of the Warburg effect are commonly associated with the generation of a malignant phenotype independently of their ability to fuel aerobic glycolysis (64). Therefore, an effort has been made to determine whether or not the Warburg effect is necessary for cancer progression, or a consequence of cancer progression.

**Metabolic reprogramming as a hallmark of cancer**

In 2011, Hanahan and Weinburg revisited their initial hypothesis, which stated that sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis are the six hallmark characteristics of cancer (65). In their updated version, they identified reprogramming energy metabolism and the Warburg effect as an emerging hallmark (64). Its designation as a hallmark is tentative; this may be a fundamental physiological change in the development of malignancy, or simply a phenotype that results as a byproduct of the activity of molecules associated with the original hallmarks of cancer. There is a growing volume of evidence that suggests that the former is true; metabolic reprogramming may be an important contributor to the malignancy of cancer cells.
Aerobic glycolysis increases oncoprotein activity

Aerobic glycolysis can positively feed back on the activation of glycolysis-enhancing oncogenic molecules. Aerobic glycolysis results in high levels of pyruvate within the cell, which causes the accumulation of active HIF1α, contributing to malignancy through upregulation of genes involved in angiogenesis (66). Additionally, increasing aerobic glycolysis and decreasing mitochondrial respiration has been shown to decrease levels of PTEN, thereby increasing activation of AKT in vitro (67). AKT plays a central role in tumourigenesis through its involvement in regulation of apoptosis, cell cycle, and angiogenesis (68). Since HIF1α and AKT are involved in generation and maintenance of aerobic glycolysis, a positive feedback loop may be established that can drastically enhance tumour malignancy. Indeed, upregulation of HIF1α and AKT have been associated with poor prognosis in colorectal and prostate/breast cancer, respectively (69,70).

Lactic acid enhances tumour malignancy

Lactic acid generation and export out of the cell is the last step in aerobic glycolysis and is also proposed to be beneficial to tumours for a number of reasons. An acid-mediated tumour invasion model suggests that increasing lactic acid in the tumour microenvironment promotes invasion and metastasis by degrading the extracellular matrix and inducing cell death in normal tissue (15). Cancer cells, specifically colorectal cancer cells, are thought to acquire resistance to acid-induced apoptosis, and are capable of invasion into the acidic microenvironments that they generate (71). An acidic microenvironment has also been shown to enhance angiogenesis through increasing VEGF expression in human cancer cells in vitro (72). Furthermore, it has been suggested
that lactic acid secreted by cancer cells has an inhibitory effect on the human immune response (73).

*Rapid proliferation is sustained by glycolytic intermediates*

Studies show that in normal human pluripotent stem cells, aerobic glycolysis is the preferred mechanism of glucose metabolism, and this shifts to preferential mitochondrial glucose oxidation as cells become differentiated and less proliferative (74). Somatic cells that exhibit higher rates of aerobic glycolysis are more quickly reprogrammed into induced pluripotent stem cells than somatic cells that show more of a preference for oxidative phosphorylation *in vitro* (75). This is in accordance with previous research suggesting that metabolic reprogramming is not a consequence of pluripotency but a necessary change for pluripotent induction (76). Human pluripotent stem cells use some of the same strategies as cancer cells in inducing this metabolic phenotype, such as upregulating glycolytic enzymes and inactivating PDH (77). The commonalities between the metabolism of cancer cells and human pluripotent stem cells are also shared with other groups of rapidly proliferating cells, such as activated T cells. T cells have been shown to upregulate and increase membrane localization of glucose transporters to facilitate aerobic glycolysis when an immune response is initiated (78).

Although glycolysis is much less efficient than oxidative phosphorylation in ATP production, evidence suggests that cancer cells do not attempt to maximize cellular ATP; in fact, cancer cells decrease levels of cellular ATP to diminish its feedback inhibition on glycolysis (79,80). The fact that ATP generation is not the main function of aerobic glycolysis in cancer cells highlights the importance of generating metabolic intermediates to sustain rapid proliferation. Pyruvate kinase is the enzyme responsible for catalyzing the
last step in glycolysis to generate pyruvate; if this step is slowed down, the cell diverts glycolytic intermediates towards pathways involved in the synthesis of macromolecules as opposed to generating ATP from conversion of pyruvate to lactate (81). In cancer cells, the M2 isoform of pyruvate kinase (PKM2) is preferentially expressed and provides a growth advantage for tumours (82). This isoform of pyruvate kinase is tightly controlled and easily manipulated by the tumour cell to inactivate its catalytic function, allowing the accumulation of the metabolic intermediates that drive proliferation (12). Colorectal cancer has been shown to have upregulated expression of PMK2, allowing maintenance of high proliferative capabilities (83).

With inactivation of PMK2, tumours experience a buildup of glycolytic intermediates, and cancer cells upregulate enzymes responsible for initiating biosynthesis of nucleic and amino acids from these accumulating glycolytic intermediates. Phosphoglycerate dehydrogenase initiates synthesis of serine and glycine from glycolytic carbon and is expressed at high levels in cancer, acting as a key molecule associated with oncogenesis (84). The pentose phosphate pathway (PPP) is responsible for, among other things, biosynthesis of nucleic acid precursor ribose 5-phosphate (R5P) from glycolytic intermediates via an oxidative and non-oxidative pathway (85). Transketolase-like 1, which controls non-oxidative R5P generation, is upregulated in human cancers and corresponds to poor prognosis (86,87). Transketolase-like 1 is important in invasion and tumour growth and its upregulation is negatively associated with patient survival in colorectal cancer (88). It has been suggested that glucose 6-phosphate dehydrogenase, which controls oxidative R5P generation, is not as important in tumour metabolism (89).
Similarities between metabolism of cancer cells and other rapidly proliferating cells such as pluripotent stem cells and active T cells suggest that the Warburg effect plays a key role in facilitating tumour proliferation. Evidence supports the concept that ATP generation is not favored in cancer cell metabolism of glucose. Furthermore, enzymes capable of diverting glycolytic intermediates from ATP-generating pathways towards biosynthetic pathways are upregulated in cancer and associated with poor prognosis. Thus, rapid glycolysis not only results in an acidic microenvironment that favors tumour progression, it is also of fundamental importance in maintaining levels of cellular building blocks like nucleic acids and amino acids to sustain rapid proliferation.

The Warburg effect contributes to apoptosis resistance

Reduction of key enzymes associated with aerobic glycolysis has been associated with increased apoptosis in cancer (90), and upregulation of glycolytic enzymes corresponds with resistance to chemotherapy-induced apoptosis in colorectal cancer cells (91,92). This indicates that altered metabolism plays a key role in the ability of cancer to evade cell death.

Reactive oxygen species (ROS) play a multi-faceted role in cancer cell development and survival (93). On one hand, enhanced generation of ROS has been shown to contribute to tumourigenesis (1,94). On the other hand, excessive ROS production has been shown to cause DNA damage, activating G2/M phase cell cycle arrest, and, if damage is sustained, inducing apoptosis in cancer cells in vitro (95). The importance of maintaining a balance between a tumourigenic and a lethal cellular concentration of ROS is, therefore, critical to cancer cell function. Aerobic glycolysis is a
key mechanism by which cancer cells protect themselves from potentially harmful oxidative stress (96).

This protection from oxidative stress is through generating reducing potential for ROS detoxification, as well as by limiting pyruvate availability for oxidative phosphorylation (13). Aerobic glycolysis plays a key role in this protection; it has been shown in vitro that ROS inhibits PKM2, which increases glucose flux through the pentose phosphate pathway and generates reducing potential for ROS detoxification (97). Specifically, increased pentose phosphate activity via PKM2 inhibition has been shown to increase the levels of cellular reduced glutathione, which corresponds with a decreased level of cellular ROS (98). Inducing changes in glucose metabolism in colorectal cancer cells results in alterations of the pentose phosphate pathway, decreased levels of reduced glutathione, and increased levels of ROS, which were associated with G2/M phase cell cycle arrest and extensive apoptosis (99).

In addition to generating reducing potential, enhancement of glycolysis also contributes to ROS protection by reducing mitochondrial respiration (13). Reduction of mitochondrial respiration allows for maintenance of mitochondrial membrane potential (MMP) in a hyperpolarized state; decreasing MMP causes generation of ROS in colorectal cancer cells in vitro (17). Inhibition of lactate dehydrogenase A in cancer has been shown to increase ROS and induce apoptosis by forcing cancer cells to metabolize pyruvate using mitochondrial respiration, thus decreasing MMP (100). Decreasing MMP by activating PDH, thereby shifting pyruvate metabolism from glycolysis to oxidative phosphorylation, also generates ROS and induces apoptosis in colorectal cancer cells in vitro (4,17). Thus, control over cellular ROS levels through generation of reduction
potential and maintenance of mitochondrial membrane hyperpolarization is a key mechanism by which the Warburg effect contributes to apoptosis resistance in cancer cells.

Aerobic glycolysis also enhances cellular resistance to apoptosis through its modulation of Bcl-2 proteins (101). The Bcl-2 protein family consists of pro- and anti-apoptotic members whose balance in expression and activity ultimately determines whether or not mitochondrial outer membrane permeabilization (MOMP) will occur, resulting in caspase-mediated apoptosis (20). Aerobic glycolysis has been shown to stabilize the anti-apoptotic Bcl-2 protein Mcl-1, while inhibiting the activity of pro-apoptotic proteins Puma, Bid, and Noxa. In cancer cells, aerobic glycolysis causes stabilization of AKT (67). Glycolysis-mediated AKT activation stabilizes the expression of Mcl-1 by preventing its proteasomal degradation (102), and glycolysis maintains normal translation of Mcl-1 (103). Stimulation of glycolysis via activated AKT has been shown to inhibit the expression of Puma in human cancer cells in vitro (104). The glycolytic enzyme hexokinase has shown AKT-dependent mitochondrial localization, where it inhibits the activity of Bid (105). In addition to AKT-dependent glycolytic regulation of Bcl-2 protein expression, it is currently thought that Noxa inhibition may be achieved via a glucose-dependent pathway (106,107).

**The Warburg effect as a therapeutic target**

The Warburg effect fundamentally contributes to cancer malignancy on many levels, and has provided the opportunity to develop more cancer-specific therapies, as altered metabolism is a phenotype shared with few other groups of cells. By interfering with the malignant phenotype that cancer cells develop via the Warburg effect, inhibition
of aerobic glycolysis in cancer treatment could greatly reduce tumour malignancy, decreasing its proliferation, apoptotic resistance, and potentially inducing apoptosis.

Directly inhibiting glycolytic enzymes is the purpose of many recently developed anti-cancer drugs. Hexokinase, the enzyme that catalyzes the first step in the glucose metabolic pathway, can be targeted using 2-deoxy-D-glucose (2DG) (108), 3-bromopyruvate (109), lonidamide (110), mannoheptulose (111), and methyl jasmonate (112). Phosphofructokinase can be targeted using 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) (113). Glyeraldehyde-3-phosphate dehydrogenase (GAPDH) can be inhibited using iodoacetamide or iodoacetate (114). PKM2 is inhibited by compounds such as TT232 (115), and LDHA can be inhibited by compounds such as galloflavin (116).

Although inhibiting glycolysis may seem ideal in the treatment of cancer cells, directly inhibiting glycolytic enzymes may not result in the desired outcome. 2DG, which functions to competitively inhibit glucose transporters and hexokinase (117), has been shown to interfere with the ability of the brain to rapidly uptake glucose more than it interferes with tumour glucose uptake (118). 3-Bromopyruvate has been shown to cause toxicity in liver and gastrointestinal system (119). Iodoacetate has shown to be ineffective in reducing proliferation or inducing apoptosis in certain cancer cells (120). Thus, it is important to develop more effective and exclusive cancer therapies that exploit the Warburg effect.

*Reversing aerobic glycolysis*

Although inhibition of glycolytic enzymes shows some promise in the treatment of cancer by suppressing the Warburg effect, this method does not guarantee specificity
to cancer cells, as other cells in the body require rapid glucose uptake and glycolysis (118,119). This is because although cancer cells show a significant increase in glucose uptake and glycolysis, the unique feature of their metabolism lies in the fact that glycolysis is not coupled to mitochondrial respiration, as is usually seen in normal cell populations (121). Therefore, instead of inhibiting glycolytic enzymes, it is of interest to revert the metabolic phenotype of cancer cells back to that of normal cells by coupling glycolysis with mitochondrial respiration.

In normal cells, pyruvate generated from glycolysis is converted into acetyl-coA and transported into the mitochondria in a reaction catalyzed by the rate-limiting enzyme PDH, where it is metabolized through pyruvate oxidation. In cancer cells, the inhibition of PDH by PDK is critical in maintaining the metabolic phenotype and malignancy of cancer (122). PDK upregulation has been linked to tumour malignancy and drug resistance in colorectal and non-small cell lung cancer (123,124). Therefore, PDK serves as a unique therapeutic target that, if inhibited, will revert the metabolic phenotype in cancer cells (11,125).

*Dichloroacetate as a metabolic reprogramming agent*

Dichloroacetate (DCA) is a small molecule inhibitor of PDK that has recently gained popularity after research published in 2007 showed its ability to preferentially promote apoptosis and reduce proliferation of non small cell lung cancer cells *in vitro* (4). Prior to this publication, the therapeutic potential of DCA in cancer was not realized, and primary interest in this compound was in the treatment of other metabolic disorders. In 1962, research showed that diisopropylammonium dichloroacetate (DIPA) reduced hyperglycemia and increased respiration in diabetic rats (126). Several years later, it was
suggested that the ability of DIPA to promote glucose oxidation exclusively in diabetic rats was due to its acid moiety, DCA (127). The ability of DCA to activate PDH in diabetic rat cells was first suggested in 1973, when an increase in glucose and pyruvate oxidation was associated with an increase in PDH-catalyzed acetyl-coA levels (128). This activation of PDH by DCA in rats was finally linked to an inhibitory effect of DCA on PDK, an enzyme that inhibits the activity of PDH (129).

In addition to diabetes, research suggests that DCA can improve cardiac function by modulating energy metabolism. Through its activation of PDH, DCA promotes the preferential oxidation of carbohydrates over fatty acids in cardiac tissue, increasing efficient energy production and therefore improving myocardial function in vivo and in vitro (130). DCA is able to enhance myocardial efficiency in patients with coronary artery disease (131), which may result in myocardial infarction and cardiac hypertrophy if untreated. Cardiac hypertrophy can also result from pulmonary arterial hypertension (PAH), in which cells exhibit a glycolytic shift that can be reversed by DCA to improve cardiac output (132). DCA has also been shown to enhance apoptosis of pulmonary artery smooth muscle cells (PASMC) by decreasing mitochondrial membrane potential in vitro (133), and decrease the PASMC proliferation that leads to PAH (134). If cardiac hypertrophy does occur, DCA can improve postischemic function and recovery in rat hearts by coupling glycolysis with glucose oxidation (135).

Through its ability to increase pyruvate flux into the mitochondria for oxidative phosphorylation by indirectly activating PDH, DCA decreases production of lactate in patients with cirrhosis (136). Therefore, DCA showed promise as a therapy for lactic acidosis, a potentially fatal disease caused by a buildup of lactic acid in body tissues and
blood (137). However, although DCA showed statistically significant changes in blood pH following treatment, these results were not translated in clinical trials; DCA has little effect on the clinical outcome of patients being treated for lactic acidosis (24,138).

*Dichloroacetate as a cancer-specific therapy*

DCA has been experimentally proven to increase glucose oxidation, reverse glycolytic shift, decrease resistance to apoptosis, inhibit rapid proliferation, and decrease lactate production in cancer cells experimentally, but its translation to clinical trials has been disappointing. However, recent evidence supporting its potential as an exclusive cancer-targeting agent has sparked renewed interest in DCA’s metabolic remodeling capabilities (139). In fact, DCA has been shown to reverse almost all of the malignant phenotypes that result from the Warburg effect in cancer.

Secretion of lactic acid by tumours results in an acidic microenvironment, which has been shown to suppress the immune system (73). DCA reduced lactic acid secretion and immunosuppression in thymoma and melanoma tumours *in vivo*, and therefore holds the potential to increase efficacy of cancer immunotherapy (140).

The ability of aerobic glycolysis to increase levels of HIF1α leads to angiogenesis *in vitro* and *in vivo* (66). In non-small cell lung cancer and mammary carcinoma, DCA treatment caused downregulation of HIF1α, decreasing its ability to initiate and sustain angiogenesis *in vitro* and *in vivo* (6). One mechanism by which glioma cells stabilize HIF1α is through IDH mutations that decrease cellular levels of α-ketogluterate (35). In glioblastoma, DCA treatment increased cellular levels of α-ketogluterate, leading to a decrease in HIF1α and an inhibition of angiogenesis *in vivo* (5). A decrease in HIF1α
activity also resulted from DCA treatment in melanoma (141) and T cell lymphoma (142).

In addition to downregulation of HIF1α, DCA increases the activity of p53 in non-small cell lung cancer, breast cancer, and T cell lymphoma cell culture (6,142). The ability of p53 to promote mitochondrial respiration is partially due to its downregulation of PDK2 (143), and increased p53 activity resulted in decreased HIF1α following DNA damage induced by oxidative stress in vitro (144,145).

Two other main phenotypes resulting from the Warburg effect are increased proliferation, and resistance to ROS-induced apoptosis. These can be reversed by DCA in non-small cell lung cancer, breast cancer, and glioblastoma in vitro and in vivo, with no negative effects on normal cell populations (4-6). Inhibition of PDK2 by siRNA mimicked the physiological effect of DCA on the cancer cells, indicating that DCA's effects were due to PDK inhibition (4). Publications also support the potential role of DCA alone or combined with other cancer therapies as an effective treatment of prostate cancer (146), endometrial cancer (147), pancreatic cancer (148), cervical cancer (149), head and neck cancer (150), gastric cancer (11), hepatocellular carcinoma (8), and liver cancer (10).

Although initiation of apoptosis is seen in many cancer cell lines in vitro, data collected in vivo indicate slowed tumour growth rather than tumour eradication, indicating a potentially more important role of slowed proliferation in DCA treatment. In rat mammary adenocarcinoma cells, DCA treatment over 4 days caused a cell-line dependent reduction in proliferation by 20-80%, without any increase in apoptosis in vitro (151). Furthermore, metastatic breast cancer in vivo showed a 58% decrease in lung
metastases following DCA treatment, corresponding to decreased proliferation in vitro (151). Further evidence of the ability of DCA to decrease proliferation without increasing apoptosis was shown in multiple myeloma, where certain cell lines were less proliferative but no more apoptotic at lower doses of DCA, and less proliferative and more apoptotic at higher doses of DCA (7). The same anti-proliferative effect of DCA at low doses compared to an apoptotic effect at higher doses was seen in a separate study on colorectal cancer cell lines (17). The ability of DCA to decrease proliferation as well as induce apoptosis may therefore be cancer cell line specific, and/or dose-dependent.

These results may lead to implementing DCA as a stand-alone treatment, but also show the potential of DCA as an agent that enhances the apoptotic effects of other established treatments. DCA diminished the ability of cancer cells to resist apoptosis through treatment with sorafenib, a small molecule inhibitor of tyrosine protein kinases, by increasing oxidative phosphorylation (8). In hypoxic cancer cells that have adapted to treatment with bevacizumab, combination treatment with DCA restores inhibition of angiogenesis, reducing tumour growth (9). In combination with cisplatin, DCA significantly reduced tumour growth in vivo when compared with cisplatin alone (10). DCA in combination with 5-fluorouracil increased apoptosis and decreased lactate production in vitro in cancer cells more effectively than 5-fluorouracil alone (11). Similar results were seen with DCA in combination with bortezomib; DCA greatly enhanced sensitivity to bortezomib, reducing proliferation, increasing ROS production, and increasing apoptosis in vitro (7).

DCA is particularly successful in brain tumours like glioma and glioblastoma, and its ability to cross the blood-brain barrier makes is ideal for effective delivery of the drug
The first clinical trials in glioblastoma were conducted in 2010 (5). In glioblastoma cell cultures derived from 49 isolated tumours, comparisons were made between pre- and post-DCA-treatment phenotypes. DCA treatment depolarized mitochondria, increased mitochondrial ROS, increased apoptosis, decreased proliferation, and decreased HIF1α expression, which corresponded with decreased tumour vasculature. In Phase II clinical trials, 5 glioblastoma patients, three of whom had exhibited disease progression after several chemotherapeutic treatments, were treated with DCA for 15 months at a therapeutic concentration of approximately 0.5 mM. All but one of the patients, who died after three months, were clinically stable after 15 months of DCA treatment. Currently, clinical trials are recruiting patients for DCA in combination with cisplatin for head and neck cancers, and clinical trials are underway for DCA treatment in glioblastomas and other neoplasms (153).

Although positive therapeutic effects of DCA are seen pre-clinically and in early clinical trials of brain cancers, treatment of colorectal cancer with DCA has not been as favorable. Treatment of colorectal cancer cells in vitro with DCA showed activity only against cancer cells with defects in electron transport chain or mitochondrial DNA, was not exclusive to cancer cells, and only induced apoptosis at concentrations well over physiological relevance (16). Other studies have confirmed that in colorectal cancer cells, DCA is required at doses up to 50 mM to induce apoptosis in vitro (17), 100 times the physiological dose used in previous clinical trials (5). Furthermore, a study from our laboratory (2010) showed that DCA had a cell line-dependent ability to induce apoptosis in colorectal cancer cell lines in vitro, and in some cases protected hypoxia-induced apoptosis and enhanced tumour growth in vivo (18). The mechanism by which colorectal
cancer cells may evade DCA-induced apoptosis is therefore of interest in determining the basis for drug resistance in certain cancer cells.

**Bcl-2 family member Mcl-1 in resistance to apoptosis**

One mechanism by which cancer cells resist apoptosis is through differential regulation of Bcl-2 proteins. Bcl-2 proteins are overexpressed in many types of cancer, and regulate a critical step in mitochondrial outer membrane permeabilization (MOMP) preceding apoptosis (20). Mcl-1, an anti-apoptotic member of the Bcl-2 family confers resistance to several types of cancer treatments and may be a key modulator of susceptibility to apoptosis-inducing agents in cancer. Upregulation of Mcl-1 in promyelocytic leukemia *in vitro* allows resistance to apoptosis induced by standard anti-cancer drugs mitoxantrone, vincristine, etoposide, and doxorubicin, while its downregulation facilitates cell death (21). ABT-737, an anti-apoptotic Bcl-2 family protein inhibitor, is ineffective in several cancer cell lines due to its low affinity for Mcl-1, indicating that Mcl-1 can promote drug resistance even if its other anti-apoptotic family members are suppressed (22). By suppressing Mcl-1 in combination with ABT-737, it is possible to overcome cancer resistance to this drug, including in colorectal cancer cell lines *in vitro* (154-156). Colorectal cancer cells also show resistance to chemotherapy-induced senescence through Mcl-1 overexpression (157). Mcl-1 is unique among the Bcl-2 family of proteins in that it is involved in not only apoptosis, but also cell cycle progression and proliferation in cancer cells, and it is highly and rapidly regulated within the cell. The rapid regulation of Mcl-1 makes it a potential facilitator of drug resistance in cancer cells, as changes in its activity can occur rapidly to protect cells against apoptosis (158).
Pre-translational modifications of Mcl-1

Among other transcriptional regulators, Mcl-1 is upregulated by the AKT survival pathway (159), which is enhanced by aerobic glycolysis in cancer (67). Following transcription, exon skipping results in production of an Mcl-1 splice variant containing exon 1 and 3, as opposed to the full-length Mcl-1 transcript that contains exon 1, 2, and 3 (160). This splice variant shows pro-apoptotic qualities as it has the capability of binding and inhibiting anti-apoptotic Mcl-1 (161). Its expression appears to be affected by the levels of exon junction complex protein Y14 (162). Intriguingly, proteins involved with cell cycle such as CDK1, aurora kinases, and PLK1, regulate splicing of Mcl-1, and increasing the expression of this splice variant generally precedes cell cycle arrest and apoptosis (163).

Post-translational modifications of Mcl-1

Another death-promoting shortened form of Mcl-1 may arise as a result of caspase-mediated cleavage, a characteristic that is not shared by the other anti-apoptotic Bcl-2 family proteins Bcl-2 and Bcl-x (164). This caspase-mediated cleavage has been shown to modify Mcl-1's subcellular location, increase its affinity for deactivating binding partners, and inhibit its ability to protect the cell against apoptosis (165). A short fragment of Mcl-1 generated by caspase-mediated cleavage is also critical in the induction of apoptosis by bortezomib (166).

Unlike other members of the Bcl-2 family, Mcl-1 has a short half-life of approximately 30 minutes, allowing it to respond rapidly to treatment (167). Proteasomal degradation of Mcl-1 can be achieved independently of ubiquitination (168) or through
ubiquitination targeting lysine residues by Mcl-1 ubiquitin ligase E3 (MULE) (169) and β-transducin-containing protein (23), and reversed by USP9X (170). Stability following inhibition of proteasomal degradation allows it to confer resistance of cancer cells to apoptosis induced by proteasomal inhibitors (171). Stabilization of Mcl-1 can also be achieved through ERK-mediated phosphorylation at threonine 92, and inhibiting ERK-mediated phosphorylation promotes sorafenib-induced apoptosis in vitro (172). Mcl-1 undergoes JNK-mediated phosphorylation at serine 121 and threonine 163, which is thought to stabilize it and protect cells against TNF-α-induced apoptosis (173), although other studies show that this phosphorylation propagates oxidative stress-induced apoptosis (174). Increasing Mcl-1 turnover rate through proteasomal degradation is possible through GSK-3-mediated phosphorylation at serine 155 and serine 159 (23). With a mutant form of Mcl-1 unable to be phosphorylated at serine 155, breast cancer cells showed marked resistance to apoptosis induced by 5-fluorouracil, cisplatin, and taxol (23). Phosphorylation at serine 159 is also thought to interfere with the ability of Mcl-1 to bind and inhibit Bim, a pro-apoptotic Bcl-2 protein (175).

Mcl-1 has multiple regulatory binding partners within the cell, and its binding patterns can determine whether or not apoptosis will occur via MOMP. Bak, a pro-apoptotic molecule responsible for forming pores in the mitochondrial outer membrane leading to cytochrome c release, caspase activation, and apoptosis, is sequestered by Mcl-1 binding, preventing its apoptotic function (176). Bax, a second pore-forming pro-apoptotic protein is indirectly inhibited by Mcl-1 following its activation (177). Mcl-1 is also capable of binding and inhibiting activated Bid (178) and Bim (179), both of which are pro-apoptotic proteins. Although some studies claim that these pro-apoptotic proteins
are responsible for directly activating Bak and Bax (180), others suggests that these effects are indirect and that Bid and Bim simply sequester anti-apoptotic proteins like Mcl-1, preventing their inhibition of Bak and Bax (181). Therefore, these pro-apoptotic proteins may have dual roles in apoptosis induction. Mcl-1 also affects the ability of Puma, a pro-apoptotic protein, to induce apoptosis in colorectal cancer cells by binding and inhibiting its function (182). Puma also potentially inhibits Mcl-1 by displacing its inhibitory grasp on Bak (183). While Bid, Bim, and Puma are capable of inhibiting several Bcl-2 proteins, Noxa is exclusively capable of binding Mcl-1 and displacing its interaction with Bak and Bim to induce apoptosis (184). This activity of Noxa is critical in sensitizing cells to bortezomib-induced apoptosis (154-156). Noxa also mediates hypoxia-induced apoptosis through its upregulation and inhibition of Mcl-1 (185), as the C-terminal sequence of Noxa is capable of targeting Mcl-1 for proteasomal degradation (186). Puma and Noxa are both upregulated by p53 in response to DNA damage (187,188), and therefore may play a role in response of cancer cells to oxidative stress. Although the precise mechanism by which interactions between Bcl-2 proteins result in apoptosis, it is clear that Mcl-1 plays an integral role in preventing apoptosis through its interactions with other Bcl-2 family members.

Role of Mcl-1 in proliferation and cell cycle progression

Although Mcl-1 is primarily associated with its role in promoting cell survival at the mitochondrial level, several reports indicate that it exhibits nuclear localization in vitro as well (25,27). It is proposed that regulating the localization of Mcl-1 between the nucleus and the mitochondria is achieved through modification of its N terminus, which determines whether it will be anti-apoptotic at the mitochondria, or anti-proliferative at
the nucleus (189). Additional reports state that phosphorylation of Mcl-1 on serine 162 is required for its mitochondrial localization, and without this phosphorylation, Mcl-1 is primarily localized to the nucleus where its ability to interact with Bak is impaired (190).

Mcl-1 levels fluctuate throughout the cell cycle due to phosphorylation at serine 64 by CDK1 and CDK2 in the G2/M phase (29), which has been shown to accelerate proteasomal degradation of Mcl-1, potentially mediating apoptosis that results from prolonged mitotic arrest (28). Treating cells with a CDK inhibitor blocked apoptosis induced by proteasomal degradation of Mcl-1, indicating that activation of CDK during mitotic arrest propagates an apoptotic response through cell-cycle dependent control over Mcl-1 (191). It has also been suggested that a shortened form of Mcl-1 translocates into the nucleus and binds to CDK1 in an inhibitory fashion; CDK1 normally plays a role in progression through G2 and M phases, and this inhibitory interaction could explain the role of Mcl-1 in reduced cell growth (25). An inhibitory role of Mcl-1 on the S phase of the cell cycle may be due to its interaction with proliferating cell nuclear antigen (PCNA); an Mcl-1 mutant lacking the PCNA binding site was not able to inhibit cell cycle progression during etoposide treatment, while cells expressing wildtype Mcl-1 were delayed in the cell cycle following treatment with etoposide (27).

It has been suggested that cell cycle arrest may overcome the induction of apoptosis (192); cancer cells may, in fact, reduce proliferation in order to overcome drug-induced apoptosis (193). Mcl-1 may play a role in this proliferation adjustment through cell cycle arrest, and has been shown to interact with and become phosphorylated by Chk1, resulting in an accumulation of cells in the G2 phase (194). Phosphorylation by Chk1 suggests a novel role for Mcl-1 in DNA damage repair; cells lacking Mcl-1 were
shown to have a greater incidence of chromosomal abnormalities following etoposide treatment, and Mcl-1 was shown to be recruited to points of DNA damage during cell cycle arrest and associate with proteins in complex that function to sense DNA damage (26). The early response gene product IEX-1 can initiate Mcl-1 localization to the nucleus to maintain G2 arrest following treatment with DNA-damaging agents, highlighting the important role of Mcl-1 in DNA damage response (195).

The role of Mcl-1 in the cell cycle may support a key link between apoptosis and proliferation. Mcl-1 is regulated throughout the cell cycle by Cdk-mediated phosphorylation and subsequent degradation, but also can play a role in cell cycle arrest. Mcl-1 may bind to PCNA to induce cell cycle arrest in response to DNA-damaging agents, and may also directly contribute to DNA repair through its phosphorylation by Chk1. This novel function of Mcl-1 will provide insight into the mechanism by which Mcl-1-mediated resistance to apoptosis is achieved.

*DCA and Mcl-1*

While there is no published evidence directly linking Mcl-1 activity to cancer cell response to DCA treatment, a role for Mcl-1 in this response may exist. Mcl-1 is a critical step in sorafenib-induced apoptosis (172). Cells treated with DCA are able to overcome resistance to sorafenib, with DCA inducing apoptosis in cancer cells combined with sorafenib that cannot be accomplished by sorafenib alone (8). Mcl-1 regulation by Noxa is critical in propagating apoptosis in response to bortezomib treatment, and the generation of a caspase-mediated Mcl-1 cleavage product is an important outcome of Noxa binding to Mcl-1 in this response (7). As with sorafenib, DCA has been shown to overcome resistance to bortezomib or enhance bortezomib-induced apoptosis in cancer.
Mcl-1 stabilization also confers resistance to 5-fluorouracil and cisplatin (23), both of which can be combined with DCA to overcome apoptosis resistance in cancer cells (10,11). The ability of DCA to enhance or overcome resistance to apoptosis induced by sorafenib, bortezomib, 5-fluorouracil, and cisplatin indicates that DCA could potentially modulate Mcl-1 activity following treatment, overcoming the ability of Mcl-1 to protect cancer cells against apoptosis. DCA induces cell cycle arrest in G2 phase of colorectal cancer cells (17). Mcl-1 may not only play a role in apoptosis at the mitochondria, but may prevent apoptosis through its nuclear function of reducing proliferation in order to overcome DNA damage (193), accounting for this cell cycle arrest.
RATIONALE

DCA has been proposed to specifically inhibit the progression of tumours while leaving normal cells unaffected due to its ability to reverse the metabolic phenotype of cancer cells back to that of normal cells. DCA has been shown to induce apoptosis and decrease proliferation, leading to clinical trials in cancers such as glioblastoma. However, experimental results of colorectal cancer treated with DCA are inconclusive; in some cases, colorectal cancer cells withstand DCA-induced apoptosis and in others, DCA enhances tumour growth and protects against hypoxia-induced apoptosis. It is due to these unexpected results that this thesis will focus on the molecular changes associated with DCA-treated colorectal cancer cells. Determining altered protein expression may shed light on unknown mechanisms by which cancer cells can resist drug-induced apoptosis. Bcl-2 family proteins are key mediators of cellular apoptosis and are commonly dysregulated in cancer cells. Thus, the first objective of this thesis was as follows:

Objective 1: Determine protein expression changes in pro- and anti-apoptotic Bcl-2 proteins in colorectal cancer cell lines exposed to DCA, and investigate how apoptosis and proliferation relate to these changes.

Mcl-1 is an important protein that modulates resistance to several cancer therapies; resistance to these therapies can be overcome by DCA treatment, leading to a potentially important role of Mcl-1 in the cellular response to DCA. Mcl-1 has been shown to protect cells against apoptosis, as well as mediate DNA damage response, cell cycle progression,
and therefore proliferation. It has been suggested that a cell may evade apoptosis by sacrificing the ability to rapidly proliferate; Mcl-1 may play an integral role in this response. The relationship between Mcl-1 activity and DCA treatment has not been explored previously, and may be valuable in determining a novel role of Mcl-1 in evading DCA-induced apoptosis. Thus, the second objective of this thesis is as follows:

**Objective 2:** Characterize Mcl-1 activity within the cell following DCA treatment by examining changes in the multiple levels at which it is regulated, and relate these changes to altered cellular behavior.

Pursuing these objectives may lead to enhanced understanding of the variable responses of cancer types to DCA treatment, and determine a mechanism by which colorectal cancer cells exhibit resistance to DCA-induced apoptosis.
MATERIALS AND METHODS

A list of suppliers for chemical and reagents is found in Appendix I, and details of solution preparation are found in Appendix II.

Tissue Culture

Human colorectal cancer cell lines HCT116 (ATCC), HCT116 p53−/− (196), and HCT116 Bax−/− (197) were grown in DMEM containing 10% FBS, 1 mM sodium pyruvate, and 50 µg/ml gentamicin in a 37°C incubator with 5% CO₂. Cells were passaged every 3-4 days at approximately 95% confluence. Cells were grown in 12- or 6-well plates, or single plates either 6 cm or 10 cm in diameter.

Cells were serum starved for approximately 24 hours prior to any treatment in DMEM containing 0.1% FBS. For induction of hypoxic responses, 150 µM cobalt chloride was added to the media along with respective treatments. Cells were treated with 20 mM DCA unless otherwise indicated. To induce apoptosis, cells were treated with either 50 µM etoposide or 385 nM 5-fluorouracil. To induce oxidative stress, cells were treated with 50 µM H₂O₂. To inhibit proteasomal activity, cells were treated with 1 µM MG132. Treatments were left on from 2 hours to 4 days; media was changed daily for cells treated longer than 24 hours.

Protein isolation and quantification

For extraction of whole cell lysate, 50-250 µl lysis buffer was used to re-suspend pelleted cells, or added directly to cell culture plates prior to scraping and transfer into a fresh tube. Once mixed via pipetting with lysis buffer, cells were incubated for 10 minutes on ice before centrifugation at 12,000 x g for 15 minutes at 4°C. Supernatant was removed and mixed in a fresh tube, and then separated into 10 µl aliquots before being
stored at -80°C. Separation of nuclear from cytosolic protein was performed using the BioVision Inc. Nuclear/Cytosolic Fractionation Kit reagents and protocol. Nuclear and cytosolic fractions were stored separately -80°C. Whole cell, nuclear, and cytosolic protein lysates were assessed equally in protein quantification and western immunoblotting, as described below.

Protein was quantified using the Bio-Rad DC™ Protein Assay Kit reagents and protocol, standardizing OD630 values of each sample to control values for 0.2, 0.5, 1, 2.5, and 5 µg/µl BSA. Based on these values, between 20-50 µg of total protein was then used for further analysis.

**SDS-PAGE and western immunoblotting**

Proteins were loaded onto 15% polyacrylamide gels and separated by electrophoresis for 1 hour and 15 minutes at 125 V in electrophoresis running buffer. For Noxa, proteins were transferred onto a methanol-activated polyvinylidene difluoride (PVDF) membrane with a pore size of 0.2 µm using wet transfer with CAPS buffer at 300 mA for 1 hour. All other proteins were transferred onto a methanol-activated PVDF membrane with a pore size of 0.45 µm using semi-dry transfer with semi-dry transfer buffer at 17 V for 25 minutes. Transfer of proteins onto PVDF membrane was confirmed by staining of membranes with amido black, which allows for all protein bands to be visualized, followed by destaining with methanol prior to blocking.

Membranes were blocked for 1 hour at room temperature with 5% (w/v) non fat milk in Tris-buffered saline/Tween 20 (TBS-T) and incubated overnight at 4°C with primary antibodies diluted in 5% (w/v) non fat milk in TBS-T. Primary antibodies and their respective concentrations were as follows: mouse-anti-α-tubulin (1:100,000), rabbit-
anti-Bid (1:2,000), rabbit-anti-Bad (1:5,000), rabbit-anti-Bax (1:5,000), rabbit-anti-Bcl-2 (1:1,000), rabbit-anti-Bcl-x (1:2,500), rabbit-anti-Mcl-1 (1:1,000), rabbit-anti-Bag-1 (1:2,000), rabbit-anti-Puma (1:1,000), mouse-anti-Noxa (1:1,000), rabbit-anti-caspase 3 (1:800), rabbit-anti-GSK3β (1:1,000), rabbit-anti-pGSK3β (1:1,000), rabbit-anti-Lamin A/C (1:1,000), and rabbit-anti-β-actin (1:1,000). After incubation with primary antibody, membranes were washed 3 times for 10 minutes with TBS-T before incubation with HRP-labeled goat-anti-mouse or goat-anti-rabbit secondary antibodies diluted 1:20,000 in 5% (w/v) non-fat milk in TBS-T for 1 hour at room temperature. After incubation with secondary antibody, membranes were washed 3 times for 10 minutes with TBS-T. Membranes were then exposed to enhanced chemiluminescence using HRP substrate Luminata™ Forte and visualized using X-ray film exposure or using the Bio-Rad ChemiDoc™ XRS+ system. Molecular weight of proteins was determined by comparison with GeneDirex® BLUeye Prestained Protein Ladder, which was loaded next to protein samples before electrophoresis. Densitometric analysis was performed using the Bio-Rad Image Lab™ Software.

**Immunofluorescence**

Fluorescence microscopy was used to determine subcellular localization of Mcl-1, and to observe the formation of apoptotic bodies following treatment. For the subcellular localization of Mcl-1, cells were grown on sterile Fisherbrand Superfrost® Plus slides for 24 hours and fixed for 15 minutes with 4% paraformaldehyde. After fixation, slides were washed 3 times for 5 minutes each time with phosphate-buffered saline (PBS) and allowed to dry overnight. Slides were then incubated for 1 hour at room temperature in blocking buffer. Following blocking, cells were incubated overnight at 4°C, in anti-Mcl-1.
diluted 1:100 in antibody dilution buffer or just in antibody dilution buffer for a negative control. The next day, slides were washed 3 times for 5 minutes each time in PBS and incubated at room temperature with a goat-anti-rabbit secondary antibody conjugated to Cy3® diluted 1:300 in antibody dilution buffer. Upon addition of the fluorescent secondary antibody, slides were kept in reduced light for all incubation periods and storage. After incubation with the secondary antibody, slides were washed 3 times for 5 minutes each time in PBS and incubated at room temperature for 7 minutes with 30 μM 4’,6-diamidino-2-phenylindole (DAPI) diluted 1:100 in PBS. Cells were then washed a final 3 times for 5 minutes each time. After the final washes, Dako Fluorescent Mounting Medium was used to apply coverslips prior to visualization.

For observation of apoptotic bodies, trypsinized adhered cells were collected along with the floating cells from the media and centrifuged at 1,000 x g for 10 minutes. Following centrifugation, cells were resuspended to a final concentration of 1 x 10^6 cells/ml in PBS, and 200 μl was added to a single cytospin funnel attached to a Fisherbrand Superfrost® Plus slide. To transfer the cell suspension to the slide, cytospins were performed at 700 x g for 6 minutes using a Shandon Cytospin 3 Cytocentrifuge. Slides were then fixed as previously described and allowed to dry overnight. The following day, cells were stained for 7 minutes with DAPI, washed, and coverslipped as previously described.

Visualization of immunofluorescent slides was done using a Leica Opti-tech microscope with a QImaging™ QICAM Fast 1394 camera, and images were captured using QCapture Pro™ software and overlaid using Adobe® Photoshop.
**Growth curve determination**

To determine the rate of proliferation and viability of cells following treatment, cell counts were performed every day for 4 days in treated and untreated cells. Cells were washed and given fresh media with or without treatment each day. Cells were plated in 12-well plates at 25,000 cells per well in triplicate. After 24 hours, cells were counted and treatment began. To count cells, every 24 hours, media was collected to account for dead or floating cells, and adhered cells were trypsinized. Trypsinized cells were added to the media and separated into single cells using a 22-gauge needle. From each replicate of both treatments, 10 µl cell suspension was added to 10 µl Trypan blue solution, added to a disposable Countess™ cell counting chamber slide and counted with a Countess™ automated cell counter. Cell number was analyzed to determine if growth rate differed between treated and untreated cells, and viability was calculated to determine if decreased growth rate was due to an increase in cell death.

**RNA isolation and PCR**

For RNA isolation, 1 ml TRIzol was added to cells on a 6 cm plate. Cells were then scraped off of the plate, transferred to a 1.5 ml centrifuge tube, and incubated for 5 minutes at room temperature. Following incubation, 0.2 ml chloroform was added to the TRIzol, and tubes were vortexed for approximately 30 seconds prior to incubation at room temperature for 10 minutes. Following incubation, cells were centrifuged at 12,000 x g for 15 minutes at 4°C, and the top of 3 resulting phases was transferred into a new tube and mixed with 0.5 ml isopropanol before being incubated overnight at -20°C. Following incubation, tubes were centrifuged at 12,000 x g for 10 minutes and the
supernatant was removed. Pellet was mixed with 75% ethanol by pipetting, and centrifuged at 7,500 x g for 5 minutes at 4°C. Supernatant was poured off and RNA was solubilized with DEPC-treated water by pipetting.

RNA was quantitated @ A260 with Thermo Scientific Nanodrop® ND-1000 and used for cDNA synthesis by reverse transcription using the Invitrogen SuperScript® First-Strand Synthesis System for RT-PCR in the Eppendorf Mastercycler®. 0.5 µl cDNA was then mixed with 0.5 µl each primer (10 µM) designed to amplify both isoforms of Mcl-1 mRNA (1050 bp/810 bp; F: 5’TAATCGGACTCAACCTCTACTGTG, R: 5’TAGATATGCCAAACCAGCTCCT), as well as 0.5 µl dNTPs (10 mM), 2.5 µl 10 X buffer and 0.2 µl Taq DNA transcriptase from Invitrogen, and RNase free water to a final volume of 25 µl. Reaction conditions for the PCR in the Eppendorf Mastercycler® are as follows: 95°C for 5 minutes followed by 36 cycles of 95°C for 30 seconds, 56°C for 20 seconds, and 72°C for 60 seconds, followed by 72°C for 5 minutes. Following amplification, PCR products were run on a 1% agarose gel in TAE buffer with 1 X RedSafe™ Nucleic Acid Staining Solution at 100 V for 20 minutes, and visualized using Bio-Rad ChemiDoc™ XRS+ System. PCR products were run alongside a FroggaBio 1Kb DNA Ladder in order to determine relative size in base pairs.

For real-time quantitative PCR (RT-qPCR), reaction conditions were optimized until an assay performance efficiency of 90-110% was achieved; standard curves were run on the experimental plate to ensure that performance efficiency was maintained. Three biological replicates were combined with 0.5 µl each respective primer (10 µM) to amplify the target gene, Mcl-1 (298 bp; F: 5’-ATGCTTCGGAAAACCTGGACAT R: 5’-
TCCTGATGCCACCTTCTAGG (198), and the housekeeping gene, \( \beta\)-actin (171 bp; F: 5'-AAGATCAAGATCATTGCTCTC R: 5'-CAACTAAGTCATAGTCCGCC (199)). To this, a mixture of 4 µl cDNA diluted 1/16, and 5 µl Bio-Rad Ssofast™ Evagreen® Supermix, was made to a final reaction volume of 10 µl with RNase free water. Reaction conditions for the RT-qPCR in the CFX96™ Real-Time System from Bio-Rad were as follows: 95°C for 30 seconds followed by 39 cycles of 95°C for 10 seconds and 59°C for 10 seconds. A melt curve was performed immediately following PCR to ensure that only the desired product was amplified; PCR products were heated at increments of 0.5°C for 5 seconds at each temperature ranging from 65°C to 95°C. Data analysis for RT-qPCR products was performed using the CFX Manager™ software from Bio-Rad.

**Flow cytometry and cell cycle analysis**

To determine cell cycle stage following treatments, flow cytometric analysis was performed. After treatment, cells were washed with ice cold 1X PBS and trypsinized. Cells were collected by centrifugation at 200 x g for 5 minutes at 4°C and washed twice with ice cold PBS. Cells were resuspended at a concentration of 5 x 10^6 cells/ml using a 22-gauge needle to generate a single cell suspension, and 1 ml of the suspension was added to 9 ml of 70% ethanol through a BD Falcon™ 40 μm nylon cell strainer to remove clumps of cells. Cells were then further fixed in the 70% ethanol for 24 hours at -20°C.

After fixation, cells were spun at 200 x g for 10 minutes at 4°C and washed with PBS. Cells were resuspended in 500 µl of a 10 µg/ml propidium iodide (PI) staining solution and incubated for 15 minutes at 37°C, protected from light. Following staining, cells were stored at 4°C in the dark for no more than one hour prior to analysis. Analysis
was performed using the BD FACScan™ system and BD CellQuest™ Pro software. Cell scatter plots were used to select relevant cells for analysis based on forward and side scatter to determine cell size and content, and intensity of PI staining was measured through the FL-2 channel. DNA content was determined by intensity of PI staining; cells with 2N DNA content were assumed to be in G1, cells with 4N DNA content were assumed to be in G2/M, and all cells in between were assumed to be in various stages of the S phase. Cyflogic™ software was used to determine percentage of cells in each phase of the cell cycle.

**Statistical Analysis**

Statistical analysis was performed using GraphPad Prism® software. Wilcoxon paired t-tests were used to determine if differences existed in Mcl-1 mRNA transcription and Mcl-1 protein expression, while an unpaired Kolmogorov-Smirnov test was used to detect significant differences in cell growth rate between untreated and treated samples. At least three biological replicates were used for each analysis, and treatments were considered significantly different if statistical tests produced a $p$-value under 0.05.
RESULTS

DCA did not increase apoptosis in HCT116 cells

As DCA has been shown to induce apoptosis in a variety of cancer cell lines (5,146,147,152), I decided to determine if HCT116 cells were vulnerable to apoptosis following treatment with 20 mM DCA. In order to visualize cells undergoing apoptosis, HCT116 cells were stained with DAPI to determine the appearance of apoptotic bodies (Figure 1) Apoptotic bodies were seen following 24 hours of treatment with etoposide, which was used as a positive control (Figure 1A). As expected, apoptotic bodies were not seen in cells growing under control conditions (Figure 1B). Cells treated with 20 mM DCA also did not show a noticeable increase in apoptotic bodies after 24 hours of exposure (Figure 1C). This suggests that DCA does not induce apoptosis in HCT116 cells, as apoptotic bodies are a key physiological change associated with apoptotic cell death.

A second test was performed to confirm that cells were not undergoing DCA-induced apoptosis, as suggested by lack of apoptotic body formation in DCA-treated HCT116 cells. Caspase 3 is cleaved upon activation, resulting in the generation of 17 and 19 kDa fragments that initiate the apoptotic cascade following death stimuli. Western blotting of HCT116 cell lysates was used to determine if cells showed an increase in caspase 3 activation following treatments, indicated by levels of cleaved caspase 3 (Figure 2). Cells treated with 385 nm 5-FU as a positive control showed apoptotic activation by the appearance of 17 and 19 kDa proteolytic fragments of caspase 3. Untreated cells did not activate caspase 3, indicated by a lack of detectable caspase 3 cleavage products. Cells treated with 20 mM DCA also showed no caspase activation,
indicated by a lack of caspase 3 cleavage. Full-length caspase 3 (35 kDa) was expressed at equal levels in control, DCA-treated, and 5-FU-treated cells, indicating that increased levels of active caspase 3 were due to increased cleavage and not increased protein expression. This suggests that 20 mM DCA is not sufficient in inducing apoptosis through the activation of caspase 3, even at doses almost 50-fold greater than those of clinical relevance.

In order to further confirm the lack of apoptosis, overall viability of HCT116 cells was assessed after 4 days of treatment with 20 mM DCA. Staining untreated and treated cells with Trypan blue allowed comparison of the total number of viable and non-viable HCT116 cells. Cells grown in control media had a viability average of 96.66%, while cells grown in media with 20 mM DCA had a viability average of 96.33%, confirming the results that suggest a lack of apoptosis in DCA-treated HCT116 cells.

**DCA decreased proliferation in HCT116 cells**

DCA has also been shown to decrease proliferation of cancer cells, sometimes independently of apoptosis induction (151). Therefore, I generated a growth curve in which total cell numbers were counted for HCT116 cells grown in control media or media containing 20 mM DCA over 4 days (Figure 3). Upon treatment with DCA, cells proliferated at a slower rate than those growing in control media, and the final number of cells in DCA-treated wells was approximately 45% of the number of cells in control wells. The reduction in cell number following 4 days of treatment was determined not to be a result of cell death, as final viability of the cells was over 90% (Figure 3).
Expression of several Bcl-2 proteins did not change with DCA treatment

The Bcl-2 protein family is comprised of pro- and anti-apoptotic members whose expression has been linked to apoptotic resistance in cancer cells (20). To determine whether or not Bcl-2 protein expression corresponds with resistance to DCA-induced apoptosis or DCA-induced decrease in proliferation, western blots were used to visualize protein levels (Figure 4). DCA (20 mM) did not alter the expression levels of pro-apoptotic proteins Bax, Bid, and Bad or anti-apoptotic proteins Bcl-2, Bcl-x, and Bag-1 in HCT116 cells. However, DCA had a time-dependent effect on Mcl-1 protein (Figure 5A). After 2.5 hours, a lower molecular weight Mcl-1 band was detected, which was weaker in DCA-treated cells than in control cells. The intensity of the full-length Mcl-1 was also significantly less in DCA-treated cells when compared to control cells after 6 hours (p = 0.0078 Figure 5B). This variation in expression of full-length Mcl-1 was maintained when cells were treated with CoCl$_2$ to mimic hypoxia, indicating that these alterations in Mcl-1 were independent of HIF1α stabilization (Figure 5B). This decrease in Mcl-1 was sustained after a period of 24 hours (Figure 5A), suggesting that the initial decrease in Mcl-1 seen following 6 hours of treatment could be a long-term effect of DCA-treatment.
Figure 1. Detection of apoptotic bodies with DCA treatment. After 24 hours of treatment with 50 μM etoposide, apoptotic bodies (arrows) were visible in HCT116 cells (A). Following 24 hours, control (B) and DCA-treated (20 mM) (C) cells had morphologically normal nuclei, and DCA treatment did not induce apoptotic body formation. Scale bars = 10 μm.
Figure 2. Caspase 3 activation following DCA treatment. HCT116 cells were treated with 20 mM DCA for 18 hours to see if caspase activation occurred, indicating initiation of apoptosis. Caspase activation, indicated by the generation of 17 and 19 kDa proteolytic fragments can be seen after 18 hours of treatment with 5-FU, the positive control, but not in control or DCA treated cells. Tubulin was used to ensure equal loading of total protein, and full-length caspase was used to ensure that increases in activated caspase was due to a relative increase in cleavage, and not due to an increase in caspase 3 expression.
Figure 3. Rates of proliferation following DCA treatment. A growth curve was established by measuring the number of cells per well of HCT116 cells untreated and treated with 20 mM DCA. The rate of growth in DCA-treated cells is slower than the rate of growth in control cells, indicating that DCA could potentially be inhibiting the ability of cells to rapidly proliferate. Values plotted represent fold-increase of cells per well relative to day 1. Each data point is the average of three biological replicates, with error bars representing standard deviation.
Identity of lower Mcl-1 band

After observations of a lower molecular weight Mcl-1 band in western blots were made, I attempted to determine the identity of this lower band by looking at potential pre- and post-translational Mcl-1 modifications. As the intensity of this lower molecular weight band varies in a time-dependent manner (Figure 5A), I performed a more extensive time course to determine that the Mcl-1 lower molecular weight band was most prominent 4 hours after reintroduction to control media or media containing 20 mM DCA after overnight serum starvation (Figure 6A). As reports have indicated that the identity of a 28 kDa Mcl-1 band could be an isoform generated due to alternative message splicing (161), mRNA from cells grown in control medium or medium containing 20 mM DCA after overnight serum starvation was collected after 4 hours. The full-length Mcl-1 mRNA contains exons 1, 2, and 3, while the alternative splice variant contains only exons 1, and 3. Therefore, primers were generated to amplify gene products starting at exon 1 and ending at exon 3, so that both isoforms would be detected. The full-length isoform, which is translated into the full-length 40 kDa Mcl-1 protein, contains 1050 base pairs, while the short isoform, which is translated into a 28 kDa Mcl-1 protein, contains 810 base pairs. To determine whether two mRNA isoforms were present in these cells, RT-PCR using primers to amplify both splice variants was performed. In both control and DCA-treated HCT116 cells, an Mcl-1 amplification product at 1050 base pairs was present, but no band was seen at 810 base pairs (Figure 6B). This suggests that only the full length isoform is present in both untreated and treated cells, and therefore the lower band seen on western immunoblotting is not likely a protein generated due to alternative mRNA splicing.
Another possible mechanism by which a lower molecular weight Mcl-1 protein may be generated is via caspase-mediated cleavage at Asp127 or Asp157 (165). Levels of low molecular weight Mcl-1 were compared with levels of cleaved caspase 3 in cells treated with 20 mM DCA for 24 hours as well as in control and 5-FU treated cells for 24 hours (Figure 6C). In cells treated with 5-FU, western blotting detected cleaved caspase and the lower molecular weight Mcl-1. In untreated cells, there was no detection of cleaved caspase or the lower molecular weight Mcl-1. However, in cells treated with 20 mM DCA, no cleaved caspase was detected but the lower molecular weight Mcl-1 was detected. A correlation between caspase 3 cleavage and the lower molecular weight Mcl-1 was not established based on these results, and therefore other mechanisms of Mcl-1 processing are likely employed in HCT116 cells.
Figure 4. Various Bcl-2 protein expression levels following DCA treatment in HCT116 cells. Whole cell lysate from control and DCA-treated (20 mM) HCT116 cells showed no differences between anti-apoptotic (Bcl-2, Bcl-x, and Bag-1) or pro-apoptotic (Bax, Bad, and Bid) protein expression following DCA treatment when compared to the loading control (β-actin). This indicates that resistance to DCA-induced apoptosis in HCT116 cells is not due to changes in protein expression of these Bcl-2 proteins.
Figure 5. Changes in Mcl-1 expression following DCA treatment. A. HCT116 cells were treated with 20 mM DCA for 2.5, 7, and 24 h and Mcl-1 expression was examined compared to control cells. After 2.5 hours, a short form of Mcl-1 seemed to decrease relative to the control levels of this protein. Full-length Mcl-1 showed an increase in DCA-treated cells relative to control. After 7 hours, full-length Mcl-1 expression decreased in DCA-treated cells relative to control. B. Mcl-1 expression levels were normalized to β-actin. A significant decrease in Mcl-1 levels occurs following 7 hours of DCA treatment. Values indicate the relative Mcl-1 expression, and error bars represent standard error of the mean. A Wilcoxon test was performed to determine statistical significance, which was given at a p-value of 0.0078 with N = 8. C. After 7 hours of treatment, patterns of Mcl-1 expression between control and DCA treated cells are consistent between cells grown in normoxia and cells grown in simulated hypoxia (addition of CoCl$_2$ to stabilize HIF1α), as is the response to DNA damage-inducing agent etoposide (50 µM).
Figure 6. Characterization of the lower molecular weight Mcl-1 band. A. Levels of the Mcl-1 lower molecular weight form appeared most strongly after 4 hours of cell culture, and are decreased in cells treated with 20 mM DCA compared with untreated and etoposide-treated (50 µM) cells. B. Using primers designed to detect both the full length (1050 bp) and splice variant (810 bp) Mcl-1 mRNA, only the full-length isoform of Mcl-1 was detected in untreated and DCA-treated (20 mM) cells. C. In untreated, DCA-treated (20 mM), and 5-FU-treated (385 nM) cells, cleaved caspase did not show a correlation with the presence of lower molecular weight Mcl-1 after 24 hours, as the lower molecular weight Mcl-1 appeared independently of cleaved caspase in DCA-treated cells, and
appeared along with cleaved caspase in 5-FU-treated cells. This suggests that the Mcl-1 lower band is generated independently of caspase-3 cleavage.
**Mcl-1 expression changes are not due to changes in mRNA levels**

To determine the reason for the decreased levels of Mcl-1 seen in DCA-treated HCT116 cells when compared to control cells, pre- and post-transcriptional mechanisms of control over Mcl-1 were considered. Using real-time quantitative RT-PCR, levels of Mcl-1 mRNA were measured in control and DCA-treated cells to see if altered transcription was the cause of decreased levels of Mcl-1 in the cell. Data from three biological replicates showed the same level of Mcl-1 mRNA in control and DCA treated cells, indicating that alteration of Mcl-1 following DCA treatment is accomplished post-transcriptionally (Figure 7A). Running the PCR products on an agarose gel allowed visualization to confirm the identity of the band based on the number of base pairs (Figure 7B).

**Mcl-1 expression levels may be due to post-translational control by the proteasome**

One mechanism by which Mcl-1 is regulated post-transcriptionally is via proteasomal degradation. In order to determine whether or not increased proteasomal degradation caused the observed decrease in Mcl-1 levels, cells were treated with or without the addition of the proteasomal inhibitor MG132. MG132 increased the amount of Mcl-1 in both control and DCA-treated cells, showing the importance of proteasomal degradation in Mcl-1 regulation (Figure 8A). Without the addition of MG132, cells showed a significant decrease in Mcl-1 following 7 hours of DCA-treatment (20 mM) when compared to control. With the addition of MG132, Mcl-1 levels between control and DCA-treated cells were not significantly different (Figure 8B). This suggests that alterations in proteasomal degradation of Mcl-1 may be the reason for altered Mcl-1 levels within the cell after DCA treatment.
Since GSK3β has the ability to regulate Mcl-1 degradation via phosphorylation (23), I checked to see if this was a mechanism involved in the response of HCT116 cells to DCA-treatment. Western immunoblotting was performed to determine whether GSK3β activation increased with DCA treatment, as GSK3β is capable of phosphorylating Mcl-1, increasing its rate of degradation. Inactive phosphorylated GSK3β was compared with active unphosphorylated GSK3β following 6 hours of treatment with 10 or 20 mM DCA, and 5-FU. Levels of both inactive and active GSK3β were unchanged following all treatments, indicating that increased GSK3β activation is not the mechanism by which DCA increases proteasomal degradation of Mcl-1 (Figure 8C).

**Expression of Puma and Noxa did not change after DCA treatment**

Puma and Noxa have also been shown to increase Mcl-1 degradation (186). After 7 hours of treatment with 20 mM DCA, western immunoblotting was performed to determine whether or not protein levels of pro-apoptotic Noxa and Puma were affected by DCA treatment, corresponding with changes in Mcl-1 protein levels. The expression of Noxa and Puma did not change with DCA treatment (Figure 9), indicating that an alteration in levels of these proteins is not responsible for the observed decrease in Mcl-1.
Figure 7. Quantification of Mcl-1 mRNA following DCA treatment. A. RT-qPCR was used to quantify changes in Mcl-1 expression following DCA treatment. The target gene Mcl-1 mRNA levels were normalized to levels of the reference gene β-actin mRNA levels and compared between samples that had been treated with 20 mM DCA and those that had not. This graph depicts the mean relative Mcl-1 mRNA expression, with error bars representing standard error of the mean. A Wilcoxon test was performed and statistical significance was not achieved (p = 0.75). B, C. PCR products were run on agarose gels to confirm that the product amplified was the same size as the target Mcl-1 (B) and β-actin (C).
Figure 8. Effect of proteasomal degradation on Mcl-1 levels in the cell. A. After treatment with proteasome inhibitor MG132 (5 µM), the amount of Mcl-1 in control cells and DCA-treated cells was approximately equal. Data points represent Mcl-1 expression relative to the loading control, β-actin, and error bars represent standard error of the mean; N = 3. Wilcoxon tests were performed to determine statistically different populations. Statistical significance was not achieved (p = 0.75), indicating that Mcl-1 expression levels do not change between control and DCA-treated cells following proteasomal inhibition. B. A representative blot shows that Mcl-1 expression after 6 hours of 20 mM DCA treatment decreases compared to levels seen in control. After the addition of MG132, Mcl-1 levels are comparable between control and DCA treated cells, indicating that proteasomal degradation plays a role in Mcl-1 changes following DCA treatment. C. Levels of activated GSK3β did not change relative to inactive pGSK3β and tubulin after 6 hours of treatment with different concentrations of DCA and 385 nM 5-FU, indicating that DCA does not change the activity of GSK3β to increase proteasomal degradation of Mcl-1. Values underneath the western blot indicate densitometric
measurements of GSK3β activation compared to inactive GSK3β, with both densitometric values normalized to tubulin.
Figure 9. Cellular levels of Noxa and Puma following DCA treatment. Expression levels of Puma (left) and Noxa (right) after 7 hours of treatment with or without 20 mM DCA. As a positive control for the expression of these proteins upon apoptosis induction, cells were treated with 385 nm 5-FU or 50 μM etoposide. Neither Puma nor Noxa showed any changes in expression following treatment with DCA compared to control.
**Mcl-1 may be linked to proliferation and cell cycle via nuclear localization**

The localization of Mcl-1 within the cell may be mitochondrial, cytosolic, or nuclear, and all locations indicate a different function of Mcl-1 within the cell (200). Subcellular localization of Mcl-1 was determined with immunofluorescence to suggest the function of Mcl-1 within the cell. The overlap of the anti-Mcl-1 antibody with DAPI indicates that Mcl-1 is primarily localized within the nucleus of HCT116 cells regardless of whether they are treated with DCA (Figure 10A, B). Mcl-1 localization in the nucleus is not homogeneous, but rather there are concentrated areas of Mcl-1 within the nucleus itself. This is consistent in cells treated with oxidative damage-inducing agent H$_2$O$_2$ for 6 hours (Figure 10C), and lost during mitosis, when Mcl-1 is not detectable by immunofluorescence (Figure 10D). In order to determine if this nuclear localization was dependent on p53 or Bax, immunofluorescence of HCT116 p53$^{-/-}$ and HCT116 Bax$^{-/-}$ cells was also performed. Mcl-1 localized to the nucleus in both HCT116 derivative cell lines, regardless of whether they were control or DCA-treated, indicating that Mcl-1 nuclear localization is independent of p53 or Bax (Figure 11).

Western immunoblotting of nuclear and cytosolic cell fractions showed that Mcl-1 is altered in the nucleus and cytosol of HCT116 cells following 20 mM DCA treatment (Figure 12). The short form of Mcl-1 is also much more prominent in cytosolic lysates, and showed a marked decrease following DCA treatment. Mcl-1 levels in the nucleus decreased following DCA treatment as well, and full-length Mcl-1 was more prominent in the nucleus than in the cytoplasm. Since the anti-apoptotic function of Mcl-1 interactions with pro-apoptotic Bcl-2 family members requires Mcl-1's presence in the
cytosol and mitochondria, nuclear localization of full-length Mcl-1 within HCT116 cells suggests an alternative function for this protein.

**Effects of DCA treatment on cell cycle arrest in HCT116 cells**

Mcl-1 has also been shown to induce cell-cycle arrest in the G2 phase (25). To determine if decrease in proliferation as well as a decrease in Mcl-1 levels following DCA treatment was related to changes in cell cycle, flow cytometry was performed. Comparing the proportion of cells in G2 in 20 mM DCA vs. control treated cells showed a slight but not significant (p > 0.05) increase in DCA treated cells (Figure 13). This suggests that decreased proliferation and altered Mcl-1 expression induced by DCA are independent of cell-cycle arrest.
Figure 10. Cellular localization of Mcl-1 in HCT116 cells. Cells were grown on slides and immunofluorescence was performed to determine the subcellular localization of Mcl-1. Control (A) and DCA-treated (20 mM; B) cells show heterogeneous nuclear staining of Mcl-1, possibly reflective of replication or repair foci. This staining pattern was consistent in H₂O₂-treated cells (C). Mcl-1 is not detectable during mitosis (D); an arrow indicates a mitotic cell. Scale bars = 10 µm.
Figure 11. Cellular localization of Mcl-1 in HCT116 cells without p53 or Bax. HCT116 p53−/− (A, B) and Bax−/− (C, D) cells were grown on slides in control (A, C) and 20 mM DCA (B, D) media for 24 hours. Immunofluorescence was performed to determine cellular location of Mcl-1. As with wildtype HCT116 cells, Mcl-1 localization is nuclear, showing heterogeneous staining indicated by concentrated areas of immunofluorescence within the nucleus. Consistency of subcellular localization of Mcl-1 between these cell lines and wildtype cells indicates that nuclear localization of Mcl-1 in HCT116 cells is independent of p53 or Bax. Scale bars = 10 µm.
Figure 12. Expression of Mcl-1 in nuclear and cytosolic cell fractions. Western blots of nuclear and cytosolic lysates from cells untreated or treated with 20 mM DCA for 6 hours. Mcl-1 expression in both fractions showed decreases following DCA treatment, and the short form of Mcl-1 appeared more strongly in the cytosolic protein fraction, which showed minimal expression of full-length Mcl-1. Lamin A/C was used as a positive marker for nuclear proteins, and was not seen in the cytosolic fraction indicating that negligible nuclear contamination occurred. Likewise, tubulin was used as a positive marker for cytosolic proteins, and was not seen in the nuclear fraction indicating that negligible cytosolic contamination occurred.
Figure 13. Cell cycle analysis of cells untreated or treated with DCA. Percentage of cells in G2/M phase was compared (A) between cells untreated and treated with 20 mM DCA for 24 hours. Cell cycle analysis was performed using flow cytometry (B: control, C: DCA), and percentage of cells in G2/M phase was compared to evaluate cell cycle arrest. Although a slight increase in the proportion of cells in G2/M phase was observed after 24 hours of treatment, this increase was not significant according to a Wilcoxon paired t-test ($p=0.25$) with N=3. Error bars represent standard error of the mean. If G2/M phase arrest was occurring in DCA-treated cells, the majority of cells would appear to be in G2/M phase. However, the majority of the cells here are in G1, which is similar to the cell cycle phase of control cells.
DISCUSSION

The research presented here shows that DCA is unable to induce apoptosis in the human colorectal cancer cell line HCT116, but reduces its proliferation following 4 days. After 6 hours of treatment with 20 mM DCA, Mcl-1 levels significantly decrease when compared with those in control cell lysates. This decrease in Mcl-1 is not due to transcriptional downregulation, but may be due to an increase in its proteasomal degradation, independent of Noxa or Puma expression or GSK3β activation. Variation in a short form of Mcl-1 was also observed, but the identity of this lower molecular weight band is inconclusive; experiments suggest that it is neither the product of an alternative splice variant, nor a product of proteolytic cleavage by caspase 3. Mcl-1 localization in HCT116 cells was determined to be nuclear, and its levels were altered following treatment with 20 mM DCA for 6 hours in both nuclear and cytosolic protein. However, its localization to the nucleus did not result in G2 arrest following treatment with 20 mM DCA.

DCA has been shown to induce apoptosis and reduce cell proliferation by reversing the Warburg effect, a characteristic associated with tumour malignancy (64). The Warburg effect is the preferential utilization of aerobic glycolysis in cancer cells, even in the presence of abundant oxygen wherein normal cells would utilize oxidative phosphorylation for energy metabolism (121). Analysis of cells isolated from human colorectal tumours show that the Warburg effect is a prominent phenotype of colorectal cancer (201). The Warburg effect is especially important in the initiation of invasion, determined by gene expression in the marginal edges of colorectal tumours (202). Although these data suggest that DCA could hold value as a cancer-specific treatment for
cancer, experiments studying the effect of DCA in colorectal cancer have not consistently supported this potential. Previous work in our laboratory indicated that in 5 different human colorectal cancer cell lines, DCA treatment had cell line-dependent effects; in some cases, DCA was not able to induce apoptosis, while in others, it was successful (18). Furthermore, DCA had a cell line-dependent cytoprotective effect against hypoxia-induced apoptosis (18).

The purpose of my study was to relate changes in Bcl-2 protein expression with phenotypic changes in colorectal cancer cell lines induced by DCA treatment. The same colorectal cancer cell lines used previously by our laboratory were used in an initial experiment to detect Bcl-2 protein expression with western immunoblotting. Although most of the Bcl-2 family member protein expression was consistent between untreated and DCA treated cells, only HCT116 cells showed altered expression of Mcl-1 following DCA treatment. Therefore, further characterization of DCA’s effect on HCT116 cells was performed in order to determine a potential relationship between Mcl-1 expression and cellular response to DCA.

Previous findings in our laboratory show that HCT116 is resistant to DCA-induced apoptosis both in normoxia and anoxia (18), and my experiments looking at apoptotic body formation, caspase 3 cleavage, and cell viability over four days of treatment confirmed that HCT116 cells did not undergo apoptosis when treated with 20 mM DCA. Concentrations of DCA above 30 mM are well above physiological concentrations and have shown nonspecific inhibition of cell growth, therefore rendering DCA an inappropriate alternative to traditional chemotherapies at high concentrations (16). Although DCA is not effective at inducing apoptosis in HCT116 cells, even at
supraphysiological concentrations, it is effective at reducing proliferation; my experimental data show that after 4 days of treatment, the cell number in DCA-treated wells was ~45% of that in control wells.

The ability of DCA to reduce proliferation independently of cell death has been reported as a dose-dependent phenomenon; in multiple myeloma cells, 5-10 mM DCA was shown to reduce proliferation independent of cell death, while 10-25 mM DCA was shown to induce superoxide production and apoptosis (7). Additionally, treatment of metastatic breast cancer cells with 1-5 mM DCA showed a decrease in proliferation, but no signs of apoptosis (151). Studies in colorectal cancer cells SW480, HT29, and LoVo showed that 20 mM of DCA was required to reduce cellular proliferation, but apoptosis was not detected until concentrations of 50 mM were used (17). At 50 mM, DCA decreased normal cell growth by approximately 70%, and apoptosis was only induced in 5% of SW480 and HT29 cells (17), compared with 12% apoptosis induction in A549 cells treated with only 0.5 mM DCA (4).

This ability of cells to withstand DCA-induced apoptosis may be due to their capacity to effectively perform oxidative phosphorylation without the overproduction of ROS. A study compared breast cancer cell line MCF7 with MCF7 rho(0), which is deficient for mitochondrial DNA encoding complex I and IV in the electron transport chain. This study showed that electron transport chain defects increased apoptosis in DCA-treated cells by 3-5 fold (16). This same study measured the efficacy of DCA against HCT116 cells and HCT116 p53−/−, which also show a decrease in complex IV activity. This electron transport defect enhanced DCA’s effect on HCT116 cells, lowering the IC₅₀ from 45 mM in HCT116 wildtype cells to 28 mM in HCT115 p53−/− cells (16).
Thus, the ability of HCT116 cells to withstand DCA-induced apoptosis may be due to their capability to oxidize glucose normally in the mitochondria. This is further supported by the ability of DCA to synergize with arsenic trioxide, which was shown to inhibit complex IV activity in the electron transport chain of human breast cancer cells in vitro (203). However, DCA alone may still decrease cellular proliferation by reducing the amount of metabolic intermediates that can be used to synthesize biomolecules required for cell growth.

DCA treatment in HCT116 cells decreased Mcl-1 expression after 7 hours and reduced the levels of a short form of Mcl-1, which was most prominent in whole cell lysate following 4 hours of treatment. Some studies report that this short form of Mcl-1 in human non small-cell lung cancer cell line A549 and leukemia cell line K562 is a pro-apoptotic splice variant, which was determined to be responsible for inhibiting Mcl-1 and freeing Bak from its inhibitory grasp (160,204). However, my experimental results of PCR amplification designed to detect the presence of both isoforms of Mcl-1 indicated that this splice variant was not present in my HCT116 cells. Another possible source of the short form of Mcl-1 is caspase-mediated cleavage. Studies show that a pro-apoptotic form of Mcl-1 is generated through cleavage by activated caspase 3 in human lymphoma cells, and this cleavage is reported to render Mcl-1 incapable of preventing apoptosis activated by Bim (164,165). My experimental results indicate no correlation between the presence of activated caspase 3 and the short form of Mcl-1; therefore, the identity of the lower molecular weight Mcl-1 is inconclusive.

Although HCT116 cells do not undergo apoptosis in response to DCA treatment, the expression of Mcl-1 after 7 hours is significantly decreased. This response is counter-
intuitive, as Mcl-1 is a pro-survival member of the Bcl-2 family, and its downregulation would presumably precede induction of apoptosis. It is possible that the role of anti-apoptotic Bcl-2 proteins in HCT116 cell survival is not mediated by Mcl-1, but rather by Bcl-x and Bcl-2, both of which were consistently expressed at much higher levels than Mcl-1 in these cells. Together, these two proteins perform functions redundant to those of Mcl-1, including the inhibition of Bak, Bax, Bim, and Puma (205). The lack of apoptosis in these cells suggests that resistance to apoptosis is occurring downstream of Mcl-1 regulation, or that Mcl-1 is involved in a cellular function independent of apoptosis - potentially in proliferation.

In order to determine the cause of decreased Mcl-1 expression after 7 hours of DCA treatment, experiments were performed that examined the various mechanisms by which Mcl-1 is regulated. Mcl-1 is under the transcriptional control of the AKT signaling pathway (159), which can be activated as a result of the Warburg effect in human leukemia and lymphoma cell lines (67). Therefore, by diminishing the Warburg effect, DCA may reduce the activation of the AKT signaling pathway in HCT116 cells, thus reducing the transcription of Mcl-1. However, RT-qPCR data indicate that after 7 hours, despite a significant decrease in Mcl-1 protein, Mcl-1 mRNA levels are expressed at identical levels within treated and untreated HCT116 cells.

The turnover of Mcl-1 in the cell occurs rapidly due to proteasomal degradation, allowing Mcl-1 to quickly respond to drug-induced changes (167). Mcl-1 degradation plays a key role in chemosensitization and apoptosis (23), and can mediate resistance to proteasomal inhibition as a cancer treatment (171). Through inhibiting proteasomal degradation in HCT116 cells, Mcl-1 downregulation resulting from DCA treatment was
alleviated, suggesting that DCA-induced downregulation of Mcl-1 is controlled by Mcl-1’s turnover rate. Mcl-1 turnover rate can be increased by a variety of mechanisms within the cell including interactions with Puma, Noxa, and GSK3β (23,182-184).

The expression of Mcl-1 binding partners Noxa and Puma was therefore determined after 7 hours of treatment with DCA. Studies in the non-small cell lung cancer cell line A549 indicate that DCA increases the amount of ROS-induced DNA damage in cells (206). Both Puma (187) and Noxa (188) are capable of responding to DNA strand break damage, in a p53-mediated mechanism (207), and could therefore be involved in the response of DCA-treated cells to ROS-induced DNA strand breaks (208). Puma can rapidly induce apoptosis in human colorectal cancer cells in vitro (209) and has been shown to interact with Mcl-1 and inhibit its function (183). Interestingly, Puma can be inhibited by AKT, which is stabilized by glycolysis (67,104), suggesting that by interfering with aerobic glycolysis, DCA may increase levels of Puma within the cell thus increasing its interaction with Mcl-1. In endometrial cancer cells, treatment with 10 mM DCA increased transcription of Puma in a p53 dependent manner (147). The interaction of Puma with Mcl-1 has been shown to increase caspase-dependent cleavage of Mcl-1 and induce its proteasomal degradation (210). However, my results indicate that Puma expression is not affected by DCA-treatment, thus changes in its expression are not responsible for promoting increased Mcl-1 degradation.

Inhibition of Noxa also occurs via a glucose-dependent pathway, indicating that interference with aerobic glycolysis by DCA may activate this protein (106,107). Noxa has been shown to displace Mcl-1 from its inhibitory grasp on pro-apoptotic proteins (184) and Noxa's interaction with Mcl-1 has been shown to precede the generation of a
caspase-mediated cleavage product of Mcl-1 in multiple myeloma cells treated with bortezomib (211). Noxa has also been reported to increase the proteasomal degradation of Mcl-1 in human embryonic kidney cells (212) and mouse embryonic fibroblasts (186). Therefore, inhibition of glycolysis by DCA may result in increased expression of Puma or Noxa, both of which have shown involvement in the generation of a short form of Mcl-1, and both of which have the ability to enhance proteasomal degradation of Mcl-1. However, this mechanism of Mcl-1 regulation might not be present in my HCT116 cells, as Noxa protein levels do not change after DCA-treatment.

Additionally, GSK3β has been shown to increase the proteasomal degradation of Mcl-1 (23). A decrease in AKT activation has been shown to decrease the phosphorylation of GSK3β, which is active in its unphosphorylated form (101). Thus, inhibition of aerobic glycolysis could reduce the activity of the AKT signaling pathway, enhancing GSK3β activation, which increases Mcl-1 degradation. However, my experimental results show no increase in the activation of GSK3β following treatment with DCA, suggesting that an alternative mechanism of enhanced proteasomal degradation may be involved in Mcl-1 regulation following DCA treatment in HCT116 cells.

Degradation of Mcl-1 also occurs due to changes in the cell cycle; Mcl-1 levels increase from the G1 phase and peak in the G2 phase, followed by accelerated degradation during mitosis due to CDK1-mediated phosphorylation in human osteosarcoma and cervical cancer cells in vitro (28). This cell-cycle dependent regulation of Mcl-1 is independent of the Bcl-2 family members (28), and suggests a possible role for Mcl-1 in proliferation or cell cycle regulation. Immunofluorescence of HCT116 cells
shows that Mcl-1 is primarily localized to the nucleus; this localization is lost during cell division. This suggests that the role of Mcl-1 may be independent of apoptosis in these cells and is instead involved in proliferation or cell cycle checkpoints.

There are several reports of Mcl-1’s role in the nucleus, including its inhibitory effect on CDK1 in the G2 phase (25,191), and its inhibitory effect on PCNA in the S phase (27). However, if Mcl-1 was inhibiting cell cycle progression, its downregulation shouldn’t correspond with a decrease in proliferation, suggesting an alternative function of Mcl-1 in the nucleus in HCT116 cells. Mcl-1 has also been shown to interact with Chk1 in response to DNA damage in the G2 phase (26,194,195). Mcl-1 is recruited to sites adjacent to DNA strand breaks and initiates DNA damage repair, resulting in an accumulation of cells in the G2 phase following oxidative damage (26). It is suggested that the involvement of Mcl-1 in DNA damage response is ATM-mediated (195), and constitutive activation of ATM in response to DNA damage has been reported following DCA treatment (206). This could explain the distinct areas of Mcl-1 accumulation within the nucleus seen in my study; it may function to localize to sites of DNA damage, and repair this damage following oxidative stress. This in turn could lead to cell cycle delay as the DNA repair machinery performs its function (26). As HCT116 cells have inherently high levels of ROS relative to normal cells (44), this DNA damage may be constant, explaining the consistent nuclear localization patterns between control and DCA-treated cells. Downregulation of Mcl-1 following DCA treatment may increase the time needed to repair DNA damage following DCA treatment, resulting in a prolonged G2 phase and decreased proliferation.
Indeed, DCA (50 mM) has been shown to induce an eight-fold accumulation of colorectal cancer cells HT29 in the G2 phase, corresponding with induction of apoptosis (17). Mcl-1 may prevent apoptosis resulting from prolonged G2 arrest by repairing ROS-induced DNA damage and allowing progression of the cell cycle. However, the cell cycle analysis I performed showed a marginal but not significant increase in cells accumulated in the G2 phase after 24 hours of DCA treatment. This indicates that the DCA-induced reduction in proliferation is probably independent of cell cycle arrest in HCT116 cells. It is possible that cells are being prolonged in the G2 phase to allow sufficient time for DNA damage repair, however this was not detected at 24 hours. The involvement of Mcl-1 in DNA damage repair may be further investigated by determining its association with other members of DNA damage repair complexes. Specifically, Mcl-1 was shown to interact with γ-H2AX and NBS1 in human cervical cancer in vitro (26). Co-immunoprecipitation or immunofluorescence to determine co-localization of either of these proteins with Mcl-1 in the nucleus would provide evidence of Mcl-1’s function in cell cycle arrest/DNA repair in HCT116 cells.

In a study of tissue samples from colorectal cancer patients, immunohistological staining indicated that Mcl-1 showed nuclear localization in all positively stained cases (213). Additionally, a strongly significant positive correlation was seen between Mcl-1 expression and tumour grade, tumour stage, and the presence of metastases (213). This suggests that downregulation of Mcl-1 could correlate with a less metastatic and proliferative phenotype, decreasing the aggressiveness of the cancer. Moreover, breast cancer cells show decreased proliferation and cell growth in vitro when Mcl-1 was
knocked down (214), and translational inhibition of Mcl-1 is associated with reduced proliferation in chronic myelogenous leukemia cells (215).

Cancer stem cells are a subpopulation within a tumour that is considered the driving force behind tumour proliferation, metastasis, and resistance to apoptosis in many types of cancer (216). They are characterized based on their stem-like phenotype, including the capacity for self-renewal and the ability to give rise to any cancer cell type found in its tumour (216). The role of Mcl-1 in cancer stem cell maintenance is heavily supported in a variety of reports. In the non-small cell lung cancer cell line A549, two populations distinguished based on stem-like characteristics (including self-renewal) showed differences in their tumorigenicity in vitro and in vivo (217). The population with the greater capacity of clonogenic and self-renewal showed significantly higher Mcl-1 expression when compared with cells that were less aggressive and showed a lesser ability to self-renew (217). Another recent study on hematopoietic stem cells also showed that Mcl-1 downregulation is associated with a decreased self-renewal capacity (218). The essential role of Mcl-1 in stem cell maintenance of human hematopoietic stem cells and progenitor cells is independent of its ability to promote cell survival (219). In that study, knockdown of Mcl-1 did not decrease cell survival in vitro, but reduced the self-renewal ability in vivo (219). Similar consequences of Mcl-1 loss were seen in human leukemic stem cells and human pluripotent stem cells (219). It is possible that Mcl-1 reduces proliferation independently of apoptosis by reducing the ability of cancer stem cells to self-renew.

HCT116 has been characterized as a highly aggressive form of colorectal cancer, consisting mainly of cancer stem cells with a high self-renewal capacity and a low
capacity for differentiation (220). It is therefore possible that the decrease in Mcl-1 expression in DCA-treated cells reduces proliferation by interfering with the capacity of the progenitor cells in this line to self-renew. HT29 cells, which were susceptible to DCA-induced apoptosis at a concentration of 50 mM (17), are characterized as having an intermediate capacity to differentiate and self-renew (220). The non-small cell lung cancer cell line A549 was used in the first report of DCA induced apoptosis in cancer and showed apoptosis at only 0.5 mM DCA (4). This cell line contains only a small side population of cells showing stem cell-like properties, with the majority of cells showing limited ability to self-renew (217). This supports the notion that the variable ability of DCA to induce apoptosis may be due to the proportion of cells within a tumour or tumour-derived cell line that are cancer stem cells.

The work presented in this thesis demonstrates that although DCA is incapable of inducing apoptosis in HCT116 cells, it significantly reduces their proliferation in vitro, corresponding with a proteasome-mediated decrease in Mcl-1 protein expression after 7 hours; this decrease is maintained after 24 hours of treatment. The resistance of HCT116 cells could be due to their functional mitochondria, ability to repair DNA damage induced by oxidative stress, or the high proportion of cancer stem cells in this population. The involvement of Mcl-1 in the response of HCT116 cells to DCA treatment may be due to its involvement in cell cycle progression or stem cell maintenance, and the pro-survival function of this protein may not be as important in HCT116 cells as its function in proliferation and self-renewal.
Implications and Future Directions

The involvement of Mcl-1 in the response of HCT116 to DCA treatment has implications for the potential mechanism by which DCA overcomes resistance to apoptosis induced by anti-cancer therapies. DCA shows a limited ability to induce apoptosis in HCT116 cells, but decreases their proliferation. Mcl-1 is downregulated after DCA treatment, which corresponds with this reduced proliferation. Mcl-1 downregulation is also associated with limiting the ability of human cancer, hematopoietic, and pluripotent stem cells to self-renew and regenerate (219). As HCT116 is comprised mainly of cancer stem cells, it is possible that Mcl-1 downregulation following DCA treatment is indicative of a loss of self-renewal capacity, corresponding to a decrease in proliferation. HCT116 is a highly aggressive cancer cell line with a large population of cancer stem cells and shows resistance to DCA treatment, whereas cancer cell lines that do not contain primarily cancer stem cells, such as colorectal cancer cell line HT29 (17,220) and non-small cell lung cancer cell line A549 (4,217), are reported to be more susceptible to DCA treatment. This implies that the population of cancer stem cells within a tumour may correspond with the ability of DCA to induce apoptosis in cancer cells. This may also explain why some cancer cells show inhibition of proliferation independently of apoptosis.

Mcl-1 has been shown to interact with a complex of proteins responsible for repairing DNA damage (26), and therefore may mediate resistance to DCA-induced apoptosis, as ROS-induced DNA damage is a mechanism by which DCA exerts its apoptotic effects (206). Although DCA decreases Mcl-1 in HCT116 cells, its main function may not be to resist apoptosis through the mitochondrial pathway, but rather to
repair DNA damage in the nucleus. Therefore, Mcl-1 may still function to overcome DNA damage in HCT116 cells, but its DCA-induced decrease in expression prolongs this process, resulting in lower rates of proliferation.

Mitochondrial competence may also explain resistance to DCA-induced apoptosis seen in cancer cells. HCT116 cells with decreased complex IV activity showed enhanced susceptibility to DCA-induced apoptosis, as did breast cancer cell lines with mtDNA damage resulting in decreased complex IV activity (16). DCA enhances apoptosis induced by cisplatin (23). Cisplatin has been shown to accumulate in the mitochondria and damage mtDNA (221), leading to complex IV damage. Thus, functioning enzymes in the electron transport chain may lead to resistance to DCA-induced apoptosis; however the indirect activation of PDH by DCA would lead to less aerobic glycolysis by enhancing glucose oxidation, thereby decreasing proliferation due to the generation of fewer glycolytic intermediates. Additionally, resistance to cisplatin can be overcome by downregulation of Mcl-1 in ovarian cancer cells, suggesting that DCA may also enhance apoptosis induced by cisplatin by reducing Mcl-1-mediated chemoresistance (222).

DCA may overcome the apoptotic resistance of cancer cells to anti-cancer therapies by decreasing the amount of Mcl-1 in the cell. Resistance to 5-fluorouracil can be conferred by Mcl-1 in osteosarcoma (223) and pancreatic cancer cells (224) in vitro. In colorectal cancer cell lines LS174T, LoVo, SW480, and HT29, DCA and 5-FU have a synergistic effect on apoptosis, which could possibly be mediated by Mcl-1 downregulation (225). Resistance to bortezomib-induced apoptosis can also be overcome by inhibition of Mcl-1 (226), and more recently by combination treatments with DCA in multiple myeloma cells (7).
As the potential for DCA in cancer treatment is a relatively recent observation, there is still much to understand about its mechanisms of action in various cancer cells. The involvement of Mcl-1 in cellular responses to DCA has not yet been previously reported, and may provide insight into the mechanism by which DCA is able to enhance apoptosis. Knowledge of this may help determine which combination therapies might benefit from DCA inclusion. For example, Mcl-1 has been shown to confer resistance to ABT-737, a general inhibitor of anti-apoptotic Bcl-2 proteins with a low affinity for Mcl-1 (154); it would be of interest to determine whether or not DCA is able to overcome resistance to this therapy. It has been reported that in HCT116 cells, Bax is required for apoptosis induction due to the inhibitory effect of Mcl-1 on Bak (205). Therefore, co-treatment of DCA with existing therapies may induce a more robust apoptotic response due to the ability of Bak to contribute to MOMP.

Mcl-1’s role in stem cell regulation and self-renewal has been recently characterized, and its involvement in altering stem cell phenotypes following DCA treatment should be further assessed. The potential of DCA to modulate cancer stem cell phenotype has not previously been reported. Further analysis of molecular changes associated with DCA treatment is therefore warranted, to include pathways involved in stem cell maintenance such as Wnt signaling and β-catenin, Notch, and Hedgehog. It has been suggested that post-treatment resistance and recurrence of glioblastoma following DCA trials may be due to cancer stem cell populations that are not eradicated by DCA (5). Thus, variable populations of stem cells within a tumour may be the reason for the wide variety of responses to DCA treatment reported in the literature. This concept should be further investigated by isolating populations of cancer stem cells within
tumour-derived cell lines and comparing the effects of DCA on these versus more
differentiated cells of the same line. If DCA is successful in inhibiting the self-renewal
potential of cancer stem cells, it may hold promise as a target for this generally
chemoresistant group of cells when continuously exposed, or if administered in
combination with a stem cell differentiating agent such as salinomycin (227).

The effect of DCA on cancer cells is largely dependent on individual
characteristics within the cell, including the cell’s ability to perform oxidative
phosphorylation in the mitochondria, the ability of the cell to respond to DNA damage
and oxidative stress, and the proportion of cancer stem cells within the cell line being
studied. As such, multiple considerations should be taken into effect when deciding
whether or not DCA will be effective as a stand-alone treatment, or if it should be used in
combination with DNA damage-inducing agents, agents interfering with mitochondrial
function, or stem cell differentiating agents.
Limitations

Techniques utilized in these experiments may limit the ability of the results presented to be recapitulated in vivo. DCA has been shown to decrease tumourigenicity not only through decreasing proliferation and increasing apoptosis, which can be seen in vitro using the techniques described in this thesis, but also by reducing angiogenesis and modulating the tumour microenvironment, which cannot be elucidated using the tissue culture systems I employed. In order to achieve a full understanding of the effects of DCA on colorectal tumours, in vivo experiments must be performed that correspond with molecular analysis derived from cell culture. In vitro studies using DCA may not accurately reflect its effect in vivo. For example, a previous study in our laboratory showed that DCA significantly increased apoptosis in SW480 cells in vitro, however these same cells showed a significant increase in tumour growth with DCA exposure in vivo (18).

Another limitation of this study is the nature by which Bcl-2 proteins are controlled within the cell. Although transcriptional changes do affect the levels of overall Bcl-2 protein expression within the cell, post-transcriptional activity such as phosphorylation, subcellular localization, and interactions with other Bcl-2 family members are responsible for a significant proportion of Bcl-2 protein activity (20). Therefore, it cannot confidently be concluded that Noxa and Puma are not responsible for Mcl-1 degradation, as changes in their expression alone is not enough to confirm these actions; instead, co-immunoprecipitation experiments analyzing the interactions of Mcl-1 with Puma, Noxa, Bim, and Bak could be performed to solidify the evidence obtained regarding their activity.
Finally, I assumed in this model that HCT116 cells were exhibiting the Warburg phenotype, however this was never confirmed in my study. Moreover, DCA-treatment on HCT116 cells was assumed to result in PDK inhibition and indirect activation of PDH, promoting glucose oxidation over aerobic glycolysis; however, DCA has variable affinities for binding PDK depending on the PDK isoform, and therefore may be less effective in HCT116 cells based on their PDK expression profile (228). Further characterization of the metabolic profile of HCT116 control and DCA-treated cells is warranted to support interpretations of the experimental results obtained.
SUMMARY AND CONCLUSIONS

The purpose of this study was to investigate the effects of DCA on human colorectal HCT116 cells, and correlate these changes to altered expression of the anti-apoptotic protein Mcl-1. DCA did not increase apoptosis in HCT116 cells, but was successful in reducing proliferation over a treatment period of 4 days. Additionally, after 7 hours of DCA treatment, Mcl-1 levels were significantly lower when compared to control cells. Mcl-1 expression was not decreased at the transcriptional level, but evidence suggested that DCA caused increased turnover of Mcl-1 within the cell due to proteasomal degradation. Noxa and Puma are two binding partners of Mcl-1 that inhibit its activity and accelerate its degradation, and their expression has been shown to change as a result of glycolysis. However, DCA did not alter levels of Noxa or Puma in HCT116 cells, suggesting an alternative mechanism of Mcl-1 degradation. GSK3β is also activated by inhibition of glycolysis, and in its unphosphorylated active state can phosphorylate Mcl-1 and increase the rate of Mcl-1 degradation by the proteasome. However, levels of activated GSK3β were unaffected by DCA treatment, indicating an alternative mechanism may be responsible for Mcl-1 degradation.

Mcl-1 showed substantial nuclear localization within HCT116 cells, and its expression in the nucleus and cytoplasm was decreased following 7 hours of DCA treatment. Mcl-1 may have a role in cell cycle delay by activating the DNA damage checkpoint, resulting in an accumulation of cells in the G2 phase, however no signs of significant cell cycle arrest were observed after 24 hours of DCA treatment. Cells may still be accumulating in the G2 phase prior to 24 hours, which can be investigated to confirm the role of Mcl-1 in the nucleus of HCT116 cells. HCT116 cells may be
sacrificing their ability to rapidly proliferate in order to recover from DNA damage that could be caused by DCA-induced reactive oxygen species, thereby evading apoptosis.

The decrease of Mcl-1 following DCA treatment could be the reason why DCA is able to enhance or overcome resistance to apoptosis when combined with drugs that are inhibited by Mcl-1. Additionally, increasing Mcl-1 expression has been shown to correlate with tumour malignancy and metastases, and may play a role in cancer stem cell maintenance. Decreases in Mcl-1 have been shown to correlate with a decreased ability of a variety of stem cells to self-renew, and may suggest that cancer stem cell renewal is inhibited following DCA treatment, accounting for reduced proliferation seen after 4 days of treatment in HCT116. Since HCT116 is a particularly aggressive form of colorectal cancer with little ability to differentiate, it could serve as a model for which therapies targeting stem cell subpopulations can be tested. DCA could hold potential as part of a combination therapy designed to inhibit proliferation and eradicate cancer stem cells, overcoming resistance and recurrence of cancer after chemotherapeutic intervention.
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### APPENDIX I – CHEMICAL LIST AND SUPPLIERS

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<tr>
<td>Normal Goat Serum</td>
<td>Life Technologies, Burlington, ON, Canada</td>
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<tr>
<td>Nuclease free water</td>
<td>BioRad, Mississauga, ON, Canada</td>
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<td>PBS</td>
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<td>PI</td>
<td>BioVision, Milpitas, CA, USA</td>
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<td>PMSF</td>
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<td>Primers</td>
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<td>Protease inhibitor cocktail II</td>
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<tr>
<td>Rabbit-anti-Bad antibody</td>
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<tr>
<td>Rabbit-anti-Bag-1 antibody</td>
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<tr>
<td>Rabbit-anti-Bax antibody</td>
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<tr>
<td>Rabbit-anti-Bcl-2 antibody</td>
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<tr>
<td>Rabbit-anti-Bcl-x antibody</td>
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<td>Rabbit-anti-Bid antibody</td>
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<tr>
<td>Rabbit-anti-caspase 3 antibody</td>
<td>Cell Signaling Technology, Danvers, MA, USA</td>
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<tr>
<td>Rabbit-anti-GSK3β antibody</td>
<td>Cell Signaling Technology, Danvers, MA, USA</td>
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<tr>
<td>Rabbit-anti-Mcl-1 antibody</td>
<td>Abcam, Burlington, ON, Canada</td>
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<tr>
<td>Rabbit-anti-pGSK3β antibody</td>
<td>Cell Signaling Technology, Danvers, MA, USA</td>
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<tr>
<td>Rabbit-anti-Puma antibody</td>
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<tr>
<td>Rabbit-anti-β-actin antibody</td>
<td>Cell Signaling Technology, Danvers, MA, USA</td>
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<tr>
<td>RedSafe ™ Nucleic Acid Staining Solution</td>
<td>FroggaBio, Toronto, ON, Canada</td>
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<tr>
<td>SDS</td>
<td>BioRad, Mississauga, ON, Canada</td>
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<td>Sodium pyruvate</td>
<td>Sigma-Aldrich, Oakville, ON, Canada</td>
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<td>Ssofast™ Eva green® Supermix</td>
<td>BioRad, Mississauga, ON, Canada</td>
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<td>SuperScript First-Strand Synthesis System</td>
<td>Sigma-Aldrich, Oakville, ON, Canada</td>
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<td>Taq DNA polymerase</td>
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<td>TEMED</td>
<td>Roche, Mississauga, ON, Canada</td>
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<td>Tris Base</td>
<td>Fisher Scientific, Nepean, ON, Canada</td>
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<td>Triton X-100</td>
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<td>TRIzol</td>
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<td>Trypan blue</td>
<td>Sigma-Aldrich, Oakville, ON, Canada</td>
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<tr>
<td>Trypsin</td>
<td>Veterinary College, Guelph, ON, Canada</td>
</tr>
<tr>
<td>Tween 20</td>
<td>Fisher Scientific, Nepean, ON, Canada</td>
</tr>
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</table>
APPENDIX II – PREPARATION OF SOLUTIONS

15% Resolving Gel 2 x 1.5 mm Gel

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>7.2 ml</td>
</tr>
<tr>
<td>40% acrylamide-bis solution</td>
<td>7.5 ml</td>
</tr>
<tr>
<td>1.5 M Tris-HCl (pH 8.8)</td>
<td>5 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>200 µl</td>
</tr>
<tr>
<td>10% APS</td>
<td>240 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

Add together in the order written and fill space between plates until ¾ full. Top off with isopropanol. Once polymerized, pour off the isopropanol and add the Stacking Gel.

5% Stacking Gel 2 x 1.5 mm Gel

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>3.2 ml</td>
</tr>
<tr>
<td>40% acrylamide-bis solution</td>
<td>1 ml</td>
</tr>
<tr>
<td>0.5 M Tris-HCl (pH 6.8)</td>
<td>1.26 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>50 µl</td>
</tr>
<tr>
<td>10% APS</td>
<td>50 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>5 µl</td>
</tr>
</tbody>
</table>

Add to resolving gel, add comb, and allow polymerization. When the gel is polymerized, it can be used right away or stored at 4°C for up to 3 days.

10 X Running buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>144.2 g</td>
</tr>
<tr>
<td>Tris base</td>
<td>30.2 g</td>
</tr>
<tr>
<td>20% SDS</td>
<td>50 ml</td>
</tr>
</tbody>
</table>

Mix in H₂O to a volume of 900 ml, bring to a pH of 8.3, top up with H₂O to a final volume of 1 L, and store at room temperature. Dilute to 1 X when needed for experimental purposes.

CAPS Buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAPS</td>
<td>4.38 g</td>
</tr>
<tr>
<td>Methanol</td>
<td>200 mL</td>
</tr>
</tbody>
</table>

Mix in H₂O to a volume of 1.7 L, bring to a pH of 11, top up to a final volume of 1.8 L with H₂O, and store at room temperature.
**Semi-dry transfer buffer**
- Glycine: 1.92 g
- Tris base: 1.46 g
- Methanol: 100 ml
- 10% SDS: 1.88 ml

Mix in H₂O to a final volume of 500 ml and store at 4°C.

**10 X TBS**
- Tris base: 24.25 g
- NaCl: 80 g

Mix above ingredients in H₂O to a volume of 900 ml, bring to a pH of 7.6, and bring to a final volume of 1 L with H₂O. Store at room temperature and dilute to 1 X for use in experiments. For TBS-T, add 1 ml Tween 20 to 1 L of 1 X TBS.

**50X TAE Buffer**
- Tris base: 242 g
- 0.5 M EDTA: 100 ml

Mix in H₂O to a final volume of 1 L, store at room temperature, and dilute to 1 X when needed.

**Immunofluorescence Blocking Buffer**
- 10 X PBS: 2.5 ml
- Normal goat serum: 1.25 ml
- Triton X-100: 75 µl

Mix in H₂O to a final volume of 25 ml, and store at 4°C for up to 2 weeks.

**Immunofluorescence Antibody dilution buffer**
- 10 X PBS: 4 ml
- Triton X-100: 120 µl
- BSA: 0.4 g

Mix in H₂O to a final volume of 40 ml, and store at 4°C for up to 2 weeks.

**PI staining solution**
- DNase-free RNase A: 2 mg
- PI (1mg/ml): 200 µg

Mix in PBS + 0.1% Triton X-100 to a final volume of 500 µl. Once prepared, protect from light and use immediately.