The Role Of Nuclear Factor-Erythroid-2-Related Factor 2 In Sensitivity To Thymidylate Synthase Inhibitors In Colon Cancer Cells

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THE ROLE OF NUCLEAR FACTOR-ERYTHROID-2-RELATED FACTOR 2 IN SENSITIVITY TO THYMIDYLATE SYNTHASE INHIBITORS IN COLON CANCER CELLS

by

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Submitted in Partial Fulfillment of the Requirements
For the Degree of Doctor of Philosophy in
Biological Sciences
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2016

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Dedication

This work is dedicated to my family who has given their unconditional support through this journey. They have provided me with strength and encouragement through all the difficult times. I would not be here today if it wasn’t for them.
Acknowledgements

I would like to first acknowledge my mentor, Dr. Frank Berger, who guided me through this journey and helped me to become a better scientist. I want to thank him for his years of support, and encouragement. I would also like to acknowledge Karen Barbour for her patience and guidance in the lab with learning all the techniques and tricks. I would also like to acknowledge her and thank her for her work on the CRISPR/CAS9 study. I would like to thank my laboratory peers and co-workers, both former and current, Katie, Stephanie, Daniel, John, Nikeya, Ufuk, Yu, Grishma, Yang, and Sapana. I want to thank my committee members as well, Dr. Marj Pena, Dr. Minsub Shim, Dr. David Reisman, and Dr. Angela Murphy for their guidance and help through this journey.

I would also like to thank my family, mom and dad, Aunt Eileen and Uncle Bryan, cousins Tracy, Robert, and Bryan, and the rest of my extended family for their constant support and encouragement. I would also like to thank my support system of friends, Kayla, Joe, Sara, Varun, Lindsey, Bradley, Melissa, Asha, Graham, Brooke, and Kortni as well as many others who have been there over the years. Some of these friends have been along this journey with me from the start while others I have met along the way. Some have even been there to talk through experiments.
Finally, I would like to thank my Carolina Crossfit and Bikram Yoga Columbia families. These two places have become second homes to me and everyone both coaches and members have become friends. They unknowingly have become a strong support system. They have been there as an outlet to relax my mind and relieve the stress of the day.
Abstract

Nuclear factor-erythroid-2-related factor 2 (NRF2), a member of the cap ‘n’ collar family of bZIP transcription factors, confers protection against oxidative and electrophilic stress. NRF2 is of great interest in cancer research, due to its role in response to chemotherapy. The class of drugs targeting thymidylate synthase (TYMS) has been useful in the treatment of colorectal cancer, among other cancers. It has long been known that inhibition of TYMS leads to depletion of thymidine levels and the onset of programmed cell death, deriving from the enzyme’s function as the sole de novo source of thymidine for DNA replication and repair. Exposing cells to TYMS inhibitors such as fluoropyrimidine antimetabolites (5-fluorouracil, or FUra; 5’-fluoro-2’-deoxyuridine, or FdUrd), as well as anti-folate analogs (raltitrexed, or RTX), induce intracellular concentrations of reactive oxygen species, which are a primary cause of drug-mediated toxicity. This prompted our focus on assessing the impact of NRF2 on cellular response to TYMS inhibitors. Using human colon tumor-derived cell line HCT116, we have shown by gene expression profiling that drug exposure induces expression of a number of genes that are regulated by NRF2. Quantitative PCR assays of several colon tumor cell lines verified that FUra, FdUrd, and RTX induce transcription of several genes known to be NRF2-targets, including AKR1B10, ALDH3A1, HSPB8, HMOX1, and SERPINE1, among others. Such induction mirrors that in response to the classical NRF2 activator tert-butylhydroquinone (tBHQ). NRF2 protein concentrations in the nucleus

vi
are increased by FdUrd treatment, though not to the same extent as with tBHQ. Reporter gene constructs were used to show that both FdUrd and tBHQ induce transcription mediated by the NRF2-binding antioxidant response element (ARE). Furthermore, chromatin-immunoprecipitation experiments revealed that TYMS inhibitors promote occupancy by NRF2 of the ARE regions of several genes; again, tBHQ had a much greater effect. Additionally, chromatin-immunoprecipitation experiments indicated that TYMS inhibitors do not alter the acetylation of histones near the promoter regions. We observed no correlation between the activity of the target gene, and the acetylation of histones. We also showed that the PI3K/AKT pathway does not affect the stability of NRF2 in HCT116 cells. Finally, we observed that increases in the apoptotic index following exposure to TYMS inhibitors were greater in cells in which the transcription factor was subjected to siRNA-mediated “knockdown” or CRISPR/Cas9-mediated “knockout”, indicating that reduced NRF2 expression sensitizes cells to TYMS inhibitors. Overall, we conclude that TYMS inhibitors activate NRF2 and its downstream target genes, thereby constraining drug response. Reducing such activation of NRF2 or its consequences may be an effective strategy to sensitizing tumor cells to chemotherapy.
Table of Contents

Dedication .................................................................................................................................................. iii
Acknowledgements ........................................................................................................................................ iv
Abstract ...................................................................................................................................................... vi
List of Tables ............................................................................................................................................... x
List of Figures ........................................................................................................................................... xi
List of Abbreviations ................................................................................................................................. xiii

Chapter 1. General Introduction .............................................................................................................. 1
  1.1 Colorectal Cancer .................................................................................................................................. 1
  1.2 Thymidylate Synthase and its inhibitors .............................................................................................. 3
  1.3 Nuclear factor-erythroid-2-related factor 2 (NRF2) ............................................................................. 5
  1.4 NRF2 and therapy .................................................................................................................................. 11
  1.5 Goals of the project ............................................................................................................................... 14

Chapter 2 Thymidylate synthase inhibitors induce NRF2 levels .............................................................. 19
  2.1 Introduction ......................................................................................................................................... 19
  2.2 Materials and Methods ......................................................................................................................... 20
  2.3 Results .................................................................................................................................................. 24
  2.4 Discussion ............................................................................................................................................ 29

Chapter 3 NRF2 modulates sensitivity to TYMS inhibitors ........................................................................ 50
List of Tables

Table 2.1 qRT-PCR Primers for mRNA analysis ................................................................. 34

Table 2.2 qRT-PCR Primers for ARE regions from ChIP experiments ......................... 35

Table 2.3 AREs located in the regions amplified by primers for ChIP experiments ....... 36

Table 3.1 Additional primers used for qRT-PCR of mRNA levels ................................. 60

Table 3.2 Sequences for the guide RNAs targeting the NRF2 gene .............................. 60
List of Figures

**Figure 1.1** Thymidylate synthase (TYMS) reaction .......................................................... 15

**Figure 1.2** Chemical structure of TYMS inhibitors ............................................................... 16

**Figure 1.3** Nuclear factor-erythroid-2-related factor 2 (NRF2) ............................................ 16

**Figure 1.4** Activation of NRF2 .......................................................................................... 17

**Figure 1.5** Schematic of Reactive Oxygens Species levels ................................................ 18

**Figure 2.1** mRNA fold changes in human colon tumor cells ............................................... 37

**Figure 2.2** Protein levels of NRF2 .................................................................................... 38

**Figure 2.3** Induction dependent on depletion of thymidine levels ....................................... 38

**Figure 2.4** Induction of NRF2 protein with FdUrd or tBHQ ............................................... 39

**Figure 2.5** NRF2 target gene induction with FdUrd or tBHQ ............................................. 40

**Figure 2.6** Lowering tBHQ concentration so NRF2 protein levels are equal .................... 43

**Figure 2.7** Depiction of ARE reporter gene in HCT116 ARE\(^7\)-Luciferase and mutant ...... 44

**Figure 2.8** Expression of the reporter gene in cells treated with FdUrd or tBHQ ............... 44

**Figure 2.9** Induction of the reporter gene when tBHQ concentration is lowered ............ 45

**Figure 2.10** Increase in occupancy at ARE regions ............................................................ 46

**Figure 2.11** Increase in occupancy is dependent on thymidine depletion ......................... 49

**Figure 3.1** NRF2 and target gene expression with siRNA targeting NRF2 ......................... 61

**Figure 3.2** Target gene expression in NRF2 knockdown ................................................... 62

**Figure 3.3** Apoptotic index in HCT116 and other colon tumor cells lines ......................... 63
Figure 3.4 Initial knockout of NRF2 with three different guide RNAs................................. 64

Figure 3.5 Analysis and mutations of clones .............................................................................. 64

Figure 3.6 Target gene expression in NRF2 null clones ............................................................... 65

Figure 3.7 Apoptotic index in NRF2 null clones ............................................................................. 66

Figure 4.1 AKT pathway and phosphorylation .............................................................................. 76

Figure 4.2 NRF2 is phosphorylated ............................................................................................... 77

Figure 4.3 TYMS inhibitors increase phospho-AKT ................................................................. 77

Figure 4.4 Apoptotic index for cells treated with MK2206 .......................................................... 77

Figure 4.5 Histone acetylation at ARE regions in HCT116 cells ............................................... 78

Figure 5.1 Current working model ......................................................................................... 84

Figure A.1 Histone acetylation at ARE regions in A24 cells ...................................................... 105

Figure A.2 Total histones at ARE regions in A24 and HCT116 cells ........................................... 106
List of Abbreviations

AKR1B10 .......................................................... Aldo-Keto Reductase Family 1 Member B10
AKR1C1 .......................................................... Aldo-Keto Reductase Family 1 Member C1
AKT ........................................................................PKB/Protein Kinase B
ALDH3A1 .......................................................... Aldehyde Dehydrogenase 3 Family Member A1
ARE ........................................................................Antioxidant Response Element
BSA ..........................................................................Bovine Serum Albumin
CBR3 ......................................................................Carbonyl Reductase 3
CH$_2$-THF ............................................................ $N^5,N^{10}$-Methylenetetrahydrofolate
ChIP ........................................................................Chromatin Immunoprecipitation
CRISPR/CAS9 .........................................................Clustered regularly interspaced short palindromic repeats/CAS9
dThd ........................................................................Thymidine
dTMP .................................................................Deoxythymidine Monophosphate
dUMP .......................................................................Deoxyuridine Monophosphate
FdUMP ........................................................................5-Fluoro-2’-Deoxyuridylic Acid
FdUrd .......................................................................5’-Fluoro-2’-Deoxyuridine
FUra ..........................................................................5-fluorouracil
GAPDH .................................................................Glyceraldehyde 3-Phosphate Dehydrogenase
GCLM ........................................................................Glutamate-Cysteine Ligase Modifier Subunit
gRNA ......................................................................Guide Ribonucleic Acid
GSK3 ................................................................. Glycogen Synthase Kinase 3
H2B K5, K12, K15 .............................................. Histone H2B Acetylated Lysine 5, 12, 15
H3 K9, K14, K18, K27 ...................................... Histone H3 Acetylated Lysine 9, 14, 18, 27
H4 K12, K16 ..................................................... Histone H4 Acetylated Lysine 12, 16
HCT116, HCT-15, SW480, DLD-1, LoVo .................. Colon Cancer Cell Lines
HMOX1 .................................................................. Heme oxygenase 1
HSPB8 .............................................................. Heat Shock Protein Family B (Small) Member 8
KEAP1 .................................................................. Kelch-like ECH-associated protein 1
LUC .......................................................................... Luciferase
MK2206 .............................................................. AKT inhibitor
mRNA .............................................................. Messenger Ribonucleic Acid
NAC ..................................................................... N-acetylcysteine
NRF2 ................................................................ Nuclear factor-erythroid-2 related factor 2
NOX ...................................................................... NADPH Oxidase
NQO1 ................................................................. NAD(P)H Quinone Oxidoreductase 1
PBS .................................................................... Phosphate Buffered Saline
qRT-PCR .......................................................... Quantitative Real Time Polymerase Chain Reaction
ROS .................................................................... Reactive Oxygen Species
RTX ....................................................................... Raltitrexed
SDS-PAGE ..................................................... Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SERPINE1 ........................................................ Serpin Family E Member 1
siRNA .................................................................. Small Interfering Ribonucleic Acid
SPP1.......................................................................................................................... Secreted Phosphoprotein 1

tBHQ.................................................................................................................................. tert-Butylhydroquinone

TUNEL........................................ Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling

TYMS ............................................................................................................................ Thymidylate Synthase
Chapter 1
General Introduction

1.1 Colorectal Cancer

Colorectal cancer (CRC) is one of the leading causes of cancer-related deaths in the western world (Mishra et al., 2013). In the United States, it is the third most commonly diagnosed cancer in both men and women. There is about a 5-6% risk of developing CRC (2016b; Mundade et al., 2014). It is estimated that there will be about 150000 new cases of colorectal cancer in 2016 (Siegel et al., 2016). The risk factors for developing colon cancer include intrinsic factors as well as those associated with lifestyle (Mishra et al., 2013). Family history, increase in age, and ethnic background are among the intrinsic risk factors. There are however many lifestyle related factors which increase the risk of CRC. Some of these include obesity, physical inactivity, certain diets, and smoking (Mundade et al., 2014; Slattery, 2000). A small amount of cases may be due to genetic predisposition (Jasperson et al., 2010; Mundade et al., 2014). The amount of somatic mutations contributing to the progression and pathogenesis of CRC is far more extensive than previously thought and is not yet fully known (Mundade et al., 2014). However, there are three main genetic mechanisms that are responsible for the sporadic CRC: chromosomal instability (CIN), microsatellite instability (MSI) and the serrated pathway (Mundade et al., 2014; Pino and Chung, 2010). The CIN mechanisms
suggest a stepwise pattern in mutations that activate oncogenes and inactivate tumor suppressor genes (Mundade et al., 2014; Vogelstein et al., 1988). One of the tumor suppressor genes that is inactivated is Adenomatous polyposis coli (APC) (Mundade et al., 2014). A mutation in the APC gene is found in 70-80% of CRC, and is one of the earliest events in sporadic CRC progression (Arvelo et al., 2015; Mundade et al., 2014). The MSI pathway accounts for about 10-15% of sporadic CRC. MSI results from impaired DNA mismatch repair (MMR) (Mundade et al., 2014). Impairment of MMR, leads to accumulation of more mutations, some of the earliest of which are mutations in the APC gene (Armaghany et al., 2012; Mundade et al., 2014). The serrated pathway, unlike the CIN and MSI pathways (initiated by APC mutations), results from mutations in protein kinase B-Raf (BRAF) and epigenetic silencing of genes involved in cell differentiation, DNA repair, and cell-cycle control (Jass et al., 2002; Leggett and Whitehall, 2010; Mundade et al., 2014).

Although the death rate from CRC has been decreasing for more than 20 years, racial and ethnic disparities in screening, treatment, and survival still persist (2016a; 2016b; Gellad and Provenzale, 2010; Mundade et al., 2014). Colon cancer, like many other cancers can be divided into stages: I, II, III, and IV. Within these stages they can be broken down even further to IIA, IIB, IIC, IIIA, IIIB, and IIIC depending on the T, N, and M stages (2016a; 2016b). T describes how far the primary tumor has grown, N indicates if and how much the lymph nodes are involved, and M indicates metastases. The five year survival rate at stage I is about 90% and drops to about 10% by stage IV (2016a; 2016b). Treatment for CRC, like many other cancers, includes surgery, radiation, chemotherapy,
and targeted therapy; the type and combination of treatments depends on the stage of the cancer (Mundade et al., 2014). Some of the common chemotherapy drugs used to treat CRC are 5-fluorouracil (FUra), Capecitabine, Oxaliplatin, and Irinotecan as well as others (2016b). Additionally, with the discovery of Oxaliplatin and Irinotecan, combination therapies have been born. Some of these common combination are FOLFOX (folinic acid, 5-fluorouracil, and Oxaliplatin), FOLFIRI (folinic acid, 5-fluorouracil, and Irinotecan), and IROX (Irinotecan, and Oxaliplatin) (Guglielmi and Sobrero, 2007).

Many of the common drugs used are anti-metabolites which target an enzyme or multiple enzymes involved in DNA synthesis. One of the key enzymes that has been a target of numerous chemotherapy drugs is thymidylate synthase (Chu et al., 2003; Ozer et al., 2015).

1.2 Thymidylate Synthase and its inhibitors

Thymidylate synthase (TYMS, EC 2.1.1.45) catalyzes the reductive methylation of 2′-deoxyuridine-5′-monophosphate (dUMP) by \( N^5N^{10} \)-methylene tetrahydrofolate (CH\(_2\)H\(_4\)PteGlu) to form dTMP and dihydrofolate (Figure 1.1) (Berger and Berger, 2006; Carreras and Santi, 1995). TYMS is the only de novo source of thymidine for the cell, making it critical for DNA synthesis, replication and repair (Ozer et al., 2015). Inhibition of TYMS leads to deficiency of dTMP, which results in DNA damage and eventually cell death. This has made TYMS an attractive target for many chemotherapeutic drugs (Barbour and Berger, 2008; Berger and Berger, 2006; Carreras and Santi, 1995; Chu et al., 2003; Longley et al., 2003; Wilson et al., 2014). TYMS inhibitors include both fluoropyrimidines and folate analogs, both of which reduce the de novo synthesis of
dTMP (Figure 1.2). fluoropyrimidines such as 5-fluorouracil (FUra) and 5'-fluoro-2'-deoxyuridine (FdUrd) are metabolized to the active molecule 5-fluoro-2'-deoxyuridylic acid (FdUMP). FdUMP is an analog of dUMP, and forms a ternary complex with CH₂H₄PteGlu; but with the fluorine atom in the place of a hydrogen, the reaction is halted, and the complex is held together by covalent bonds (Barbour and Berger, 2008; Carreras and Santi, 1995; Ozer et al., 2015). FdUMP can also be phosphorylated to 5-fluoro-2'-deoxyuridine-5'-triphosphate (FdUTP), which can be incorporated into DNA to lead to inhibition of DNA synthesis and function (Chu et al., 2003). fluoropyrimidines have been used to treat ovary, breast, and gastrointestinal tract cancers for decades (Chu et al., 2003; Longley et al., 2003). Folate-based inhibitors were designed based on the structure of CH₂H₄PteGlu (Bertino, 1997; Carreras and Santi, 1995; Takemura and Jackman, 1997). Some examples of folate based inhibitors are raltitrexed (RTX), LY231514 [(2R)-2-[[4-[2-(2-amino-4-oxo-1,7-dihydropyrrolo[2,3-d]pyrimidin-5-yl)ethyl]benzoyl]amnio]pentanedioic acid], ZD9331 [(2S)-2-[[4-[(2,7-dimethyl-4-oxo-1H-quinazolin-6-yl)methyl-prop-2-ynlamino]-2-fluorobenzoyl]amino]-4-(2H-tetrazol-5-yl)butanoic acid], and GW1843U89 [(S)-2-(5-(((3-methyl-1-oxo-1,2dihydrobenzo[f]quinazolin-9-yl)methyl)amino)-1-oxoisindolin-2-yl)pentanedioic acid]. These have advanced to clinical trials and have been shown to reduce tumors (Bertino, 1997; Takemura and Jackman, 1997).

Even though TYMS inhibitors have been used to treat cancer for decades, the mechanism by which this occurs is still not fully known. Several studies have shown that the induction of reactive oxygen species (ROS) in FUra-treated cells, derived from an
increase in expression of the enzyme ferredoxin reductase (FDXR), is a key to drug-mediated cell death (Hwang et al., 2001; Liu and Chen, 2002). Along with these studies, there were many which showed there was an increased ROS levels after exposure to FUra (Akhdar et al., 2009; Lamberti et al., 2012; Matsunaga et al., 2010). Most recently, it was shown that NADPH oxidase (NOX) is an important source of the increases in ROS levels in cells treated with TYMS inhibitors. Furthermore, drug-treated cells mount a protective response that includes activation of the transcription factor nuclear factor-erythroid-2-related factor 2 (NRF2), and many of its downstream target genes (Akhdar et al., 2009; Ozer et al., 2015).

1.3 Nuclear factor-erythroid-2-related factor 2 (NRF2)

NRF2 is a member of the cap ‘n’ collar family of transcription factors and contains a basic leucine zipper DNA binding domain at its C-terminus (Lau et al., 2008). NRF2 is organized into seven domains, Neh1-7 (Figure 1.3) (Keum and Choi, 2014; Sykiotis et al., 2011). Neh 1 is the DNA binding and dimerization domain. Here NRF2 also interacts with other transcription factors. This domain also contains the nuclear localization signal (Jain et al., 2005; Lau et al., 2008). The Neh 2 domain, which lies at the N-terminal end, binds with Kelch-like ECH associated protein 1 (KEAP1). KEAP1 is a negative regulator of NRF2 (Keum and Choi, 2014; Niture et al., 2014). The Neh3 domain, which lies at the C-terminal end, is a transcriptional activation domain. This domain recruits chromatin remodeling protein CHD6 (Keum and Choi, 2014; Lau et al., 2008; Nioi et al., 2005). Neh 4 and 5 are critical for antioxidant response element (ARE) transactivation through binding to transcriptional coactivators (Keum and Choi, 2014; Lau et al., 2008). The Neh
6 and Neh 7 domains bind to β-TrCP and RARα, respectively (Keum and Choi, 2014; McMahon et al., 2004; Wang et al., 2013), both of which repress NRF2. Cellular levels of NRF2 are strictly regulated by KEAP1, which serves as a link between NRF2 and Cul3-type E3 ubiquitin ligase, which is required for poly-ubiquitination (Furukawa and Xiong, 2005; Huang et al., 2015; Keum and Choi, 2014; Kobayashi et al., 2004; McMahon et al., 2003; Zhang et al., 2004). KEAP1 acts as a major sensor for oxidative or electrophilic stress because of the large number of cysteine residues (Dinkova-Kostova et al., 2002; Huang et al., 2015).

There are two main models of what happens when one or more of these cysteines are modified by stress. The first model is the complete breakdown of the NRF2-KEAP1 complex due to the modification of cysteine residues on KEAP1. The other newer model is the change of conformation of the NRF2-KEAP1 complex, which prevents NRF2 from being ubiquitinated (Eggler et al., 2005; Huang et al., 2015). At this point NRF2 accumulates in the nucleus and activates its downstream target genes (Figure 1.4). The genes that NRF2 regulates are those of phase II cytoprotective enzymes, various drug metabolizing enzymes, transporters, and cellular reducing equivalents such as heme oxygenase-1 (HMOX1), NAD[P]H:quinone oxidoreductase-1 (NQO1), and aldo-keto reductase 1 B10 (AKR1B10) (No et al., 2014). NRF2 is not only important in combating oxidative stress, but has been shown to play a role in many other cellular processes. Many of the downstream targets of NRF2 contain an antioxidant response element sequence (ARE) in the promoter region which binds NRF2 through the Neh 1 (Lau et al., 2008). Additionally, Bach1 (a transcription factor
belonging to the cap ‘n’ collar, b-Zip family of proteins) forms a heterodimer with small Maf proteins (b-Zip proteins which lack a transcriptional activation domain), and competes with NRF2 for binding to the ARE (Dhakshinamoorthy et al., 2005; Niture et al., 2014); because NRF2 can no longer associate with the ARE, the expression levels of the cytoprotective genes are reduced to basal levels.

Although KEAP1 is the major regulator of NRF2 activation and levels within the cell, there is evidence of post-translational phosphorylation and acetylation (Lau et al., 2008; Sun et al., 2009). A number of studies have examined upstream regulators of NRF2 phosphorylation, including protein kinase C δ (PKCδ), mitogen activated protein kinases (MAPKs), PKR-like endoplasmic reticulum kinase (PERK), phosphatidylinositol 3-kinase (PI3K), casein kinase 2, the SRC family of tyrosine kinases, and glycogen synthase kinase-3 (GSK-3) (Apopa et al., 2008; Huang et al., 2015; Jain and Jaiswal, 2007; Niture et al., 2011). The relevance of many of these to the regulation of NRF2 is still to be determined, because of the large percentage of residues that can be phosphorylated (Cuadrado, 2015). A study done by Bloom et al. demonstrated that phosphorylation at Serine 40 of NRF2 by PKCδ is necessary for NRF2 release from KEAP1, but it is not required for either NRF2 stabilization/accumulation in the nucleus or transcriptional activation (Bloom and Jaiswal, 2003). Serine 40 lies within the Neh 2 domain, which is responsible for the interaction between NRF2 and KEAP1. The effects of MAPKs on NRF2 signaling depend on the specific MAPK, and can either promote or inhibit NRF2 activation (Huang et al., 2015; Lee et al., 2013). PI3K signaling promotes nuclear translocation of NRF2 and induces the expression of ARE-containing genes (Huang et al.,
2015; Kang et al., 2002). In contrast, GSK-3β, inhibits NRF2 by promoting its degradation (Cuadrado, 2015). GSK-3β catalyzes the phosphorylation of NRF2 at the Neh 6 domain and decreases its stability by creating a degradation domain, which is recognized by the ubiquitin ligase adapter β-TrCP and tagged for proteasome degradation by the Cullin1/Rbx1 complex (Chowdhry et al., 2012; Cuadrado, 2015; Rada et al., 2012). It is thought that this process starts with phosphorylation at Serines 342 and 347 by an unknown kinase, and followed by GSK-3β-catalyzed phosphorylation at Serines 335 and 338 (Cuadrado, 2015). This is then recognized by β-TrCP, and lysine residues are ubiquitinated.

Currently, there are at least two mechanisms to control the cellular levels of NRF2: the first is redox-dependent through KEAP1, while the second is dependent on phosphorylation within the Neh 6 domain (Cuadrado, 2015). Under normal conditions, NRF2 levels are kept low (Taguchi et al., 2011), as are its downstream targets. Following either oxidative stress or signaling cascade leading to phosphorylation, NRF2 levels will increase and begin to accumulate in the nucleus. At this point, NRF2 heterodimerizes with co-factors and binds to the ARE, resulting in activation of cytoprotective genes (Kaspar et al., 2009; Taguchi et al., 2011). These cytoprotective genes will alleviate the oxidative stress, and remove damaged proteins to promote cell survival (Shelton and Jaiswal, 2013). Following this induction, because of the tight regulation of NRF2, the cell responds by exporting NRF2 out of the nucleus for subsequent degradation (Shelton and Jaiswal, 2013; Taguchi et al., 2011).
NRF2 plays a major role in protecting the cell against oxidative stress and DNA damage. (Kaspar et al., 2009; Shelton and Jaiswal, 2013). There have been many studies which have shown that activation of NRF2, through either genetic alterations or activating drugs, inhibit carcinogenesis. Thus, NRF2 has been viewed as a tumor suppressor (Sporn and Liby, 2012). Additionally studies with NRF2-null mice showed that the anti-carcinogenic activity of chemopreventive drugs in these animals was either significantly decreased or totally abolished (Sporn and Liby, 2012). Many of the chemopreventive drugs that have been used in studies are natural products that are easily obtained in foods. tert-Butylhydroquinone (t-BHQ), along with sulforaphane (SF), are well-known NRF2 inducers and can be found in foods as either a preservative or in cruciferous vegetables respectively (Huang et al., 2015). These compounds induce NRF2 by reacting with the cysteine residues on KEAP1 (Shelton and Jaiswal, 2013; Sporn and Liby, 2012). Some in-vivo studies demonstrated that there was an increase in tumor burden in NRF2-knockout mice (Aoki et al., 2001; Iida et al., 2004; Ramos-Gomez et al., 2001; Shelton and Jaiswal, 2013). This is further evidence that NRF2 may act as a tumor suppressor. Additionally, NRF2 has been shown to be both upregulated by other tumor suppressor proteins as well as being targeted for degradation by oncoproteins (Shelton and Jaiswal, 2013). Taking the above information in total, it is reasonable to call NRF2 an anticancer protein especially in premalignant states (Shelton and Jaiswal, 2013).

However, considering the protective effects that NRF2 exerts on the cell, it is logical to think that it can also provide protection to a cancer cell, thereby acting as a tumor promoter. NRF2 has been labeled a double-edged sword in cancer (Hayes and
McMahon, 2006). One study showed that oncogenes K-Ras, B-Raf and Myc targeted the increased transcription of NRF2 in cancer cells (DeNicola et al., 2011; Niture et al., 2014; Shelton and Jaiswal, 2013). When these genes were overexpressed in mice, there was an increase in basal expression of NRF2, and a decrease in ROS, allowing the cancer cells to escape from oxidative death. Additionally, NRF2 has been known to regulate many genes that control the hallmarks of cancer (Shelton and Jaiswal, 2013). Another study showed that NRF2 is able to regulate many of the enzymes involved in programming nucleotide synthesis and cell proliferation (Mitsuishi et al., 2012; Niture et al., 2014; Shelton and Jaiswal, 2013). Mitsuishi et al., found that the PI3K-AKT pathway also contributes to the activation of NRF2 (Mitsuishi et al., 2012). A third finding which connects NRF2 to being a tumor promoter is the upregulation of antiapoptotic factors such as Bcl-2 and Bcl-xL (Niture et al., 2014; Shelton and Jaiswal, 2013). The increase in these factors leads to a decrease in apoptosis, and cancer cell and tumor survival. There is also increasing evidence that NRF2 is constitutively elevated in many types of cancer cells and tumors from patients (Jiang et al., 2010; Kim et al., 2010; Lister et al., 2011; Ohta et al., 2008; Padmanabhan et al., 2006; Rushworth et al., 2012; Singh et al., 2006; Zhang et al., 2010). This upregulation can be due to mutations in either KEAP1 or NRF2, or even hypermethylation of the KEAP1 gene (Padmanabhan et al., 2006; Singh et al., 2006). Loss of function mutation of KEAP1 as well as hypermethylation has been seen in lung, breast, ovarian, liver, gastric, and prostate cancers (Huang et al., 2015; Padmanabhan et al., 2006; Singh et al., 2006). Additionally, overexpression has been linked to poorer prognosis in cancer patients (Hu et al., 2013; Sasaki et al., 2013; Solis et
al., 2010). In all, the above studies show that NRF2 can be an oncogene or tumor promoting factor.

1.4 NRF2 and therapy

The constitutive activation of NRF2 creates a microenvironment that is conducive for the tumor cells to survive, however, it also helps protect these cells from therapeutic intervention (Shelton and Jaiswal, 2013). Because NRF2 upregulates many genes that are involved in pumping drugs and toxic chemicals out of the cell, the cancer cells which upregulate NRF2 can become resistant to drug therapy (Kim et al., 2007; Okawa et al., 2006; Vollrath et al., 2006). Studies have also shown suppression of NRF2 activity in cancer can increase the efficacy of chemotherapy (Samatiwat et al., 2015; Zhong et al., 2013).

Because of the dual functions of NRF2, it would be best described as a protooncogene (Shelton and Jaiswal, 2013). Varmus et al. first described this term in 1976, defining a protooncogene as a normal gene that typically controls cell growth, but when it obtains a gain of function mutation, or its expression is altered, becomes oncogenic (Bishop and Department of Microbiology and Immunology and the G.W. Hooper Research Foundation, 2016; Stehelin et al., 1976). NRF2, when expressed at normal levels, is beneficial to the cell to defend against oxidative stress and prevent cancer; however, when it becomes overexpressed, it provides a prosurvival benefit to a precancerous cell that can become a malignant tumor that is resistant to chemotherapeutics (Niture et al., 2014; Shelton and Jaiswal, 2013). Figure 1.5 illustrates the difference in reactive oxygen species levels depending on the stage and level of
NRF2. It has long been understood that cancer cells contain a higher level of ROS, which is why many chemotherapeutic drugs work by increasing the ROS in cancer cells. These cells are closer to that threshold so a small increase can push them to death, whereas non-cancerous cells, even with an increase in ROS, still remain below the threshold. Those cancer cells which have a high basal level of NRF2 when treated with chemotherapeutics are able to stay just below the threshold with the high levels of antioxidants. These cancer cells become resistant to the chemotherapeutic drugs.

Because oxidative stress and inflammation are major causes of many diseases, the KEAP1-NRF2-ARE pathway is an important defense (Abed et al., 2015). As stated above, NRF2 plays an important role in the prevention of cancer. The NRF2 pathway has also been shown to play a role in many neurodegenerative diseases such as Alzheimer’s and Parkinson’s Disease (Giudice et al., 2010; Sandberg et al., 2014). It has also been shown that low levels of NRF2 might be involved in the developing oxidative stress in diabetes (Giudice et al., 2010; Haan, 2011). In addition to the role NRF2 might play in the development of diabetes, the NRF2-ARE pathway also appears to have a role in complications that arise from diabetes such as diabetic nephropathy (Haan, 2011).

Oxidants and oxidative stress have been shown to play a role in the development of chronic obstructive pulmonary disease and other respiratory diseases, as well as cardiovascular disease (Giudice et al., 2010). As a result, activation of NRF2 has been shown to be protective against this. In addition, NRF2’s role in many other diseases is still being investigated. As a result of the role that activation and higher levels of NRF2 play in the prevention of many diseases, there has been much effort placed on the
search for and development of activators/inhibitors of the KEAP1-NRF2 interaction. The KEAP1-NRF2 pathway can be activated by cellular stress, and both endogenous and exogenous chemical inducers (Niture et al., 2010). Many of the ARE-inducers that have been identified come from natural sources. There are also synthetic and small molecule inducers that are indirect inhibitors of the KEAP1-NRF2 interaction, but there are some direct inhibitors of this interaction (Abed et al., 2015; Magesh et al., 2012). These indirect inhibitors act on other proteins involved in the activation of NRF2, such as Maf proteins.

High levels of NRF2 play an important part in preventing many diseases. Thus, there has been a lot of effort in finding NRF2 inducers. It is also known that higher levels of NRF2 in cancer cells may cause drug resistance, so that recent efforts to find small molecule inhibitors of NRF2 have occurred (Kensler and Wakabayashi, 2010; Magesh et al., 2012; Wang et al., 2008). Many of these compounds are isolated from natural sources such as brusatol and wogonin (No et al., 2014). Most have not been studied thoroughly and may function in a cell-type specific manner. Some of them have also been shown to be activators as well as inhibitors of NRF2 (Paredes-Gonzalez et al., 2015). Since NRF2 can be activated through AKT signaling and contains an internal degron which is phosphorylated by GSK-3β, AKT-directed agents have been looked at as possible indirect inhibitors of NRF2. Two inhibitors of the AKT pathway, LY294002 and MK-2206, showed decreases in NRF2 and its downstream genes (Chowdhry et al., 2012).
1.5 Goals of the project

Because of the role NRF2 plays in chemoresistance, and the fact that many common chemotherapeutic drugs increase the oxidative stress levels within the cell, the response to these drugs may be determined by the cellular levels of NRF2. This creates an opportunity to determine a way to reduce the levels of NRF2 and increase the efficacy of the chemotherapy. Therefore, we have undertaken a study of NRF2 and response to TYMS inhibitors. The first aim of the current study focuses on the effect of TYMS inhibitors on NRF2 and its downstream targets in human colon cancer cells. The second aim of the study examines the effect of NRF2 knockdown or knockout on sensitivity of human colon tumor cells to TYMS inhibitors. Finally, the third aim of the study looks at post translational modifications. The first half investigates phosphorylation and a specific AKT inhibitor (MK-2206) to determine if the phosphorylation state of NRF2 in human colon tumor cells has an effect on the drug-induced apoptosis. The second half determines if the acetylation of histones within the ARE region is altered by drug exposure.
Figure 1.1. **Thymidylate synthase (TYMS) reaction.** TYMS catalyzes the transfer of a methyl group from \(N^5, N^{10}\)-methylene-THF to 2’deoxyuridine-5’-monophosphate (dUMP) to form 2’-deoxythymidine-5’-monophosphate (dTMP). The methyl group is denoted in red.
Figure 1.2. **Chemical structure of TYMS inhibitors.** Fluoropyrimidines; 5-FU and FdUrd and Folate analog; Raltitrexed (RTX). Fluoropyrimidines form a stable ternary complex with TYMS rendering it inactive. The folate analogs were designed based off the structure of $N^5, N^{10}$-methylene tetrahydrofolate.

Figure 1.3. **Nuclear factor-erythroid-2-related factor 2 (NRF2).** Structure and function of the 7 Neh domains. Neh2 binds to KEAP1, Neh 4 and 5 are the ARE transactivation domain along to binding to other coactivators. The Neh 6 and 7 domains bind to proteins which repress NRF2 activity and Neh 1 and 3 are the DNA binding and dimerization domain and transcriptional activation domain, respectively.
Figure 1.4. Activation of NRF2. Left side shows a cell under basal conditions where NRF2 is bound to KEAP1, ubiquitinated and degraded. Right side shows a cell undergoing electrophilic or oxidative stress. Here NRF2 is activated and translocates into the nucleus to activate transcription of target genes.
Figure 1.5. Schematic of Reactive Oxygen Species levels. It has been long understood there is a threshold between survival and death of cells with regard to levels of reactive oxygen species in the cell. Chemotherapeutic drugs have been shown to increase the ROS levels within cells to push these cells past the threshold. The top two arrows show normal cells and the ROS levels with and without drug treatment. All lie well below the threshold. The middle two arrows show cancer cells with normal NRF2 levels. Here the cells without drug have a higher basal level of ROS than normal cells and when treated with drugs pass the threshold. The bottom two arrows show cancer cells with high levels of NRF2. These cells without drug have a higher level of ROS but when treated they do not pass the threshold likely because of the high levels of NRF2.
Chapter 2
Thymidylate synthase inhibitors induce NRF2 levels

2.1 Introduction

TYMS inhibitors have been used clinically for many years but the exact mechanism is still not yet fully understood. Previous studies in our lab investigated the role of oxidative stress in response to TYMS inhibitors. There have been many studies that have shown FUra treatment increased intracellular ROS levels, but it was not determined if this was due to the inhibition of TYMS or other effects of the antimetabolite. We were able to show that the increase in ROS levels is due to the inhibition of TYMS and depletion of dTMP pools. We extended the study and found that the increase in ROS levels by TYMS inhibitors promotes apoptosis, indicating that oxidative stress is important to the cytotoxicity of these inhibitors. We then wanted to determine the mechanism by which the ROS levels are increased by TYMS inhibitors. We tested the role of the enzyme NOX, which catalyzes the formation of superoxide. It was found that NOX activation is mostly due to the inhibition of TYMS. Additionally, the activation of NOX is a contributing factor to the apoptotic response to TYMS-targeted agents.

In addition to the results above, there was also interest in determining the impact of TYMS inhibitors on the cellular transcriptome. Microarray analysis revealed 1975 genes whose levels of expression were altered by treatment with FUra. 1119 of
these were induced, while 856 were repressed. While narrowing the list down to redox-related genes, many of them are known to be regulated by the transcription factor, NRF2. NRF2 controls the expression of genes conferring protection against oxidative stress and electrophilic stress. Additional studies have shown common chemotherapy drugs inducing NRF2 levels in breast, and lung cancers.

Here we extend the findings above and demonstrate that TYMS inhibitors, FdUrd, FUra, and RTX, induce protein levels of NRF2 as well as increasing many target genes. We also demonstrate that this induction is due to the depletion of thymidine levels in the cell by adding exogenous thymidine. Additionally, we demonstrate that TYMS inhibitors activate NRF2 target genes through the ARE as well as increasing the occupancy of many of these ARE regions.

2.2 Materials and Methods

**Cell Culture:** Human colon tumor cell line HCT116 was generously given to us by Dr. Michael G. Brattain. SW480 and HCT-15 cell lines were obtained from American Type Culture Collection (ATCC); (Manassas, VA). HCT116 ARE\textsuperscript{7}-Luciferase and HCT116 MUT-ARE\textsuperscript{7}-Luciferase cell lines were kindly given to us by Dr. Minsub Shim. All cells were grown in RPMI-1640 medium (Cellgro) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals) and 100 U/mL penicillin, 100 mg/mL streptomycin, and 250 ng/mL amphotericin B (Cellgro) at 37\textdegree C in a humidified 5% CO\textsubscript{2} atmosphere. HCT116 ARE\textsuperscript{7}-Luciferase cells and the mutant were grown with 100\textmu g/mL Hygromycin B (InvivoGen, San Diego, CA, USA).
**Drug Treatment:** Cells were treated with TYMS inhibitors FUrA, FdUrd (Sigma-Aldrich Co., St. Louis, MO, USA), and RTX (AstraZeneca, Macclesfield, Cheshire, UK) at the indicated concentrations and times. 10µM Folinic Acid (Leucovorin, Sigma-Aldrich Co., St. Louis, MO, USA) was added to the cells along with FdUrd. When exogenous thymidine (deoxythymidine (dThd), Sigma-Aldrich Co., St. Louis, MO, USA) was added to the cells, it was co-incubated with TYMS inhibitors. Cells were treated with tBHQ at the indicated times and concentrations.

**Western Blotting:** Cells were counted, and total protein extracts were lysed directly in 1X sodium dodecyl sulfate (SDS) lysis buffer (62.5mM TRIS pH6.8, 10% Glycerol, 2% Sodium dodecyl sulfate, 0.01% Bromophenol blue with 10µL/mL 2-Mercaptoethanol added prior to use). For every 1x10^6 cells 125µL of 1X SDS was added. The appropriate concentration was determined experimentally. Extracts were sonicated at 40% amplitude on ice in bursts of 10 seconds three times using a QSonica sonicator. For cytoplasmic and nuclear extracts, cells were counted, harvested and prepared with the NE-Per® Nuclear and Cytoplasmic Extraction Reagents according to manufacturer’s instructions. Nuclei were isolated, and 1X SDS buffer was added to the nuclear pellet at 125µL 1X SDS per 2x10^7 cells. Nuclear suspensions were then sonicated on ice at 40% amplitude in 10 second bursts three times. Cytoplasmic extract concentrations were quantified using the Bradford assay (Bio-Rad, Hercules, CA, USA) with BSA as a protein standard. Lysates were run on a 4-15% gradient gel (Bio-Rad, Hercules, CA, USA), electrophoresed in SDS-PAGE buffer, transferred to nitrocellulose membranes and probed with the appropriate antibody overnight at 4°C. The membrane was washed
three times for 10 minutes with 1X PBS/0.05% Tween and incubated with the appropriate secondary antibody at a dilution of 1:1000 in 5% non-fat milk in 1X PBS/0.05% Tween for 1 hour at room temperature. ECL kit (GE Healthcare Life Sciences, Amersham ECL, Pittsburgh, PA, USA) was used to visualize the antigen-antibody complexes by chemiluminescence after washing the membrane. Antibodies were: anti-NRF2 rabbit monoclonal (12721, D1Z9C XP®, Cell Signaling Technology, Danvers, MA, USA) (1:1000 in 5% non-fat milk in 1X PBS/0.05% Tween), and anti-Luciferase mouse monoclonal (sc-74548, C-12, Santa Cruz Biotechnology, Dallas, TX, USA) (1:500 in 5% non-fat milk in 1X PBS/0.05% Tween). Anti-Lamin A rabbit polyclonal (sc-20680, H-102, Santa Cruz Biotechnology, Dallas, TX, USA) (1:2000 in 5% non-fat milk in 1X PBS/0.05% Tween) and anti-Lactate Dehydrogenase goat monoclonal (100-173, Rockland Antibodies & Assays, Gilbertsville, PA, USA) (1:2000 in 5% non-fat milk in 1X PBS/0.05% Tween) were used to control for equal loading of cytoplasmic and nuclear fractions, respectively. To control for equal loading in whole cell extracts anti-beta Actin-HRP mouse monoclonal (ab49900, AC-15, HRP, Abcam Inc. Cambridge, MA, USA) (1:20000) was used.

**RNA Extraction and qRT-PCR:** RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, Maryland, USA), according to the manufacturer’s protocol. RNA concentrations were spectrophotometrically measure at 260 and 280nm absorbance. For each reaction 1μg of RNA was reverse transcribed in a Thermal Cycler with iscript cDNA synthesis kit (BioRad, Hercules, CA, USA) according to the manufacturer’s protocol. The reactions were incubated for 5 minutes at 25°C, 30 minutes at 42°C, and then 5 minutes at 85°C.
with a final hold at 4°C. 1μl of cDNA was amplified using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s protocol. Reactions were run for one cycle at 95°C for 10 minutes to activate the SYBR Green, followed by 40 cycles at 95°C for 15 seconds, 60°C for 20 seconds and 72°C for 40 seconds followed by a dissociation curve using an Applied Biosystems 7300 Real Time PCR System. Relative mRNA levels were normalized to GAPDH, and calculated by the 2^{-\Delta\Delta Ct} method. Relative changes among samples were expressed as fold-changes. Primers (Integrated DNA Technologies Inc., Coralville, IA, USA) used for qPCR are listed in Table 2.1.

**Chromatin Immunoprecipitation (ChIP):** Chromatin Immunoprecipitation was carried out using the SimpleChIP® Plus Enzymatic Chromatin IP Kit with magnetic beads (Cell Signaling Technologies, Danvers, MA, USA), according to the manufacturer’s protocol. The protocol was optimized for HCT116 cells. Each immunoprecipitation reaction used 5x10^6 cells and 0.75μL of micrococcal nuclease. Chromatin was prepared and its concentration and level of shearing was verified. Concentrations were spectrophotometrically measured at 260 nm and 280 nm absorbance and the shearing was verified by running samples on a 1% agarose gel. Ideal shearing should show a 5 band pattern sizes no smaller than 100 bp and no larger than 900 bp. 10μg of cross-linked chromatin was used in each reaction. Anti-NRF2 was used at a dilution of 1:50, and 1μl of Normal Rabbit IgG antibody was used as a control. 2μl of the final DNA was amplified using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Reactions were run using the Applied Biosystems 7300 Real Time PCR System for
one cycle at 95°C for 10 minutes to activate the SYBR Green, followed by 40 cycles at
95°C for 15 seconds, 60°C for 1 minute, and 1 cycle of 95°C for 15 seconds, 60°C for 1
minute, 95°C for 15 seconds, and 60°C for 15 seconds. The levels of the IPs were
compared to the levels of the input samples to determine the % input using the
following equation. Percent Input = 2% × 2^[C(T) Input Sample − C(T) IP Sample], C(T) = C_T = Threshold
cycle of PCR reaction. Each sample was compared to its appropriate input. These values
were then compared to the IgG background control sample. Data was ultimately
expressed as a signal to noise ratio. The signal being the % input of the treatment group
and the noise being the % input of the IgG. Primers (Integrated DNA Technologies Inc.,
Coralville, IA, USA) used for qPCR are listed in Table 2.2. Regions used for ARE for each
gene are listed in Table 2.3.

Statistics: Two-tailed student T tests were used to determine the statistical significance
of the chromatin immunoprecipitation experiments. Results were considered significant
if p value was less than 0.05. These are show by placing an asterisk (*) above the bar.

2.3 Results

TYMS inhibitors induce NRF2 and target genes

Previous studies have shown chemotherapy drugs to increase NRF2 levels in a variety of
cancer cells (Bekele et al., 2016; Kang et al., 2014; Kwak et al., 2002; Takano et al., 2015;
Wang et al., 2014). Additionally we found that expression of several NRF2 target genes
was increased when cells were treated with TYMS inhibitors (Ozer et al., 2015). To verify
and extend these findings we treated HCT116, SW480, and HCT-15 cells with 10μM
FdUrd for 24 hours. Cells were harvested, RNA was extracted and qRT-PCR was used to
analyze the mRNA levels of NRF2 target genes. The concentration and time points were picked based upon previous studies in the lab. We were able to verify the microarray result. Figure 2.1 shows the increases in expression of NRF2 target genes in response to FdUrd. To further extend this, HCT116 cells were treated with 10μM FdUrd for 24 hours and cells were harvested for preparation of whole cell, cytoplasmic and nuclear protein extracts. Figure 2.2A illustrates the increase in total NRF2 protein when cells are treated with FdUrd. Additionally, the protein is exclusively nuclear, the concentration of which increases following drug exposures (Figure 2.2B).

Because TYMS is the sole source of dTMP for the cell, inhibition of this enzyme abolishes the production of thymidine which leads to DNA damage and eventually apoptosis. Addition of exogenous thymidine to the cell, decreases the dependency on TYMS, leading to decreased effects of TYMS-directed drugs. Ozer et al., demonstrated that the addition of thymidine reduced the levels of ROS as well as inhibiting the drug-induced apoptosis linking both of these to the depletion of thymidine levels (Ozer et al., 2015). In order to determine if the increase in target genes was a result of the depletion of thymidine, exogenous thymidine was added to cells simultaneously with FdUrd, and mRNA levels of target genes were measured. Induction by FdUrd was ameliorated with addition of exogenous thymidine (Figure 2.3), indicating that the induction of these target genes is due to the depletion of thymidine in the cell. Additionally, NAC, an antioxidant, was added to determine if the increase in ROS levels played a role in the induction. When NAC was added simultaneously with FdUrd to cells, we saw no
reduction in target gene expression (data not shown). This indicated that the intracellular levels of ROS do not have any effect on the induction of NRF2 target genes.

**tBHQ induces NRF2 to a greater extent than TYMS inhibitors**

We compared induction of NRF2 target genes by TYMS inhibitors to that of a known NRF2 inducer, tBHQ. HCT116 cells were treated with either 100μM tBHQ, or 10μM FdUrd for 3, 6, 12, 24, or 48 hours. Cells were harvested and protein extracts were prepared and analyzed. Figure 2.4A shows modest and slow increase in NRF2 protein following FdUrd treatment, as opposed to a rapid and large increase when cells were treated with tBHQ. Nuclear concentrations of NRF2 were greater in tBHQ treated cells as well (Figure 2.4B). Target gene mRNA levels were concentration and time-dependent in the cell lines (Figure 2.5A, B, C). We wanted to determine the effect on target gene induction when tBHQ concentration is lowered and NRF2 protein induction equals induction by FdUrd. After determining the necessary concentration, we looked at the effect on target gene induction. At low concentrations of tBHQ, we saw similar induction of NRF2 protein as when the cells are treated with FdUrd (Figure 2.6A). At 1, 5, and 10μM tBHQ, which showed about equal protein levels, showed almost no induction in target genes (Figure 2.6B). This experiment indicated to us that the induction of NRF2 protein levels by TYMS inhibitors was not just an increase in concentration. TYMS inhibitors may also be involved in post translational modifications of NRF2. tBHQ induction of NRF2 and its target genes is dependent on the concentration of NRF2 protein. Here we have two separate pathways of the concentration dependent (tBHQ)
and concentration independent (TYMS inhibitors) activation of NRF2 and its target genes.

**TYMS inhibitors induce NRF2 target genes through the ARE element**

To determine if induction of NRF2 target genes by TYMS inhibitors occurs through the ARE element, which is the known binding region of NRF2, HCT116 ARE\(^7\)-Luciferase and HCT116 ARE\(^7\)-MUT-Luciferase cells were used. These cells contain a stably transfected reporter gene comprised of 7 tandem ARE sequences with a 13 bp separator (Figure 2.7). The ARE sequences are from the consensus ARE sequence which has been determined experimentally. The mutant cells have the 7 tandem ARE sequences mutated so they are inactive. The mutant cells were used as a control to verify that the induction we see is due to activation of the ARE. These cells were treated with 10μM FdUrd or 100μM tBHQ for increasing times. Peak induction with FdUrd occurred at 48 hours, whereas it only took 6 hours to reach peak induction with tBHQ (Figure 2.8A and 2.8B respectively). Although induction of the reporter gene by FdUrd did not occur for 48 hours, it was higher than that of tBHQ. To further extend the earlier findings when we lowered the concentration of tBHQ to have equal expression of NRF2 protein levels to that of TYMS inhibitors, we repeated this experiment in the HCT116 ARE\(^7\)-Luciferase cells. Here we used the three lowest concentration of tBHQ, 1, 5 and 10μM, and treated the cells for 6 hours, along with FdUrd for 48 hours. When we analyzed the reporter gene expression, tBHQ treated cells showed no induction as these lower concentrations (Figure 2.9). Again, these findings indicate that TYMS inhibitors are inducing NRF2 through a non-canonical pathway. The mutant construct was used in all experiments as
a control to verify induction was specific to the ARE. In all experiments the mutant construct showed no induction with TYMS inhibitors or tBHQ, indicating that the induction of the reporter gene is specific to the ARE.

**TYMS inhibitors increase NRF2 occupancy of the ARE**

Previous studies have shown anti-cancer drugs increase the occupancy of NRF2 in the ARE region of the promoter of some NRF2 target genes (Chorley et al., 2012; Kwak et al., 2002). We wanted to extend these findings to colon cancer cells treated with TYMS inhibitors. HCT116, SW480 and HCT-15 cells were treated with 10μM FdUrd or 100μM tBHQ for 3, 6, 12, 24, or 48 hours. Proteins were crosslinked to chromatin using formaldehyde, and chromatin was then sheared. Antibodies to either NRF2 or Normal Rabbit IgG was incubated with the chromatin preparation, co-precipitating DNA was isolated, and DNA samples were analyzed by qRT-PCR for the presence of the ARE regions. Figure 2.10A shows an increase in occupancy of ARE regions from each of 6 genes by NRF2 in HCT116 following exposure to FdUrd or tBHQ. Figures 2.10B and 2.10C show the increase in occupancy of these regions by NRF2 in SW480 and HCT-15, respectively. As seen in the figures, the occupancy is increased by TYMS inhibitors as early as 3 hours for some of the regions. Like previously, the effect of tBHQ was greater than FdUrd.

To determine if this increase in ARE occupancy was due to the depletion of thymidine pools, we added exogenous thymidine simultaneously with FdUrd for 3, 6, and 12 hours. Figure 2.11 shows that the increase in occupancy is dependent on the
depletion of thymidine levels. When exogenous thymidine is added with FdUrd there is no increase in occupancy.

2.4 Discussion

The current data shows that TYMS inhibitors increase and activate NRF2 and its target genes as shown in Figures 2.1 and 2.2. This increase is due to the depletion of thymidine levels in the cells, as determined by the absence of induction when exogenous thymidine is added (Figure 2.3). However, when these cells were treated with N-acetylcysteine (NAC), an antioxidant, there was no reversal of induction with FdUrd, indicating that the induction is not due to the increases in ROS levels. These results indicate that the depletion of thymidine levels plays a role in the activation of NRF2 whereas the intracellular levels of ROS have no role in the activation of NRF2.

Furthering this, when the effects of tBHQ, a classical NRF2 activator, were compared to TYMS inhibitors, the protein induction levels varied quite a bit, with tBHQ inducing the protein concentration very quickly and to a much greater extent. FdUrd on the other-hand took 48 hours to show peak induction and was much lower than that of tBHQ as seen in Figure 2.4. These results indicate the tBHQ can activate NRF2 much faster and to a greater extent. The mechanism by which tBHQ activates NRF2 is well known. tBHQ modifies the cysteines on KEAP1 allowing for the activation of bound NRF2 and inhibits the degradation of newly synthesized NRF2. While TYMS inhibitors do increase the protein levels of NRF2, we do not know the mechanism driving this induction. Taking the induction of target genes into the mix, we saw a wide variation of induction between tBHQ and TYMS inhibitors. Taking both the protein levels and the
target gene levels into account, it is clear that the concentration of NRF2 protein levels may not be the sole factor in induction of downstream targets. TYMS inhibitors may very well be activating co-factors, inactivating inhibitors, and/or post-translational modifiers of NRF2 and/or histones. To further extend the theory that TYMS inhibitor induction and activation of NRF2 is not solely an increase in concentration, we lowered the concentration of tBHQ so that the NRF2 protein levels were equal to that of TYMS inhibitors. At these lower concentrations of tBHQ, target genes showed no induction (Figure 2.6B). These results further extend the theory that TYMS inhibitors are activating NRF2 through a mechanism other than increased concentration.

Because NRF2 is known to activate its target genes through the ARE element within gene promoter regions, we sought to determine if TYMS inhibitors were working through the ARE. As seen in Figure 2.8, both tBHQ and FdUrd induce the reporter construct, indicating that they induce the target genes through the ARE. This reporter gene allows us to isolate the ARE element from the rest of the promoter region and determine the differences in induction by TYMS inhibitors and tBHQ. We have shown that they both induce the reporter gene, but we want to further get at the question as to the difference in induction between TYMS inhibitors and tBHQ. Furthering the earlier findings, we used the lower concentrations of tBHQ (1, 5, and 10μM). Our results showed that at these lower concentrations of tBHQ where there was an equal concentration of NRF2, there was no induction of the reporter gene (Figure 2.9). The results along with the findings from earlier, strongly indicate that the concentration of NRF2 may not be the most important factor in activation and induction of target genes.
Since there are limited studies on regulation of the occupancy of the ARE region by anti-cancer drugs, we decided to expand the study and determine if TYMS inhibitors are increasing the occupancy of the ARE element by NRF2. We utilized ChIP technology to undertake this part of the study. Cells treated with TYMS inhibitors, showed an increase in occupancy of ARE regions tested from each of 5 distinct genes. There were differences observed between cell lines as seen in Figure 2.10. The differences are likely due to the heterogeneity of the cell lines. Interestingly the increased occupancy of the ARE does not necessarily have any bearing on the induction of the target gene. For example, for the NQO1 gene, FdUrd increases the occupancy of its ARE but there is little to no induction of mRNA levels. This indicates that occupancy does not necessarily imply activity of the target gene. This can also be an additional way in which TYMS inhibitors are activating NRF2. This increase in occupancy of the ARE is due to the depletion of dTMP levels in the cell, based on the observation that exogenous thymidine inhibits the increase in occupancy.

Previous studies are in accordance with our initial results that show TYMS inhibitors increase the protein levels of NRF2 as well as target genes (Bekele et al., 2016; Kang et al., 2014; Kwak et al., 2002; Takano et al., 2015; Wang et al., 2014). We were the first to investigate the difference in activation of NRF2 by TYMS inhibitors and the classical NRF2 activators. This is important because recently the role of NRF2 in chemoresistance has come to light. By better understanding how these anti-cancer drugs are able to activate NRF2, we may better be able to combat this resistance. In addition to the modest increase in NRF2 protein levels, we demonstrated that TYMS
inhibitors are likely doing more than just increasing NRF2 levels. The microarray showed many genes that were not involved in redox metabolism but were upregulated by treatment with FUra. These genes may be involved in activating the co-factors, such as Maf, or inactivating inhibitors like Bach1 (Blank, 2008; Hur and Gray, 2011; Moon and Giaccia, 2015). Additionally, they may also be involved in post-translational modifiers of NRF2 or histones near the promoter regions of target genes (Choudhury et al., 2010; Kawai et al., 2011; Sun et al., 2009). One study done in breast cancer cells showed that with treatment of FUra there was an increase in acetylation of H3 and H4 (Hernandez-Vargas et al., 2006). A few studies have also shown the importance of signaling and phosphorylation of NRF2 (Apopa et al., 2008; Bloom and Jaiswal, 2003; Jain et al., 2008). We will later show that FdUrd increases the phosphorylation of AKT, however we rule out the PI3K/AKT pathway in the activation of NRF2. Because FdUrd increases active AKT, it is possible that other signaling molecules may be affected by FdUrd as well. Two of these possible mechanisms are investigated further in chapter 4 and the appendix. Furthermore, we were the first to determine that TYMS inhibitors increase the occupancy of the ARE, that the increase is due to the depletion of thymidine levels, and that this occupancy does not imply activation of the gene. tBHQ also increased the occupancy and to a greater extent than TYMS inhibitors. This increase in occupancy is likely due in part to the increase in NRF2 protein levels. However, this is not the sole factor for this increase, as the increase in some of the genes can be seen as early as 3 hours with FdUrd treatment, before a detectable increase in NRF2 protein concentration. This again points to TYMS inhibitor involvement in a non-canonical
activation of NRF2. This data additionally indicates that co-factors are likely involved in the activation of target genes, and TYMS inhibitors may be involved in activating these or altering the activity of the promoter region. Here again we see the possibility of TYMS inhibitors altering the acetylation near the promoter regions (Choudhury et al., 2010; Hernandez-Vargas et al., 2006).

In short, TYMS inhibitors increase NRF2 protein levels, target genes, and occupancy of the ARE in a thymidine dependent manner. This activation is not solely concentration dependent, and it likely involves a non-canonical pathway. We have determined that TYMS inhibitors increase NRF2 levels, we wanted to extend on this and determine if this induction leads to an increased resistance against chemotherapy. Here we will knockdown NRF2 through siRNA technology and further knockout NRF2 through CRISPR/CAS 9 technology.
Table 2.1. **qRT-PCR Primers for mRNA analysis**. Primers above were used to analyze the levels of mRNA of the NRF2 target genes.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers (5’ to 3’)</th>
</tr>
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<tbody>
<tr>
<td>AKR1B10</td>
<td>sense: 5’ – ACCTGTTCATCGTCAGCAAG – 3’</td>
</tr>
<tr>
<td></td>
<td>antisense: 5’ – CATCCCCAGACTTTGAATCCC – 3’</td>
</tr>
<tr>
<td>AKR1C1</td>
<td>sense: 5’ – GCGTGGAGAAGTAAAGATG – 3’</td>
</tr>
<tr>
<td></td>
<td>antisense: 5’ – CTTGTTGAAGTAAAGATG – 3’</td>
</tr>
<tr>
<td>ALDH3A1</td>
<td>sense: 5’ – TGCTACGTGGACAAGAAGCTG – 3’</td>
</tr>
<tr>
<td></td>
<td>antisense: 5’ – CACCAATTGTTCTGATCGAG – 3’</td>
</tr>
<tr>
<td>CBR3</td>
<td>sense: 5’ – AAGCTGAGATGACACTAGTAAAGAC – 3’</td>
</tr>
<tr>
<td></td>
<td>antisense: 5’ – AAGCCCTTAAACACTGCAAAC – 3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>sense: 5’ – TCGCTGAGCAGGAGAAAGAAG – 3’</td>
</tr>
<tr>
<td></td>
<td>antisense: 5’ – GAGAGTGGGTCGCTGCTG – 3’</td>
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<td>HMOX1</td>
<td>sense: 5’ – TCAGGCAGGAGGTGATAGAAG – 3’</td>
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<td>antisense: 5’ – TGCGGTGTCATGGGTCAAG – 3’</td>
</tr>
<tr>
<td>HSPB8</td>
<td>sense: 5’ – AAAGATGGGATAAGTGGGAGGTG – 3’</td>
</tr>
<tr>
<td></td>
<td>antisense: 5’ – GGGAAAGTGGAGAATACTG – 3’</td>
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<tr>
<td>NQO1</td>
<td>sense: 5’ – TCAACGAGAGGCTAGGTC – 3’</td>
</tr>
<tr>
<td></td>
<td>antisense: 5’ – TACAGCATGGTTGAGGAGG – 3’</td>
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<tr>
<td>SERPINE1</td>
<td>sense: 5’ – GTGGAAGGTTCGAGGGGAGGAAGGTG – 3’</td>
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<tr>
<td></td>
<td>antisense: 5’ – GAAATGGAGGCAATTCAAG – 3’</td>
</tr>
<tr>
<td>SPP1</td>
<td>sense: 5’ – CAGTGATTGCTTTGCTCC – 3’</td>
</tr>
<tr>
<td></td>
<td>antisense: 5’ – ATTCTGCTTGAGATGGGTC – 3’</td>
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Table 2.2. *qRT-PCR Primers for ARE regions from ChIP experiments*. The above primers were used to identify if TYMS inhibitors were increasing the occupancy of the ARE regions in the promoters of the genes.

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<thead>
<tr>
<th>Genes</th>
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<td>AKR1C1</td>
<td>sense: 5’ – CCAGGAGTGTCGCAAGGT – 3’&lt;br&gt;antisense: 5’ – CCCTACAATCTACTCGGGTTGATG – 3’</td>
</tr>
<tr>
<td>CBR3</td>
<td>sense: 5’ – CTTAGGCGATAGACGGGCAAG – 3’&lt;br&gt;antisense: 5’ – GAGCCCATACTCAACCAATCAG – 3’</td>
</tr>
<tr>
<td>GCLM</td>
<td>sense: 5’ – CTCGGCTAGATTCTGCTT – 3’&lt;br&gt;antisense: 5’ – CGGGAGAGCTGATTCCAA – 3’</td>
</tr>
<tr>
<td>HMOX1 – 4KB</td>
<td>sense: 5’ – GAATGCGTGAACGCAGATTCC – 3’&lt;br&gt;antisense: 5’ – CCTGCGTACCATGGAAGC – 3’</td>
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<tr>
<td>HMOX1 – 9KB</td>
<td>sense: 5’ – CCGCAGTGAATCTCCATCCGA – 3’&lt;br&gt;antisense: 5’ – ATGCTCGAGATCCACTCCA – 3’</td>
</tr>
<tr>
<td>NQO1</td>
<td>sense: 5’ – CCCTTTAGCCTTTGCGACGAA – 3’&lt;br&gt;antisense: 5’ – TGCCACCAGGGAAAGTGTGTAT – 3’</td>
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</tbody>
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Table 2.3. AREs located in the regions amplified by primers for ChIP experiments. Promoter regions contain 1-4 ARE consensus sequences. All regions are located upstream of the transcriptional start site except for GCLM which is located in the 5’ untranslated region.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence 1</th>
<th>Sequence 2</th>
<th>Sequence 3</th>
<th>Sequence 4</th>
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</thead>
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<td>-6178 A G G G T G A C T C A G C A T C T</td>
<td>-6194</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBR3</td>
<td>-2479 T A T G T G A C C C T G C A G A G</td>
<td>-2463</td>
<td></td>
<td></td>
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<tr>
<td>GCLM</td>
<td>147 A C A A T G A C T A A G C A G A A</td>
<td>131</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMOX1 4KB</td>
<td>-3899 C T G G T G A C T C A G C A A A A</td>
<td>-3915</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMOX1 9KB</td>
<td>-8949 A C C G T G A C T C A G C G A A A</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-8978 A C C G T G A C T C A G C G A A A</td>
<td>-8994</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-9007 G C G G T G A C T T A G C G A A A</td>
<td>-9023</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-9036 A C C G T G A C T C A G C A G G A</td>
<td>-9052</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NQO1</td>
<td>-389 A C A G T G A C T C A G C A G A A</td>
<td>-373</td>
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</table>
Figure 2.1. mRNA fold changes in human colon tumor cells. The above NRF2 target genes are a representative subset of several genes tested. HCT116 untreated is set as the control and the 4 other cell lines are set relative to it. Untreated cells are indicated by the white bars and FdUrd treated cells are indicated by the black bars. A variety of basal levels and induced levels can be seen across the 5 cell lines and 6 genes. FdUrd increases NRF2 target genes to varying degrees throughout the 5 cell lines. NQO1 a well-known NRF2 target gene does not show induction with FdUrd treatment in any of the cell lines.
Figure 2.2. Protein levels of NRF2. A shows the increase in NRF2 total protein when cells are treated with FdUrd for either 24 or 48 hours. Actin is used as a loading control. B shows the increase in nuclear localization of the NRF2 protein when cells are treated with FdUrd. Here Lamin and LDH are used as loading controls as well as to verify cytoplasmic and nuclear extracts were clean.

Figure 2.3. Induction dependent on depletion of thymidine levels. Thymidine alone has no effect on the levels of target genes. FdUrd shows induction and when FdUrd and exogenous thymidine are added together the induction by FdUrd alone is suppressed. This indicates that the induction is due directly to the inhibition of TYMS.
Figure 2.4. **Induction of NRF2 protein with FdUrd or tBHQ.** A shows a time course from 3-48 hours of the induction of total NRF2 protein with either FdUrd or tBHQ. FdUrd induction peaks at 48 hours and tBHQ between 3 and 6 hours. B shows total NRF2 protein levels in three cell lines. Induction by FdUrd varies amongst cell lines yet tBHQ is still stronger. C shows the localization of NRF2 protein mostly to the nucleus with some in the cytoplasm with tBHQ treatment.
Figure 2.5. **NRF2 target gene induction with FdUrd or tBHQ.** A HCT116, B SW480, and C HCT-15 show the relative increase in NRF2 target genes when cells are treated with FdUrd or tBHQ for 3-48 hours. When cells are treated with FdUrd there is a slow increase in many of the target genes, however with tBHQ treatment many of the target genes are induced quicker and to a greater extent.
Figure 2.6. **Lowering tBHQ concentration so NRF2 protein levels are equal.** A shows the western to determine a concentration of tBHQ where the NRF2 induction matches that of FdUrd. B shows the mRNA levels corresponding to the lower tBHQ concentrations. There is little to no induction of target genes when the tBHQ concentration is lowered to 1 or 5μM which is where the NRF2 protein levels equal those by FdUrd.
Figure 2.7. **Depiction of the ARE reporter gene in HCT116 ARE$^7$-Luciferase and mutant.** There are 7 repeat ARE consensus sequences with a 13 basepair spacer between and a minimal promoter with luciferase as a reporter. The mutant has those 7 ARE consensus sequences mutated to be inactive.

![Diagram of ARE reporter gene in HCT116 ARE$^7$-Luciferase and mutant](image)

Figure 2.8. **Expression of the reporter gene in cells treated with FdUrd or tBHQ.** A shows the induction of the reporter gene by FdUrd which peaks at 48 hours. B shows the induction by tBHQ compared to FdUrd from 6-48 hours. The tBHQ induction peaks at 6. Although the tBHQ induction peaks earlier than FdUrd, FdUrd induces the reporter gene to a greater extent.

![Expression of reporter gene in cells treated with FdUrd or tBHQ](image)
Figure 2.9. **Induction of the reporter gene when tBHQ concentration is lowered.** As seen in the earlier figures when using the lower concentration of tBHQ concentration there is no induction of the reporter gene. Lanes 7-12 shows the cell line with the mutant ARE reporter to verify that the induction is due to the ARE itself.
Figure 2.10. Increase in occupancy at ARE regions. A HCT116, B SW480, and C HCT-15 show the increase in occupancy at 6 or 5 ARE regions respectively from 3-48 hours in cells treated with either 10μM FdUrd or 100μM tBHQ. FdUrd increase the occupancy at all promoter regions analyzed but again tBHQ increases the occupancy to a greater extent. * indicates p value <0.05.
Figure 2.11. **Increase in occupancy is dependent on thymidine depletion.** Three target genes ARE region were tested and it shows that the increase in occupancy by FdUrd is dependent on the deprivation of thymidine as the increase disappears when thymidine is added simultaneously with FdUrd. Again, this indicates that the increase is due directly to the inhibition of TYMS.
Chapter 3
NRF2 modulates sensitivity to TYMS inhibitors

3.1 Introduction

NRF2 has been described as a double-edged sword for its early protective properties in preventing cancer and generating resistance to chemotherapy (Hayes and McMahon, 2006). There has been much research towards finding activators of NRF2 to prevent cancer and many other chronic diseases where NRF2 has been implicated. There has been little effort in looking for inhibitors of NRF2, and the few that have been found are not effective in all cells. They may activate in one cell line, inhibit in another, and do nothing in a third (Paredes-Gonzalez et al., 2015).

Recent studies have shown several oncogenes to upregulate NRF2 expression (DeNicola et al., 2011; Niture et al., 2011; Shelton and Jaiswal, 2013). This upregulation decreases the levels of ROS in the cell, allowing escape from cell death. NRF2 itself has been implicated in upregulating anti-apoptotic factors leading to a decrease in drug-induced apoptosis (Niture et al., 2014; Shelton and Jaiswal, 2013). Similar studies have implicated NRF2 in reprogramming of nucleotide synthesis to maintain cell growth (Mitsuishi et al., 2012; Niture et al., 2014; Shelton and Jaiswal, 2013). Most recently, NRF2 has been shown to be constitutively activated in many cancers, resulting in a poorer prognosis (Hu et al., 2013; Jiang et al., 2010; Kim et al., 2010; Lister et al., 2011;
Ohta et al., 2008; Padmanabhan et al., 2006; Rushworth et al., 2012; Sasaki et al., 2013; Singh et al., 2006; Solis et al., 2010; Zhang et al., 2010). A few studies have shown that suppressing NRF2 in cancer cells can increase the sensitivity to chemotherapeutic drugs (Samatiwat et al., 2015; Zhong et al., 2013).

Two recent studies with TYMS inhibitors showed that NRF2 and antioxidants decreased the efficacy of FUra on apoptosis (Fu et al., 2014; Kang et al., 2014). These studies did not further investigate the mechanism behind the involvement of NRF2 in this resistance. Because of recent studies showing that over-expression of NRF2 creates an environment resistant to many chemotherapeutic agents, there is much interest in trying to find a way to inhibit/decrease expression in cancer to re-sensitize the tumors to these drugs. Many chemotherapeutic drugs work by increasing ROS-mediated toxicity in the cell to lead to cell death. NRF2 in cancer cells can decrease the impact of ROS, thereby preventing cell death. Because a few studies have shown that suppression of NRF2 will lead to an increase in cellular apoptosis, we decided to determine if the apoptotic index in response to TYMS inhibitors is modulated by activation of NRF2.

The previous chapter showed that TYMS inhibitors increase levels of NRF2 as well as its target genes. Therefore, we determine if down-regulating NRF2 expression affects apoptotic response to drug exposure. siRNA targeting NRF2 was used to “knockdown” NRF2 and CRISPR/CAS9 technology was used to completely “knockout” expression of the protein.
3.2 Materials and Methods

**Cell Culture:** HCT116, SW480, HCT-15, HCT116 ARE\(^7\)-Luciferase, and HCT116 ARE\(^7\)-MUT-Luciferase cell lines were used. HCT116/200 is a TS-overproducing derivate of HCT116 and has previously been described in (Berger et al., 1988). HCT116 p53\(^{+/+}\), and HCT116 p53\(^{-/-}\) was generously given to us by Dr. Philip Buckhaults. Cells were maintained under the same conditions as previously stated in Section 2.2.

**Drug Treatment:** TYMS inhibitors and folinic acid were used as stated in section 2.2.

**siRNA Transfection:** All cell lines were transfected with either a control scrambled siRNA or a mixture of 4 targeting NRF2 siRNAs (Dharmacon, Lafayette, CO, USA). Cells were transfected according to the recommended protocol from Dharmacon with the following changes: final concentration of siRNA was 50nM and DharmaFECT 4 transfection reagent was used (Dharmacon, Lafayette, CO, USA). After 24 hours exposure to the siRNA and transfection reagent, fresh media was added and cells were harvested directly or treated further with TYMS inhibitors prior to harvest.

**Western Blotting:** Cell preparation and western blotting was carried as described in Section 2.2

**RNA Extraction and qRT-PCR:** RNA isolation and qRT-PCR was as described in Section 2.2. Additional primers used are listed in Table 3.1.

**Measurement of Apoptotic Cell Death:** To quantitate apoptotic cell death, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays were applied, using the In-Situ Cell Death Detection Kit, POD (Roche Applied Science, Indianapolis, IN, USA). Cells were transfected with siRNA for 24 hours and then treated
with TYMS inhibitors for 48 hours at the indicated concentrations. Cells were stained according to the manufacturer’s protocol following hematoxylin counterstaining. Slides were observed under a light microscope at 400X magnification, photos were taken and the apoptotic cells were counted manually based on the staining and morphology. Those that exhibited brown nuclear staining due to the fragmented nuclear chromatin were determined to be apoptotic. The apoptotic index was calculated as the ratio of apoptotic cells/total cells. At least 1,000 cells from several microscopic fields were counted in each determination.

**CRISPR/CAS9:** Three guide RNAs which targeted different regions of NRF2 were cloned into a lentiviral vector by the Viral Vector Facility at the USC School of Medicine along with a vector control by Dr. Boris Cantor. The sequences were created very specifically to minimize off-target effects. Sequences can be found in Table 3.2. HCT116 cells were plated in 6 well plates at a concentration of 250,000 or 500,000 cells in 3 mL of media. The next day 1 mL of fresh media along with 10μl of concentrated virus was added to each well. Cells were incubated with the virus for 8 hours at which point an additional 1 mL of media was added. Cells were then allowed to grow for 48 hours, after which fresh media containing 5μg/mL of puromycin was added as a selection agent. Selection agent was applied for about 2 weeks, after this cells were grown without puromycin. After 2 weeks, cells were tested for NRF2 expression by Western blotting, to determine the knockout efficiency. One population, termed 143 showed the most efficient reduction in NRF2 expression. Individual clones were generated by plating a single cell in each well of a 96 well plate. Cells were allowed to grow and were expanded. The clones were treated
with tBHQ (100μM for 6 hours) to identify those which completely lacked NRF2. These clones (A5, A15, A17, and A24) were then further tested and 400bp of the DNA around the gRNA was PCRed and sent to be sequenced to verify the mutations in the NRF2 gene. Truncated protein sequences were predicted based on the mutations found in the DNA sequences.

**Statistics:** Two-tailed student T tests were used to determine the statistical significance of the TUNEL assay experiments. Results were considered significant if p value was less than 0.05 and is indicated by an asterisk (*).

3.3 Results

**Knockdown of NRF2 decreases target genes and increases apoptosis**

A few studies have shown that by suppressing NRF2 cells and tumors that were previously resistant to chemotherapy are now re-sensitized (Lee et al., 2015; Ma et al., 2012; Samatiwat et al., 2015; Wang et al., 2008). To extend these findings and determine if NRF2 is a constraining factor in response to TYMS inhibitors in colon cancer, we used a mixture of siRNAs targeting NRF2 to knock down its expression. These siRNAs were created by Dharmacon to limit off target effects. NRF2 expression was knocked down about 70% by introduction of a mixture of siRNAs specifically for the transcription factor (Figure 3.1A). Furthermore, many of its target genes were decreased about 50% (Figure 3.1B). Figure 3.2 shows that in the transfected cells target gene expression in cells treated with 10μM FdUrd for 48 hours are still induced even though NRF2 levels are reduced. To measure apoptosis, cells were treated with TYMS inhibitors for 48 hours and the TUNEL assay was used. HCT116 cells transfected with NRF2 specific
siRNAs and treated with TYMS inhibitors showed an increase in apoptotic index about 1.7-fold as compared to drug-treated cells exposed to the control siRNAs (Figure 3.3A). We repeated this in other cell lines, including HCT116/200 (which is resistant to FdUrd) to determine if this finding was universal. HCT116/200 showed a similar result as HCT116, about a 1.7-fold increase. However, SW480 and HCT-15 did not exhibit a significant increase in drug-induced apoptosis in the following siRNA knockdown of NRF2 (Figure 3.3B). We noted that both SW480 and HCT-15 are mutant for p53, so we determined if this had any bearing on the results. We compared HCT116 p53+/+ and HCT116 p53−/− (Figure 3.3B) and found that the wild type p53 cell line showed about a 1.7-fold increase in drug-induced apoptosis whereas the p53 null cell line did not show a significant increase in apoptosis in the knockdown. Therefore, we believe that there may be a NRF2 dependent pathway for drug-induced apoptosis and a NRF2 independent pathway. These 2 pathways may also have to do with the status of p53 in the cells.

**Knockout of NRF2 sensitizes cells to TYMS inhibitors**

Three guide RNAs specific for NRF2 were initially tested for the ability to reduce NRF2 expression following tBHQ treatment (Figure 3.4). From the three guide RNAs it was clear that the population termed 143, showed the best initial knockdown of NRF2. Since there was still NRF2 present, this population was cloned to find the cells which completely lacked any NRF2 expression. The clones were then treated with tBHQ to determine NRF2 expression. Figure 3.5A shows the western and the 4 clones A5, A15, A17, and A24 were chosen for further analysis. These clones are identified in the boxes. To verify that the NRF2 gene was mutated, these 4 clones were sequenced (Figure 3.5B).
Sequencing revealed that both alleles were mutated by either an insertion or deletion. The mutations in the DNA predicted truncated protein sequences. The clones were treated with 1μM FdUrd or 100μM tBHQ and the target gene expression was analyzed by RT-PCR. The basal levels of target genes were reduced, some to almost undetectable levels, and induction by both TYMS inhibitors and tBHQ was wiped out (Figure 3.6). Furthermore, the clones were treated with 100nM FdUrd for 48 hours to determine the apoptotic index. The concentration of FdUrd was lowered following initial experiments with the clones which showed these cells exhibited a higher sensitivity to TYMS inhibitors and a lower concentration was required to determine differences in apoptosis. The 4 clones when treated with TYMS inhibitors showed a 2-4-fold increase in the apoptotic index when compared to the drug-treated control vector (Figure 3.7).

3.4 Discussion

Extending from what we found in the previous chapter that NRF2 and its target genes are induced by TYMS inhibitors, here we determined that by reducing NRF2 levels, we are able to increase cellular sensitivity to TYMS inhibitors. This is in line with several other studies that showed reducing NRF2 levels by siRNA or shRNA increases the sensitivity to chemotherapeutic drugs (Lee et al., 2015; Ma et al., 2012; Samatiwat et al., 2015; Wang et al., 2008).

Initially we knocked down NRF2 and saw a modest increase in drug-induced apoptosis (Figure 3.3). Here we also found that the status of p53 may have an impact on whether the cells exhibit an NRF2 dependent or independent drug-induced apoptosis. HCT116 and HCT116 p53+/+ both showed a significant increase in apoptosis whereas
SW480, HCT-15 and HCT116 p53\(^{-}\) did not. The latter of the three cells lines either have no p53 or a mutant p53. However, this is not the only difference between HCT116 and SW480 cell lines. p53 is a tumor suppressor protein, and the loss of wild-type p53 activity is frequently found in many different types of tumors (Muller and Vousden, 2014). Wild-type p53 is a powerful inducer of apoptosis in tumor cells. The reasoning here is in line with why we believe the wild-type cell lines exhibited an increase in apoptosis when NRF2 was reduced, whereas those with mutant or lacking p53 did not show such an increase. NRF2 expression may be more important in the sensitivity to chemotherapeutic agents when p53 is wild-type. Some of the limitations with only using a knockdown is there is still a small amount of expression of NRF2 in the cells. Additionally, within the population of cells there may be some which reduced NRF2 expression to very low levels, while others are unaffected. While we saw a modest increase in apoptosis, we expected to see a greater increase, and this modest increase may be due to the small amount of remaining NRF2, or the variation in transfection efficiency of the population of cells. Additionally, the drug-induced expression of the target genes may also be due to the small amount of NRF2. To more closely determine if NRF2 has a role in the sensitivity to chemotherapy agents, we decided to knockout NRF2 expression totally.

Since there were no available NRF2 null cells we decided to use the new CRISPR/CAS9 technology to create such cells. By using this technology, one must consider that there may be off target effects of the guide RNA. Additionally, there may be effects from the selection agent. With the use of a specialized software and the Viral
Vector Facility at USC School of Medicine the lentiviral vector guide RNA’s had limiting off target effects. The overall population of HCT116 cells from the three initial guide RNA’s all had some NRF2 remaining. After choosing the population termed 143, which showed the least expression of NRF2, we cloned this population, identified the clones with no detectable NRF2 protein, and further studied these to determine the effects on the inducibility of target genes, and the effect on apoptosis. Four clones were chosen to be analyzed, to reduce any clonal effects. Both the basal levels and the inducibility of the target genes are decreased to almost undetectable (Figure 3.6). Furthering this, we used the TUNEL assay to determine drug-induced apoptosis in the CRISPR/CAS9 clones. Here we saw that the NRF2 null clones showed a dramatic 2-4-fold increase in drug-induced apoptosis (Figure 3.7). These findings are currently being repeated in SW480, HCT116 p53+/+ and HCT116 p53-/- cells. Initial findings in SW480 cells indicate that the drug-induced apoptosis does not increase in the NRF2 null clones in this line, and that many of the target genes while reduced and not inducible with tBHQ, are inducible with FdUrd. Taking these findings along with the ones found using the siRNA for NRF2, it still appears that there may be two paths for the apoptosis. In HCT116 cells it appears that decreasing the levels of NRF2 is important to the sensitivity of chemotherapeutic agents (NRF2 dependent), whereas in SW480 cells decreasing the levels of NRF2 do not impact the sensitivity to chemotherapeutic agents (NRF2 independent). Again, this may be due to the status of p53. As stated earlier, HCT116 is wild-type and SW480 is mutant for p53. Again, this is not the only difference between these two cell lines. Further studies are currently being undertaken in our lab using the HCT116 p53+/+ and HCT116 p53-/- cells to
investigate if the initial finding with the siRNA still holds true and if p53 status has a larger role in the resistance. This is an important finding in furthering the research into NRF2’s role in resistance as well as if the status of p53 also plays a role (Rotblat et al., 2012). Some recent studies have shown there to be a positive feedback loop between NRF2 and p53, while other studies have shown MDM2 as a target of NRF2 (Kalo et al., 2012; You et al., 2011). The discrepancies between the reports indicate that the relationship between p53 and NRF2 may be dependent on the cellular and biological context. Some possible future directions may be to also look into possible restoration of p53 if the finding holds up to be true to determine if by re-establishing wild-type p53 in the NRF2 knockout cells we can increase sensitization to chemotherapeutic drugs. By looking into the relationship between NRF2 and p53 and drug-induced apoptosis, this will help to determine the correct choice of treatment for patients depending on the molecular signature of the tumor.
Table 3.1. Additional primers used for qRT-PCR of mRNA levels.

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<th>Genes</th>
<th>Primers (5’ to 3’)</th>
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<td>NRF2</td>
<td>sense: 5’ − ACATCCAGTCAGAAACCAGTG − 3’</td>
</tr>
<tr>
<td></td>
<td>antisense: 5’ − AAGTGCCGAAGAAACCTC − 3’</td>
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Table 3.2. Sequences for the guide RNAs targeting the NRF2 gene. Above indicate the three sequences created to target the NRF2 gene in the CRISPR/CAS9 experiments.

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<th>gRNAs</th>
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</tr>
<tr>
<td>pBK142 (142)</td>
<td>5’ − TCGATGTGACGGTATATC − 3’</td>
</tr>
<tr>
<td>pBK143 (143)</td>
<td>5’ − GACTGGGCTCTCGATGTGAC − 3’</td>
</tr>
</tbody>
</table>
Figure 3.1. NRF2 and target gene expression with siRNA targeting NRF2. A shows the downregulation of NRF2 with an NRF2 targeting siRNA. Consistent downregulation of about 70% was achieved. B shows the subsequent target gene expression when NRF2 is downregulated. Target gene expression was reduced about 40-50%.
Figure 3.2. **Target gene expression in NRF2 knockdown.** Target genes are reduced when NRF2 is knocked down, however there is still induction when cells are treated with TYMS inhibitors.
Figure 3.3. **Apoptotic index in HCT116 and other colon tumor cell lines.** A shows the apoptotic index for HCT116 with a siRNA for NRF2 with TYMS inhibitors. There is a significant increase for all TYMS inhibitors with a siRNA for NRF2. The drug-induced apoptosis is due to inhibition of TYMS, because when thymidine is simultaneously added with FdUrd, apoptosis is inhibited. B shows the apoptotic index in HCT116 as well as other cells lines. A significant increase was seen in HCT116, and HCT116/200 but not in SW480 or HCT-15. Furthermore, HCT116 p53+/+, the parent to HCT116 p53−/−, showed a significant increase in apoptosis whereas the HCT116 p53−/− did not. The last two cell lines were obtained from another lab which can account for the differences in HCT116 and HCT116 p53+/+ apoptotic index. * indicates p value <0.05.
Figure 3.4. **Initial knockout of NRF2 with three different guide RNAs.** The western shows the levels of NRF2 after cells were transduced with three different guide RNAs targeting NRF2. The guide RNA 143 showed the most efficient knockout of NRF2.

![Western blot images showing NRF2 and Actin levels for different guide RNAs and treatments.](image)

Figure 3.5. **Analysis and mutations of clones.** A shows the western analysis of a variety of isolated clones. All clones lacking NRF2 are indicated in red and were analyzed. Those boxed in are presented in further detail here. As seen in the western there is no NRF2 present even when treated with tBHQ. B shows the four different mutations around exon 4 in the DNA that were found in the four clones.

![Western blot images showing NRF2 and Actin levels for different clones and treatments.](image)

<table>
<thead>
<tr>
<th>Clone</th>
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</tr>
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</tr>
<tr>
<td>15</td>
<td>Δ17/Ins 1</td>
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<td>24</td>
<td>Δ91/Ins 1</td>
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</table>
Figure 3.6. **Target gene expression in NRF2 null clones.** Three NRF2 target genes in the four NRF2 null clones plus the vector were analyzed for their expression basally and when treated with both TYMS inhibitors and tBHQ. Basal levels were reduced and there were no drug induced levels with either FdUrd or tBHQ.
Figure 3.7. **Apoptotic Index in NRF2 null clones.** TUNEL experiments showing the apoptotic index in the vector and four clones. The apoptosis increases in NRF2 null clones from about 1.8-3.36-Fold depending on the clone. All four clones showed a significant increase in apoptosis. * indicates p value <0.05.
Chapter 4
Post translational modifications

4.1A Introduction

We already know that TYMS inhibitors induce both oxidant and antioxidant factors. Our microarray analysis showed many genes that were induced by FUra, so it is reasonable to believe that TYMS inhibitors are inducing many other changes within the cell as well. Some of these other changes could be activating signaling pathways such as the PI3K/AKT pathway (West et al., 2002). It has been described that AKT itself could be activated due to oxidative stress in the cell (West et al., 2002). NRF2 itself has been known to have many post translational modifications, both for activation as well as suppression. Recent studies have shown NRF2 to be phosphorylated at many serine residues (Apopa et al., 2008; Bloom and Jaiswal, 2003; Rada et al., 2012). Further investigations showed that serine 40 and serine 347 are both important sites (Bloom and Jaiswal, 2003; Rada et al., 2012). Serine 40 is phosphorylated by PKCδ and is important in stabilization of NRF2 (Bloom and Jaiswal, 2003). Serine 40 is located in the Neh2 domain, which is the domain that binds to KEAP1. When NRF2 is unable to bind to KEAP1, it is stabilized and activation of target genes occurs. Serine 347 on the other hand is located in the Neh6 domain, which has an internal degron domain of NRF2 (Rada et al., 2012). When phosphorylation occurs at this site, NRF2 becomes destabilized and
there is an increase in it degradation. Phosphorylation within the Neh6 domain creates a recognition motif for the ubiquitin E3 ligase adapter β-TrCP, which tags NRF2 for proteasomal degradation by a Cullin1/Rbx1 complex (Cuadrado, 2015). The PI3K/AKT pathway has been implicated in the phosphorylation at this site (Rada et al., 2012). In short when AKT is activated by phosphorylation at serine 473, GSK3 is inactivated and cannot phosphorylate NRF2, thereby stabilizing the transcription factor (Figure 4.1).

Some preliminary studies which led us to investigate NRF2 phosphorylation was the banding pattern of NRF2 on Western blots. Because of the studies discussed above, we wanted to determine if this multiple banding pattern was due to phosphorylation of NRF2. By treating HCT116 extracts with λ phosphatase (which removes all phosphates), the multiple banding pattern disappeared and one band with a faster mobility resulted (Figure 4.2). Some of the studies that showed the importance of NRF2 phosphorylation utilized specific or non-specific inhibitors. One of these inhibitors, MK2206, inhibits the three isoforms of AKT (AKT1, AKT2, and AKT3), whereas many of the other inhibitors were not specific, leading to possible off-target effects (Chowdhry et al., 2012; Simioni et al., 2013). By inhibiting the phosphorylation of AKT, the downstream target GSK3 remains active and therefore can phosphorylate NRF2 at serine 347 to promote degradation (Rizvi et al., 2014).

Here, we show that TYMS inhibitors increase the phosphorylation of AKT, thereby activating it. As a consequence, downstream target GSK3 is inactive and cannot phosphorylate NRF2, rendering it relatively stable. We find that the AKT inhibitor, MK2206, inhibits phosphorylation of AKT, but does not affect drug-induced apoptosis.
4.2A Materials and Methods

**Cell Culture:** HCT116 cells were used. Cells were maintained under the same conditions as previously stated in Section 2.2.

**Drug Treatment:** TYMS inhibitors and folinic acid were used as stated in section 2.2. MK2206 (SelleckChem, Houston, TX, USA) was used to inhibit phosphorylation of AKT. Appropriate concentration of MK2206 was determined experimentally. Cells were pre-treated with MK2206 for 3 hours before treatment with FdUrd.

**Western Blotting:** Cell preparation and western blotting was carried out just as described in Section 2.2. Anti-AKT rabbit monoclonal (9272, Cell Signaling Technology, Danvers, MA, USA) at a dilution of 1:2000 in 5% non-fat milk in 1X PBS/0.05% Tween, and anti-pAKT (S473) rabbit monoclonal (4060 (D9E) XP® Cell Signaling Technology, Danvers, MA, USA) at a dilution of 1:1000 in 5% non-fat milk in 1x PBS/0.05% Tween antibodies were used.

**Measurement of Apoptotic Cell Death:** To determine apoptotic cells, HCT116 cells were pre-treated with MK2206 for 3 hours followed by treatment with FdUrd for 48 hours. TUNEL assay was performed as described in Section 2.2

4.3A Results

**TYMS inhibitors induce phosphorylation of AKT**

We treated HCT116 protein extracts with λ phosphatase to determine if the multiple banding pattern of NRF2 was due to phosphorylation. Figure 4.2 shows a multiple banding pattern in both basal and drug treated cells, and when these extracts were treated with λ phosphatase it resulted in a single band with a slightly faster mobility.
This indicated that NRF2 is phosphorylated under basal and treated conditions. Since it is known that the AKT pathway can be activated by oxidative stress conditions within the cell, we determined if TYMS inhibitors had any effect on the PI3K/AKT pathway. Additionally, this could provide a mechanism behind observation of a small increase in NRF2 protein but larger increase in target genes in response to drug exposure. HCT116 cells were treated with 10μM FdUrd for various times. Figure 4.3 shows that FdUrd increases the phosphorylation of AKT, while total AKT remains unchanged.

**MK2206 inhibits AKT phosphorylation but has no effect on drug-induced apoptosis**

Initial experiments were done to determine a concentration of MK2206 where phosphorylation of AKT was inhibited but the amount of cell death was minimal after 4 days. We then sought to determine if these conditions increase drug-induced apoptosis. We found that apoptosis remained unchanged by pre-treatment of MK2206 (Figure 4.4), indicating that the PI3K/AKT pathway is not important in the activation of NRF2 by TYMS inhibitors. We verified that pAKT was inhibited as seen by the insert in Figure 4.4.

**4.4A Discussion**

Extending our findings from the previous two chapters which showed that TYMS inhibitors induce NRF2 and its target genes, but not solely through increase in concentration of the transcription factor, we looked for post-translational modifications which might be involved. We initially found that NRF2 is phosphorylated; however, we do not know which sites. This initial finding led us to look for pathways which might be involved in this phosphorylation. A few studies led us to both the PKCδ and PI3K/AKT pathways (Bloom and Jaiswal, 2003; Cuadrado, 2015; Rada et al., 2012). Initial studies
revealed that TYMS inhibitors increased phosphorylation of AKT (Figure 4.3), which is in line with a study conducted by another lab where they showed FUra increased phosphorylation of AKT (Jin et al., 2016). The increased phosphorylation leads to increased activation and ultimately downstream inhibition of GSK3. Only active GSK3 will phosphorylate NRF2 at the internal degron and lead to decreases in its concentration. This pathway could be one of the mechanisms by which TYMS inhibitors activate NRF2 and its target genes without large increases in the NRF2 protein concentration.

We used MK2206, which is an AKT inhibitor, to determine if by inhibiting this activation of pAKT we can increase the active GSK3, which would phosphorylate and decrease the concentration of NRF2, leading to an increase in drug-induced apoptosis. TUNEL assays determined that there was no change in drug-induced apoptosis following MK2206 treatment, even though the pAKT levels were inhibited completely (Figure 4.4). Also, there were no changes in levels of NRF2 or the banding pattern of NRF2 on Western blots. This is quite the opposite result from many other studies which used MK2206 in conjunction with chemotherapy drugs and saw either a reduction in tumor burden or an increase in apoptosis (Agarwal et al., 2014; Duan et al., 2014; Hirai et al., 2010; Jin et al., 2016; Liu et al., 2011; Ma et al., 2013; West et al., 2002). We expected our results to line up with those of the studies listed above; however, our results lead us to believe that the activation or inhibition of the PI3K/AKT pathway does not have any impact in the stability of NRF2 in HCT116 cells.
In sum, these results revealed that NRF2 is phosphorylated. Additionally, we determined that the PI3K/AKT pathway does not have any impact in the stability of NRF2, because its inhibition does not alter drug-induced apoptosis.

4.1B Introduction

The first part of this chapter showed that TYMS inhibitors are inducing many factors in the cell that could help create a cellular environment resistant to chemotherapy. Our results indicate that the PI3K/AKT pathway is not involved in regulating stability of NRF2; however, NRF2 has been shown to be acetylated at several lysine residues as well (Chen et al., 2014; Kawai et al., 2011; Sun et al., 2009). There have been some studies that have shown histone acetyltransferases (HATs) to be co-activators/co-factors that bind NRF2. NRF2 associates with one acetylase, CREB binding protein (CBP) (Katoh et al., 2001), which acetylates both non-histone proteins as well as histones (Kawai et al., 2011). Since NRF2 was shown to be associated with CBP, we decided to determine if CBP is involved in activating NRF2. We found by co-immunoprecipitation assays that NRF2 and CBP are bound in HCT116 cells (data not shown). TYMS inhibitors did not disrupt this interaction. We then decided to determine if CBP was bound to NRF2 at ARE’s. Using ChIP technology, we were not able to detect any binding at the 6 ARE’s previously described in chapter 2.

Changes in histone acetylation generally indicate changes in gene transcription, increased acetylation causing chromatin to loosen leading to transcription. In contrast, a decrease means reduced transcription (Eberharter and Becker, 2002). If we can identify
a change in acetylation at the promoter regions, then we can look for other possible histone acetyltransferases.

In the following experiments, we used ChIP technology to determine the acetylation patterns of histones at ARE regions which we have already shown to bind NRF2. We determined that there is a no correlation between the acetylation pattern and the activity of the gene.

4.2B Materials and Methods

Cell Culture: HCT116, cells were used. Cells were maintained under the same conditions as previously stated in Section 2.2.

Drug Treatment: TYMS inhibitors and folinic acid were used as stated in section 2.2.

Chromatin Immunoprecipitation (ChIP): Chromatin Immunoprecipitation was carried out using the SimpleChIP® Plus Enzymatic Chromatin IP Kit with magnetic beads (Cell Signaling Technologies, Danvers, MA, USA), according to the manufacturer’s protocol with the optimized procedure states in section 2.2. Antibodies used were anti-H2B K5 (12799 (D5H15) XP®), anti-H2B K12 (9072 (D7H4)), anti-H2B K15 (9083 (D8H1) XP®), anti-H3 K9 (9649 (C5B11)), anti-H3 K14 (7627 (D4B9)), anti-H3 K18 (13998 (D8Z5H)), anti-H3 K27 (8173 (D5E4) XP®), anti-H4 K12 (13944 (D2W60)), anti-H4 K16 (13534 (E2B8W)), anti-H2B (12364 (D2H6)), anti-H3 (4620 (D2B12) XP®), and anti-H4 (14149 (D2X4V)). All antibodies were purchased from Cell Signaling Technologies. Primers are the same listed in Table 2.2. Results were analyzed the same as stated in section 2.2 with the addition of determining the ratio of acetylated histone to total histone after the background noise was taken in account.
**Statistics:** Two-tailed student T tests were used to determine the statistical significance of the chromatin immunoprecipitation experiments. Results were considered significant if p value was less than 0.05. These are show by placing an asterisk (*) above the bar.

4.3B Results

**TYMS inhibitors affect the acetylation of histones**

HCT116 cells were treated for 12 hours with 10μM FdUrd, chromatin was isolated, and ChIP technology was used to assess extents of histone acetylation at ARE regions previously shown to bind NRF2 in Chapter 2. We observed that the acetylation of histones varies slightly in response to FdUrd depending on the ARE. As seen in Figure 4.5, the acetylation patterns for the 6 different regions does not change by a significant amount. There appears to be little to no correlation between activity of the gene and acetylation at the promoter region.

4.4B Discussion

It has long been understood that an increase in histone acetylation within a promoter region is linked to actively transcribing genes, whereas a decrease in acetylation or increase in methylation indicates reduced transcription (Eberharter and Becker, 2002). Here, we tested the effect of TYMS inhibitors on patterns of histone acetylation in ARE regions of NRF2 target genes. There was no significant increase in histone acetylation at those regions following FdUrd treatment, in contrast to what we were expecting. We expected to see an increase AREs of FdUrd-induced genes, and no change at those regions of genes that showed no response to drug. There appears to be no correlation between the histone acetylation and FdUrd response at AREs. This could mean that
increase in acetylation may not be important in activating these genes. A study by Deckert and Struhl revealed that individual activators have distinct patterns of histone acetylation, and transcriptional activation may not necessarily be associated with increased acetylation (Deckert and Struhl, 2001). There are still more experiments that need to be done to fully understand if there really is no correlation with histone acetylation and activity of the gene. The experiments include adding additional time-points to be tested. By adding additional time-points, we would be able to determine if the histone acetylation occurs at earlier or later time-points. We do observe that the target genes are induced at varying time-points so it could be very likely that the increases in acetylation may occur early on, prior to 12 hours.
Figure 4.1. **AKT pathway and phosphorylation.** Top part of figure shows AKT active, which inactivates GSK3, leaving NRF2 not phosphorylated in the Neh6 domain. The lower part of the figure depicts when AKT is inactive, leaving GSK3 active and able to phosphorylate NRF2 at serine 347, leading to degradation.
Figure 4.2. **NRF2 is phosphorylated.** By treating extracts with λ phosphatase it indicated that NRF2 both in untreated and treated cells is phosphorylated. The triple banding pattern seen disappeared in those extracts treated with λ phosphatase and resulted in a single band with a slightly faster mobility.

Figure 4.3. **TYMS inhibitors increase phospho-AKT.** Treating cells with FdUrd increases the phosphorylation of AKT at S473. Total AKT remains unchanged.

Figure 4.4. **Apoptotic index for cells treated with MK2206.** TUNEL assays indicate there is no change in apoptosis when cells are pre-treated with MK2006 to inhibit phosphorylation and activation of AKT. Levels of pAKT (S473) were checked and remain inhibited under the conditions which cells were analyzed for apoptosis.
Figure 4.5. **Histone acetylation at ARE regions in HCT116 cells.** This figure shows the patterns of histone acetylation when HCT116 cells were treated with 10μM FdUrd for 12 hours. The acetylation patterns seem to vary from gene to gene, but remain somewhat constant over the 9 acetylated histones tested. There was no significant increase or decrease across the 9 acetylated histones. The few significant changes are indicated by an *. The * indicates a p value <0.05.
TYMS is the sole de novo source of thymidine for the cell (Ozer et al., 2015). This has made it an attractive target for cancer chemotherapy for years (Barbour and Berger, 2008; Berger and Berger, 2006; Carreras and Santi, 1995; Chu et al., 2003; Longley et al., 2003; Wilson et al., 2014). Deprivation of intracellular thymidine leads to DNA damage and ultimately cell death (Ozer et al., 2015). TYMS inhibitors such as FdUrd, FUra, and RTX have been used to treat cancers for over 50 years now and are still one of the most common therapies. Many chemotherapy drugs kill cells by increasing the ROS levels past the threshold of survival (Akhdar et al., 2009; Lamberti et al., 2012; Matsunaga et al., 2010). Until recently the mechanism behind this was not well understood. Previous studies done in our lab have shown that treatment with TYMS inhibitors increases ROS levels in the cells by activating NADPH Oxidase 2 (Ozer et al., 2015). Further studies showed that by treating the cells with antioxidants, drug-induced apoptosis is ameliorated, indicating that it is due to the rise in ROS levels within the cell (Ozer et al., 2015). In addition to the higher levels of ROS, it was also found that TYMS inhibitors are also inducing antioxidants which are combating the increases in ROS levels, thereby diminishing the effect of the drugs (Ozer et al., 2015). Many of these antioxidant and drug
detoxifying genes were linked to transcription factor NRF2. Here we investigate the effect of NRF2 on drug-induced apoptosis.

We found that TYMS inhibitors increase the protein concentration of NRF2 and many of its target genes. Extents of these increases vary among the genes and among different colon cancer cell lines. We also showed that the increase in NRF2 concentration was much lower than that caused by a common activator of NRF2, tBHQ. NRF2 activation by TYMS inhibitors is due to the deprivation of thymidine in the cell, but not to the increased ROS levels, as addition of thymidine ameliorated the induction but NAC had no effect. Additionally, we showed that both tBHQ and FdUrd induce the target genes through the ARE region. Even though tBHQ induces the concentration of NRF2 at the protein level much more effectively than FdUrd, the induction of the reporter gene by FdUrd is higher than that of tBHQ. Thus, indicating that TYMS inhibitors are inducing NRF2 through mechanisms other than just concentration. ChIP assays revealed that FdUrd increases the occupancy of the ARE region. This increase in occupancy at the ARE does not necessarily mean activation of the target gene. Thus, indicating that binding of NRF2 to the ARE is required but not sufficient for induction of target genes. There was an increase at the NQO1 ARE, NQO1 is not induced by TYMS inhibitors.

To determine if the induction in NRF2 is creating an environment that is resistant to chemotherapy, we knocked down NRF2. With this knockdown, basal levels of target genes are decreased and there is a modest increase in drug-induced apoptosis. We also found that the status of p53 may have a role in whether reducing NRF2 levels can sensitize cells to chemotherapeutic drugs. There may be an NRF2 dependent
mechanism and an NRF2 independent mechanism. To determine if this modest increase is due to the small amount of NRF2 remaining in the cells, we knocked out NRF2 using the new CRISPR/CAS9 technology. With the knockout of NRF2, many of the target genes were not expressed, even in the presence of TYMS inhibitors. Drug-induced apoptosis levels were increased from 2-4-fold in the different clones. We also found here that the two mechanisms NRF2 dependent and independent are apparent. HCT116 showed the increase in drug-induced apoptosis, whereas SW480 did not show a significant increase and the target genes were inducible by TYMS inhibitors but not tBHQ. This indicated that reduction in NRF2 levels re-sensitizes cells to TYMS inhibitors.

While tBHQ induces NRF2 levels to a greater extent than FdUrd, target genes, as well as the reporter contract are induced to the same or an even higher level than tBHQ. It was clear that FdUrd is activating NRF2 through means other than solely concentration. Several previous studies have shown that NRF2 is phosphorylated at many serine residues. These phosphorylated sites have different functions, some activate NRF2, some prevent ubiquitination and degradation, while still others target NRF2 for degradation (Bloom and Jaiswal, 2003; Rada et al., 2012). NRF2 is phosphorylated at Serine 347 by GSK3, which is an internal degron that leads to degradation of NRF2 and lower levels in the cell (Cuadrado, 2015). Initial studies indicated that NRF2 is phosphorylated in untreated cells. We also found that TYMS inhibitors activate AKT, which inactivates GSK3 and inhibits phosphorylation at the internal degron. This may be one way TYMS inhibitors are inducing NRF2 independent of an increase in its concentration. Using a specific inhibitor for AKT, we demonstrated that
even with inhibition of the AKT pathway and ultimately activation of GSK3 and phosphorylation of NRF2 in the internal degron, there was no increase in drug-induced apoptosis. Thus indicating that the PI3K/AKT pathway does not have any impact in the stability of NRF2, or drug-induced apoptosis.

Previous studies as well as studies in our lab showed that NRF2 interacts with CBP. We were not able to determine if CBP alters acetylation of NRF2 or binds to the ARE with NRF2. We instead decided assess alterations in acetylation at the lysine residues of histones bound to AREs. We found that the acetylation patterns of the histones do not correlate with activity of the gene.

Overall we can conclude that TYMS inhibitors are increasing NRF2 through a non-canonical pathway. TYMS inhibitors do not appear to increase acetylation of histones near the AREs. The PI3K/AKT pathway does not appear to have an impact on chemotherapeutic drug sensitivity. The increases in NRF2 and target genes are a constraining factor in TYMS inhibitor resistance, as shown by the increases in drug-induced apoptosis in the NRF2 knockout cells. There is also an NRF2 dependent mechanism and NRF2 independent mechanism with regard to sensitivity to chemotherapeutic agents. Figure 5.1 shows a working model of our conclusions. Further studies are necessary to have a better understanding of what else TYMS inhibitors are doing to increase the target gene expression with the small increase in protein levels of NRF2. Additionally, more studies looking at additional time-points in the histone acetylation are necessary to fully understand if the histone acetylation patterns really have no correlation with activity of the gene, or if the acetylation increases or decreases
occur at earlier or later time-points. Additionally further studies are needed to determine if p53 plays a role in the NRF2 dependent and independent mechanism of drug-induced apoptosis.

The major implications of these findings are there are other ways NRF2 may be induced that are not just by concentration. We were also the first lab to show that TYMS inhibitors increase NRF2 occupancy at the ARE, and that this increase does not indicate activation of the gene. Also in contrast with many studies, the PI3K/AKT pathway does not have any impact on the stability of NRF2, or drug-induced apoptosis in HCT116 cells. Because NRF2 is phosphorylated at many sites there are other pathways which could be involved and could be investigated for further studies. We also found the unlike long understood concept that increased histone acetylation in a promoter region loosens chromatin and is linked with activity of the gene, there was no change in acetylation of histones in the AREs of actively transcribing genes. Finally, we demonstrated the NRF2 is a constraining factor in sensitivity to chemotherapeutic drugs. Additionally, there is likely an NRF2 dependent and independent mechanism for drug-induced apoptosis.
Figure 5.1. **Current working model.** Based on our findings, this is a working model of TYMS inhibitors and their impact on NRF2. Previously, we found that TYMS inhibitors increase ROS, which leads to cell death (1), but we now know that they are also increasing and activating NRF2 and its target genes to promote cell survival (2). Ideally, we want to eliminate pathway 2 (cell survival) and increase pathway 1 (cell death) to sensitize cancer cells to chemotherapy.
References

(2016a) AJCC - American Joint Committee on Cancer.

(2016b) American Cancer Society | Information and Resources for Cancer: Breast, Colon, Lung, Prostate, Skin.


Apopa PL, He X and Ma Q (2008) Phosphorylation of Nrf2 in the transcription activation domain by casein kinase 2 (CK2) is critical for the nuclear translocation and transcription activation function of Nrf2 in IMR-32 neuroblastoma cells. J Biochem Mol Toxicol 22(1): 63-76.


Bloom DA and Jaiswal AK (2003) Phosphorylation of Nrf2 at Ser40 by protein kinase C in response to antioxidants leads to the release of Nrf2 from INrf2, but is not required for Nrf2 stabilization/accumulation in the nucleus and transcriptional activation of antioxidant response element-mediated NAD(P)H:quinone oxidoreductase-1 gene expression. J Biol Chem 278(45): 44675-44682.


Chowdhry S, Zhang Y, McMahon M, Sutherland C, Cuadrado A and Hayes JD (2012) Nrf2 is controlled by two distinct [beta]-TrCP recognition motifs in its Neh6 domain, one of which can be modulated by GSK-3 activity. Oncogene 32(32): 3765-3781.


Haan JBd (2011) Nrf2 Activators as Attractive Therapeutics for Diabetic Nephropathy.


Appendix A
Histone acetylation in NRF2 null cells

As stated earlier in chapter 4, acetylation of histones may not be playing a large role in the activation of the target genes. There was no correlation in the acetylation of histones and the activity of the gene in HCT116 cells. This is unlike what has been commonly understood in the literature where increase in acetylation means increase in activity of the genes and decrease in acetylation means reduced activity. After this, we thought to investigate if the knockout of NRF2 had an effect on the histone acetylation patterns.

We used a single clone (A24) and treated the cells for 12 hours with 10μM FdUrd. We isolated chromatin, and performed chromatin-immunoprecipitation. Figure A.1. shows the 6 ARE regions analyzed by ChIP. Here we see a larger increase overall in histone acetylation. When comparing the A24 to HCT116 it is clear that the histone acetylation pattern is different. It is also worth to note that when comparing just the total histones there was a large decrease in total histones at the ARE regions in the A24 clone but not much change in total histones in the HCT116 cells when treated with TYMS inhibitors (Figure A.2.). The increases in histone acetylation seen in the A24 clone may be due to the decreases in total histones since they are expressed as a ratio. The clones as stated earlier are more sensitive to TYMS inhibitors so it is possible that there...
is more damage to the DNA and histones are just being lost. It may also be due to the loss of non-acetylated histone proteins while the acetylated histones remain intact on the chromatin, thereby exhibiting an increase in acetylated histones. There are more studies that need to be done in order to determine if NRF2 is involved in the histone acetylation patterns. Additional time-points should be looked at as well as lower concentrations of FdUrd because of the higher sensitivity that the A24 clones exhibit. It would also be good to look at additional clones to verify that the results are not due to clonal isolation.
Figure A.1. **Histone acetylation at ARE regions in A24 cells.** This figure shows the patterns of histone acetylation when A24 NRF2 null cells were treated with FdUrd for 12 hours. The acetylation patterns seem to vary slightly from gene to gene, but overall there appears to be an increase in acetylated histones across the 6 promoter regions analyzed. * indicates $p$ value <0.05.
Figure A.2. **Total histones at ARE regions in A24 and HCT116 cells.** All cells were treated with 10μM FdUrd for 12 hours. A shows the decrease in total histones in the A24 clone when treated with FdUrd, whereas B shows the change in histones in HCT116 cells. Here there is no change or a slight increase in total histones when treated with FdUrd.