Novel Drug 2-benzoyl-3-phenyl 6,7-dichloroquinoxaline 1,4-dioxide Induces Colon Cancer Cell Apoptosis Through HIF-1α Pathway

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Novel Drug 2-benzoyl-3-phenyl 6,7-dichloroquinoxaline 1,4-dioxide Induces Colon Cancer Cell Apoptosis Through HIF-1α Pathway

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DEDICATION

This thesis is dedicated in loving memory of Alexi Pavlov and Dr. Raja Fayad.
ACKNOWLEDGEMENTS

I first need to thank my parents Theodore and Elisabeth and my brother Nick without whom I would never have made it this far. Even though he will never be able to read this, I would like to thank Dr. Fayad for everything he taught me before his untimely passing, I could not have imagined spending my time with anyone else. I would like to thank my thesis committee members; Dr. James Carson, Dr. J. Larry Durstine, and Dr. Ho-Jin Koh whom have given their time and expertise to better me as a scientist and a person. I would like to thank the lab members, both past and present, of the IIIC who have assisted me and throughout the completion of this degree; Arpit Saxena and Kamaljeet Kaur, and any not specifically mentioned here. Finally I would like to thank my friends and family abroad who have always been there to support me.

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ABSTRACT

Recent developments in the field of cancer genomics have shown transcription factor HIF-1α as a major player in the survival and proliferation of colorectal tumors. Hypoxia targeted drug engineering has led to significant advancements in cancer treatments as a method of directly utilizing the hypoxic regions against the tumor. Novel drug DCQ (2-benzoyl-3-phenyl 6,7-dichloroquinoxaline 1,4-dioxide) has shown promising anti-tumor results in-vitro and in-vivo. The purpose of this study was to utilize a tumor xenograft and genetic mouse model of colorectal cancer to investigate the safety, clinical effectiveness, and mechanism of action of DCQ.

Methods: 10 week old Balb/c mice were injected subcutaneously with 2 million CT-26 cells and were monitored for tumor growth over 14 days before receiving treatment. \( Apc^{min/+} \) mice were clinically evaluated from 8 weeks of age and began treatments at 16 weeks of age. DCQ treatment given at a 17mg/kg dose and 100μL DMSO injection as control. Injections were given bi-weekly over a four week period. Results: DCQ caused significant decrease in tumor weight (p<0.05) and final tumor area (p<0.05) in Balb/c mice at time of sacrifice than control and \( Apc^{min/+} \) mice showed significantly lower clinical score after 1 week of therapy along with decreased large tumor size (p<0.05) and number (p<0.05). Histological analysis showed increased total apoptotic area (p<0.05) in tumor tissue sections and tumor specific apoptosis in colon tissue in both models. Western blot analysis of Balb/c showed a decreased nuclear expression of HIF-1α (p<0.05) and increased expression of pro-apoptotic genes dephosphorylated-Bad (p<0.001), cleaved caspase-9 (p<0.05), and Bax (p<0.05) paralleled with a decrease in anti-apoptotic Bcl-2 gene (p<0.05). Conclusions: DCQ induces tumor specific apoptosis through mechanisms involving down regulation of HIF-1α and increased intracellular
apoptosis in Balb/c mice and Apc\textsuperscript{min/+} mice. Novel drug DCQ may potentially have use as a chemothrapeutic agent to reduce the pathology of sporadic intestinal and colorectal cancers.

KEYWORDS: Cancer, Colon, DCQ, Drug
# Table of Contents

**DEDICATION** ........................................................................................................ iii

**ACKNOWLEDGEMENTS** ............................................................................................... iv

**ABSTRACT** .................................................................................................................. v

**LIST OF TABLES** .......................................................................................................... ix

**LIST OF FIGURES** ....................................................................................................... x

**LIST OF ABBREVIATIONS** ........................................................................................ xi

**CHAPTER 1: INTRODUCTION AND AIMS** ................................................................. 1

**CHAPTER 2: REVIEW OF LITERATURE** ................................................................. 12

  2.1 COLORECTAL CANCER ......................................................................................... 13

  2.2 MODELS OF STUDYING COLORECTAL CANCER ........................................... 14

  2.3 CHEMOTHERAPY TREATMENTS ....................................................................... 18

  2.4 HYPOXIA IN THE TUMOR MICROENVIRONMENT .......................................... 20

  2.5 2-BENZOYL-3-PHENYL 6,7-DICHLOROQUINOXALINE 1,4-DIOXIDE .............. 30

**CHAPTER 3: NOVEL DRUG 2-BENZOYL-3-PHENYL 6,7-DICHLOROQUINOXALINE 1,4-DIOXIDE INDUCES COLON CANCER CELL APOPTOSIS THROUGH HIF-1[ALPHA] PATHWAY** ........ 36

  3.1 ABSTRACT ............................................................................................................ 37

  3.2 INTRODUCTION .................................................................................................. 39

  3.3 METHODS ......................................................................................................... 43

  3.4 RESULTS .......................................................................................................... 49

  3.5 DISCUSSION ....................................................................................................... 55
LIST OF TABLES

Table 3.1 Apc\textsuperscript{min/+} mouse clinical score guidelines ..........................................................65
Table A.1 Animal treatment groups for experiment #1 ........................................................................89
Table A.2 Animal clinical score guidelines .........................................................................................89
Table A.3 Animal treatment groups for experiment #2 ........................................................................99
LIST OF FIGURES

Figure 1.1 Working Model ........................................................................................................8
Figure 2.1 2-benzoyl-3-phenyl 6,7-dichloroquinoxaline 1,4-dioxide..............................31
Figure 3.1 Experimental Design ..............................................................................................64
Figure 3.2 DCQ therapy decreases tumor xenograft weight and area .................................64
Figure 3.3 DCQ increases apoptosis in tumor xenografts of Balb/c mice .........................65
Figure 3.4 DCQ decreases proliferation in tumor xenografts of Balb/c mice .................66
Figure 3.5 DCQ decreases nuclear HIF-1α protein expression in tumor xenografts of Balb/c mice .....................................................................................................................67
Figure 3.6 DCQ increases pro-apoptotic proteins Cleaved Caspase-9, Dephosphorylated-Bad, and Bax and decreases anti-apoptotic protein Bcl-2 in tumor xenografts of Balb/c Mice .................................................................................................................................68
Figure 3.7 DCQ treatment does not induce liver, kidney, or brain apoptosis in Balb/c mice ........................................................................................................................................69
Figure 3.8 DCQ improves Apc\textsuperscript{min/+} mouse clinical score after 4 weeks of treatment ......70
Figure 3.9 DCQ reduces Apc\textsuperscript{min/+} mouse intestinal tumor count and size .............71
Figure 3.10 DCQ induces colon tumor apoptosis and decreases tumor proliferation of Apc\textsuperscript{min/+} mice .................................................................................................................................72
Figure 3.11 DCQ does not induce normal colon epithelium apoptosis in Apc\textsuperscript{min/+} mice ..72
Figure 3.12 DCQ decreases nuclear expression of HIF-1α in colon tumors of Apc\textsuperscript{min/+} mice ........................................................................................................................................73
Figure 3.13 DCQ increases pro-apoptotic proteins Cleaved Caspase-9 and Bax and decreases anti-apoptotic protein Bcl-2 in colon tumors Apc\textsuperscript{min/+} mice .................................................................................................................................73
Figure 3.14 DCQ treatment decreases liver apoptosis and does not induce kidney or brain tissue apoptosis in Apc\textsuperscript{min/+} mice .................................................................................................................................74
LIST OF ABBREVIATIONS

AKT .......................................................................................................................... Protein Kinase B
APAF1 .............................................................. Apoptotic protease activating factor 1
AOM .......................................................................................................................... Azoxymethane
Apc ............................................................................................................................ Adenomatous polyposis coli
Arnt ........................................... Aryl hydrocarbon receptor nuclear translocator protein
Bad .............................................................. Bcl-2-associated death promoter
Bax ............................................................................................................................ Bcl-2-associated X protein
Bcl-2 .......................................................................................................................... B-cell lymphoma 2
Bcl-xL ...................................................................................................................... B-cell lymphoma-extra large
CT-26 ............................................................. CT-26 murine colon adenocarcinoma
Cul2 ............................................................................................................................ Cullin-2
DCQ ................................................................. 2-benzoyl-3-phenyl 6,7-dichloroquinoxaline 1,4-dioxide
DMH .......................................................................................................................... 1,2-dimethylhydrazine
DNA ............................................................................................................................ Deoxyribonucleic acid
Dephos-Bad ...................................................... Dephosphorylated-Bcl-2-associated death promoter
EGL ............................................................................................................................. Egg Laying Nine-9
EPO ............................................................................................................................ Erythropoietin
FDA ............................................................................................................................. US Food and Drug Administration
GLUT1 ...................................................................................................................... Glucose Transporter-1
HCR ............................................................................................................................. Hypoxia cytotoxicity ratio
HIF-1 .......................................................................................................................... Hypoxia Inducible Factor-1
HIF-1α................................................................. Hypoxia Inducible Factor-1 alpha
HIF-1β................................................................. Hypoxia Inducible Factor-1 beta
hr ............................................................................. hours
IL-6 ................................................................. Interleukin 6
kDa ............................................................................. Kilodalton
LDHA .............................................................. Lactate Dehydrogenase A
LLC ........................................................................... Lewis Lung Carcinoma
mg/kg ........................................................................ milligrams per kilogram
min ............................................................................... minute
Min ............................................................................ Apc
MOMP ........................................................ mitochondrial outer membrane permeabilization
mRNA ........................................................................ messenger RNA
NFκB ........................................................................ Nuclear Factor-kappa B
NOS ........................................................................ Nitric Oxide Synthase
ODDD ........................................................ Oxygen-dependent degradation domain
PAS .............................................................................. Per Arnt Sim
PBS ................................................................. Phosphate buffered saline
PBS-T ............................................................. Phosphate buffered saline and tween
Per .............................................................................. Period circadian protein
PHD(s) .................................................................. Prolyl Hydroxylase Domain(s)
PI3K .......................................................................... Phosphoinositide 3-kinase
QdNOs ....................................................................... Quinoxaline di-N-oxides
RBX1 .......................................................................... RING-box protein 1
SDS ........................................................................... Sodium dodecyl sulfate
Sim .............................................................................. Single-minded protein
TNFα................................................................. Tumor Necrosis Factor alpha
WHO............................................................... World Health Organization
V........................................................................ Volts
VEGF............................................................... Vascular Endothelial Growth Factor
VLH .................................................................... von Hippel Lindau protein
CHAPTER 1

INTRODUCTION AND AIMS
In 2012 the WHO reported cancer as the leading cause of death worldwide accounting for nearly 8.2 million deaths. In the United States, cancer related deaths were reported as second highest behind heart disease (Torre et al., 2015). Colorectal cancer is the second most prevalent cancer in both men and women in the United States in terms of incidence and death rates. The American Cancer Society estimates approximately 51,000 Americans will die of colon cancer and nearly 150,000 new cases will be diagnosed in 2014 (Siegel, DeSantis, & Jemal, 2014). It is currently estimated that the average American has a 5% lifetime risk of developing colorectal cancer and men are at higher risk than women are with a morbidity rate of 59 per 100,000 people versus 44 for women. The incidence of colorectal cancer increases with advancing age in both genders, with the highest risk occurring in those over 69 years of age. The financial burden of colorectal cancer has had a large impact on the current health care system. In 2010, the direct cost of colorectal cancer was estimated to be $12.2 billion dollars, $10.7 being due to lost productivity and premature death (Siegel et al., 2014; Torre et al., 2015). Thus, colorectal cancer has had a major impact on both the economy and population of the United States.

The initial incidence of hypoxia in the tumor environment arises because of oxygen diffusion limitations in avascular primary tumors (Lunt, Chaudary, & Hill, 2008; Mathonnet et al., 2014; Ryan, Lo, & Johnson, 1998; Zeng, Liu, Pan, Singh, & Wei, 2014). As a result of increased hypoxia in the tumor environment, the tumor microvasculature proliferates and serves to benefit tumor growth and metastasis. Normal responses to hypoxia typically see increased microvasculature proliferation in the tissue but with eventual compensation by the body through increased red blood cell count and increased oxygen carrying capacity of the blood (Lunt et al., 2008; Yasuda, 2008). In the
tumor environment, the increased microvasculature proliferation in the tumor tissue is highly abnormal and often fails to correct for the oxygen deficit. This persistent hypoxia leads to spatial disorganization of the tumor vascular networks and eventually leads to intercapillary distances that are beyond the diffusion range of oxygen (~200\(\mu\)m) (Brocato, Chervona, & Costa, 2014; Kunz & Ibrahim, 2003).

Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric transcription factor that plays a critical role in the cellular response to hypoxia. HIF-1 was discovered by the identification of a hypoxia response element (HRE; 5′-RCGTG-3′) in the 3′ enhancer of the gene for erythropoietin (EPO), a hormone that stimulates erythrocyte proliferation and undergoes hypoxia-induced transcription (Semenza, Nejfelt, Chi, & Antonarakis, 1991). HIF-1 regulates the transcription of a broad range of genes that facilitate responses to a hypoxic environment, including genes regulating angiogenesis, erythropoiesis, cell cycle, metabolism, and apoptosis. The HIF-1 complex consists of two subunits, HIF-1\(\alpha\) and HIF-1\(\beta\), both of which are basic helix-loop-helix proteins of the PAS family. HIF-1\(\alpha\) is seen to accumulate under hypoxic conditions whereas HIF-1\(\beta\) is constitutively expressed in both hypoxic and normoxic conditions. When expressed under hypoxic conditions, HIF-1\(\alpha\) is seen to play a vital role as a mediator for solid tumor expansion including activation of angiogenic factor VEGF. HIF-1\(\beta\) is the Aryl hydrocarbon Receptor Nuclear Translocator (ARNT), which is essential for the xenobiotic response (Wolff et al., 2013). Under normoxic conditions, undifferentiated HIF-1 is targeted by an E3 ubiquitin ligase containing the von Hippel Lindau protein (VLH). The human genome contains EGL9 (Egg Laying Nine-9) homologues that are named EGLN1, EGLN2, and EGLN3 (also known as PHD2, PHD1, and PHD3 respectively). Prolyl Hydroxilase
domain-containing proteins (PHDs) post-translationally modify HIF-1, allowing interactions with VHL to occur. All three proteins of PHDs can hydroxylate HIF-1α at one of two proline sites within the ODDD (Pro-402 and Pro-564). VHL is part of a larger protein complex that includes Elongin-B, Elongin-C, Cul2, RBX1, and a ubiquitin-conjugating enzyme (E2). This complex, along with ubiquitin-activating enzyme (E1), regularly mediates ubiquitylation of HIF-1 and its subunits (Ke & Costa, 2006).

In hypoxic conditions, HIF-1α subunits are not recognized by pVHL, this leads to accumulation of HIF-1α which dimerize with HIF-1β and translocate into the nucleus. In the nucleus, these proteins act as transcriptional regulators with cofactors such as CBP/p300 and DNA polymerase II complex to bind to hypoxia-responsive elements (HREs) and activate transcription of target genes. The understanding of HIF-1α target genes is well understood, HIF-1α-activated genes include Vascular Endothelial Growth Factor (VEGF), Glucose Transporter-1 (GLUT1), Lactate Dehydrogenase (LDHA), Erythropoietin (EPO), and Nitric Oxide Synthase (NOS) (He, Jiang, Zhang, & Wu, 2014; Zeng et al., 2014).

Defective apoptosis is frequently associated with malignancies originating from B-lymphocytes. Bcl-2 family proteins govern the mitochondrial outer membrane permeabilization (MOMP) in the intrinsic regulation of apoptosis. In the presence of a trophic factor, PI-3K is activated and leads to the activation of protein kinase B (PKB or Akt) which hydrolyzes ATP to phosphorylate pro-apoptotic protein Bcl-2-associated death promoter (Bad). Phosphorylated BAD at this point forms a 14-3-3 heterodimer which prevents its binding to Bcl-2 which leaves Bcl-2 to form a heterodimer with Bcl-xL. The formation of this dimer does not allow for the formation and opening of Bax/Bak.
heterodimer ion channel to promote the release of cytochrome c to the cytoplasm. Absence of cytochrome c does not allow for the formation of the cytochrome c/APAF-1/procaspsase-9 multiprotein complex which does not allow for the initiation of apoptosis. Therefore, apoptosis is actively inhibited through the blocking of this mechanism which is stimulated by the presence of a trophic factor (Erler et al., 2004; Wei et al., 2001; Xin & Deng, 2006; Zhang et al., 2004).

Quinoxaline di-N-oxides (QdNOs) were approved in the late 1960's for use as feed additives in livestock farming to promote animal growth and as an effort to replace banned therapeutic antibiotics, penicillin, and tetracyclines (Diab-Assaf, Haddadin, Yared, Assaad, & Gali-Muhtasib, 2002; Gali-Muhtasib, Diab-Assaf, & Haddadin, 2005). Approval of QdNOs for use was due to the large body of evidence showing that quinoxaline antibiotics are inhibitors of DNA synthesis and had increased effectiveness in anaerobic conditions. The structural design of QdNOs is the key to the increased effectiveness of DNA synthesis inhibition in these types of antibiotics. In the presence of an electron rich environment, a free radical intermediate is created by the transfer of an electron from this electron rich environment to the electron-poor nitrogen centers of the 1,4-di-N-oxide moiety. In a hypoxic environment, this transfer is more likely to occur due to the highly reducing conditions of hypoxic cells, leading to hypoxic cells acting as a targeting element to cytotoxicity of these compounds (Diab-Assaf, Haddadin, Yared, Assaad, & Gali-Muhtasib, 2002; H. U. Gali-Muhtasib, Diab-Assaf, & Haddadin, 2005).

Administration of quinoxaline di-N-oxides to tumor cultures has shown promising evidence of tumor specific apoptosis and decreased proliferation through interactions with the HIF-1α pathway. Preliminary research from the American University of Beirut
has shown tumor specific cytotoxicity mechanisms of quinoxaline 1,4-dioxides (QdNOs) cultured under hypoxic conditions. The QdNOs were found to possess a 50- to 100-fold greater cytotoxicity to human T-84 colorectal cells cultured under hypoxia compared to anoxic environment. It was reported that the hypoxia cytotoxicity ratio (HCR), the ratio of equitoxic concentrations of the drug under aerobic/anoxic condition, was highly structure related and dependent on the nature of the substituents' on the QdNO heterocycle. The most cytotoxic, 2-benzoyl-3-phenyl-6,7-dichloro derivative of the quinoxaline 1,4-dioxide, or DCQ, was potent at a dose of 1μM with an HCR of 100 and significantly reduced the levels of HIF-1α transcript and protein. It was further hypothesized that the C-6 and C-7-chlorine of DCQ might play a significant role in the selective hypoxic cytotoxicity of the drug (Diab-Assef et al., 2002; H. Gali-Muhtasib et al., 2000).

Administration of DCQ in both tumor cell cultures and healthy epithelial cells at concentrations of 5μM and 10μM show promising results to tumor specific induction of apoptosis via HIF-1α pathway and up-regulation of pro-apoptotic apoptosis genes (El-Khatib et al., 2010; H. U. Gali-Muhtasib et al., 2005; Ghattass et al., 2014). While there is evidence of direct effectiveness of DCQ in cell cultures and a suggested safe concentration of 17mg/kg, there is still an incomplete understanding of the in-vivo effectiveness, overall safety, and direct mechanisms of action that account for the tumor specific targeting of DCQ. The overall goal of this proposal is to determine clinical effectiveness of DCQ in inducing apoptosis of colorectal tumor cells and systemic safety of the 17mg/kg dosage in an implant and genetic model of colorectal cancer. Our central hypothesis is that DCQ administered at a concentration of 17mg/kg will be effective at
reducing tumor size and burden in both models of colorectal cancer and reduce HIF-1α expression leading to up-regulated tumor specific mitochondrial induced apoptosis without showing signs of systemic toxicity.

**Specific Aim #1** will evaluate the safety and effectiveness of the 17mg/kg DCQ dosage in a xenograft and genetic model of colorectal cancer.

**Specific Aim #2**: will determine the mechanism of action of DCQ in the two animal models of colorectal cancer.

**Working Model**: Initially the proposal will examine the overall effectiveness of DCQ on decreasing tumor burden and improving animal health of the 17mg/kg dosage in a CT-26 xenograft model and \( Apc^{\text{min/+}} \) model of colorectal cancer (AIM 1). Next, the proposal will examine if DCQ therapy works through similar mechanisms in both models to induce tumor specific apoptosis (AIM 2). The \( Apc^{\text{min/+}} \) and CT-26 xenograft animal models differ in rate of cancer progression and overall clinical tumor burden relative to body size, however both serve as effective models of studying colorectal cancer. Understanding the effectiveness of DCQ in both a xenograft and genetic model of colorectal cancer will serve as an important step for progression of DCQ to human trials in addition to furthering our understanding of the role of QdNOs in the prevention and treatment of colorectal cancer.
Figure 1.1 Working Model
Specific Aim #1 will evaluate the safety and effectiveness of the 17mg/kg DCQ dosage in a xenograft and genetic model of colorectal cancer.

**Rationale:** It has been previously shown that DCQ is effective in causing tumor specific apoptosis and can repress tumor growth in multiple human cancer cell lines through activation mechanisms involving down-regulation of HIF-1α pathway (Diab-Assef et al., 2002; El-Khatib et al., 2010; Ghattass et al., 2014; H. U. Gali-Muhtasib et al., 2005; Haykal et al., 2008, 2009). Toxicity studies from the American University of Beirut have suggested a concentration of 17mg/kg dose will be effective in causing tumor cell apoptosis while staying below systemic toxic levels, therefore preserving essential healthy tissue in both the GI system and the entire body. The 17mg/kg dosage has previously been shown to be effective in a breast cancer xenograft study, however, there is lack of clinical data from this study and DCQ has yet to be investigated *in-vivo* for clinical effectiveness in a colorectal cancer model (Ghattass et al., 2014). Thus, we would like to investigate the safety and clinical effectiveness of the 17mg/kg dose in a tumor implant and genetic model of colorectal cancer.

**Aim 1.1:** Observation of tumor size and weight in Balb/c mice implanted with CT-26 tumor xenografts over a four week therapy period with DCQ administration at a concentration of 17mg/kg.

**Aim 1.2:** Clinical score of Apc\(^{min/+}\) mice over a four week therapy period with DCQ administration at a concentration of 17mg/kg.

**Aim 1.3:** Safety of DCQ will be determined by assessment of other vital organs for presence of increased apoptosis and abnormal phenotype.
Specific Aim #2: will determine the mechanism of action of DCQ in the two animal models of colorectal cancer.

Rationale: Studies in multiple human cell lines from the American University of Beirut have shown consistent results of increased mitochondrial induced apoptosis with DCQ treatment. The CT-26 xenograft and $Apce^{min/+}$ transgenic mouse are common experimental models of colorectal cancer. The mutated Apc gene causes activation of the Wnt/β-catenin pathway which is responsible for tumorigenesis in the colon and small intestines (Bienz & Clevers, 2000; Leclerc, Deng, Trasler, & Rozen, 2004; Newton, Kenneth, Appleton, Näthke, & Rocha, 2010; Näthke & Rocha, 2011). Although this is a specific genetic model of cancer there is genetic variability in the tumor formation and therefore each tumor site is expected to respond differently to drug treatment. HIF-1α and the Apc gene have been shown to have cross-communication at the transcriptional level which serves as a driving force for intestinal tumorigenesis (Mehl et al., 2005; Newton et al., 2010; Yamulla et al., 2014). Since it has been determined that DCQ works through interruption of the HIF-1α pathway, we are interested in mechanistic differences between and within the murine CT-26 tumor xenograft model and $Apce^{min/+}$ genetic model of colorectal cancer.

Aim 2.1: Activation of DCQ will be determined through expression of HIF-1α. Decreased HIF-1α will indicate the hypoxic environment of the tumor environment is promoting transformation of DCQ to its active intermediate.
**Aim 2.2:** Presence of tumor specific apoptosis and expression of pro-apoptotic and anti-apoptotic genes will be performed to compare between the two models of colorectal cancer and indicate mechanisms of mitochondrial induced apoptosis.

**Aim 2.3:** Histological analysis of tumor containing sections of colon and intestine in \( Apo^{-/-} \) mice will indicate tumor specific induction of apoptosis by DCQ.
2.1 Colorectal Cancer

In 2012 the WHO reported cancer as the leading cause of death worldwide accounting for nearly 8.2 million deaths. In the United States, cancer related deaths were reported as second highest behind heart disease (Torre et al., 2015). Colorectal cancer is the second most prevalent cancer in both men and women in the United States in terms of incidence and death rates. The American Cancer Society estimates approximately 51,000 Americans will die of colon cancer and nearly 150,000 new cases will be diagnosed in 2014 (Siegel et al., 2014). It is currently estimated that the average American has a 5% lifetime risk of developing colorectal cancer and men are at higher risk than women are with a morbidity rate of 59 per 100,000 people versus 44 for women. The incidence of colorectal cancer increases with advancing age in both genders, with the highest risk occurring in those over 69 years of age. The financial burden of colorectal cancer has had a large impact on the current health care system. In 2010, the direct cost of colorectal cancer was estimated to be $12.2 billion dollars, $10.7 being due to lost productivity and premature death (Siegel et al., 2014; Torre et al., 2015). Thus, colorectal cancer has had a major impact on both the economy and population of the United States.

Clinical Symptoms of Colorectal Cancer

Colorectal cancer is a degenerative disease that takes years to develop in the humans. Incidence of colorectal cancer is suggested to be onset by chronic stress and poor lifestyle management, the latter of which is typically a lack of physical activity and poor diet. In the early stages of the disease, symptoms of colorectal cancer may be minimal or not present at all. The quantity and severity of symptoms associated with
colorectal cancer typically accompany the progression of the disease. In a clinical setting, symptoms of colorectal cancer are broken down into local and systemic. Local symptoms are those which have a direct effect on the colon or rectum and are seen in the various stages of the disease. These symptoms include changes in bowel habits, constipation, diarrhea, alternating diarrhea and constipation, rectal bleeding or bloody stools, abdominal bloating or cramping, incomplete bowel emptying, and thinner stools. Systemic symptoms are those that effect the entire body and are typically seen in the later stages of the disease. These symptoms include unexplained weight loss, unexplained loss of appetite, nausea or vomiting, anemia, jaundice, and weakness and fatigue. Due to the delayed onset of both local and systemic symptoms past the initial onset of the disease regular screening is recommended on a per 5 year basis for everyone over 50 years old.

2.2 Models of Studying Colorectal Cancer

There are many models of studying colorectal cancer that are being utilized to understand and investigate development, progression, and potential therapies of the condition. Utilization of both genetic and tumor implantation models are common in animal research. Cell culture methods have also been utilized, especially for drug development, to understand a direct impact of a specific factors and secreted cytokines on proliferative and necrotic regulation. Human models are only used in later progressions of drug development, therefore many investigators use rodent or in vitro models to study treatments and mechanisms involved in colorectal cancer.

Non-rodent species

Canine use has become attractive model for comparative oncology research. Similar to rodent models, there are many similarities between canine colorectal cancer
and the human disease. One of the benefits to canine intestinal cancer models is the onset of tumors is more commonly found in the large intestine rather than the small intestine. This more closely mimics the descending colon/rectal cancers that are most frequently seen in human onset of the disease (Johnson & Fleet, 2013). Evaluations of canine colorectal adenomas have revealed cytoplasmic and nuclear accumulation of β-catenin, which further suggests a dysregulation of the WNT signaling pathway is an important driver in the onset of colorectal cancer in canine species (McEntee & Brenneman, 1999). Despite the similarities between canine and human colorectal cancer, the prevalence in canines is less than 1% and therefore severely limits the effectiveness of using this model (Johnson & Fleet, 2013; McEntee & Brenneman, 1999).

Sheep are also an effective model of studying human colorectal cancer due to the similarities of lesions and metastatic behavior between the human disease. Despite this similarity, it has been shown that 100% of the intestinal adenocarcinomas developed in sheep are found in the small intestine. Along with this limitation, the unique physiological characteristics of the sheep ruminant forestomachs has been shown as a significant weakness of this model (Johnson & Fleet, 2013; Munday, Brennan, Jaber, & Kiupel, 2006).

CT-26 Adenocarcinoma

Another commonly used model is the CT-26 adenocarcinoma model of colorectal cancer. CT-26 cells can either be cultured or injected subcutaneously in Balb/c mice, which is known as a Xenograft model. Tumors typically develop within 14 days and the mice begin to show severe symptoms of tumor burden. While other animal models develop multiple intestinal polyps over the course of time, the CT-26 Xenografts develop
into large visible tumors that can account for more than 15% of the animal's total body weight. This model typically mimics the symptoms of tumor burden which include increased circulating IL-6 and insulin resistance (Aulino et al., 2010). Since this model typically shows hallmarks of human cancer conditions and allows for rapid studies to be conducted, it is often incorporated in preliminary studies for drug and therapy development. The accelerated development of the large tumor makes the tumor burden less like the human condition, but is still valid as a model of studying colorectal cancer (Aulino et al., 2010; Bonetto et al., 2011, 2012).

Diet-Induced Rodent Neoplasia Models

Epidemiological evidence has shown diet as a key modulator to the incidence of colorectal cancer in humans (Lee et al., 2012, 2013; Magalhães, Peleteiro, & Lunet, 2012; Shivappa et al., 2014). Several rodent studies have been conducted to examine the influence of poor diet on the incidence of colorectal cancer. This diet typically is designed to mimic the poor "Western diet" that has become problematic to the current health of many Americans. There are several modifications of a western diet. The "Western diet" is designed to increase dietary fat intake from 5% to 20%, decrease dietary calcium from 0.5% to 0.05%, and decrease vitamin D intake to 100 IU/kg from 1,000 IU/kg (Johnson & Fleet, 2013; Newmark, Lipkin, & Maheshwari, 1990). Feeding the Western diet for as little as 12 weeks was shown to induce hyperplasia of colonic crypts in rats and mice (Newmark et al., 1990). Longevity studies have also shown that after 2 years of being fed the Western diet, 70% of the mice developed dysplastic crypts and small polypoid lesions, most of which were found in the distal colon (Risio et al., 1996). Another study has also shown that a 2 year feeding of a Western diet low in fiber,
folate, methionine, and choline content lead to 25% of C57BI/6J mice developing intestinal tumors and showed lesions demonstrating evidence of adenocarcinoma (Johnson & Fleet, 2013; Newmark et al., 2001; Risio et al., 1996). Feeding the Western diet to mice has also been shown to induce a transcript profile in normal colonic mucosa that was similar to the pattern loss of Apc allele in Apc$^{138N/+}$ mice (Yang et al., 2008). The Western diet model of spontaneous colorectal cancer is an attractive model however the changes in physiology seen in many studies do not reflect the etiology of human colorectal cancer (Johnson & Fleet, 2013).

**Chemical-Induced Models**

A large number of chemicals have been shown to have mutagenic potential and are used to controllably induce cancer. 1,2-dimethylhydrazine (DMH) and its metabolite azoxymethane (AOM), are the two most commonly used carcinogens to induce colorectal cancer in rodents (Bissahoyo et al., 2005). DMH and AOM are alkylating agents that are typically injected intraperitoneally or subcutaneously over several weeks to induce tumorigenesis. The majority of these tumors are seen in the distal colon and are seen to harbor mutations in the β-catenin gene. This mutation affects the N-terminal amino acids of the β-catenin gene product and leads to resistance of the regulatory degradation, this stabilizes β-catenin and increases WNT signaling to drive tumor development (Bienz & Clevers, 2000; Komiya & Habas, 2008; Sebio, Kahn, & Lenz, 2014). This model therefore is useful for studying the gene-gene and gene-environment interactions that influence the pathophysiology of colorectal cancer. However it is suggested that this model is not viable due to the little evidence supporting the induction of human colorectal cancer resulting from exposure to alkylating agents (Johnson & Fleet, 2013).
The $Apc^{\text{min/+}}$ mouse has been one of the leading models for preclinical colorectal cancer research over the past 20 years. The phenotype of these mutant mice showed severe clinical symptoms of intestinal cancer from the onset of a usually fatal case of regenerative anemia attributed to multiple intestinal neoplasms, or "Min." The Min mouse has a naturally occurring T-to-A nonsense mutation at nucleotide 2,549 of the Adenomatous polyposis coli ($Apc$) gene that pares the Apc protein at the codon 850, this predisposes the mice to multiple intestinal adenomas (Moser, Pitot, & Dove, 1990). The Min mutation is autosomal dominant, and homozygosity for the mutant allele is embryonic lethal. Heterozygous $Apc^{\text{min/+}}$ mice typically become anemic by 60 days of age and progress through worsening clinical symptoms of intestinal cancer and usually die by 120 days of age (Johnson & Fleet, 2013; Moser et al., 1990).

The use of the $Apc^{\text{min/+}}$ mouse model has been widely used to study carcinogenesis or to test a developing treatment and delivery methods, the $Apc^{\text{min/+}}$ mouse has also been used to study genetic modifiers of colorectal cancer risk or Modifiers of Min (Mom) (Balmain, Gray, & Ponder, 2003; Johnson & Fleet, 2013).

### 2.3 Chemotherapy Treatments

Current chemotherapy treatments have undergone a significant improvement in tumor cell targeting over the last 60 years. Since the discovery of the DNA replication blocking mechanisms of Aminopterin, the predecessor of current treatment methotrexate, research into blockading functions of cell growth have been the major focus for the development of anti-cancer therapies. As the incidence of different types of cancer was evolved in the medical community, there was an increased demand of treatment methods.
Present therapies are able to be controlled with early detection and regular chemotherapies. As various mechanisms of cancer development and treatment were discovered, so paralleled the evolution of chemotherapy treatments. The development of chemotherapy treatments have begun to focus on drug combinations, delivery techniques, tissue specificity, and overcoming drug resistance by carcinomas (Brown & Wilson, 2004; McKeown, Cowen, & Williams, 2007; Silvestris et al., 2013).

The medical field has seen significant advancements in chemotherapy treatments over the last 40 years, however, one of the most common problems in drug design is the engineering of compounds to attack specifically the tumor environment and malignant tissue sites. Clinically, it is seen that many chemotherapy treatments can improve clinical score and wellbeing of the patient but have detrimental effects to healthy tissue. Many of the detrimental effects of chemotherapy treatments can be attributed to the non-specific targeting of tissue by the drug compound. The toxic effects of these compounds increases stress on the body and can ultimately lead to death in the patient (Bai & Wang, 2014; Gogvadze, Orrenius, & Zhivotovsky, 2009; Mathonnet et al., 2014; McKeown et al., 2007; Pettersen et al., 2014; Silvestris et al., 2013).

**Phases of Drug Development**

The progression of drug testing requires many pre-clinical and clinical steps that are highly regulated by the FDA. The first step in drug development is pre-clinical animal and in vitro based experiments that show effective and safe delivery methods and propose methods for progression to human testing. At this stage, the FDA can decide whether the drug is reasonably safe for progression to human trials or to take the drug back to formula. Once in clinical trials the drug is considered an investigational new drug and
must pass three phases of clinical testing that are overseen by both medical research and non-research based scientists and physicians.

Phase 1 testing is usually conducted in healthy volunteers in an effort to determine frequent side effects and how the drug is metabolized and excreted. The typical number of subjects for this phase is usually between 20 and 80. Phase 2 studies begin once the drug is determined safe for healthy humans and experiments begin to focus on drug effectiveness in humans. This phase tests a variety of patients with certain diseases or conditions and is compared in a controlled study against a placebo drug or current form of treatment. In Phase 2, the number of subjects can be expanded to at most 300 and is usually determined based on the range of conditions being investigated. At the end of Phase 2, if a drug has shown evidence of effectiveness in a certain population, Phase 3 can be initiated as a follow-up study with larger subject populations. The number of subjects in Phase 3 can be anywhere from a few hundred to 3,000. After passing all three phases of human clinical trials, postmarket and commitment studies are required by a sponsor and are monitored by the FDA. This typically allows the drug to enter longevity studies in humans to determine any long term side effects that may arise from continuous use of the drug. Once passed by the FDA the drug can be submitted to the FDA as a new drug and within six months the drug can be passed and distributed for use in clinical medicine (Kinch, 2014).

2.4 Hypoxia in the Tumor Microenvironment

The progression of tumor development in colorectal cancer shows a wide range of abnormal physiological characteristics that aide in the maintenance of tumor tissue. As a
solid tumor grows, the rate of cancerous cell proliferation surpasses the ability of the existing vasculature to supply growth factors, nutrients, and oxygen and to remove the catabolic waste produced by these cells. The result of these events is a change in the tumor environment and leads to an imbalance between supply and demand (Cairns, Harris, & Mak, 2011; Faubert et al., 2014). Accompanying this change introduces regions of hypoxia, low glucose, and low pH (Kunz & Ibrahim, 2003; Lunt et al., 2008; Ryan et al., 1998; Zeng et al., 2014). Most solid tumors contain hypoxic or anoxic regions that are believed to contribute to local resistance to therapy treatments in addition to promoting tumor metastasis.

**Hypoxia**

The initial incidence of hypoxia in the tumor environment arises because of oxygen diffusion limitations in avascular primary tumors (Lunt et al., 2008; Mathonnet et al., 2014; Ryan et al., 1998; Zeng et al., 2014). As a result of increased hypoxia in the tumor environment, the tumor microvasculature proliferates and serves to benefit tumor growth and metastasis. Normal responses to hypoxia typically see increased microvasculature proliferation in the tissue but with eventual compensation by the body through increased red blood cell count and increased oxygen carrying capacity of the blood (Lunt et al., 2008; Yasuda, 2008). In the tumor environment, the increased microvasculature proliferation in the tumor tissue is highly abnormal and often fails to correct for the oxygen deficit. This persistent hypoxia leads to spatial disorganization of the tumor vascular networks and eventually leads to intercapillary distances that are beyond the diffusion range of oxygen (~200um) (Brocato et al., 2014; Kunz & Ibrahim, 2003).
In the tumor environment, hypoxia has many effects on tumor biology. Many of these effects include: selection of genotypes favoring survival under hypoxia-re-oxygenation injury; pro-survival changes in gene expression that suppress apoptosis and support autophagy; and the anabolic switch in central metabolism (Brown, 2007; Lunt et al., 2008; Semenza, 2000; Weinmann, Jendrossek, Güner, Goecke, & Belka, 2004). Hypoxia also enhances receptor tyrosine kinase-mediated signaling, tumor angiogenesis, vasculogenesis, an epithelial-to-mesenchymal transition, invasiveness, metastasis, as well as suppressing immune reactivity (Bagnall et al., 2014; Chakraborty, John, & Nag, 2014; Dewhirst, 2003; Kaidi, Williams, & Paraskeva, 2007; Li & O’Donoghue, 2008; Vaupel & Mayer, 2005). Due to the continuous effects of the hypoxic environment on tumor development, hypoxia is implicated in multiple mechanisms in resistance to chemotherapy (Zeng et al., 2014; Brocato et al., 2014; Kunz & Ibrahim, 2003; Lunt et al., 2008; Palazon, Goldrath, Nizet, & Johnson, 2014).

**Hypoxia Inducible Factor-1**

Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric transcription factor that plays a critical role in the cellular response to hypoxia. HIF-1 was discovered by the identification of a hypoxia response element (HRE; 5'-RCGTG-3') in the 3' enhancer of the gene for erythropoietin (EPO), a hormone that stimulates erythrocyte proliferation and undergoes hypoxia-induced transcription (Semenza et al., 1991). HIF-1 regulates the transcription of a broad range of genes that facilitate responses to a hypoxic environment, including genes regulating angiogenesis, erythropoiesis, cell cycle, metabolism, and apoptosis. The HIF-1 complex consists of two subunits, HIF-1α and HIF-1β HIF-1, both of which are basic helix-loop-helix proteins of the PAS family. HIF-1α in seen to
accumulate under hypoxic conditions whereas HIF-1β is constitutively expressed in both hypoxic and normoxic conditions. When expressed under hypoxic conditions, HIF-1α is seen to play a vital role as a mediator for solid tumor expansion including activation of angiogenic factor VEGF. HIF-1β is the Aryl hydrocarbon Receptor Nuclear Translocator (ARNT), which is essential for the xenobiotic response (Chai et al., 2014; Unwith, Zhao, Hennah, & Ma, 2014; Wolff, Jelkmann, Dunst, & Depping, 2013).

Under normoxic conditions, undifferentiated HIF-1 is targeted by an E3 ubiquitin ligase containing the von Hippel Lindau protein (VHL). The human genome contains EGL9 (Egg Laying Nine-9) homologues that are named EGLN1, EGLN2, and EGLN3 (also known as PHD2, PHD1, and PHD3 respectively). Prolyl Hydroxylase domain-containing proteins (PHDs) post-translationally modify HIF-1, allowing interactions with VHL to occur. All three proteins of PHDs can hydroxylate HIF-1α at one of two proline sites within the ODD (Pro-402 and Pro-564). VHL is part of a larger protein complex that includes Elongin-B, Elongin-C, Cul2, RBX1, and an ubiquitin-conjugating enzyme (E2). This complex, along with ubiquitin-activating enzyme (E1), regularly mediates ubiquitylation of HIF-1 and its subunits (Ke & Costa, 2006).

In hypoxic conditions, HIF-1α subunits are not recognized by pVHL, this leads to accumulation of HIF-1α which dimerize with HIF-1β and translocate into the nucleus. In the nucleus, these proteins act as transcriptional regulators with cofactors such as CBP/p300 and DNA polymerase II complex to bind to hypoxia-responsive elements (HREs) and activate transcription of target genes. The understanding of HIF-1α target genes is well understood, HIF-1α-activated genes include Vascular Endothelial Growth Factor (VEGF), Glucose Transporter-1 (GLUT1), Lactate Dehydrogenase (LDHA),
Erythropoietin (EPO), and Nitric Oxide Synthase (NOS) (He et al., 2014; Zeng et al., 2014).

**HIF-1α and Tumor Cell Metabolism**

In a hypoxic environment, the cell relies on anaerobic components of energy metabolism to survive. Working at a high rate and with a high power output, the cell enhances its anaerobic glucose metabolism pathways, which in turn inhibit mitochondria regulation of metabolism. Cytoplasmic presence of undifferentiated HIF-1 and differentiated HIF-1α is understood to enhance the activity of glucose transporter 1, hexokinase, phosphoglucone isomerase, aldolase, phosphoglycerate kinase, phosphoglucomutase, enolase, pyruvate kinase, and lactate dehydrogenase. Essentially, the presence of HIFs in the cytoplasm upregulates every enzyme responsible for anaerobic metabolism. This increased cellular rate of glycolysis and decreased presence and activity of citric acid cycle enzymes is linked with tumor malignancy and can contribute to tumor metastasis. This is thought to be especially true of the hypoxic regions present in solid tumors. (Cairns et al., 2011; Kaelin, 2011; Pettersen et al., 2014; Zeng et al., 2014). A high rate of glucose metabolism is highly regulated in the stabilization of HIF-1α which activates transcription of glucose transporters and key glycolytic enzymes such as; lactate dehydrogenase A (LDHA), phosphoglycerate kinase-1 (PGK-1), and hexokinase-1 (HK1). Transcription of these components acts to further facilitate the increase in anaerobic glycolysis and the glycolytic switch (Cairns et al., 2011; Yasuda, 2008; Zeng et al., 2014).

In normal tissue, approximately 10% of cellular energy is obtained through anaerobic glycolysis, whereas mitochondrial aerobic respiration accounts for the other
90%. In solid tumors, the glycolytic switch increases anaerobic glycolysis contribution to more than 50%. However, when tumors are introduced to a normoxic environment mitochondrial function does not increase, this mechanism is suggested to be due to increased expression of HIF-1α. (Cairns et al., 2011; Racker & Spector, 1981; WARBURG, 1956; Zeng et al., 2014). Increased pyruvate dehydrogenase activity mediated through HIF-1α facilitates an increased turnover ratio of pyruvate to lactate. Furthermore, HIF-1α mediates and increased expression of pyruvate dehydrogenase kinase 1 (PDK-1) which functions to block the action of pyruvate dehydrogenase. Pyruvate dehydrogenase is responsible for changing pyruvate to acetyl CoA, which is then transported to the mitochondria to begin aerobic cellular respiration. Blocking of pyruvate dehydrogenase along with increased expression of anaerobic metabolic enzymes serves as a two-fold inhibition of aerobic respiration and requires solid tumor to rely primarily on glucose metabolism for fuel (Lum et al., 2007). HIF-1α is not a simple transcription factor inducer of glycolytic genes, but instead reprograms the fate of intracellular glucose; this is described as the Warburg effect (Cairns et al., 2011; Kaelin, 2011; Racker & Spector, 1981; WARBURG, 1956).

Growth factor-dependent induction of HIF-1α appears to partially contribute to the ability of growth factors and oncogenic mutations to the ability of growth factors and oncogenic mutations in the PI3K/Akt signal transduction pathway to induce a metabolic conversion to predominantly anaerobic glycolysis (Carmeliet et al., 1998; Lum et al., 2007; Newton et al., 2010; Palazon, Goldrath, Nizet, & Johnson, 2014). The chronic inhibition and low activity of the mitochondria is postulated to results in disruption of normal functioning of the mitochondria. The low-oxygen environment in tumor cells can
also result in dysregulation of genes involved in normal mitochondrial function, especially genes involved in control of apoptosis. Evidence attributes a relationship between decreased function of the mitochondria to an over-expressed form of mitochondrial-bound hexokinase which also contributes to high rates of glycolysis (Faubert et al., 2014; Lum et al., 2007; Mathupala, Ko, & Pedersen, 2009; Sun et al., 2013; Unwin et al., 2003). A study conducted by Christofk et al., 2008 showed evidence of this effect could be due to mutations in the Von Hippel-Lindau tumor suppressor gene, the gene responsible for targeting and ubiquitylation of HIF-1. This further suggesting HIF-1α may be a key regulator to tumor apoptosis resistance in addition to sustaining proliferative stimuli.

**HIF-1α and the Apc gene**

It is well established that the key response to hypoxia is activation of the transcription factor HIF-1α. However, in the tumor microenvironment there is sufficient interplay between the HIF-1α regulated gene transcripts and total expression of HIF-1α (Carmeliet et al., 1998; Ke & Costa, 2006; Newton et al., 2010; Näthke & Rocha, 2011; Palazon, Goldrath, Nizet, & Johnson, 2014). The Apc gene plays a significant role in regulation of the fundamental processes that govern normal gut epithelium. It is best known for control of the Wnt/β-catenin pathway, where it is responsible for the regulation of β-catenin which thereby regulates that transcriptional activity of T-cell factor (TCF)/lymphoid-enhancing factor (LEF) transcription factors(Bienz & Clevers, 2000).

Although not extensively investigated, the functional relationship between HIF-1α and the Wnt/β-catenin pathway has been established in recent investigations. HIF-1α can
specifically interfere with co-activation of TCF/LEF transcription mediated by β-catenin and furthermore can bind and regulate NF-κB activity which is theorized to contribute to chronic inflammation seen in the colon tissue (Bienz & Clevers, 2000; Kaidi et al., 2007; Sebio et al., 2014). Functional cross-talk has also been reported between HIF-1α and the Apc gene at the transcriptional level. Newton et al., 2010 reported a consistent direct transcriptional repression of Apc by HIF-1α. It was seen that HIF-1α bound to a hypoxia-responsive element, dubbed HRE, in the APC promoter region in the presence of hypoxia. This relationship was also confirmed in an APC depletion model which showed an increased HIF-1α expression and activity. The increase of HIF-1α was also determined to be mediated by NF-κB and was regulated by increased β-catenin levels which were increased due to the depletion of the Apc gene.

*Intracellular induced apoptosis and HIF-1α*

In the absence of a trophic factor the phosphatidylinositol-4, 5-bisphosphate 3-kinase (PI-3K)/ protein kinase B (PKB) signaling pathway is not activated and the phosphorylation of Bad does not occur. This allows for Bad to bind with anti-apoptotic gene B-cell lymphoma 2 (Bcl-2) and prevents the heterodimerization of Bcl-2 with anti-apoptotic sister gene B-cell lymphoma-extra large (Bcl-xL) on the mitochondrial membrane. Absence of this heterodimerization signals for the heterodimerization of Bcl-2-associated X protein (Bax) and Bcl-2 homologous antagonist killer (Bak) on the outer mitochondrial membrane which acts as an ion channel and causes an influx of various ions across a membrane potential. This ion flux influences the release of cytochrome c from the mitochondria into the cytoplasmic space. The release of cytochrome c from the mitochondria initiates the formation of multi-protein complex containing cytochrome c,
apoptotic protease activating factor 1 (APAF1), and procaspase-9. Formation of this complex represents the "point of no return" in the apoptosis pathway and cleaves procaspase-9 to form active cleaved caspase-9 which activates the downstream caspase cascade and will result in programmed cell death. The release of second mitochondrial-derived activator of caspases (SMAC), also known as direct inhibitor of apoptosis protein (IAP)-binding protein with low pI (DIABLO), is released by the mitochondria to further stimulate caspase cascades by inhibiting apoptosis inhibitor proteins (IAPs) (Erler et al., 2004; Wei et al., 2001; Xin & Deng, 2006; Zhang et al., 2004).

Defective apoptosis is frequently associated with malignancies originating from B-lymphocytes. Bcl-2 family proteins govern the mitochondrial outer membrane permeabilization (MOMP) in the intrinsic regulation of apoptosis. In the presence of a trophic factor, PI-3K is activated and leads to the activation of protein kinase B (PKB or Akt) which hydrolyzes ATP to phosphorylate pro-apoptotic protein Bcl-2-associated death promoter (Bad). Phosphorylated BAD at this point forms a 14-3-3 heterodimer which prevents its binding to Bcl-2 which leaves Bcl-2 to form a heterodimer with Bcl-xL. The formation of this dimer does not allow for the formation and opening of Bax/Bak heterodimer ion channel to promote the release of cytochrome c to the cytoplasm. Absence of cytochrome c does not allow for the formation of the cytochrome c/APAF-1/procaspase-9 multiprotein complex which does not allow for the initiation of apoptosis. Therefore, apoptosis is actively inhibited through the blocking of this mechanism which is stimulated by the presence of a trophic factor (Erler et al., 2004; Moreno-Galindo et al., 2014; Wei et al., 2001; Xin & Deng, 2006; Zhang et al., 2004).
HIF-1α presents a multi-influential role on the inhibition of intracellular apoptosis in the tumor microenvironment. Found on chromosome 14q23.2, HIF-1α is actively expressed in a hypoxic environment (Carmeliet et al., 1998; Erler et al., 2004; Kaidi et al., 2007; Kunz & Ibrahim, 2003; Poitz et al., 2011; Wolff et al., 2013). Paradoxically, cell adaptation to hypoxia leads not only to cell proliferation/survival but also to cell death in some circumstances. Hypoxia has been shown to induce apoptosis, where HIF-1 plays a complex role (Bristow & Hill, 2008; Kaidi et al., 2007; Rouschop et al., 2010; Zhou et al., 2014). Genetic studies using embryonic stem cells harboring a deletion of HIF-1α have showed decreased apoptosis compared with wild type cells when challenged with low oxygen (Carmeliet et al., 1998; Erler et al., 2004). Activation of caspase-3 and Apaf-1-mediated caspase-9, and the release of cytochrome c, have been reported in several cell types under hypoxic conditions (Favaro, Lord, Harris, & Buffa, 2011; McClintock et al., 2002; Pettersen et al., 2014; Rouschop et al., 2010). It has also been demonstrated that the expression of HIF-1α and HIF-1β significantly correlated with apoptosis and the pro-apoptotic factors, such as caspase-3, Fas, and Fas ligand (Volm & Koomägi, 2000). Furthermore, hypoxia depresses anti-apoptotic protein Bcl-2, whereas the pro-apoptotic protein Bcl-2/adenovirus EiB 19-kDa interacting protein 3 (BNip3) and its homolog Nip3-like protein X (NIX) were up-regulated in a HIF-dependent manner (Bruick, 2000; Kunz & Ibrahim, 2003). Some genes involved in cell cycle control, such as p53 and p21, were also found to be HIF-dependent (Carmeliet et al., 1998; Diab-Assef et al., 2002; Erler et al., 2004). In addition, p53 has been implicated in regulating hypoxia-induced apoptosis through induction of apoptosis-related genes such as Bax,
NOXA, PUMA, and PERP (Bai & Wang, 2014; Kunz & Ibrahim, 2003; Rosado-Berrios, Vélez, & Zayas, 2011; Weinmann et al., 2004).

In addition to the above classes of genes, HIF-1 also regulated many other target genes implicated in diverse processes such as adipogenesis (Lin, Lee, Shim, Chun, & Yun, 2010; Yun, Maecker, Johnson, & Giaccia, 2002), carotid body formation (Favaro et al., 2011; Kline, Peng, Manalo, Semenza, & Prabhakar, 2002), B lymphocyte development, and immune reactions (El Awad et al., 2000; Hellwig-Bürgel, Rutkowski, Metzen, Fandrey, & Jelkmann, 1999; Rosado-Berrios et al., 2011). Although there are some studies showing a role of HIF-2α in the VEGF induction (Duan, Zhang-Benoit, & Fong, 2005; Xue & Shah, 2013; Xue et al., 2012), no direct target genes have yet been identified for HIF-2α or HIF-3α. However, a recent study using a genetic “knock-in” strategy has shown that targeted replacement of HIF-1α with HIF-2α results in expanded expression of HIF-2α-specific target genes, the most significant find being Oct-4, a transcription factor essential for maintaining stem cell pluripotency (Covello et al., 2006).

2.5 2-benzoyl-3-phenyl 6,7-dichloroquinoxaline 1,4-dioxide

Quinoxaline di-N-oxides (QdNOs) were approved in the late 1960’s for use as feed additives in livestock farming to promote animal growth and as an effort to replace banned therapeutic antibiotics, penicillin, and tetracyclines (Diab-Assef et al., 2002; H. U. Gali-Muhtasib et al., 2005). Approval of QdNOs for use was due to the large body of evidence showing that quinoxaline antibiotics are inhibitors of DNA synthesis and had increased effectiveness in anaerobic conditions. The structural design of QdNOs is the key to the increased effectiveness of DNA synthesis inhibition in these types of
antibiotics. In the presence of an electron rich environment, a free radical intermediate is created by the transfer of an electron from this electron rich environment to the electron-poor nitrogen centers of the 1,4-di-N-oxide moiety. In a hypoxic environment, this transfer is more likely to occur due to the highly reducing conditions of hypoxic cells, leading to hypoxic cells acting as a targeting element to cytotoxicity of these compounds (Diab-Assef et al., 2002; El-Khatib et al., 2010; H Gali-Muhtasib et al., 2000; H. U. Gali-Muhtasib et al., 2005; Hala Gali-Muhtasib et al., 2004; Ganley et al., 2001).

![Figure 2.1 2-benzoyl-3-phenyl 6,7-dichloroquinoxaline 1,4-dioxide.](image)

DCQ administration has shown promising evidence of tumor specific apoptosis and decreased proliferation through interactions with the HIF-1α pathway. Preliminary research from the American University of Beirut has shown tumor specific cytotoxicity mechanisms of quinoxaline 1,4-dioxides (QdNOs) cultured under hypoxic conditions. The QdNOs were found to possess a 50- to 100- fold greater cytotoxicity to human T-84 colorectal cells cultured under hypoxia compared to an oxic environment. It was reported that the hypoxia cytotoxicity ratio (HCR), the ratio of equitoxic concentrations of the drug under aerobic/anoxic condition, was highly structure related and dependent on the
nature of the substituents' on the QdNO heterocycle. The most cytotoxic, 2-benzoyl-3-phenyl-6,7-dichloro derivative of the quinoxaline 1,4-dioxide was potent at a dose of 1μM with an HCR of 100 and significantly reduced the levels of HIF-1α transcript and protein. It was further hypothesized that the C-6 and C-7-chlorine of DCQ might play a significant role in the selective hypoxic cytotoxicity of the drug (Diab-Assef et al., 2002; H Gali-Muhtasib et al., 2000).

In a supplemental study, DCQ was tested for IC₅₀ and compared to QdNOs; AMQ and BPQ. It was seen that the IC₅₀ of AMQ, BPQ, and DCQ were 100, 20, and 1μM, respectively. Thus, the concentration of DCQ to induce 50% growth inhibition was 20-fold lower than that of BPQ and 100-fold lower than that of AMQ. It was also seen that these compounds were not cytotoxic to normal intestinal IEC-6 or Moe K cells at their IC₅₀ concentration (Diab-Assef et al., 2002; H. U. Gali-Muhtasib et al., 2005). To investigate the effect of DCQ on tumor cells, H. U. Gali-Muhtasib et al., 2005 exposed cultured T-84 human colon carcinoma cells to 1μM DCQ for a 48 hour period. Upon treatment with DCQ, the accumulation of sub-G₁ peak of hypo-diploid cells was evident and was seen in more than 35% of total cells. TUNEL assay of cultured cells also showed 60% of cultured cells were positive for dsDNA strand breaks compared to 35% for BPQ and 10% for AMQ. These findings suggested DCQ works by targeting the tumor microenvironment to induce anti-proliferative effects specifically in the tumor cells through an increase in cell apoptosis pathways (Diab-Assef et al., 2002; H. U. Gali-Muhtasib et al., 2005). It was also seen that DCQ significantly increased pro-apoptotic protein Bax and decreased anti-apoptotic protein Bcl-2 in T-84 cells. This indicating
activation of mitochondrial induced apoptosis of the cell as a specific mechanism of action leading to tumor cell apoptosis.

DNA damage, in particular dsDNA breaks, imposes a threat to the survival of cells if left unrepaired. At the early stages of the DNA damage response, Ataxia telangietasia mutated kinase (ATM) is autophosphorylated on Ser1981 as a response to the presence of dsDNA breaks. ATM activation, in turn, proceeds to phosphorylate p53, thereby blocking its interaction with E3 ubiquitin-protein ligase (MDM2), causing its stabilization. Stabilized p53 stimulates the expression of cyclin-dependent kinase inhibitor p21. Through its various interactions, p21 inhibits G1/S and G2/M transitions which lead to cell cycle arrest and repair or apoptosis. Thus, increased expression of p53 levels that are paralleled by increased p-ATM and p21 are due to this pathway activation and lead to cell-cycle arrest, repair, and cell death. Malignant tumor cells harbor a defective p53 and are resistant to this pathway, this favors their clonal outgrowth and contributes to the resistance of normal cell cycle responses to DNA damage, a cause of chemotherapy and radiation resistance (Kaluzová, Kaluz, Lerman, & Stanbridge, 2004; Lakin & Jackson, 1999; Vilenchik & Knudson, 2003).

In an attempt to investigate the specific cytotoxic mechanisms of DCQ, El-Khatib et al., 2010 treated cultured HCT116 (p53<sup>+/+</sup>), HCT116 (p53<sup>−/−</sup>), and HCT116 (p21<sup>−/−</sup>) cells to 0, 5, and 10μM DCQ in DMSO media for 6, 12, and 24 hours under normoxic and hypoxic conditions. p53 protein increased in response to DCQ in all three cell lines with the exception of the p53<sup>+/+</sup> cells exposed to hypoxia, the same pattern was also seen for p21 protein expression. Caspase-2 expression was increased in p53<sup>+/+</sup> cells and was further increased by 8-10 fold under hypoxia. In p53<sup>−/−</sup> showed no increase in caspase-2
and a decreased expression was seen in p21−/− cells. Although no direct interaction between p53 and caspase-2 has been observed, it is theorized that a functional connection between these two proteins is essential for the initiation of drug-induced apoptosis (Erler et al., 2004; Lakin & Jackson, 1999; Seth, Yang, Kaushal, Shah, & Kaushal, 2005). Overall, the apoptotic effects of DCQ in p53+/+ cells correlated with an increase in the pro-apoptotic caspase-2 protein, inhibition of pro-survival protein p53-induced protein with a death domain (PIDD-C), and increase in p-ATM expression.

The p21 gene is transcriptionally activated by p53 and is responsible for the p53-dependent checkpoint which induces cell cycle arrest in the event of DNA damage. Enforced p21 expression is known to result in a consistent, but partial, protection of cells from apoptosis (Bunz et al., 1998; Kaluzová et al., 2004; Lakin & Jackson, 1999; et al., 2005). A significant increase was observed in both p53+/+ and p53−/− cells when treated with DCQ in a hypoxic environment, this suggesting that p21 activation is independent of p53 in the case of DCQ. The decrease in the expression of pro-survival PIDD-C protein coupled with the increase in pro-apoptotic caspase-2 in p53+/+ cells appeared to have committed the cells to apoptosis. Interestingly, in p53−/− and p21−/− cells, apoptotic cell death occurred independent of caspase-2 activation and/or PIDD-C down-regulation (El-Khatib et al., 2010). This suggesting that the apoptotic mechanism involved in the tumor selective cell death of DCQ is independent of p53 and p21 proteins, further suggesting the involvement of other apoptotic mechanisms.

Administration of DCQ in both tumor cell cultures and healthy epithelial cells at concentrations of 5μM and 10μM show promising results to tumor specific induction of apoptosis via HIF-1α pathway and intracellular apoptosis (El-Khatib et al., 2010; H. U.
Gali-Muhtasib et al., 2005; Ghattass et al., 2014). While there is evidence of direct effectiveness of DCQ in cell cultures and a suggested safe concentration of 17mg/kg dosage, there is still an incomplete understanding of the effectiveness, safety, and direct mechanisms of action that account for the tumor specific targeting of DCQ. The overall goal of this proposal is to determine clinical effectiveness of DCQ in inducing apoptosis of colorectal tumor cells and systemic safety of the 17mg/kg dosage in an implant and genetic model of colorectal cancer. Our central hypothesis is that DCQ administered at a concentration of 17mg/kg will be effective at reducing tumor size and burden in both models of colorectal cancer and continue to work through the HIF-1α pathway and induce mitochondrial apoptosis without showing signs of systemic toxicity.
CHAPTER 3

NOVEL DRUG 2-BENZOYL-3-PHENYL 6,7-DICHLOROQUINOXALINE 1,4-DIOXIDE INDUCES COLON CANCER CELL APOPTOSIS THROUGH HIF-1[ALPHA] PATHWAY

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3.1 Abstract

Recent developments in the field of cancer genomics have shown transcription factor HIF-1α as a major player in the survival and proliferation of colorectal tumors. Presence of hypoxic areas in tumors facilitate the differentiation and nuclear translocation of HIF-1α which leads to transcription of hypoxic genes causing vascularization and metastasis of tumors. Hypoxia targeted drug engineering has led to significant advancements in cancer treatments as a method of directly utilizing the hypoxic regions against the tumor. Novel drug DCQ (2-benzoyl-3-phenyl 6,7-dichloroquinoxaline 1,4-dioxide) has shown promising anti-tumor results *in-vitro* and *in-vivo*. The purpose of this study was to utilize a tumor xenograft and genetic mouse model of colorectal cancer to investigate the safety, clinical effectiveness, and mechanism of action of DCQ. 

**Methods:** 10 week old Balb/c mice were injected subcutaneously with 2 million CT-26 cells and were monitored for tumor growth over 14 days before receiving treatment. *Apc*<sup>min/+</sup> mice were clinically evaluated from 8 weeks of age and began treatments at 16 weeks of age. DCQ treatment given at a 17mg/kg dose and 100μL DMSO injection as control. Injections were given bi-weekly over a four week period. At sacrifice, all tissues were extracted for histological and protein analysis. 

**Results:** DCQ caused significant decrease in tumor weight (p<0.05) and final tumor area (p<0.05) in Balb/c mice at time of sacrifice than control. *Apc*<sup>min/+</sup> mice showed significantly lower clinical score after 1 week of therapy along with decreased large tumor size (p<0.05) and number (p<0.05). Histological analysis showed increased total apoptotic area (p<0.05) in tumor tissue sections and tumor specific apoptosis in colon tissue in both models. Western blot analysis of Balb/c tumors and *Apc*<sup>min/+</sup> colon tumors showed a decreased nuclear expression of HIF-1α (p<0.05) and
increased expression of pro-apoptotic genes dephosphorylated-Bad (p<0.001), cleaved caspase-9 (p<0.05), and Bax (p<0.05) paralleled with a decrease in anti-apoptotic Bcl-2 gene (p<0.05). Conclusions: DCQ induces tumor apoptosis involving down regulation of HIF-1α and increased intracellular apoptosis in Balb/c mice and Apc$^{min/+}$ mice. Novel drug DCQ may potentially have use as a chemotherapeutic agent to reduce the pathology of sporadic intestinal and colorectal cancers.

KEYWORDS: Cancer, Colon, DCQ, Drug
3.2 Introduction

In 2012 the WHO reported cancer as the leading cause of death worldwide accounting for nearly 8.2 million deaths. In the United States, cancer related deaths were reported as second highest behind heart disease (Torre et al., 2015). Colorectal cancer is the second most prevalent cancer in both men and women in the United States in terms of incidence and death rates. The American Cancer Society estimates approximately 51,000 Americans will die of colon cancer and nearly 150,000 new cases will be diagnosed in 2014 (Siegel, DeSantis, & Jemal, 2014). The financial burden of colorectal cancer has had a large impact on the current health care system. In 2010, the direct cost of colorectal cancer was estimated to be $12.2 billion dollars, $10.7 being due to lost productivity and premature death (Siegel et al., 2014; Torre et al., 2015). Thus, a need does exist for the development of safer and more effective therapeutic treatments in order to prevent loss of life from ineffective treatments.

The initial incidence of hypoxia in the tumor environment arises because of oxygen diffusion limitations in avascular primary tumors (Lunt, Chaudary, & Hill, 2008; Mathonnet et al., 2014; Ryan, Lo, & Johnson, 1998; Zeng, Liu, Pan, Singh, & Wei, 2014). As a result of increased hypoxia in the tumor environment, the tumor microvasculature proliferates and serves to benefit tumor growth and metastasis. Normal responses to hypoxia typically see increased microvasculature proliferation in the tissue but with eventual compensation by the body through increased red blood cell count and increased oxygen carrying capacity of the blood (Lunt et al., 2008; Yasuda, 2008). In the tumor environment, the increased microvasculature proliferation in the tumor tissue is highly abnormal and often fails to correct for the oxygen deficit. This persistent hypoxia
leads to spatial disorganization of the tumor vascular networks and eventually leads to intercapillary distances that are beyond the diffusion range of oxygen (~200μm) (Brocato, Chervona, & Costa, 2014; Kunz & Ibrahim, 2003).

Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric transcription factor that plays a critical role in the cellular response to hypoxia. HIF-1 was discovered by the identification of a hypoxia response element (HRE; 5′-RCGTG-3′) in the 3′ enhancer of the gene for erythropoietin (EPO), a hormone that stimulates erythrocyte proliferation and undergoes hypoxia-induced transcription (Semenza, Nejfelt, Chi, & Antonarakis, 1991). HIF-1 regulates the transcription of a broad range of genes that facilitate responses to a hypoxic environment, including genes regulating angiogenesis, erythropoiesis, cell cycle, metabolism, and apoptosis. The HIF-1 complex consists of two subunits, HIF-1α and HIF-1β, both of which are basic helix-loop-helix proteins of the PAS family. HIF-1α is thought to accumulate under hypoxic conditions whereas HIF-1β is constitutively expressed in both hypoxic and normoxic conditions. When expressed under hypoxic conditions, HIF-1α plays a vital role as a mediator for solid tumor expansion including activation of angiogenic factor VEGF. HIF-1β is the Aryl hydrocarbon Receptor Nuclear Translocator (ARNT), which is essential for the xenobiotic response (Wolff et al., 2013). Under normoxic conditions, undifferentiated HIF-1 is targeted by an E3 ubiquitin ligase containing the von Hippel Lindau protein (VHL). The human genome contains EGL9 (Egg Laying Nine-9) homologues that are named EGLN1, EGLN2, and EGLN3 (also known as PHD2, PHD1, and PHD3 respectively). Prolyl Hydroxilase domain-containing proteins (PHDs) post-translationally modify HIF-1, allowing interactions with VHL to occur. All three proteins of PHDs can hydroxylate HIF-1α at one of two proline sites.
within the ODD (Pro-402 and Pro-564). VHL is part of a larger protein complex that includes Elongin-B, Elongin-C, Cul2, RBX1, and an ubiquitin-conjugating enzyme (E2). This complex, along with ubiquitin-activating enzyme (E1), regularly mediates ubiquitylation of HIF-1 and its subunits (Ke & Costa, 2006).

In hypoxic conditions, HIF-1α subunits are not recognized by pVHL, this condition leads to accumulation of HIF-1α which dimerize with HIF-1β and translocate into the nucleus. These proteins in the nucleus act as transcriptional regulators with cofactors such as CBP/p300 and DNA polymerase II complex to bind to hypoxia-responsive elements (HREs) and activate transcription of target genes. The functioning of HIF-1α target genes is well understood. HIF-1α-activated genes include Vascular Endothelial Growth Factor (VEGF), Glucose Transporter-1 (GLUT1), Lactate Dehydrogenase (LDHA), Erythropoietin (EPO), and Nitric Oxide Synthase (NOS) (He, Jiang, Zhang, & Wu, 2014; Zeng et al., 2014). Although not extensively investigated, the functional relationship between HIF-1α and the Wnt/β-catenin pathway has been established in recent investigations. HIF-1α can specifically interfere with co-activation of TCF/LEF transcription mediated by β-catenin and furthermore can bind and regulate NF-κB activity which is theorized to contribute to chronic inflammation seen in the colon tissue (Bienz & Clevers, 2000; Kaidi et al., 2007; Sebio et al., 2014). Functional cross-talk has also been reported between HIF-1α and the Apc gene at the transcriptional level. Newton et al., 2010 reported a consistent direct transcriptional repression of Apc by HIF-1α. It was seen that HIF-1α bound to a hypoxia-responsive element, dubbed HRE, in the APC promoter region in the presence of hypoxia. This relationship was also confirmed in an APC depletion model which showed an increased HIF-1α expression and activity. The
increase of HIF-1α was also determined to be mediated by NF-κB and was regulated by increased β-catenin levels which were increased due to the depletion of the Apc gene.

Quinoxaline di-N-oxides (QdNOs) were approved in the late 1960's for use as feed additives in livestock farming to promote animal growth and as an effort to replace banned therapeutic antibiotics, penicillin, and tetracyclines (Diab-Assef, Haddadin, Yared, Assaad, & Gali-Muhtasib, 2002; Gali-Muhtasib, Diab-Assaf, & Haddadin, 2005). Approval of QdNOs for use was due to the large body of evidence showing that quinoxaline antibiotics are inhibitors of DNA synthesis and had increased effectiveness in anaerobic conditions. The structural design of QdNOs is the key to the increased effectiveness of DNA synthesis inhibition in these types of antibiotics. In the presence of an electron rich environment, a free radical intermediate is created by the transfer of an electron from this electron rich environment to the electron-poor nitrogen centers of the 1,4-di-N-oxide moiety. In a hypoxic environment, this transfer is more likely to occur due to the highly reducing conditions of hypoxic cells, leading to hypoxic cells acting as a targeting element to cytotoxicity of these compounds (Diab-Assef, Haddadin, Yared, Assaad, & Gali-Muhtasib, 2002; H. U. Gali-Muhtasib, Diab-Assaf, & Haddadin, 2005).

Administration of quinoxaline di-N-oxides to tumor cultures has shown promising evidence of tumor specific apoptosis and decreased proliferation through interactions with the HIF-1α pathway. Preliminary research from the American University of Beirut has shown tumor specific cytotoxicity mechanisms of quinoxaline 1,4-dioxides (QdNOs) cultured under hypoxic conditions. The QdNOs were found to possess a 50- to 100- fold greater cytotoxicity to human T-84 colorectal cells cultured under hypoxia compared to an oxic environment. Diab-Assef et. al., 2002 reported that the hypoxia cytotoxicity ratio
(HCR), the ratio of equitoxic concentrations of the drug under aerobic/anoxic condition, was highly structure related and dependent on the nature of the substituents' on the QdNO heterocycle. The most cytotoxic, 2-benzoyl-3-phenyl-6,7-dichloro derivative of the quinoxaline 1,4-dioxide, or DCQ, was potent at a dose of 1µM with an HCR of 100 and significantly reduced the levels of HIF-1α transcript and protein. Others have further hypothesized that the C-6 and C-7-chlorine of DCQ might play a significant role in the selective hypoxic cytotoxicity of the drug (Diab-Assef et al., 2002; H Gali-Muhtasib et al., 2000). While there is evidence of direct effectiveness of DCQ in cell cultures and a suggested maximal tolerated dose of 17 mg/kg, incomplete understanding of the in-vivo effectiveness still exist, as does the overall safety, and direct mechanisms of action that account for the tumor specific targeting of DCQ. The goal of this study is to determine clinical effectiveness of DCQ in inducing apoptosis of colorectal tumor cells and systemic safety of the 17 mg/kg dosage in a xenograft and genetic model of colorectal cancer. We hypothesized that DCQ administered at a concentration of 17 mg/kg is effective at reducing tumor burden in both models of colorectal cancer and reduce HIF-1α expression along with up-regulated tumor specific intracellular induced apoptosis without showing signs of healthy tissue toxicity.

3.3 Methods

Animals

Sixteen female Balb/c mice and ten Apc<sup>min/+</sup> mice were bred and housed at the Arnold School of Public Health animal facility at the University of South Carolina. Mice were grouped housed, given access to food and water ad libitum, and kept on a 12-hour light-dark cycle. The study was approved by the Institutional Animal Care and Use
Committee at the University of South Carolina and was carried out in compliance with the Guide for Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD). The study used two separate animal models to investigate both an implant model of colorectal cancer and a genetic model of sporadic intestinal and colorectal cancer. DCQ treatment was designed to model an intense cycle of single agent chemotherapy at a progressed stage of colorectal cancer.

*Experimental Design: Balb/c Xenograft mice*

The study designed did incorporate two separate animal models to investigate both an implant model of colorectal cancer and a genetic model of sporadic intestinal and colorectal cancer. At 10 weeks of age, Balb/c mice were randomly assigned (n=8/group) to a DMSO control group or a DCQ treatment group. Mice in each group were injected sub-dermally (s.d.) with $2 \times 10^6$ CT-26 cells into the subcutaneous area of the right flank, in incomplete media, and allowed tumors to grow for 2 weeks before receiving 4 weeks of bi-weekly treatments (Figure 1A). Mice weight was determined daily, and the date of death was recorded. Changes in tumor size were monitored bi-weekly by measurement of tumor size (length and width) with a caliper device.

*Experimental Design: Apc<sup>min/+</sup> mice*

At 8 weeks of age, Apc<sup>min/+</sup> mice were assigned (n=5/group) to a DMSO control group or a DCQ treatment group and began receiving treatments at 16 weeks of age. Mice were given DCQ at a 17 mg/kg dosage dissolved in 100 μL DMSO as a treatment or 100 μL DMSO as control which were administered intraperitoneally (i.p.), twice per week for 4 weeks (Figure 1B). Toxicity studies by the American University of Beirut have shown that 17 mg/kg in 100 μL DMSO is a tolerated dosage based on mice behavior
and body weight. Clinical score was kept for Apc^{min/+} mice from 8 weeks of age through 20 weeks of age, and clinical scores were determined based on the predetermined guidelines of the Integrated Immunology of Inflammation and Cancer lab (Table 1).

_Tissue Collection_

Balb/c mice treated with DMSO or DCQ were sacrificed after 4 weeks of therapy. Apc^{min/+} mice treated with DMSO were sacrificed after 4 weeks of therapy as well. Apc^{min/+} mice treated with DCQ were sacrificed after 5 weeks of therapy due to continually improved health. At the time of sacrifice, mice were first injected with 1mg of Bromodeoxyuridine (BrdU) dissolved in sterile PBS, mice then underwent DEXA scan and were anesthetized 2 hours (hr) later with an isofluorane-oxygen gas mixture and sacrificed by cervical dislocation. The colon was removed and flushed with PBS containing 5000 IU/mL and 5000 IU/mL penicillin and streptomycin (CELLGRO), respectively. From Apc^{min/+} mice, two 2 mm^2 sections of the descending colon were dissected and stored at −80°C for Western blot analysis and at 37 °C, 5% CO2 with overnight incubation for tissue culture. A single tumor containing section of colon was fixed in 10% formalin (AZER SCIENTIFIC) for a day then embedded in paraffin wax for immunohistochemistry staining. Remaining sections of all tissues were snap-frozen in dry ice, and stored in -80 °C until further analysis (Saxena et al., 2014). Tissues analyzed for HIF-1α, especially tumor xenografts, were excised and frozen or stored in formalin, in under 1 minute (min) due to the rapid degradation of HIF-1α in a normoxic environment (Lin et al., 2006).
Tumor quantification.

Formalin-fixed intestinal sections from all animals were rinsed in deionized water, briefly stained in 0.1 % methylene blue, and counted by the same investigator who was blinded to the treatments. Tumors were counted under a dissecting microscope and were categorized according to size (>2 mm, 1–2 mm, and <1 mm).

Western Blot Analysis

Briefly, tumor xenografts, small intestine tumors, and colon tumors were homogenized using the Nuclear extraction kit (ab113474) from Abnova to separate cytoplasmic and nuclear proteins. Protein concentration was determined by the Bradford method (Bradford, 1976). Crude homogenates (10-50μg) were separated on 10-15 % polyacrylamide gels by SDS-PAGE gel electrophoresis. Gels were transferred to nitrocellulose membranes in cold transfer buffer over a 3 hour period. Equal protein loading of the gels was assessed by ponceau staining. Membranes were then blocked in 5 % milk-PBST for one hour at room temperature. Primary antibodies for Hypoxia Inducible Factor-1α (HIF-1α; Abnova) in the nucleus, and cytoplasmic cleaved caspase-9, dephosphorylated-Bad (d-Bad), Bax, and Bcl-2 were incubated at 1:500 to 1:1000 dilutions in 5 % milk-PBST overnight at 4°C. HIF-1α was incubated for 2.5 hours at room temperature. Secondary anti-rabbit IgG conjugated antibodies were incubated with membranes at a 1:2000 dilution in 5 % milk-PBST for 75 (min) at room temperature. All antibodies were purchased from cell signaling unless otherwise stated. Enhanced chemiluminescence was used to visualize the antibody-antigen interaction and developed by the SYNGENE G:BOX Chemi XX6. Digitally developed blots were analyzed by
measuring the integrated optical density (IOD) of each band using digital imaging software (GeneTools from Syngene, Cambridge, UK).

Colon Histopathology

To analyze colon histopathology, tumor containing sections of the descending colon were stained with hematoxylin and eosin stains. Tumor containing sections of the descending colon represent advanced stage tumors (Saxena et al., 2014). Briefly, tissues were sectioned at 5μm thickness and deparaffinized by standard procedure using xylene and gradation of ethanol. Sections were then washed for 5 min in dH2O, stained with filtered hematoxylin for 5min, then again washed 6x5 min in dH2O. Sections were washed in acid alcohol mix (160 mL 100 % EtOH, 1 mL acetic acid, and volumed to 200 mL with dH2O) for 6 dips then again washed in dH2O for 4 dips. Sections were then washed in Ammonia H2O (0.6 mL Ammonium OH volumed to 200 mL with dH2O) for 6 dips and then again washed in dH2O for 10 min. The sections were then stained with Eosin for 3min and dehydrated in the following alcohol progression; 1x2 min 70 % EtOH, 1x2 min 95 % EtOH, 2x3 min 100 % EtOH, and 2x5 min xylene. Slides were then air dried overnight and mounted for viewing with mounting media. Digital photographs were taken from each section at a 40x and 20x magnification with a Nikon E600 Widefield Epifluorescence and Darkfield Microscopy System at the University of South Carolina School of Medicine.

Apoptosis Detection by TUNEL Assay

Terminal deoxynucleaotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling (TUNEL) technique was used as per manufacturer instruction (R&D systems) to detect DNA strand breaks in situ. Briefly, tumor xenografts from Balb/c mice and colon
tumors from Apc<sup>min/+</sup> mice were sectioned at 5 μm thickness and deparaffinized by standard procedure using xylene and gradation of ethanol. Negative controls were performed by substituting PBS for TdT enzyme, which exhibited no immunostaining. Enumeration of apoptotic nuclei was made on slides of sections from each mouse using digital photographs taken at 4x and 20x magnification with a Nikon E600 Widefield Epifluorescence and Darkfield Microscopy System at the University of South Carolina School of Medicine. All nuclei counted showing a brown labeling. The incidence of apoptotic nuclei was given a percentage of total area of entire tumors in tumor xenografts (total apoptotic area) and counted within the tumor in the case of colon tumors from Apc<sup>min/+</sup> mice. Staining was performed in three separate experiments and counting was performed by the same investigator who was blinded to the treatments. Reliability of counting was obtained by calculating the coefficient of variance between each of the three data sets and was determined to be less than 10%.

**Cell Proliferation by BrdU Assay**

Immunohistochemistry was performed to detect proliferating cells marked with Bromodeoxyuridine by BrdU IN-SITU Detection Kit from BD Pharmingen (550803). Briefly, tumor xenografts from Balb/c mice and colon tumors from Apc<sup>min/+</sup> mice were sectioned at 5μm thickness and deparaffinized by standard procedure using xylene and gradation of ethanol. Negative controls were performed by substituting PBS for anti-BrdU primary antibody incubation, which exhibited no immunostaining. Enumeration of positively marked cells was made on slides of sections from each mouse using digital photographs taken at 4x and 20x magnification with a Nikon E600 Widefield Epifluorescence and Darkfield Microscopy System at the University of South Carolina School of Medicine.
School of Medicine. Positive cells were indicated by a brown labeling. The incidence of proliferating cells was given a percentage of total area of entire tumors in tumor xenografts (total proliferative area) and counted in the case of colon tumors. Staining was performed in three separate experiments and counting was performed by the same investigator who was blinded to the treatments. Reliability of counting was obtained by calculating the coefficient of variance between each of the three quantified data sets and was determined to be less than 7%.

Statistical Analysis

Two-way analysis of variance (ANOVA), Two-way repeated measure ANOVA and One-way ANOVA with Tukey post-hoc analysis was used to analyze within group data. Balb/c mice and Apc\(^{\text{min/}+}\) mice were not compared. Western blot and histology data statistical analysis was calculated by Student's \(t\)-test using SigmaStat version 3.5 (Systat Software Inc., Richmond, CA). Results are reported as means ± standard error of the mean. Significance was set with an alpha value of \(p < 0.05\).

3.4 Results

*DCQ reduces tumor xenograft weight and area after 4 weeks of treatment in Balb/c mice.*

We investigated the effectiveness of a 17 mg/kg DCQ dosage given semiweekly to Balb/c mice with CT-26 tumor xenografts. Figure 1A shows photographs of DMSO and DCQ treated mice at the beginning and after the 4 week treatment period. After 4 weeks of treatment, DCQ treated mice showed decreased tumor weight (\(p < 0.05\)) and area (\(p < 0.05\)) compared to DMSO treated mice (Figure 1 B-C). This data is consistent with the results obtained by where tumor volume was shown to be lower in DCQ treated Balb/c mice with MDA-MB-231 tumor xenografts (Ghattass, K, et. al., 2014).
**DCQ induces tumor apoptosis and decreases proliferation.**

Two important hallmarks of cancer that contribute to the malignancy of tumor development is the ability for the cancerous mass to resist apoptotic signaling and sustain proliferative signaling. An ideal chemotherapeutic drug would have the ability to induce both tumor cell apoptosis and block the tumor cells’ ability to proliferate. To evaluate the effect of DCQ on inducing tumor apoptosis, a standard TUNEL staining assay was performed on excised tumor xenograft sections (Figure 2a). Balb/c mice treated with DCQ showed a significantly greater percent apoptotic area within the tumor mass (p < 0.05) compared to DMSO treated mice (Figure 2b). To assess the ability of DCQ to block the sustained proliferation of tumor masses, a standard BrdU staining assay was performed on tumor xenograft sections (Figure 3). Balb/c mice treated with DCQ also showed a decreased percent proliferative area within the tumor mass (p < 0.05) compared to DMSO treated mice (Figure 3b).

**DCQ decreases nuclear expression of HIF-1α transcription protein.**

The structural design of DCQ is the key to the increased effectiveness of DNA synthesis inhibition. In the presence of an electron rich environment, a free radical intermediate is created by the transfer of an electron from this electron rich environment to the electron-poor nitrogen centers of the 1,4-di-N-oxide moiety. In a hypoxic environment, this transfer is more likely to occur due to the highly reducing conditions of hypoxic cells, leading to hypoxic cells acting as a targeting element to cytotoxicity of the compound, (Ke & Costa., 2006) this is especially true in models of colorectal cancer where tumors thrive in a low oxygen environment. To assess the extent of hypoxic signaling within the tumor xenografts, nuclear HIF-1α expression was evaluated via...
Western blot analysis (Figure 4a). DCQ treated Balb/c mice decreased both HIF-1α (p<0.05) and undifferentiated HIF-1 (p<0.05) protein levels within the nucleus of the tumor cells (Figure 4b,c).

**DCQ induces tumor cell apoptosis through intracellular death pathway.**

Gali-Muhtasib et al., 2005 showed DCQ was the most effective QdNO to induce tumor cell apoptosis in cultured T-84 human carcinoma cells and did so by increasing intracellular pro-apoptotic protein Bax while decreasing anti-apoptotic protein Bcl-2. To confirm activation of this pathway, we mapped the pro-apoptotic and anti-apoptotic markers of the intracellular death pathway in the excised tumor xenografts (Figure 5a) via Western blot analysis. In this circumstance, DCQ increased pro-apoptotic genes cleaved caspase-9 (p<0.05), dephosphorylated-Bad (p<0.001), and Bax (p<0.05) after 4 weeks of treatment (Figure 5b, c, d). DCQ decreased anti-apoptotic protein Bcl-2 (p<0.05), and this treatment effectively reducing the ratio of Bcl-2 to Bax (Figure 5e). Loading of equivalent amounts of protein for all samples was confirmed by ponceau stain. On the basis of quantification of the data by scanning densiometry, DCQ was found to increase intracellular death pathway signaling.

**4 weeks DCQ treatment at 17mg/kg may not induce liver, kidney, or brain apoptosis.**

In an effort to test the safety of the determined maximal tolerated dose of 17mg/kg, brain, liver, and kidney were excised from mice at the time of sacrifice and stained for apoptotic activity via standard TUNEL staining assay. Representative photos of DMSO and DCQ treated mice in Figure 6 show no positive indications of apoptotic activity in either mouse. These results indicate that DCQ treatment at 17mg/kg may not induce toxicity to these organs during the 4 week treatment period.
Improved clinical score after 4 weeks DCQ treatment in 16 week old Apc\textsuperscript{min/+} mice

After confirming the effectiveness and safety of the 17 mg/kg dosage in the tumor xenograft model, the effectiveness and safety of the same dosage in a genetic model of intestinal/colorectal cancer was determined. Apc\textsuperscript{min/+} mice began semiweekly clinical evaluation (Table 1) at 8 weeks of age and began semiweekly DCQ or DMSO treatments at 16 weeks of age which lasted 4 weeks. Figure 8a represents the clinical score tallied across the entire study, statistical significance was only calculated from the beginning of the treatment period, 16 weeks of age. Although the breeding group used for the DCQ treatment showed a higher clinical score prior to treatment and had a significantly higher score at the first treatment visit (p < 0.05); by week 17, mice receiving DCQ treatment showed a continuous downward progression in clinical score which tallied to be significantly less than the DMSO treated mice (p < 0.05) at all but one time point (week 16.5) throughout the treatment period. Decreased clinical score was attributed to a decreased scoring in diarrhea and fecal hemoccult (data not shown). At 20 weeks of age, DCQ treated mice were determined to still be “healthy” by the Arnold School of Public Health animal facility staff and were given another week of treatment before sacrifice. DCQ treated mice were all sacrificed at 21 weeks of age. Clinical score data for DMSO treated mice was only tallied to 19 weeks of age due to decreased health of the animals and many needing to be sacrificed before reaching the 20 week threshold. The data point at 19 weeks of age for the DMSO treated group represents the last point which all mice within the DMSO treatment group clinical score was tallied. One-way ANOVA of Apc\textsuperscript{min/+} mice within the DCQ treatment group revealed a progressive increase in clinical score relative to time from 8 weeks to 16 weeks of age (p < 0.001), prior to DCQ administration. From 16 weeks of age to sacrifice, during the DCQ treatment period,
mice showed significant decrease (p < 0.001) in clinical score relative to time which indicates a change in clinical score was induced by DCQ treatment.

**DCQ decreases intestinal tumor count and size**

To investigate the overall effectiveness of DCQ, we excised the entire small intestine and colon and counted tumors after staining with 0.1% methylene blue. No morphological differences in the colon were observed between the DMSO and DCQ treated mice (Figure 8a). However, DCQ treated mice did show a decreased total tumor number (p < 0.001) and large tumor size (p < 0.001) compared to DMSO treated mice (Figure 8b,c). No differences were seen in small and medium tumor size between the two groups.

**DCQ induces colon tumor specific apoptosis while preserving normal colon epithelium**

We showed in the Balb/c mouse xenograft model that DCQ given at a 17 mg/kg dosage effectively increased tumor tissue apoptosis and decreased proliferation while not indicating toxicity to liver, kidney and brain tissue. (Figure 2, 3, and 6). To further elucidate the effectiveness and safety of DCQ, apoptosis and proliferation data was obtained in identified tumors of Apc\textsuperscript{min/+} mice within the colon (Figure 9). Tissue sections were specifically cut to obtain a large amount of colon tissue in an effort to obtain the surrounding normal colon epithelium for analysis which would be also used to observe colon histopathology and any apoptotic activity occurring within the normal colon epithelium (Figure 10). Within the tumor, DCQ treated mice had significantly greater apoptotic activity (p < 0.001) and significantly lower proliferative activity (p < 0.05), these findings paralleling the finding in the Balb/c xenograft model. Observing the obtained TUNEL stain of the surrounding normal colon tissue, no increased apoptotic
activity was observed, indicating DCQ only induced apoptosis to the tumor tissue (Figure 10). The WT TUNEL stain was performed as a negative control.

*DCQ targets HIF-1α pathway and induces intracellular death in colon tumors of Apc\textsuperscript{min/+} mice*

Functional cross-talk has been reported between HIF-1α and the *Apc* gene at the transcriptional level. Newton et. al., 2010 reported a consistent direct transcriptional repression of *Apc* by HIF-1α in multiple cell lines. HIF-1α when bound to a hypoxia-responsive element, dubbed HRE, in the APC promoter region in the presence of hypoxia. Therefore, the Apc\textsuperscript{min/+} model of colorectal cancer provided an interesting target for testing the effectiveness of DCQ on reducing the hypoxic signaling within the tumor tissue. Colon tumors excised from the DCQ treated Apc\textsuperscript{min/+} mice showed nearly non-detectable nuclear HIF-1α protein expression (\(p < 0.05\)) when compared to DMSO treated mice (Figure 11a, b).

Along with these findings, we also saw an increased pro-apoptotic protein Bax (\(p < 0.05\)) and decreased anti-apoptotic protein Bcl-2 (\(p < 0.05\)) in DCQ treated mice compared to DMSO treated controls (Figure 12 a, b, c). Thus, effectively decreasing the Bcl-2 to Bax ratio within the tumor. These results were parallel to the data obtained from the Balb/c xenograft tumors.

*DCQ treated mice Apc\textsuperscript{min/+} mice experience less liver apoptosis and no indications of kidney or brain apoptosis*

As was done with the Balb/c treated mice, we investigated the safety of the 17 mg/kg DCQ dosage by TUNEL staining excised liver, kidney, and brain tissue from the treated Apc\textsuperscript{min/+} mice. Similar to the Balb/c mice, no apoptosis was detected in the kidney or brain tissue of DMSO or DCQ treated mice (Figure 14 a). However, DCQ treated mice
showed a significantly lower (p < 0.01) amount of apoptotic cells compared to DMSO treated control mice (Figure 14 a,b). These results show less cell death was occurring in the livers of DCQ treated mice.

3.5 Discussion

Despite advancements in cancer prevention and treatment methods the burden of colorectal cancer costs the United States over 51,000 lives and $12.2 billion dollars in 2012 alone, $10.7 billion being due to lost productivity and premature death. Two of the most significant and most investigated hallmarks of cancer are the tumor’s ability to sustain an abnormally high level of proliferative signaling and to resist apoptotic signaling. A major contributor to these hallmarks is the chronic hypoxia present in the tumor environment which activates the HIF-1α pathway. This hypoxic response is especially true in the already hypoxic environment of the mammalian colon. Identification of this trait has led to drug engineering that is aimed at targeting the hypoxic environment as a method of directly utilizing the hypoxic regions of against the tumor. The current study was aimed at investigating the safety and effectiveness of the novel hypoxia targeting drug 2-benzoyl-3-phenyl 6,7-dichloroquinoxaline 1,4-dioxide (DCQ). We report the novel finding that the maximal tolerated dose of 17 mg/kg is both effective in inducing colorectal tumor cell apoptosis and blocking tumor cell proliferation in two models of colorectal cancer and is safe at current dosage by showing a lack of normal tissue death. This study also indicated DCQ is capable of reducing critical hypoxia signaling protein HIF-1α and leads to up-regulation of pro-apoptotic and reduction of anti-apoptotic genes involved in intracellular apoptosis signaling within the tumor.
Previous investigations completed at the American University of Beirut have shown that DCQ is a hypoxia activated compound that is effective at inducing tumor specific apoptosis and also has increased effectiveness when supplemented with acute doses of radiation \textit{in-vitro} (Diab-Assef et al., 2002; El-Khatib et al., 2010; Gali-Muhtasib, Diab-Assaf, et al., 2004; Haykal et al., 2008, 2009; Itani et al., 2007). The most recent investigation completed at the American University of Beirut identified a maximal tolerated dosage in mice of 17 mg/kg in healthy mice (Ghattass et al., 2014). The next step in further progressing the understanding of DCQ was to design an investigation that would translate the compound’s ability to effectively reduce tumor burden in animals at a progressed stage of colorectal cancer. This study, therefore, was designed to evaluate two animal models of colorectal cancer which investigated the clinical safety and effectiveness in both a tumor xenograft model and a genetic model of colorectal cancer.

Balb/c implanted with CT-26 tumor xenografts given 4 weeks of a semiweekly 17mg/kg DCQ treatment showed reduced tumor size and weight. Tumor tissue was stained for presence of apoptosis and proliferation, and the data indicate that tumors of DCQ treated mice had significantly greater apoptosis and lower proliferation compared to control mice. Similar results are reported for the 20 week old \textit{Apc}^{\text{min/+}} mice treated with the same 4 week semiweekly treatment plan as Balb/c mice. The two animal models, Balb/c implant mice and \textit{Apc}^{\text{min/+}} mice were not compared. DCQ treated \textit{Apc}^{\text{min/+}} mice showed significantly lower clinical score after just one week of treatment and continued a downward trend in clinical score until time of sacrifice. The average life of an \textit{Apc}^{\text{min/+}} mouse is approximately 120 days or 18 weeks of age. DMSO treated control mice to survive just past 19 weeks of age but the life of all DCQ treated mice was extended to 21
weeks of age. The major factor of the reduction in clinical score was through the reduced diarrhea and fecal hemoccult scores, both significant clinical indications of advanced intestinal and colorectal cancer. Collectively, these results demonstrate that the 17 mg/kg dosage is effective in reducing tumor burden in both an implanted tumor model and a genetic model of intestinal and colorectal cancer. We would like to note that although tumor counting from the intestines of the Apc<sub>min/+</sub> mice showed a decreased total tumor count and large tumor size in DCQ treated mice, total number of tumors reported was noticeably lower than most previously reported studies involving Apc<sup>min/+</sup> mouse models (Balmain, Gray, & Ponder, 2003; Johnson & Fleet, 2013; Moser et al., 1990).

The data from this study provide evidence that DCQ treatment in Apc<sub>min/+</sub> mice induced tumor apoptosis and did not induce normal colon tissue death. Clinically, this finding is exciting because many drugs failing to accomplish this feat. This supports the previous findings by the group at the American University of Beirut that DCQ works in a tumor specific manner to induce only tumor cell apoptosis while preserving normal colon tissue. Protein analysis in tumors from both animal models indicate the down-regulation of nuclear transcription protein HIF-1α with DCQ treatment. The key moiety of the DCQ compound is at the 1,4-dioxide structure. In a hypoxic environment, electron transfer is more likely to occur to the 1,4-dioxide moiety due to the highly reducing conditions of the hypoxic cells, this allows a compound such as DCQ to utilize the hypoxic areas of tumors as a targeting element to the cytotoxicity of these compounds. A study by (Newton et. al, 2010) provided evidence of functional cross-talk between HIF-1α and the Apc gene at the transcriptional level. This relationship was confirmed in an Apc depletion model which showed an increased HIF-1α expression and activity in tumor cells. In the
present study, the nearly non-detectable HIF-1α protein expression in DCQ treated Apc<sup>min/+</sup> mice are an exciting find, this data indicating that DCQ has the ability to reduce critical nuclear transcription factor HIF-1α in the tumor environment <i>in-vivo</i>. However, we cannot suggest any specific mechanistic results without further mechanistic studies that would confirm the effectiveness of DCQ in reversing the increased hypoxia in tumors with Apc gene deletion.

Previously established is that the HIF-1α pathway, when highly expressed in a tumor environment, increases transcription of trophic factors such as VEGF, which can lead to the inhibition of intracellular apoptosis through phosphorylation of the PI-3kinase. Once activated, the PI-3kinase will ultimately lead to the phosphorylation of pro-apoptotic protein Bad which increases the Bcl-2:Bax ratio and prevents cell apoptosis. An investigation by Gali-Muhtasib, Diab-Assaf, et al., 2004 at the American University of Beirut reported DCQ increased pro-apoptotic protein Bax and decreased anti-apoptotic protein Bcl-2 in multiple human cell lines. We have evidence that <i>in-vivo</i> this response is activated in the tumor cells of both a xenograft and genetic model of intestinal/ colorectal cancer after 4 weeks of semiweekly DCQ treatment at 17 mg/kg. This study serves as the translational step to the animal use of the drug and indicates DCQ induces tumor cell apoptosis by increasing intracellular expression of pro-apoptotic proteins and decreasing anti-apoptotic proteins and warrants further animal and mechanistic investigations to observe the complete anti-tumor mechanisms of DCQ.

Another interesting find was the decreased incidence of liver apoptosis in Apc<sup>min/+</sup> mice treated with DCQ. Liver toxicity and hypertrophy is a trademark trait of colorectal cancer progression and cancer cachexia (Narsale et al., 2015). The decreased liver
apoptosis suggests a decreased tumor burden as a result of DCQ treatment, however, the data does not indicate a direct relationship between DCQ and decreased liver apoptosis and warrants further investigations to observe if DCQ has a direct effect on preserving liver function.

Conclusion

The involvement of hypoxia in the progression of colorectal cancer has led to the development of hypoxia targeting chemotherapeutics, however, none of which have shown to be safe and effective in eradicating colorectal tumors. In the present study, novel compound DCQ is shown as safe and effective in reducing tumor burden in xenograft and genetic models of colorectal cancer. DCQ, given semiweekly at 17 mg/kg for 4 weeks was effective at inducing tumor specific apoptosis and reducing tumor tissue proliferation while preserving normal colon tissue and without inducing apoptosis to vital organs. We also showed the down-regulation of critical hypoxia gene HIF-1α and the up-regulation of pro-apoptotic gene paralleled by decreased anti-apoptotic genes involved in regulation of the intracellular apoptosis pathway within the tumors of both Balb/c and Apc\textsuperscript{min/+} mice treated with DCQ. Additional experiments are needed to be performed to establish the complete understanding of the anti-tumor mechanisms of DCQ, and should be pursued as a future line of review for understanding the use of DCQ as a legitimate chemotherapeutic compound.
Table 3.1 *Apc*\(^{\text{min/+}}\) mouse clinical score guidelines. Clinical score was monitored on all ten *Apc*\(^{\text{min/+}}\) mice from 8 weeks of age until sacrifice. Body weight loss was determined as percent (%) loss from 8 weeks of age. Fecal Hemoccult and diarrhea was evaluated during every clinical visit.

<table>
<thead>
<tr>
<th>Body Weight Loss</th>
<th>Fecal Hemoccult</th>
<th>Diarrhea</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% = 0</td>
<td>No blood = 0</td>
<td>Hard Stool = 0</td>
</tr>
<tr>
<td>6-10% = 1</td>
<td>Some blood = 2</td>
<td>Soft Stool = 2</td>
</tr>
<tr>
<td>11-15% = 2</td>
<td>Severe bleeding = 4</td>
<td>Runny and soft = 4</td>
</tr>
<tr>
<td>16-20% = 3</td>
<td></td>
<td></td>
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<tr>
<td>&gt;20% = 4</td>
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3.6 Figure Legend

**Figure 3.1 Experimental Design.** (A) At 10 weeks of age, sixteen Balb/c mice were randomly assigned to a DMSO (control) or DCQ treatment group and implanted with 2 x 10^6 CT-26 cells. Tumors were allowed to grow for 2 weeks before receiving bi-weekly injections of either DMSO or DCQ over a 4 week period. (B) At 8 weeks of age, ten Apc\textsuperscript{min/+} mice were randomly assigned to a DMSO (control) or DCQ treatment group and began clinical evaluation. At 16 weeks of age, mice began receiving a bi-weekly injection of either DMSO or DCQ over a 4 week period. Clinical evaluations were taken bi-weekly through the course of the experiment. # = CT-26 tumor implantation, ** = Begin Apc\textsuperscript{min/+} clinical evaluation, * = DMSO or 17mg/kg DCQ injection.

**Figure 3.2 DCQ therapy decreases tumor xenograft weight and area.** Balb/c with flank tumor and final tumor weight and area. (A) Images of Balb/c mice with flank tumor at beginning of therapy (Day 1) and after 4 weeks (Day 28) of DMSO or DCQ injection. (B) Subcutaneous tumors removed and weighed at time of sacrifice in grams (g). (C) Final tumor area measured prior to sacrifice in millimeters squared (mm^2). Values are means ± standard error. *p<0.05.

**Figure 3.3 DCQ increases apoptosis in tumor xenografts of Balb/c mice.** Tumor Apoptosis: (A) TUNEL assay of tumor tissues imaged at 4x and 20x of DMSO and DCQ injected mice. (B) Percent total apoptotic area of tumor tissue. Arrows indicate examples of positively labeled cells. Values are means ± standard error. *p<0.05.

**Figure 3.4 DCQ decreases proliferation in tumor xenografts of Balb/c mice.** Tumor Proliferation: (A) BrdU assay of tumor tissues imaged at 4x and 20x of DMSO and DCQ injected mice. (B) Percent total proliferative area of BrdU positive cells. Arrows indicate examples of positively labeled cells. Values are means ± standard error. *p<0.05.

**Figure 3.5 DCQ decreases nuclear HIF-1α protein expression in tumor xenografts of Balb/c mice.** (A) Nuclear expression of HIF-1α protein is decreased in mice given DCQ treatment compared to DMSO control. Quantification of HIF-1α (B) and HIF-1 (C) levels is shown as integrated optical density as determined by densiometry analysis. Values are means ± standard error. *p<0.05.

**Figure 3.6 DCQ increases pro-apoptotic proteins Cleaved Caspase-9, Dephosphorylated-Bad, and Bax and decreases anti-apoptotic protein Bcl-2 in tumor xenografts of Balb/c Mice.** (A) Cytoplasmic expression of Cleaved Caspase-9, Dephosphorylated-Bad (Dephos-Bad), and Bax pro-apoptotic proteins and anti-apoptotic protein Bcl-2 in DMSO and DCQ treated Balb/c mice. (B-E) Quantification of Cleaved-Caspase-9, Dephos-Bad, Bax, and Bcl-2 is shown as integrated optical density as determined by densiometry analysis, respectively. Values are means ± standard error. * p < 0.05, # p < 0.001.
Figure 3.7 DCQ treatment may not induce liver, kidney, or brain apoptosis in Balb/c mice. Photographs of liver, kidney, and brain of Balb/c mice with CT-26 xenografts given DMSO or DCQ. Absence of brown labeled cells indicates no increased apoptosis in DCQ treated mice.

Figure 3.8 DCQ improves Apc^{min/+} mouse clinical score after 4 weeks of treatment. (A) Clinical scores for DMSO and DCQ treated Apc^{min/+} mice during the 13 weeks of study. Weight loss, diarrhea and blood in stools were used as parameters to calculate the clinical score (Table 1). Statistical significance was only tested from 16 weeks of age to sacrifice between both treatment groups. (B) Photographs of mouse showing regressed rectal prolapse after 1 week of DCQ treatment. Values are means ± standard error. * p < 0.05.

Figure 3.9 DCQ reduces Apc^{min/+} mouse intestinal tumor count and size. (A) Representative photographs of methylene blue-stained intestinal tissues for DMSO and DCQ treated Apc^{min/+} mice. Difference in total poly number (B) and polyp size (C) were measured in Apc^{min/+} treated with DMSO and DCQ. Values are means ± standard error. * p <0.05.

Figure 3.10 DCQ induces colon tumor apoptosis and decreases tumor proliferation of Apc^{min/+} mice. TUNEL and BrdU assays were performed to observe and measure apoptotic activity and proliferative cells in identified colonic tumors of Apc^{min/+} mice given DMSO and DCQ treatements. Average positively labeled apoptotic cells (A) and proliferating (B) cells in identified colonic tumors of DMSO and DCQ treated mice. (C) Representative photographs of TUNEL and BrdU assayed colonic tumors. Arrows indicate examples of positively labeled cells. Values are means ± standard error. * p < 0.05, # p < 0.001.

Figure 3.11 DCQ does not induce normal colon epithelium apoptosis in Apc^{min/+} mice. Identified normal colon tissue of WT, DMSO and DCQ treated mice was stained with hematoxylin and eosin and also observed for apoptosis via TUNEL assay. TUNEL assay WT representative was treated as a negative control.

Figure 3.12 DCQ decreases nuclear expression of HIF-1α in colon tumors of Apc^{min/+} mice. (A) Nuclear expression of HIF-1α protein is decreased in mice given DCQ treatment compared to DMSO control. (B) Quantification of HIF-1α levels is shown as integrated optical density as determined by densiometry analysis. Values are means ± standard error. * p < 0.05.

Figure 3.13 DCQ increases pro-apoptotic proteins Cleaved Caspase-9 and Bax and decreases anti-apoptotic protein Bcl-2 in colon tumors Apc^{min/+} mice. (A) Cytoplasmic expression of Bax pro-apoptotic protein and anti-apoptotic protein Bcl-2 in DMSO and DCQ treated Balb/c mice. Quantification of Bax (B) and Bcl-2 (C) is shown
as integrated optical density as determined by densiometry analysis, respectively. Values are means ± standard error. * p < 0.05.

**Figure 3.14 DCQ treatment decreases liver apoptosis and does not induce kidney or brain tissue apoptosis in Apc\textsuperscript{min/+} mice.** (A) Photographs of liver, kidney, and brain of Apc\textsuperscript{min/+} mice treated with DMSO or DCQ. A decreased amount of apoptotic cells in the liver was noticed indicating less apoptosis and liver toxicity in DCQ treated mice. Arrows indicate examples of positively labeled cells. (B) Average positively labeled apoptotic cells of liver tissue measured. Values are means ± standard error. * p < 0.001.
Figure 3.1 Experimental Design.

Figure 3.2 DCQ therapy decreases tumor xenograft weight and area.
Figure 3.3 DCQ increases apoptosis in tumor xenografts of Balb/c mice.
Figure 3.4 DCQ decreases proliferation in tumor xenografts of Balb/c mice.
Figure 3.5 DCQ decreases nuclear HIF-1α protein expression in tumor xenografts of Balb/c mice.
Figure 3.6 DCQ increases pro-apoptotic proteins Cleaved Caspase-9, Dephosphorylated-Bad, and Bax and decreases anti-apoptotic protein Bcl-2 in tumor xenografts of Balb/c Mice.
Figure 3.7 DCQ treatment may not induce liver, kidney, or brain apoptosis in Balb/c mice.
Figure 3.8 DCQ improves Apc<sup>min/+</sup> mouse clinical score after 4 weeks of treatment.
Figure 3.9 DCQ reduces Apc^{min/+} mouse intestinal tumor count and size.
Figure 3.10 DCQ induces colon tumor apoptosis and decreases tumor proliferation of Apc\textsuperscript{min/+} mice.

Figure 3.11 DCQ induces tumor specific apoptosis and decreases tumor proliferation in colon tumors of Apc\textsuperscript{min/+} mice.
Figure 3.12 DCQ decreases nuclear expression of HIF-1α in colon tumors of Apc\textsuperscript{min/+} mice.

Figure 3.13 DCQ increases pro-apoptotic protein Bax and decreases anti-apoptotic protein Bcl-2 in colon tumors of Apc\textsuperscript{min/+} mice.
Figure 3.14 DCQ treatment decreases liver apoptosis and does not induce kidney or brain tissue apoptosis in Apc\textsuperscript{min/+} mice.
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APPENDIX A

DETAILED AIMS AND METHODOLOGY
Specific Aim #1 will evaluate the safety and effectiveness of the 17mg/kg DCQ dosage in a xenograft and genetic model of colorectal cancer.

**Rationale:** It has been previously shown that DCQ is effective in causing tumor-specific apoptosis and can repress tumor growth in multiple human cancer cell lines through activation mechanisms involving down-regulation of HIF-1α pathway (Diab-Assef et al., 2002; El-Khatib et al., 2010; H. U. Gali-Muhtasib et al., 2005; Ghattass et al., 2014; Haykal et al., 2008, 2009). Toxicity studies from the American University of Beirut have suggested a concentration of 17mg/kg dose will be effective in causing tumor cell apoptosis while staying below systemic toxic levels, therefore preserving essential healthy tissue in both the GI system and the entire body. The 17mg/kg dosage has previously been shown to be effective in a breast cancer xenograft study however DCQ has yet to be investigated *in-vivo* for clinical effectiveness in a colorectal cancer model (Ghattass et al., 2014). Thus, we would like to investigate the safety and clinical effectiveness of the 17mg/kg dose in a tumor implant and genetic model of colorectal cancer.

**Experimental design for specific aim #1.** Experiment 1 will determine the safety and clinical effectiveness of DCQ in 10 week old Balb/c mice implanted with CT-26 murine colorectal tumor cells and in 16 week old *Apc*<sup>min/+</sup> mice.

At approximately 10 weeks of age, Balb/c mice will be randomized to a control group or treatment group. 2 million CT-26 cells, kindly provided by Dr. Marj Pena, were injected subcutaneously into the right flank of each mouse and allowed to grow for 2 weeks. After 2 weeks DCQ will be dissolved in DMSO to create a 17mg/kg
concentration in a 100μL dose and injected twice a week for four weeks. Control mice will receive a 100μL injection of sterile DMSO twice a week for four weeks. At 10 weeks of age, Balb/c mice will be approximately 20g and will be able to withstand the tumor burden and chemotherapy treatments. Apc\textsuperscript{min/+} mice were aged to 8 weeks of age and randomized into control and treatment groups. Twice a week, clinical evaluations will be conducted to measure body weight, food and water consumption, fecal hemoccult and diarrhea, and a description of any abnormal behavior or side effects that might be arising as part of the therapy. At 16 weeks of age, mice in their respective groups will receive a bi weekly 100μL injection of either DMSO or 17mg/kg of DCQ. Min mice have initiated body weight loss and intestinal polyp development and show a near maximum clinical score by 15-16 weeks of age, making it an ideal time point to assess the effectiveness of DCQ on the treatment of colorectal cancer. All mice will be housed in standard cages for the duration of the study. Body weight and tumor dimensions in Balb/c mice and clinical score in Apc\textsuperscript{min/+} mice will be measured at the time of each treatment period and at the time of sacrifice. At sacrifice, brain, liver, kidney, spleen, and plasma will be collected from all mice, tumor xenografts will also be taken from Balb/c mice, and colon and small intestine will also be collected from Apc\textsuperscript{min/+} mice. Sections of tumor xenografts, brain, liver, kidney, colon, and small intestine from respective mice will be fixed in 10% formalin for 24 hours, preserved in 70% ethanol, and then embedded in paraffin wax to be sectioned for immunohistochemistry. All tissues extracted will also be weighted and frozen for protein analysis.
Experiment #1 will determine the safety and effectiveness of DCQ in a tumor xenograft and genetic animal model of colorectal cancer.

**Animals:** Balb/c male mice will be bred with female Balb/c mine in the USC Discovery building Arnold School of Public Health animal resource facility. All mice will be provided with standard rodent chow (Harlan Teklad Rodent Diet, #8604, Madison, WI) and water *ad libitum*. Body weights will be measured throughout the course of the study. At 10 weeks of age, mice will be injected by a member of Dr. Marj Pena's lab with 2 million cultured CT-26 murine colorectal cancer cells each and assigned into one of two groups; control or DCQ treatment. *Apc*<sup>min/+</sup> male mice on a C57BL/6 background will be bred with female C57BL/6 mice in the Discovery building Arnold School of Public Health animal resource facility. Animals will be genotyped as heterozygous for the *Apc* gene. All mice will be provided with standard rodent chow (Harlan Teklad Rodent Diet, #8604, Madison, WI) and water *ad libitum*. All mice will undergo clinical evaluation and receive clinical scores based on the protocol and guidelines of the Fayad lab. At 8 weeks of age, mice will be assigned to one of two groups; control or DCQ treatment. DCQ was synthesized from 5,6-dichlorobenzofurazan oxide and dibenzoylmethane *via* the Beirut reaction by Dr. Makhluf J. Haddadin and provided by Dr. Hala Gali-Muhtasib (American University of Beirut, Beirut, Lebanon). After two weeks of tumor growth, Balb/c mice will begin a bi-weekly treatment period. DCQ treatment will consist of a bi-weekly 100μL intraperitoneal injection of DCQ dissolved in DMSO at a concentration of 17mg/kg body weight. Balb/c control mice will receive a bi-weekly vehicle injection of 100μL of DMSO. Balb/c mice will be sacrificed at 16 weeks of age after 4 weeks of
treatment. At 16 weeks of age, $Apc^{min/+}$ mice will receive either a bi-weekly intraperitoneal 100μL injection of DMSO or DCQ at a concentration of 17mg/kg body weight. All mice will be sacrificed at 20 weeks of age after a 4 week treatment period. Based on previous drug development preliminary studies and on animal data from our lab, 5-6 mice per group are sufficient to detect a difference in clinical and experimental data.

Table A.1 Animal treatment groups for experiment #1.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>Age (weeks)</th>
<th>N</th>
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<tbody>
<tr>
<td>Balb/c</td>
<td>DMSO</td>
<td>10-16</td>
<td>5-6</td>
</tr>
<tr>
<td>Balb/c</td>
<td>DCQ</td>
<td>10-16</td>
<td>5-6</td>
</tr>
<tr>
<td>$Apc^{min/+}$</td>
<td>DMSO</td>
<td>8-20</td>
<td>5-6</td>
</tr>
<tr>
<td>$Apc^{min/+}$</td>
<td>DCQ</td>
<td>8-20</td>
<td>5-6</td>
</tr>
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Table A.2 Animal clinical score guidelines

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<th>Diarrhea</th>
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<td>&gt;20% = 4</td>
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</table>
**Primary Outcomes**

Tumor size and weight:  
Tumor size will be evaluated at each treatment period in Balb/c mice. At sacrifice, tumor will be removed from the mice and weighed.

Clinical Score:  
Clinical score in \( Apc^{min/+} \) mice will be evaluated from 8 weeks of age through time of sacrifice.

Systemic toxicity: Brain, liver, and kidney will be analyzed for presence of apoptosis via TUNEL assay. Serum levels of IL-6 will be assessed via ELISA also to determine relative levels of systemic inflammation.

Secondary outcomes:

Body composition: DEXA scan will be performed on each mouse prior to sacrifice and be used to determine between group differences in body composition after 4 weeks of therapy.

**Specific Methodology Aim #1:**

**Animals:** Balb/c male mice will be bred with female Balb/c mice in the Discovery building Arnold School of Public Health animal resource facility. \( Apc^{min/+} \) male mice on a C57BL/6 background will be bred with female C57BL/6 mice in the Discovery building Arnold School of Public Health animal resource facility. Resulting heterozygous mice from the \( Apc^{min/+} \) cross will develop intestinal and colonic tumors as early as 8 weeks of
age and typically have a life span of 20-22 weeks of age. All animals will be housed in standard cages and the room will be maintained on a 12:12 light:dark cycle with the light period starting at 0700. Mice will be provided with standard rodent chow (Harlan Teklad Rodent Diet #8604) and water *ad libitum*. Body weight and tumor size will be monitored during the course of the study, final tumor weight will be measured at time of sacrifice.

All animals at time of sacrifice will be injected with 1mg of BRDU dissolved in phosphate buffered saline (PBS) and undergo DEXA scan prior to blood draw and cervical dislocation. All animal experimentation is approved by the University of South Carolina's Institutional Animal Care and Use Committee. Tumor growth will be allowed to occur for 2 weeks until treatment periods will begin. At this point, DCQ will be prepared in DMSO so as to obtain a concentration of 17mg/kg in a 100μl injection normalized to the average weight of the mice.

**Genotyping:** All animals from the Apc\textsuperscript{min/+} cross will be genotyped using a tail snip. At 4-5 weeks of age; animals will be weaned, numbered, and a small tail snip (~1-2mm) collected. The tail snip is digested in 200μL of tail digest buffer and 5μL of proteinase K. Tails are incubated overnight in a water bath set at 55°C. After incubation, samples are heat shocked at 95°C in a dri-bath for 10 minutes. Heterozygosity of the *Apc* gene will be determined *via* a PCR reaction (*Apc* forward 5' TGAGAAAGACAGAAGTTA 3', reverse 5' TTCCACTTTGGCATAAGGC 3'). PCR products are run out on a 5% polyacrylamide gel and exposed to UV light. Presence of a band indicates heterozygosity of the *Apc* gene.

**Tissue Collection:** Mice will be anesthetized *via* exposure to an isofluorane and oxygen mixture. Blood will be collected from the retro-orbital sinus using a capillary tube. Blood
will be spun at 4°C, 10,000 rpm, for 10 minutes. Plasma will then be pipetted off and stored at -80°C until analysis. Brain (br), liver (l), kidney (k), spleen (sp), and tumor xenografts (tmr) from Balb/c mice will be excised, rinsed in PBS, frozen in a dry ice box, and stored at -80°C until further analysis. Parts of each tissue will also be fixed in 10% formalin for 24 hours, preserved in 70% ethanol, and then embedded in paraffin wax for histological sectioning. Small intestine, and colon from the \textit{Apc^{min/+}} mice will be chosen for sections containing tumors will be fixed in 10% formalin for 24 hours, preserved in 70% ethanol, and then embedded in paraffin wax for histological sectioning, brain (br), liver (l), kidney (k), spleen (sp) will also be collected. Parts of each tissue will also be excised, rinsed in PBS, frozen in a dry ice box, and stored at -80°C until further analysis. Remaining sections of the small intestine will be rinsed with PBS and opened longitudinally. Sections will be fixed in 10% formalin for 24 hours and then preserved in 70% ethanol and used for intestinal tumor counts in the \textit{Apc^{min/+}} mice.

**Polyp count:** Intestinal polyps will be counted by staining in methylene blue. Formalin fixed intestinal sections from all \textit{Apc^{min/+}} mice were rinsed in deionized water, briefly stained in 0.1% methylene blue, and counted by the same investigator who was blinded to the treatments. Polyps were counted under a dissecting microscope, using forceps to pick through the intestinal villi and identify polyps. Polyps were categorized as \( \geq 1 \text{ mm} \) or \(< 1 \text{ mm} \) in the small intestine and \( > 2 \text{ mm} \), 1-2 mm, and \(< 1 \text{ mm} \) in the large intestine. After polyps were counted, intestinal sections were placed in 70% ethanol for further analysis. Pilot data yielded an interassay coefficient of variation of 4%. 

92
**Plasma IL-6:** Plasma IL-6 will be measured using a mouse specific ELISA kit. Approximately 25-50μL of plasma will be incubated. A standard curve will be used to determine the levels of circulating IL-6 in all samples after treatment with DCQ or DMSO. An ELISA plate will be coated with capture antibody using a coating buffer and set to incubate in 4°C overnight. After washing the plate 100μL of blocking solution is added for 1 hour. After three washes, 100μL of standard or diluted sample is added to the wells in duplicate and incubated for 1hr. After 7 washes, 100μL of TMB cocktail is added to each well and stored in the dark for 30 minutes. 50μL of Stop solution is added to the wells and the plate is read at 450nm and 570nm according to the manufacturer's instructions.

**Hematoxylin and Eosin Staining (H&E):** Transverse sections (~5μm) will be cut from the paraffin embedded kidney and liver of Balb/c and Apc<sup>min</sup>/+ mice on a rotary microtome. Cut tissues will be allowed to expand on a 40°C -45°C water bath and then attached to (+) charged saline coated microscope slides. Excess paraffin is removed by placing slides in a 65°C oven for 1hr and then stored at 4°C until staining. Sections will be warmed to room temperature for 5-10minutes. Tissue morphology will be analyzed by observation and comparison to healthy tissue control sections. Sections are deparaffinized using the following washing progression; 3x5min xylene washes, 2x5min 100% EtOH, 1x3min 95% EtOH, and 1x3min 70% EtOH. Sections are then washed for 5min in dH₂O, stained with filtered hematoxylin for 5min, then again washed 6x5 in dH₂O. Sections are washed in acid alcohol mix (160mL 100% EtOH, 1mL acetic acid, and volumed to 200mL with dH₂O) for 6 dips then again washed in dH₂O for 4 dips. Sections are then washed in
Ammonia H₂O (0.6mL Ammonium OH volumed to 200mL with dH₂O) for 6 dips and then again washed in dH₂O for 10min. The sections will then be stained with Eosin for 3min and dehydrated in the following alcohol progression; 1x2min 70% EtOH, 1x2min 95% EtOH, 2x3min 100% EtOH, and 2x5min xylene. Sections are allowed to air dry for 30min and then mounted for viewing with Permount mounting media. Digital photographs will be taken from each section at a 4x, 20x and 40x with a Nikon E600 Widefield Epifluorescence and Darkfield Microscopy System at the University of South Carolina School of Medicine, and morphology examined.

**TUNEL Assay:** Transverse sections (~5μm) will be cut from the paraffin embedded brain, kidney, and liver of Balb/c and Apem/+ mice on a rotary microtome. Cut tissues will be allowed to expand on a 40°C -45°C water bath and then attached to (+) charged saline coated microscope slides. Excess paraffin is removed by placing slides in a 65°C oven for 1hr and then stored at 4°C until staining. Refrigerated sections will be warmed to room temperature for 5-10 minutes. Sections will be stained for apoptotic tissue using Apop-Tag kit from Millipore (S7100). Sections are deparaffinized using the following washing progression; 3x5min xylene washes, 2x5min 100% EtOH, 1x3min 95% EtOH, and 1x3min 70% EtOH. Sections are then washed for 5min in 1X PBS and covered in 20μg/ml proteinase K diluted from stock in fresh 1X PBS for 15min to remove any possible contaminants and inactivate nucleases that would degrade apoptotic tissue. After a 2x2min wash in dH₂O the sections are blocked in 3% H₂O₂ for 5min at room temperature. After blocking the sections are washed 2x5min in PBS and incubated for 2.5hrs in a humidified chamber in a working solution of TdT enzyme at 37°C. Enzymatic
reaction is stopped using a working strength stop solution for 10min and then washed for
3min in PBS. Anti-dioxigenin conjugate is then applied to each section and incubated for
30min at room temperature in a humidified chamber. After incubation sections are
washed 4x2min in PBS and detection reagent DAB is applied to develop the desired
intensity of potentially positive cells. After washing in 3x1min and 1x5min dH₂O, slides
are counterstained for 30sec with 5% methyl green and washed in dH₂O until
counterstain no longer fades. Slides are then mounted for viewing with Permount
mounting media after a minimum 3hr drying period. Digital photographs will be taken
from each section at a 4x, 20x and 40x with a Nikon E600 Widefield Epifluorescence and
Darkfield Microscopy System at the University of South Carolina School of Medicine,
and percent apoptotic tissue will be determined with imaging software (ImageJ,
Bethesda, MD).

**Specific Aim #2: will determine the mechanism of action of DCQ in the two animal
models of colorectal cancer.**

**Rationale:** Studies in multiple human cell lines from the American University of
Beirut have shown consistent results of increased mitochondrial induced apoptosis with
DCQ treatment. The CT-26 xenograft and 
\[Ap{c}^{\text{min/+}}\] transgenic mouse are common
experimental models of colorectal cancer. The mutated Apc gene causes activation of the
Wnt/β-catenin pathway which is responsible for tumorgenesis in the colon and small
intestines (Bienz & Clevers, 2000; Leclerc et al., 2004; Newton et al., 2010; Näthke &
Rocha, 2011). Although this is a specific genetic model of cancer there is genetic
variability in the tumor formation and therefore each tumor site is expected to respond
differently to drug treatment. HIF-1α and the Apc gene have been shown to have cross-communication at the transcriptional level which serves as a driving force for intestinal tumorigenesis (Mehl et al., 2005; Newton et al., 2010; Yamulla et al., 2014). Since it has been determined that DCQ works through interruption of the HIF-1α pathway, we are interested in mechanistic differences between and within the murine CT-26 tumor xenograft model and Apc\textsuperscript{min/+} genetic model of colorectal cancer.

**Experimental design for specific aim #2.** Experiment 2 will evaluate the mechanism of action of DCQ inducing tumor specific apoptosis in a xenograft and genetic model of colorectal cancer.

The same mice from Aim #1 will be used for Aim #2. At approximately 10 weeks of age, Balb/c mice will be randomized to a control group or treatment group. 2 million CT-26 cells, kindly provided by Dr. Marj Pena, were injected subcutaneously into the right flank of each mouse and allowed to grow for 2 weeks. After 2 weeks DCQ will be dissolved in DMSO to create a 17mg/kg concentration in a 100\(\mu\)L dose and injected twice a week for four weeks. Control mice will receive a 100\(\mu\)L injection of sterile DMSO twice a week for four weeks. At 10 weeks of age, Balb/c mice will be approximately 20g and will be able to withstand the tumor burden and chemotherapy treatments. Apc\textsuperscript{min/+} mice were aged to 8 weeks of age and randomized into control and treatment groups. Twice a week, clinical evaluations will be conducted to measure body weight, food and water consumption, fecal hemoccult and diarrhea, and a description of any abnormal behavior or side effects that might be arising as part of the therapy. At 16 weeks of age, mice in their respective groups will receive a bi weekly 100\(\mu\)L injection of either DMSO or
17mg/kg of DCQ. Min mice have initiated body weight loss and intestinal polyp development and show a near maximum clinical score by 15-16 weeks of age, making it an ideal time point to assess the effectiveness of DCQ on the treatment of colorectal cancer. All mice will be housed in standard cages for the duration of the study. Body weight and tumor dimensions in Balb/c mice and clinical score in Apc\textsuperscript{min/+} mice will be measured at the time of each treatment period and at the time of sacrifice. At sacrifice, brain, liver, kidney, spleen, and plasma will be collected from all mice, tumor xenografts will also be taken from Balb/c mice, and colon and small intestine will also be collected from Apc\textsuperscript{min/+} mice. Sections of tumor xenografts, brain, liver, kidney, colon, and small intestine from respective mice will be fixed in 10\% formalin for 24 hours, preserved in 70\% ethanol, and then embedded in paraffin wax to be sectioned for immunohistochemistry. All tissues extracted will also be weighted and frozen for tissue analysis of hypoxia markers and intracellular apoptosis markers.

**Experiment #2** will determine the mechanism of action of DCQ in two animal models of colorectal cancer.

**Animals:** The same mice from Aim #1 will be used in Aim #2. Balb/c male mice will be bred with female Balb/c mine in the USC Discovery building Arnold School of Public Health animal resource facility. All mice will be provided with standard rodent chow (Harlan Teklad Rodent Diet, #8604, Madison, WI) and water *ad libitum*. Body weights will be measured throughout the course of the study. At 10 weeks of age, mice will be injected by a member of Dr. Marj Pena's lab with 2 million cultured CT-26 murine colorectal cancer cells each and assigned into one of two groups; control or DCQ
treatment. \( Apc^{min/+} \) male mice on a C57BL/6 background will be bred with female C57BL/6 mice in the Discovery building Arnold School of Public Health animal resource facility. Animals will be genotyped as heterozygous for the \( Apc \) gene. All mice will be provided with standard rodent chow (Harlan Teklad Rodent Diet, #8604, Madison, WI) and water \textit{ad libitum}. All mice will undergo clinical evaluation and receive clinical scores based on the protocol and guidelines of the Fayad lab. At 8 weeks of age, mice will be assigned to one of two groups; control or DCQ treatment. DCQ was synthesized from 5,6-dichlorobenzofurazan oxide and dibenzoylmethane \textit{via} the Beirut reaction by Dr. Makhluf J. Haddadin and provided by Dr. Hala Gali-Muhtasib (American University of Beirut, Beirut, Lebanon). After two weeks of tumor growth, Balb/c mice will begin a bi-weekly treatment period. DCQ treatment will consist of a bi-weekly 100µL intraperitoneal injection of DCQ dissolved in DMSO at a concentration of 17mg/kg body weight. Balb/c control mice will receive a bi-weekly vehicle injection of 100µL of DMSO. Balb/c mice will be sacrificed at 16 weeks of age after 4 weeks of treatment. At 16 weeks of age, \( Apc^{min/+} \) mice will receive either a bi-weekly intraperitoneal 100µL injection of DMSO or DCQ at a concentration of 17mg/kg body weight. All mice will be sacrificed at 20 weeks of age after a 4 week treatment period. Based on previous drug development preliminary studies and on animal data from our lab, 5-6 mice per group are sufficient to detect a difference in clinical and experimental data.
Table A.3 Animal treatment groups for experiment #2.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>Age (weeks)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balb/c</td>
<td>DMSO</td>
<td>10-16</td>
<td>5-6</td>
</tr>
<tr>
<td>Balb/c</td>
<td>DCQ</td>
<td>10-16</td>
<td>5-6</td>
</tr>
<tr>
<td>Apc^{min/+}</td>
<td>DMSO</td>
<td>8-20</td>
<td>5-6</td>
</tr>
<tr>
<td>Apc^{min/+}</td>
<td>DCQ</td>
<td>8-20</td>
<td>5-6</td>
</tr>
</tbody>
</table>

**Primary outcomes:**

**Activation of DCQ through HIF-1α pathway:**

The chemical structure of 2-benzoyl-3-phenyl 6,7-dichloroquinoxaline 1,4-dioxide is activated into a free radical intermediate in an electron rich environment. In a hypoxic environment, this transfer is more likely to occur due to the highly reducing conditions of hypoxic cells, leading to hypoxic cells acting as a targeting element to the cytotoxicity of these compounds. Presence of hypoxia inducible factor-1 alpha (HIF-1α) indicates vasculogenesis and angiogenesis of proliferating cells, a common characteristic of tumor cells. Intra-tumoral HIF-1α expression will be measured using western blot analysis of nuclear extracts to determine the level of environmental hypoxia present in the tumors found in the intestinal and colonic region.

**Presence of apoptosis and decreased proliferation:**

TUNEL and BRDU staining of xenograft tumors, small intestines, and colon tissue will be used to analyze the percentage of apoptotic tissue and decreased proliferation. This
will also allow for analysis of tumor region specificity of DCQ via analysis of healthy tissue surrounding small-intestine and colon tumors.

**Intracellular apoptosis:**
Mapping of the intracellular pro-apoptotic and anti-apoptotic genes will be measured using western blot analysis of cytoplasmic extracts of dephosphorylated-Bad, Bcl-2, Bax, and cleaved caspase-9.

**Secondary Outcomes:**

**Point of Infiltration:** Hematoxylin and Eosin (H&E) staining will be performed on liver and kidney tissue sections from each animal model to determine point of infiltration of metastatic tumors.

**Specific Methodology Aim #2**

**Animals:** The same mice from Aim #1 will be used for Aim #2. Balb/c male mice will be bred with female Balb/c mice in the Discovery building Arnold School of Public Health animal resource facility. Apc\(^{min/+}\) male mice on a C57BL/6 background will be bred with female C57BL/6 mice in the Discovery building Arnold School of Public Health animal resource facility. Resulting heterozygous mice from the Apc\(^{min/+}\) cross will develop intestinal and colonic tumors as early as 8 weeks of age and typically have a life span of 20-22 weeks of age. All animals will be housed in standard cages and the room will be maintained on a 12:12 light:dark cycle with the light period starting at 0700. Mice will be provided with *ad libitum* and standard rodent chow (Harlan Teklad Rodent Diet #8604). Food and water consumption in addition to body weight and clinical score will be monitored during the course of the study for the Apc\(^{min/+}\) mice. All animals at time of
sacrifice are injected with 1mg of BRDU dissolved in phosphate buffered saline (PBS) and undergo DEXA scan prior to blood draw and cervical dislocation. All animal experimentation is approved by the University of South Carolina's Institutional Animal Care and Use Committee. Average mouse weight will be recorded through the first 8 weeks of clinical evaluations (up to 16 weeks of age). At this point, DCQ will be prepared in DMSO so as to obtain a concentration of 17mg/kg in a 100μl injection normalized to the average weight of the mice.

**Tissue Collection:** Mice will be anesthetized *via* exposure to an isofluorane and oxygen mixture. Blood will be collected from the retro-orbital sinus using a capillary tube. Blood will be spun at 4°C, 10,000rpm, for 10 minutes. Plasma will then be pipetted off and stored at -80°C until analysis. Brain (br), liver (l), kidney (k), spleen (sp), and tumor xenografts (tmr) from Balb/c mice will be excised, rinsed in PBS, frozen in a dry ice box, and stored at -80°C until further analysis. Parts of each tissue will also be fixed in 10% formalin for 24 hours, preserved in 70% ethanol, and then embedded in paraffin wax for histological sectioning. Small intestine, and colon from the *Apc*<sup>min/+</sup> mice will be chosen for sections containing tumors will be fixed in 10% formalin for 24 hours, preserved in 70% ethanol, and then embedded in paraffin wax for histological sectioning, brain (br), liver (l), kidney (k), spleen (sp) will also be collected. Parts of each tissue will also be excised, rinsed in PBS, frozen in a dry ice box, and stored at -80°C until further analysis. Remaining sections of the small intestine will be rinsed with PBS and opened longitudinally. Sections will be fixed in 10% formalin for 24 hours and then preserved in 70% ethanol and used for intestinal tumor counts in the *Apc*<sup>min/+</sup> mice.
**Protein Extraction:** Tumor xenografts from Balb/c mice and tumor containing colon tissue from $Apc^{min/+}$ mice will be weighted and homogenized using a dounce homogenizer. A nuclear extraction kit (ab113474) will be used to homogenize samples and separate nuclear and cytoplasmic extracts. Samples were weighted between 35 and 45 mg. 5mL diluted ENE1 buffer and $5\mu L$ DTT was added to each sample for every gram on tissue weighted. Once grinded, samples were incubated on ice for 15 minutes and centrifuged for 10min at 12,000rpm at $4^\circ C$. The resulting supernatant was aliquoted as the cytoplasmic extract and the pellet was kept for nuclear extract. $10\mu L$ of 1:1000 diluted DTT in ENE2 and PIC in ENE2 buffer was added to the nuclear pellet for every 2mg of tissue and incubated on ice for 15min with 5sec cortex every 3min. After incubation, samples were centrifuged for 10min at 14,000rpm at $4^\circ C$ and resulting supernatant was aliquoted as nuclear extract. Protein content is determined using the Bradford Assay (Bradford, 1976).

**Western Blot:** Nuclear and cytoplasmic protein extracts will be separated by SDS-PAGE and transferred to nitrocellulose membranes. Bax, Bcl-2, Cleaved caspase-9, dephosphorylated-Bad, and HIF-1$\alpha$ (nuclear) will be probed for. Tissue homogenates, $60\mu g$ for cytoplasmic and $10\mu g$ for nuclear, are separated on 10% to 15% SDS-polyacrylamide gels. The gels are transferred to nitrocellulose membranes in 3hrs at 220mA in cold transfer buffer with the transfer apparatus kept in a $4^\circ C$ refrigerator. After transfer, membranes are washed with dH$_2$O and stained with ponceau S to ensure equal loading and proper transfer. Membranes are blocked in 5% PBS with 0.1% Tween 20 (PBST) milk for 1hr at room temperature and placed in primary antibody at dilutions of
1:500 to 1:1000 in 5% PBST milk overnight to 72hrs at 4° C, HIF-1α requires a 2.5 hour primary antibody incubation at room temperature. Secondary anti-rabbit IgG-conjugated secondary antibodies are incubated with the membranes at 1:2000 for 1hr in 5% PBST milk at room temperature. Enhanced chemiluminescence is used to visualize the antibody-antigen interactions and develop the blot using the SYNGENE G:BOX Chemi XX6. Digitally developed blots are analyzed by measuring the integrated optical density (IOD) of each band using digital imaging software (GeneTools from Syngene, Cambridge, UK).

**Hematoxylin and Eosin Staining (H&E):** Transverse sections (~5μm) will be cut from the paraffin embedded small intestine and colon from the Apcmin/+ mice on a rotary microtome. Cut tissues will be allowed to expand on a 40°C -45°C water bath and then attached to (+) charged saline coated microscope slides. Excess paraffin is removed by placing slides in a 65°C oven for 1hr and then stored at 4°C until staining. Sections will be warmed to room temperature for 5-10minutes. Sections will be stained for morphological analysis using the H&E staining protocol as describe in Aim 1.

**TUNEL Assay:** Transverse sections (~5μm) will be cut from the paraffin embedded tumor xenografts, brain, kidney, and liver of Balb/c mice on a rotary microtome. Cut tissues will be allowed to expand on a 40°C -45°C water bath and then attached to (+) charged saline coated microscope slides. Excess paraffin is removed by placing slides in a 65°C oven for 1hr and then stored at 4°C until staining. Refrigerated sections will be warmed to room temperature for 5-10minutes. Sections will be stained for apoptotic tissue using Apop-Tag kit from Millipore (S7100) as described in Aim 1.
BrdU Staining: Transverse sections (~5μm) will be cut from the paraffin embedded brain, kidney, and liver of Balb/c mice on a rotary microtome. Cut tissues will be allowed to expand on a 40°C -45°C water bath and then attached to (+) charged saline coated microscope slides. Excess paraffin is removed by placing slides in a 65°C oven for 1hr and then stored at 4°C until staining. Sections will be warmed to room temperature for 5-10 minutes. Sections will be stained for proliferating tissue by tagging of BrdU present in actively dividing tissue using BRDU IN-SITU Detection Kit from BD Pharmingen (550803). Sections are deparaffinized using the following washing progression; 3x5min xylene washes, 2x5min 100% EtOH, 1x3min 95% EtOH, and 1x3min 70% EtOH. Sections are then washed for 5min in 1X PBS, blocked with 3% H₂O₂ for 10 minutes at room temperature, and then washed again 3x5min in PBS. Antigen retrieval is performed by preparing working solution of retrieval agents from the kit and autoclaving sections in the Liquid 20 cycle in the Public Health Research Center autoclave. After antigen retrieval, sections are cooled for 20 min and washed 3x5 min in 1X PBS. Primary antibody is prepared in a 1:10 dilution with diluent buffer and sections are incubated with primary antibody for 90 min in a humid chamber at 37°C. After primary antibody incubation, sections are washed 3x2 min in 1X PBS and then covered with RTU Streptavidin-HRP secondary antibody and incubated for 30min in a humid chamber at room temperature. After incubation sections are washed 4x2min in PBS and detection reagent DAB is applied to develop the desired intensity of potentially positive cells. After washing in 3x1min and 1x5 min dH₂O, slides are counterstained for 30 sec with 5 % methyl green and washed in dH₂O until counterstain no longer fades. Slides are then mounted for viewing with Permount mounting media after a minimum 3 hr drying period.
Digital photographs will be taken from each section at a 4x, 20x and 40x with a Nikon E600 Widefield Epifluorescence and Darkfield Microscopy System at the University of South Carolina School of Medicine, and percent apoptotic tissue will be determined with imaging software (ImageJ, Bethesda, MD).